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Obesity

miRNA Signatures of Insulin Resistance in Obesity

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Objective: Extracellular microRNAs (miRNAs) represent functional biomarkers for obesity and related disorders; this study investigated plasma miRNAs in insulin resistance phenotypes in obesity.

Methods: One hundred seventy-five miRNAs were analyzed in females with obesity (insulin sensitivity, $n = 11$; insulin resistance, $n = 19$; type 2 diabetes, $n = 15$) and without obesity ($n = 12$). Correlations between miRNA level and clinical parameters and levels of 15 miRNAs in a murine obesity model were investigated.

Results: One hundred six miRNAs were significantly (adjusted $P \leq 0.05$) different between controls and at least one obesity phenotype, including miRNAs with the following attributes: previously reported roles in obesity and altered circulating levels (e.g., miR-122, miR-192); known roles in obesity but no reported changes in circulating levels (e.g., miR-378a); and no current reported role in, or association with, obesity (e.g., miR-28-5p, miR-374b, miR-32). The miRNAs in the latter group were found to be associated with extracellular vesicles. Forty-eight miRNAs showed significant correlations with clinical parameters; stepwise regression retained let-7b, miR-144-5p, miR-34a, and miR-532-5p in a model predictive of insulin resistance ($R^2 = 0.57$, $P = 7.5 \times 10^{-8}$). Both miR-378a and miR-122 were perturbed in metabolically relevant tissues in a murine model of obesity.

Conclusions: This study expands on the role of extracellular miRNAs in insulin-resistant phenotypes of obesity and identifies candidate miRNAs not previously associated with obesity.

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Introduction

The pandemic levels of obesity represent a major public health challenge. Obesity is a systemic disorder and risk factor for multiple diseases, including type 2 diabetes (T2D), hypertension, and cardiovascular disease. Insulin resistance (IR) is strongly associated with obesity and T2D; however, not all individuals with obesity develop IR and T2D.

MicroRNAs (miRNAs) are small noncoding RNAs (19-23 nucleotides) that inhibit translation and/or direct messenger RNA degradation (1). They are involved in the pathogenesis of complex diseases, including obesity (2) and T2D (3). miRNAs are present in blood, in which they are packaged in extracellular vesicles (EVs) (4); are associated with low-density lipoprotein (LDL) or high-density lipoprotein (HDL) (5); or are bound by RNA-binding protein argonaute-2 (6) to prevent their degradation. As such, their potential as disease biomarkers has been increasingly interrogated. *In vitro* miRNAs transported in association with exosomes or HDL can be delivered to recipient cells in their active form and can modulate target messenger RNAs (4,5), altering cell function and key cell signaling processes. Therefore, although there is still much to understand about the functional role of circulating miRNAs *in vivo*, they represent a novel cell-cell communication network, mediating cross talk between organs.

Following the first report of circulating miRNAs associated with T2D in 2010 (7), studies have reported associations between circulating miRNA levels and obesity in adults (8,9), young adults (10), and children (11) and between these levels and related metabolic disorders, including metabolic syndrome (12,13), prediabetes (14,15), and T2D (9,15,16). Obesity is strongly associated with IR, and although several of these studies have investigated potential correlations between IR

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TABLE 1 Clinical and anthropometric data for the obesity study cohort

	IS ($n = 11$)	IR ($n = 19$)	T2D ($n = 15$)	IS vs. IR[a]	IS vs. T2D[a]	IR vs. T2D[a]
Age (y)	41 (± 5)	40 (± 6)	45 (± 8)	NS	NS	NS
BMI (kg/m^2)	42.6 (± 4.0)	44.7 (± 3.9)	44.2 (± 4.2)	NS	NS	NS
Fasting glucose (mmol/L)	4.6 (± 0.3)	4.7 (± 0.4)	9.3 (± 4.4)	NS	0.002	7.5×10^{-5}
Fasting insulin (pmol/L)	41.9 (± 9.2)	105.9 (± 35.6)	142.0 (± 67.7)	6.4×10^{-5}	6.2×10^{-5}	NS
HOMA2 IR	0.8 (± 0.2)	1.9 (±0.6)	3.4 (± 3.0)	4.8×10^{-5}	0.009	NS
McA	7.8 (±1.0)	5.4 (±0.6)	4.7 (± 1.0)	6.8×10^{-9}	4.9×10^{-8}	0.04
HbA1c (DCCT %)	5.4 (± 0.3)	5.4 (± 0.3)	7.5 (± 1.7)	NS	0.0007	1.0×10^{-5}
Total cholesterol (mmol/L)	5.14 (± 1.05)	5.11 (± 0.80)	5.02 (± 1.0)	NS	NS	NS
Triglycerides (mmol/L)	0.94 (±0.29)	1.57 (±0.63)	2.29 (± 1.35)	0.0004	0.003	0.04
LDL (mmol/L)	3.06 (± 1.0)	3.08 (±0.65)	3.11 (± 1.26)	NS	NS	NS
HDL (mmol/L)	1.64 (± 0.31)	1.33 (±0.18)	1.21 (± 0.22)	0.002	0.0004	NS
Systolic BP (mm Hg)	132.4 (± 9.9)	134.2 (± 13.9)	143.9 (± 13.0)	NS	0.02	0.04
Diastolic BP (mm Hg)	70.4 (± 10.1)	76.0 (± 10.4)	74.6 (± 7.9)	NS	NS	NS

