



Profiling of Circulating MicroRNAs Reveals Common MicroRNAs Linked to Type 2 Diabetes That Change With Insulin Sensitization

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OBJECTIVE

This study sought to identify the profile of circulating microRNAs (miRNAs) in type 2 diabetes (T2D) and its response to changes in insulin sensitivity.

RESEARCH DESIGN AND METHODS

The circulating miRNA profile was assessed in a pilot study of 12 men: 6 with normal glucose tolerance (NGT) and 6 T2D patients. The association of 10 circulating miRNAs with T2D was cross-sectionally validated in an extended sample of 45 NGT vs. 48 T2D subjects (65 nonobese and 28 obese men) and longitudinally in 35 T2D patients who were recruited in a randomized, double-blinded, and placebo-controlled 3-month trial of metformin treatment. Circulating miRNAs were also measured in seven healthy volunteers before and after a 6-h hyperinsulinemic-euglycemic clamp and insulin plus intralipid/heparin infusion.

RESULTS

Cross-sectional studies disclosed a marked increase of miR-140-5p, miR-142-3p, and miR-222 and decreased miR-423-5p, miR-125b, miR-192, miR-195, miR-130b, miR-532-5p, and miR-126 in T2D patients. Multiple linear regression analyses revealed that miR-140-5p and miR-423-5p contributed independently to explain 49.5% ($P < 0.0001$) of fasting glucose variance after controlling for confounders. A discriminant function of four miRNAs (miR-140-5p, miR-423-5p, miR-195, and miR-126) was specific for T2D with an accuracy of 89.2% ($P < 0.0001$). Metformin (but not placebo) led to significant changes in circulating miR-192 (49.5%; $P = 0.022$), miR-140-5p (−15.8%; $P = 0.004$), and miR-222 (−47.2%; $P = 0.03$), in parallel to decreased fasting glucose and HbA_{1c}. Furthermore, while insulin infusion during clamp decreased miR-222 (−62%; $P = 0.002$), the intralipid/heparin mixture increased circulating miR-222 (163%; $P = 0.015$) and miR-140-5p (67.5%; $P = 0.05$).

CONCLUSIONS

This study depicts the close association between variations in circulating miRNAs and T2D and their potential relevance in insulin sensitivity.

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Biomarkers in medical care are benchmarks in the body, preferably in blood circulation, that can be reliably evaluated to indicate the presence of physiological and metabolic disturbances (1). Several components associated with the pathophysiology of type 2 diabetes (T2D) have been uncovered in the last decades, being the result of alterations in insulin secretion coupled with changes in insulin action in insulin-sensitive tissues (i.e., muscle, liver, and adipose tissue) and modulated by a complex multifactorial web of relationships (2). In this new scenario, virtually all tissues and systems are active players, modulating both insulin action and response. The existence of this complex system aimed at regulating energy balance, in close association with the immune system and chronic inflammation, calls for a broader view of this paradigm (2).

In this context, it has been postulated that circulating microRNAs (miRNAs) could act as a new mode of communication between insulin-sensitive tissues (3). miRNAs are a class of evolutionally conserved noncoding RNAs of 19–22 nucleotides that function as negative regulators of gene expression (4). Previous studies have identified the altered expression of miRNAs in insulin-sensitive tissues from obese and/or T2D patients, suggesting a potential role for these small RNA molecules in the complications associated with the metabolic complications (5,6). During the last 5 years, it has been demonstrated that miRNAs are not only intracellular molecules, since they are also detectable outside the cells in body fluids (e.g., in serum, plasma, saliva, urine, and milk) (7). They are protected from degradation by RNAses because they are contained in small membranous vesicles (e.g., exosomes, exosome-like vesicles, microparticles, and apoptotic bodies) (8), packaged within HDLs (9), and linked to RNA-binding proteins (e.g., argonaute 2 and nucleophosmin 1) (10). It has been suggested that extracellular miRNAs have specific physiological functions, depending on their cellular origin and regulating immune function, cell migration and differentiation, and other

aspects of cell-to-cell communication (7,11). This has sparked the concept that extracellular miRNAs contained in body fluids could be useful as biomarkers for the detection and classification of diseases (12). Indeed, their presence in plasma and the possibility of detecting and analyzing small interindividual variations make circulating miRNAs excellent potential biomarkers for complex systemic diseases such as T2D (8,13, 14). Thereby, body fluid miRNAs could provide an integrated view of the metabolic profile of T2D patients (15).

By means of both cross-sectional and longitudinal analyses in men, which included placebo-controlled treatments with metformin, acute insulin (hyperinsulinemic-euglycemic clamp), and insulin infusion plus intralipid and heparin, known to induce insulin resistance (16), this study aimed to identify the circulating miRNAs associated with T2D and their response to changes in insulin sensitivity.