Data presented as mean ± standard deviation (SD). Threshold of $P \leq 0.05$ considered significant. For healthy females ($n = 12$), mean age was 44 (± 9), which was not significantly different from any of the three phenotypic groups above. Mean BMI was 22.9 ± 2.6, which was significantly different from the IS ($P = 3.4 \times 10^{-12}$), IR ($P = 7.8 \times 10^{-17}$), and T2D ($P = 3.9 \times 10^{-14}$) phenotypes.
[a]*t* test *P* values presented for the comparisons between clinical groups.
BP, blood pressure; DCCT (Diabetes Control and Complications Trial units); NS, not significant; HOMA, homeostasis model assessment; McA, McAuley Index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; IS, insulin sensitivity; IR, insulin resistance; T2D, type 2 diabetes.

and miRNA levels, none to our knowledge has specifically interrogated insulin sensitivity (IS) phenotypes in obesity. Here, we investigate circulating plasma miRNAs in individuals with three obesity phenotypes (IS, IR, and T2D) and examine potential tissues of origin for miRNAs of interest in a mouse model of obesity.

Methods

Sample inclusion

Subjects gave written informed consent, and the study complied with the Helsinki Declaration. The Central Regional Ethics Committee, Wellington, New Zealand, approved the study of individuals with obesity (WGT/00/04/030). Samples from individuals without obesity were obtained from within the Institute of Environmental Science and Research under institutional ethics. All individuals self-reported as having European ancestry.

Samples from individuals with obesity were obtained from patients undergoing gastric bypass. Individuals were fasted overnight, blood was collected prior to gastric bypass in EDTA tubes and then centrifuged at 1300 *g* for 10 minutes, and plasma was removed and stored at −80 °C. Study participants were females with severe obesity grouped into three categories: individuals with T2D and those without T2D who were classified as having either IS or IR. IS was assessed using two indices: the HOMA-IR (homeostasis model assessment), using the HOMA Calculator version 2.2.3 from the Diabetes Trials Unit (University of Oxford, Oxford, UK; https://www.dtu.ox.ac.uk/homacalculator), and the McAuley Index (McA) (17), calculated as EXP(3.29 − [0.25 × ln(insulin mU/L)] − [0.22 × ln(BMI)] − [0.28 × ln(triglycerides mmol/L)]). Individuals without diabetes were classified as having IS if they had a HOMA-IR ≤ 1.0 and a McA > 6.3; they were classified as having IR if they had a HOMA-IR ≥ 1.0 and a McA < 6.3. Individuals with T2D were either diagnosed at the time of gastric bypass ($n = 6$, fasting glucose mmol/L ≥ 7 and/or HbA1c ≥ 6.5 % DCCT [Diabetes Control and Complications Trial units]) or had been previously diagnosed and were taking oral hypoglycemics ($n = 8$) or had diet-controlled T2D ($n = 1$). Clinical and anthropometric data are presented in Table 1. Fasted plasma samples were also obtained from 12 nondiabetic (self-reported) age- and sex-matched (44 ± 9 years; female) controls with BMI of 22.9 ± 2.6 kg/m^2. Age did not differ significantly between individuals with and without obesity. Additional clinical information was not available for controls.

Animal model

Experiments were conducted in accordance with the Beth Israel Deaconess Medical Center Animal Ethics Committee and the Institutional Animal Care and Use Committee regulations. Male C567BL/6 mice (Charles River Laboratories, Inc.) were fed normal chow ($n = 5$) or a 45% high-fat high-sucrose (HFHS) diet ($n = 5$) from 11 weeks of age for a total of 20 weeks. Mice were housed in a barrier facility for the entirety of the experiment and allowed free access to food and water. At euthanasia, mice fed normal chow had a mean body weight of 33.6 ± 2.8 g (± SD), while HFHS diet-fed mice were 53.1 ± 2.5 g. Animals were euthanized by ketamine/xylazine overdose, and blood and organs were collected immediately. Total blood was collected in EDTA microcentrifuge tubes and centrifuged at 1,000 *g* for 15 minutes to separate plasma. Plasma was centrifuged at 2,000 *g* for 15 minutes. Heart, liver, subcutaneous adipose, pericardial adipose, and visceral adipose tissues were harvested, washed in phosphate-buffered saline (PBS), and snap-frozen on dry ice. For RNA extraction/analysis and statistical analyses, see the Supporting Information Methods.

Results

Circulating miRNAs in human plasma

We compared the levels of 170 detectable miRNAs in plasma between three groups with obesity (IS, IR, and T2D) and controls

TABLE 2 Summary of the literature for the 18 miRNAs showing significantly different plasma levels in all obesity groups compared to lean controls

	Changes in circulating levels in human studies of obesity and T2D	Tissue expression and relevant functional roles in animal and cell models
miR-23a	Decreased serum levels in patients with T2D and prediabetes (15); increased in whole blood of patients with metabolic syndrome and hypercholesterolemia (12)	NA
miR-23b	NA	Decreased in mice with diet-induced obesity, upregulated with low-fat feeding (31); increased exosome-bound miR-23b released from adipocytes cultured from visceral fat from subjects with obesity vs. lean BMI (S1)
miR-26a	NA	Promoted brown fat adipogenesis through targeting ADAM17 (S2); decreased in mice with diet-induced obesity, upregulated with low-fat feeding (31); decreased liver expression in subjects with overweight vs. lean BMI; overexpression in high-fat diet-fed mice improved IS (S3)
miR-28-5p	NA	NA
miR-30e-3p	NA	Downregulated in white adipose tissue of mice fed high-fat diet (S4)
miR-151-5p	Decreased serum levels in young adults with obesity vs. nonobesity (10)	Decreased expression in white adipose tissue of subjects with obesity vs. lean BMI (S5)
miR-151-3p	Decreased serum levels in young adults with obesity vs. nonobesity (10)	NA
miR-181a	Increased in serum of male patients with T2D (S6); decreased in monocytes of patients with obesity; weight loss normalized expression levels (S7)	Targets IDH1 and decreases expression of lipid synthesis genes; transgenic mice had lower body weight (S8); targets SIRT1 (S6)
miR-197	Increased in whole blood of patients with metabolic syndrome and hypercholesterolemia (12); decreased in patients with T2D (7); expression levels inversely correlated with risk of glycemic progression in Asian Indians (S9)	NA
miR-374b	NA	NA
miR-584	Increased in whole blood of patients with metabolic syndrome and hypercholesterolemia (12)	NA
let-7d	NA; let-7 in general but none specific to let-7d	NA
let-7e	NA; let-7 in general but none specific to let-7e	NA
let-7f	Decreased in patients with T2D; increased with diabetic treatment (S10)	Decreased in mice with diet-induced obesity; upregulated with low-fat feeding (31)
miR-32	NA	NA
miR-101	Increased in serum of patients with T2D vs. normal glucose tolerance	NA
miR-144	Upregulated in whole blood of patients with T2D vs. impaired fasting glucose (S11); higher plasma expression associated with T2D in Swedes but not Iraqis (16)	Targets IRS1 (S11); liver levels increased in patients with morbid obesity and nonalcoholic steatohepatitis; targets ABCA1 (S12)
miR-365	NA	Regulated brown fat adipogenesis (S13,S14)

References S1-S14 can be found in Supporting Information.
NA, not applicable.

TABLE 3 Top five miRNAs in each patient group showing greatest positive and negative fold-change difference from controls

	miRNA	Log2 fold change (control)	KS test statistic	Bonferroni adjusted *P* value[2]	Changes in circulating levels in human studies of obesity and T2D	Tissue expression and relevant functional roles in animal and cell models
IS	**miR-144**	5.1	1	0.0003	Upregulated in whole blood of patients with T2D vs. impaired fasting glucose (S11); higher plasma expression associated with T2D in Swedes but not Iraqis (16)	Targets IRS1 (S11); liver levels increased in patients with morbid obesity and nonalcoholic steatohepatitis; targets ABCA1 (S12)
	miR-365	4.0	1	0.0037	NA	Regulated brown fat adipogenesis (S13,S14)
	miR-32	3.4	1	0.0002	NA	NA
	miR-451	3.1	0.83	0.0476	Serum levels downregulated following Roux-en-Y gastric bypass surgery in patients with low BMI (S15); serum levels increased in patients with non-alcoholic fatty liver disease (25)	Increased in heart of mice with high-fat diet-induced obesity, knockout reduced cardiac lipotoxicity; targets CAB39 (S16)
	miR-150	2.1	1	0.0003		Obese knockout mice had exacerbated IR (S17); upregulated in pancreatic islet cells of mice with diabetes (S18)
	let-7f	−3.8	0.91	0.0058	Decreased in patients with T2D; increased with diabetic treatment (S10)	Decreased in mice with diet-induced obesity; upregulated with low-fat feeding (31)
	let-7e	−3.6	1	0.0005	NA; let-7 in general but none specific to let-7e	Decreased in mice with diet-induced obesity; upregulated with low-fat feeding (31)
	miR-409-3p	−3.5	0.83	0.0476	NA	NA
	miR-151-5p	−3.0	0.91	0.0058	NA	NA
	miR-374b	−2.8	0.91	0.0058	NA	NA
IR	**miR_144**	5.3	1	0.0002	As above	As above
	miR-193b	3.8	1	0.0005	Increased in prediabetes but not T2D; levels normalized with chronic exercise (14)	Targets CREB5, NRIP1, and NFYA; stimulated adiponectin secretion in adipocytes and white adipose tissue (S19); indirectly regulated CCL2 production in white adipose tissue (5); regulated brown fat adipogenesis (14)
	miR-365	3.8	1	0.0001	NA	As above
	miR-451	3.2	1	2.4×10^{-6}	As above	As above
	miR-122	3.1	0.92	7.5×10^{-5}	Highly associated with obesity and IR (8,10,11); reduced after weight loss (11); associated with hepatic steatosis (25)	Decreased expression following bariatric surgery in rats with shifts in metabolic profile; targets CS, GLUT1, G6PD, FASN, PRKAB1, and ALDOA (S20)
	miR-409-3p	−4.0	0.92	0.0002	NA	NA
	let-7f	−4.0	1.00	2.4×10^{-6}	As above	As above
	let-7e	−3.9	1.00	2.0×10^{-5}	As above	As above
	miR-1974	−3.7	1.00	2.4×10^{-6}	NA	NA
	miR-382	−3.7	0.83	0.0020	NA	NA

TABLE 3 (continued).

	miRNA	Log2 fold change (control)	KS test statistic	Bonferroni adjusted *P* value[2]	Changes in circulating levels in human studies of obesity and T2D	Tissue expression and relevant functional roles in animal and cell models
T2D	**miR-193b**	5.1	0.93	0.0063	As above	As above
	miR-144	4.1	1	0.0006	As above	As above
	miR-136	3.8	0.83	0.0349	NA	NA
	miR-34a	3.5	0.85	0.0049	Increased circulating levels in patients with T2D; significant with meta-analysis across controlled studies (24)	Suppressed FGF1 and SIRT1 and inhibited brown fat formation in mice with obesity (S21); targets NAMPT and SIRT1 in liver (S22); disrupted beta-KL/FGF19 signaling in liver (S23)
	miR-32	3.4	1	2.0×10^{-5}	NA	NA
	let-7d	−3.7	0.92	0.0005	let-7 in general but none specific to let-7d	NA
	let-7c	−3.6	0.83	0.0197	let-7 in general but none specific to let-7c	Decreased in mice with diet-induced obesity; upregulated with low-fat feeding (31)
	let-7e	−3.4	1	2.0×10^{-5}	As above	As above
	let-7f	−3.2	1	2.0×10^{-5}	As above	As above
	miR-485-3p	−2.8	0.93	0.0003	NA	NA

References S10-S23 can be found in Supporting Information.
KS, Kolmogorov-Smirnov test statistic; NA, not applicable.

(Supporting Information Figure S1). Eighteen miRNAs showed a significant difference (adjusted $P \leq 0.05$) in levels in all groups with obesity compared to controls (14 downregulated, 4 upregulated). These included miRNAs with the following attributes: previously reported roles in obesity and altered circulating levels associated with disease (e.g., miR-144, miR-151-5p); roles in obesity but no reports of changes in plasma abundance (e.g., miR-365, miR-23b, miR-26a); and no current reported role in, or association with, obesity (miR-28-5p, miR-374b, miR-32) (Table 2).

Significant differences ($P \leq 0.05$) were observed for IS versus IR (miR-335 and miR-423-5p), IS versus T2D (let-7b), and IR versus T2D (miR-19b, miR-22, miR-22-5p, miR-136, miR-152, miR-484). Table 3 presents the top five miRNAs with the greatest positive and negative fold-change difference between controls and each subgroup with obesity.

We interrogated potential associations between plasma miRNAs (ΔCt [cycle threshold]) and 11 clinical parameters using the Pearson correlation. Forty-eight miRNAs showed a significant correlation (adjusted $P \leq 0.05$, absolute $r \geq 0.3$) with at least one clinical parameter (Supporting Information Table S1). No significant correlations were observed with fasting cholesterol, and only one significant miRNA-trait correlation was observed for diastolic blood pressure (miR-145) and for fasting LDL (miR-17). Table 4 presents the miRNA-trait correlations with absolute $r \geq 0.4$.

We further explored which miRNA(s) were the best predictors of trait by performing stepwise regression analyses of all miRNAs significantly associated with a given trait (Table 5). Notably, we observed four miRNAs (let-7b, miR-144-5p, miR-34a, and miR-532-5p) retained in a model strongly predictive of IR ($R^2 = 0.57$, $P = 7.5 \times 10^{-8}$). To investigate the potential contribution of clinical and anthropometric variables (Table 1) to these models (Table 5), we performed additional stepwise regression analyses by including these variables and the miRNAs from the respective final models (Table 5); miRNAs were retained in the final model for all clinical traits except fasting glucose, HbA1c, and HDL (as expected, the strong correlation between HbA1c and fasting glucose, $R^2 = 0.86$, $P = 2.2 \times 10^{-16}$, dominated the respective models). For IR (McA), three of the four miRNAs (let-7b, miR-144-5p, and miR-34a) were retained, and fasting lipids (total cholesterol, HDL, and LDL) were included in the final model ($R^2 = 0.70$, $P = 8.6 \times 10^{-10}$).

To determine extracellular compartmentalization, we investigated miRNAs found in our study but not previously implicated in obesity (Table 2) in plasma EVs isolated from six females with obesity. Additionally, we measured the expression of two miRNAs previously associated with non-EV fractions as controls (miR-146a (18) and miR-92 (19)). All but one of the miRNAs of interest tested (miR-23b) were undetectable (mean Ct ≥ 35) in the non-EV fraction but detectable in the EV fraction: miR-374b (Ct 21.6 ± 1.5), let-7d (Ct 22.1 ± 1.1), let-7f (Ct 22.1 ± 1.3), miR-32 (Ct 27.4 ± 0.9), miR-26a (Ct 21.1 ± 1.2), miR-30e (Ct 25.0 ± 1.3), and miR-365 (Ct 30.6 ± 0.8). One miRNA, miR-23b, was detected in both the EV (Ct 18.9 ± 1.1) and flow (Ct 28.5 ± 4.4) fractions, a finding similar to that observed for the control miRNAs (miR-146a: EV = Ct 21.0 ± 1, flow = Ct 25.7 ± 4.1 and miR-92a: EV = Ct 23.2 ± 0.8, flow = Ct

TABLE 4 Correlations between plasma miRNA level and clinical traits passing absolute $r \geq 0.4$ and adjusted $P \leq 0.05$

	Clinical trait								Direction of fold change vs. control[a]		
miRNA	Glucose	HbA1c	Insulin	McA	HOMA-IR	Triglycerides	HDL	Systolic BP	IS	IR	T2D
let-7b				−0.44 (0.006)						↓	↓
let-7c								0.4 (0.013)		↓	↓
let-7d			0.47 (0.003)	−0.43 (0.008)	0.41 (0.012)				↓	↓	↓
miR-21						−0.43 (0.007)			↓	↓	
miR-22	−0.41 (0.011)	−0.42 (0.009)				−0.43 (0.007)					
miR-22-5p						−0.42 (0.010)					
miR-25			−0.45 (0.005)								
miR-30a						−0.41 (0.013)					
miR-34a			−0.44 (0.006)	0.49 (0.002)	−0.43 (0.008)	−0.49 (0.002)				↑	↑
miR-99a						−0.45 (0.005)					
miR-122			−0.46 (0.004)	0.56 (2×10^{-4})	−0.41 (0.013))	−0.41 (0.012)				↑	↑
miR-155								0.41 (0.012)		↓	↓
miR-192			−0.42 (0.011)							↑	
miR-193b						−0.46 (0.004)				↑	↑
miR-194						−0.41 (0.011)				↑	
miR-210	−0.42 (0.010)	−0.43 (0.009)		0.48 (0.003)		−0.41 (0.012)					
miR-215						−0.44 (0.007)			↑	↑	
miR-378			−0.54 (4×10^{-4})	0.42 (0.009)	−0.52 (8×10^{-4})	−0.47 (0.003)	0.49 (0.002)			↑	
miR-505						−0.50 (0.001)					
miR-532-5p						−0.44 (0.007)				↑	
miR-660						−0.45 (0.005)					

Correlation analyses performed on normalized miRNA Ct levels (ΔCt). Higher Ct indicates lower miRNA level. All blood measures assayed on fasted samples.
[a]In cases in which a significant (adjusted $P \leq 0.05$) difference detected between miRNA levels in control and obesity groups, direction of fold change indicated.

TABLE 5 Results from stepwise linear regression of miRNAs against trait

	miRNA final model			Final model including clinical variables		
	miRNAs loaded in final model	Adjusted R^2	R^2 *P* value	Variables loaded in final model	Adjusted R^2	R^2 *P* value
McA	let-7b, miR-144-5p, miR-34a, and miR532-5p	0.57	7.5×10^{-8}	let-7b, miR-144-5p, miR-34a, total cholesterol[a], HDL[a], and LDL[a]	0.70	8.6×10^{-10}
HOMA-IR	let-7g and miR-378	0.27	5.3×10^{-4}	miR-378, HbA1c, HDL[a], and LDL[a]	0.47	5.9×10^{-6}
Fasting insulin	let-7d, let-7g, miR-122, miR-15b, miR-193b, miR-210, miR-335, miR-34a, miR-374a, miR-374b, miR-378, and miR-421	0.50	2.1×10^{-4}	let-7d, miR-193b, miR-335, miR-34a, miR-374a, glucose[a], HbA1c, diastolic BP, and HDL[a]	0.53	2.1×10^{-5}
Fasting glucose	let-7g and miR-22	0.27	5.1×10^{-4}	Age, insulin[a], HbA1c, and LDL[a]	0.88	2.2×10^{-16}
HbA1c	miR-136, miR-155, and miR-505	0.37	5.4×10^{-5}	Glucose[a], insulin[a], HOMA-IR, systolic BP, HDL[a], and LDL[a]	0.91	2.2×10^{-16}
Fasting triglycerides	miR-125b, miR-193b, miR-215, miR-22, miR-27b, miR-34a, miR-502-3p, and miR-532-5p	0.47	7.8×10^{-5}	miR-125b, miR-193b, miR-215, miR-27b, miR-34a, BMI, insulin[a], HOMA-IR, HbA1c, diastolic BP, and LDL[a]	0.64	1.1×10^{-6}
Fasting HDL	miR-22 and miR-378	0.26	7.7×10^{-4}	Glucose[a], insulin[a], HOMA-IR, McA, HbA1c, total cholesterol[a], and LDL[a]	0.57	1.5×10^{-6}
Systolic BP	miR-155 and miR-193b	0.24	0.0014	miR-155, miR-193b, BMI, glucose, HOMA-IR, diastolic BP, and total cholesterol[a]	0.40	3.4×10^{-4}

[a]Indicates fasting blood measures.

29.8 ± 4.6). Thus, the majority of these miRNAs are EV associated, and miR-23b may also be associated with either argonaute-2 proteins or lipoprotein complexes.

Analysis of candidate miRNAs in mouse models of obesity

Fifteen miRNAs were selected for further analysis based on (1) no previous report of a role in obesity or T2D (let-7d, let-7e, miR-32, and miR-28-5p), (2) a potential role in obesity but no previous report of changing circulating levels (miR-365), or (3) a reported role in obesity and a correlation with at least one clinical parameter in the current study (miR-19a, miR-34a, miR-122, miR-155, miR-192, miR-193b, miR-378a, miR-451, and miR-484); let-7f was also included because of the reported association with T2D and because many studies to date have not differentiated among let-7 family members (see Tables 2-3). We compared miRNA levels in mice on normal chow versus an HFHS diet for 20 weeks (the body weight of HFHS mice increased by 27.2 ± 2.1g, compared to controls [7.4 ± 2.2g]).

We observed significantly different levels of plasma miRNAs between control and HFHS mice for miR-122 and miR-192 ($P = 0.008$). Performing an additional a priori analysis informed by the direction of change in the human study also revealed a significant difference in the level of miR-378a ($P = 0.04$) (Figure 1).

To evaluate potential tissue origins or targets of these miRNAs, we investigated the levels of miRNA-122, miRNA-192, miR-378a, let-7d, let-7e, and let-7f in metabolically relevant tissues (subcutaneous adipose, visceral adipose, pericardial adipose, liver, and left-ventricle heart tissues) (Figure 2). We observed a significant decrease in miR-378a in visceral adipose tissue of HFHS mice compared to control mice ($P = 0.008$). Given the small sample size, and in order to reveal additional differences that might warrant future investigation, we also applied a less stringent (one-tailed) statistical analysis. We observed significant increases ($P = 0.04$) in miRNA levels in the HFHS mice compared to the normal chow group for miR-378a and let-7f in pericardial fat and miR-122 and let-7e in subcutaneous fat, and we observed significant decreases for let-7e and let-7f in heart tissue and for let-7f in liver tissue.

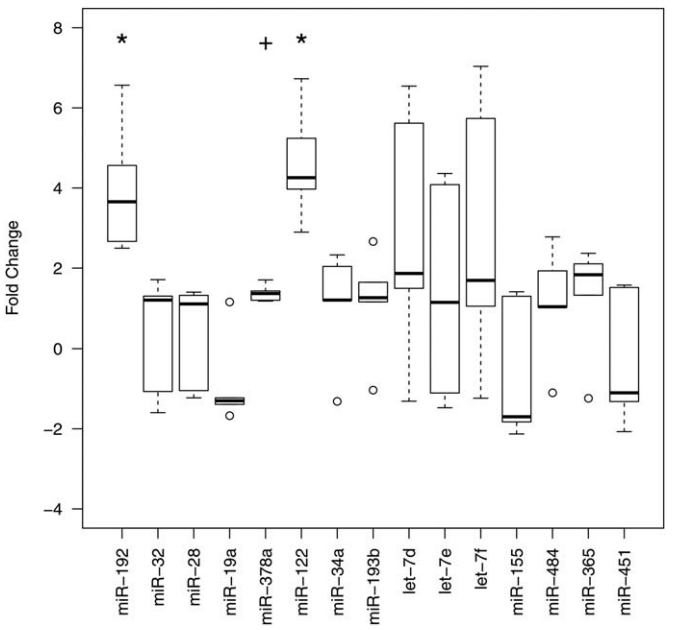

Figure 1 Levels of 15 plasma miRNAs in a mouse model of obesity, fold change relative to controls. Fold change was calculated using the formula $2^{-\Delta\Delta Ct}$. * indicates significance at $P \leq 0.05$ (two-tailed), and + indicates significance at $P \leq 0.05$ (one-tailed).

Discussion

This study investigated plasma miRNA profiles (Exiqon-targeted panel) in 45 individuals with obesity (IS = 11, IR = 19, T2D = 15) compared to controls (BMI 22.9 ± 2.6 kg/m^2). Eighteen miRNAs showed differential abundance in all obesity phenotypes (Table 2). Although several of these miRNAs had been associated with obesity and/or T2D, three (miR-28-5p, miR-374b, and miR-32) had not. Significant differential plasma abundance was also observed for three miRNAs in a murine obesity model, with differential expression of two of these seen in metabolically relevant tissues. Strong associations were observed between circulating miRNA levels and clinical traits (Table 4, Supporting Information Table S1). Stepwise regression analyses revealed four miRNAs contributing significantly to a model predictive of IR ($R^2 = 0.57$, $P = 7.5 \times 10^{-8}$) (Table 5); the association of circulating levels of these miRNAs with IR in humans has not previously been reported. Further stepwise regression analyses investigating the addition of clinical variables identified a model with increased predictive strength ($R^2 = 0.70$, $P = 8.6 \times 10^{-10}$) (Table 5) that retained three of the four miRNAs and included fasting lipids. This study provides additional evidence for a plasma miRNA signature in obesity and for signatures that may differentiate metabolic subphenotypes of IS and IR in obesity; it highlights specific avenues for future research, in terms of both potential circulating miRNA biomarkers and identification of miRNAs with previously unrecognized roles in obesity.

Alterations in circulating miRNAs in association with obesity (8,11,20) and related metabolic disorders (7,12-14) have been reported. Of the 18 circulating miRNAs showing significant alterations in all individuals with obesity compared to those without (Table 2), dysregulation of 11 has previously been reported in obesity, T2D, or metabolic syndrome in humans, whereas 7 have no previous reports of associations between circulating levels in humans and these disorders (miR-23b, miR-26a, miR-28-5p, miR-30-3p, miR-374b, miR-365, and miR-32). Altered levels of miR-26a, miR-23b, and miR-30e-3p have been shown in murine obesity models, and changes in miR-26a and miR-23b have been observed in human tissues. To our knowledge, miR-28-5p, miR-374b, and miR-32 have not been implicated in previous obesity-related studies, including perturbed levels in human patients (circulating or tissue) or mechanistic studies in animal or cell models.

Not all individuals with obesity develop IR or T2D, and it is not fully understood why some are relatively metabolically "healthy" or at least resistant to metabolic complications such as IR. Stepwise regression analyses of the miRNAs whose plasma level showed strong associations with IR (absolute $r \geq 0.3$, adjusted $P \leq 0.05$) (Supporting Information Table S1) identified four miRNAs

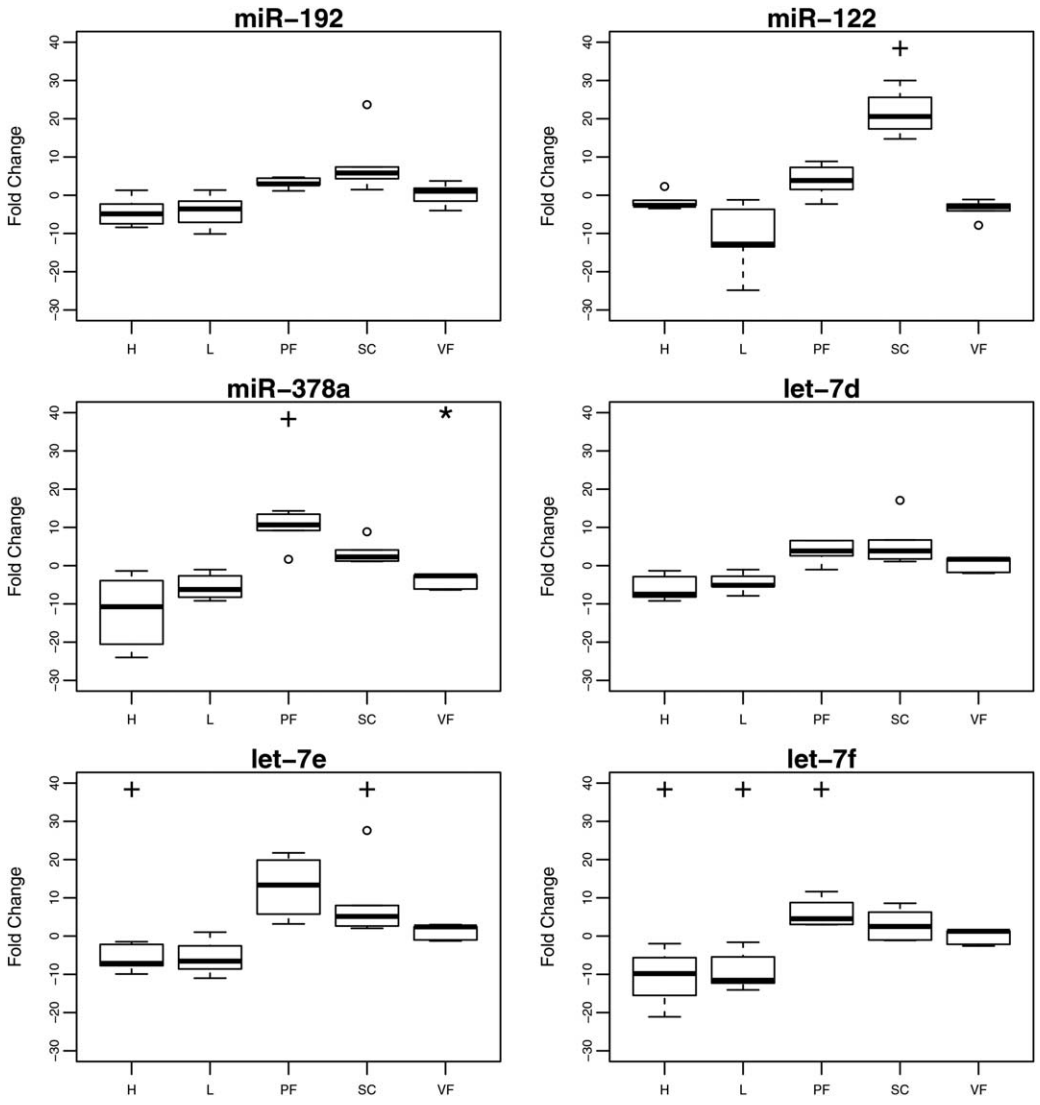

Figure 2 Tissue miRNA expression levels in a mouse model of obesity, fold change relative to controls. Levels of miR-192, miR-122, miR-378a, let-7d, let-7e, and let-7f in heart (H), liver (L), pericardial adipose (PF), subcutaneous adipose (SC), and visceral adipose (VF) tissues. * indicates significance at $P \leq 0.05$ (two-tailed), and + indicates significance at $P \leq 0.05$ (one-tailed). Fold change was calculated using the formula $2^{-\Delta\Delta Ct}$.

contributing significantly to a model predictive of IR ($R^2 = 0.57$, $P = 7.5 \times 10^{-8}$) (Table 5). To our knowledge, these four miRNAs (let-7b, miR-144-5p, miR-34a, and miR-532-5p) have not been directly linked with IR in humans. When clinical variables were added, three of the four miRNAs (let-7b, miR-144-5p, and miR-34a) were retained in a final model that included fasting lipids ($R^2 = 0.70$, $P = 8.6 \times 10^{-10}$, Table 5). Of these miRNAs, all but miR-144-5p have been implicated in obesity and/or T2D. A recent analysis of miRNA-sequencing data revealed a decrease in miR-144-5p in blood in Alzheimer's disease (21), a disorder in which IR is implicated, which is consistent with our observations of a negative correlation between plasma miR-144 level and IR. We observed an increase in plasma miR-34a in individuals with obesity and T2D compared to individuals without these conditions, and we also observed a positive correlation between plasma miR-34a level and IR; this is consistent with previous reports of increased circulating levels of miR-34a in T2D (22-24). Perturbed circulating levels of miR-34a have also been observed in obesity (22) and nonalcoholic fatty liver disease (25), along with reported roles for miR-34a in pancreatic beta-cell function (26) and hepatic lipid metabolism (27). Animal studies have suggested a role for let-7b in diabetic renal dysfunction (28-30), and decreased circulating levels have been seen in mouse obesity models (31), which is consistent with our observation of a negative correlation between circulating let-7b and IR.

We observed strong correlations (absolute $r \geq 0.4$, adjusted $P \leq 0.05$) between circulating levels of miR-122, let-7d, miR-210, and miR-378 and IR (Table 4). Plasma miR-122 levels positively correlated with IR, which is consistent with a study in males that reported that serum miR-122 levels correlated positively with HOMA-IR ($r = 0.401$) (10). While circulating levels of miR-210, miR-378, and let-7d have not been associated with IR to date,

studies have suggested a potential role. Increased miR-210 has been associated with pancreatic beta-cell apoptosis in diabetes mouse models (32), and we observed a positive correlation between plasma miR-210 and IR. For let-7d, we observed a negative correlation between plasma level and IR. The expression of let-7d has been shown to be increased in skeletal muscle in T2D and to modulate IL-13 secretion (33), raising the possibility that let-7d may be retained by muscle rather than secreted into the circulation in IR states, or, alternatively, that let-7d is delivered to and/or taken up by skeletal muscle from the circulation in the IR state; these are options that warrant further investigation. The miRNA miR-378a modulates *PPARGC-1β* and is encoded within an intron of the gene (34). It is upregulated by leptin, IL-6, and TNF-α in human adipocyte cell cultures (35) and after exercise in human skeletal muscle (36), with animal studies implicating it in brown adipose tissue thermogenesis (37), hepatic insulin signaling (38), and obesity and adipogenesis (39). We observed a positive correlation between plasma miR-378a level and IR.

Although the association of circulating miRNA levels with clinical factors may hint at a functional role, it does not indicate causality but highlights these miRNAs for further analyses. This is pertinent because miRNAs bound to lipoproteins or EVs can transfer between cell types and functionally affect downstream processes. We investigated the presence of eight miRNAs whose circulating levels had not previously been associated with obesity in EVs; all were present at significant levels, adding weight to their candidacy for further investigation.

We observed similar changes in three circulating miRNAs in our human study and murine model of obesity, including miR-122 and miR-192, for which associations with obesity and related disorders in humans and animal models have been reported (8,10,11). Additionally, we saw an increased abundance of plasma miR-378a in HFHS mice; as described above, this miRNA has several potential roles in obesity and IR, but changes in circulating levels in humans have not previously been reported. This replication of our observations suggests that miR-122, miR-192, and miR-378a may be conserved biomarkers for obesity or metabolic state. In addition, we observed differential expression of these miRNAs across metabolically relevant tissues, including visceral, subcutaneous, and pericardial fat tissues (all implicated in obesity physiology) (Figure 2). In particular, miR-378a was significantly decreased in visceral adipose tissue but increased in pericardial adipose, demonstrating that miRNA expression patterns are not only tissue specific (e.g., liver and heart tissue) but are also different across fat deposits. Changes in tissue miRNA levels may reflect changes in transcription rate, degradation of the miRNAs, uptake from the circulation or surrounding cells, or release of the miRNAs from the cell or tissue. Further studies are required to investigate the mechanisms behind the expression changes we observed and any functional role these miRNAs may have.

Potential limitations of this study included the small sample size. However, a stringent adjusted *P* value was used for our analyses, and our observation of both significant changes in several circulating miRNAs consistent with previous reports in the literature and three miRNAs in a mouse model of obesity provide additional confidence in the results. Future studies with larger patient numbers are needed to validate these findings and confirm both the biomarker status for the miRNAs reported here and any potential functional role in disease biology. Although the differences in observed miRNA levels can arise through issues with sample age and/or quality, our robust quality control analysis and our observation that 11 of 18 circulating miRNAs that showed significant alterations in all individuals with obesity compared to those without (Table 2) have previously been reported in obesity, T2D, or metabolic syndrome in humans provide confidence in our results. Our analysis was performed on miRNAs interrogated by an Exiqon serum/plasma focus panel; therefore, additional miRNAs associated with IR may have been missed by this targeted approach. Our study also focused on females, and although the mouse study investigated circulating miRNAs in male mice and we observed an increase in miR-122, miR-192, and miR-378a in both the human female cohort and male mice, further studies are needed to explore the miRNAs reported here in larger cohorts including both sexes. Finally, although the spin column EV-RNA isolation method employed here is relatively specific for isolation of EV-RNA over non–EV-RNA (19), small amounts of non–EV-RNA may also be captured by the filter, raising the possibility that some of the obesity-associated miRNAs may also be bound to proteins or lipoproteins.

Conclusion

We have identified miRNAs whose circulating levels are significantly perturbed in obesity. Roles for several of these have previously been reported in obesity and related disorders, highlighting the possibility that other miRNAs identified by this study also have functional roles in obesity. Levels of three miRNAs in plasma of a mouse model were consistent with the human data. Forty-eight miRNAs were significantly associated with a clinical trait, and four miRNAs were retained in a model strongly predictive ($R^2 = 0.57$) of IR after stepwise regression. When fasting lipids were added to this model, three of the four miRNAs were retained, and the strength increased ($R^2 = 0.70$). These data provide additional evidence for the role of miRNAs in obesity and related disorders, and they highlight specific avenues for future research. O

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