RESEARCH DESIGN AND METHODS

The study design included the evaluation of circulating miRNAs in association with T2D in a four-step procedure: 1) discovery study (or identification sample), in which samples from 12 age-matched men with or without T2D were used for profiling miRNAs in plasma; 2) cross-sectional validation study, in which the most relevant circulating miRNAs for T2D were validated in 93 subjects (45 with normal glucose tolerance [NGT] and 48 T2D patients), including the men used during the discovery study; 3) longitudinal validation study, in which the miRNAs of interest were assessed in a randomized, placebo-controlled, and double-blinded validation study consisting of 18 placebo and 17 metformin-treated T2D patients; and 4) the analysis of miRNAs in plasma samples collected from young healthy male volunteers before and after insulin infusion (clamp) and when insulin was accompanied by intralipid and heparin, which induces insulin resistance.

Cross-Sectional Studies

An identification sample of 12 men (6 obese subjects [mean \pm SD BMI 33.3 ± 3.9 kg/m²], age 47 ± 5 years) with NGT (fasting glucose 101.2 ± 12.5 mg/dL,

HbA_{1c} 31.2 ± 1.9 mmol/mol) and 6 obese and T2D patients (BMI 41.9 ± 10.4 kg/m², age 43 ± 3 years, fasting glucose 124.6 ± 12.9 mg/dL, and HbA_{1c} 37.7 ± 4.7 mmol/mol) was recruited and studied in the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain) for profiling miRNAs in plasma using TaqMan Low Density Arrays (Applied Biosystems, Darmstadt, Germany).

Plasma specimens from an extended sample of 93 Caucasian men (45 NGT and 48 subjects with T2D), including the subjects used for the discovery study, were prospectively recruited from normal population and T2D outpatient clinics on the basis of a stable metabolic control in the previous 6 months, as defined by stable HbA_{1c} values during this period, as previously described (17). This extended sample was used for validating the results assessed in the identification sample (Table 1).

The study protocol was approved by the Ethics Committee and the Committee for Clinical Investigation of the Hospital Universitari Dr. Josep Trueta. All subjects provided written informed consent before entering the study.

Metformin Study

The most relevant circulating miRNAs in T2D were also analyzed by individual TaqMan hydrolysis probes before (baseline) and 3 months after treatment in a randomized, placebo-controlled, and double-blinded study consisting of 18 placebo and 17 metformin-treated T2D patients (16 men and 19 women, age 53 ± 8 years, fasting glucose 131.2 ± 21.4 mg/dL, and HbA_{1c} 51.9 ± 5.3 mmol/mol) (Table 3).

After screening, diabetic patients were assigned to either therapy with metformin (Acyfabrik S.A., Madrid, Spain) or matching placebo after simple randomization procedures. Inclusion criteria were 1) age 18–65 years; 2) T2D diagnosis in the previous 6 months, as defined by the American Diabetes Association criteria; 3) absence of systemic and metabolic disease other than T2D and absence of infection within the previous month; 4) absence of diet or medication that might interfere with glucose homeostasis, such as antibiotics and glucocorticoids;

Table 1—Clinical characteristics and relative circulating miRNA concentrations of subjects included in the cross-sectional analysis (n = 93)

	NGT	T2D	<i>P</i> (Student <i>t</i> test)
Nonobese subjects			
<i>N</i> (only men)	35	30	
Age (years)	48.1 ± 10.1	54 ± 10	0.022
BMI (kg/m ²)	25.2 ± 1.8	26.4 ± 2.4	0.031
SBP (mmHg)	120.2 ± 11.9	139.1 ± 18.6	<0.0001
DBP (mmHg)	76.6 ± 8.4	79.7 ± 10.3	0.190
Fasting glucose (mg/dL) ^a	90.7 ± 9.2	212.6 ± 61	<0.0001
HbA _{1c} (%) ^b	4.73 ± 0.35	7.67 ± 1.46	<0.0001
Total cholesterol (mg/dL) ^c	206.5 ± 46.2	198.1 ± 40.5	0.441
HDL cholesterol (mg/dL) ^c	51.3 ± 12.6	44 ± 13.5	0.027
LDL cholesterol (mg/dL) ^c	133.6 ± 42.2	101.7 ± 32.6	0.001
Fasting triglycerides (mg/dL) ^d	91 ± 59.3	257.3 ± 142.7	<0.0001
Creatinine (mg/dL)	1.01 ± 0.1	1.08 ± 0.52	0.545
LBP (μg/mL)	14.1 ± 11.2	53.3 ± 23	<0.0001
CFB (μg/mL)	237.6 ± 61.3	310.8 ± 104.8	0.002
miR-140-5p ^e	0.47 ± 0.23	1.64 ± 1.13	<0.0001
miR-142-3p ^e	0.19 ± 0.13	1.27 ± 1.12	<0.0001
miR-222 ^e	0.82 ± 0.38	1.74 ± 1.34	<0.0001
miR-423-5p ^e	6.18 ± 5.18	2.15 ± 1.42	<0.0001
miR-125b ^e	2.21 ± 2.37	0.37 ± 0.34	<0.0001
miR-192 ^e	4.25 ± 4.1	1.21 ± 1.26	<0.0001
miR-195 ^e	0.66 ± 0.74	0.59 ± 1.23	0.001
miR-130b ^e	2.69 ± 2.42	1.07 ± 0.92	0.002
miR-532-5p ^e	3.47 ± 3.08	1.65 ± 1.17	0.005
miR-126a ^e	0.94 ± 0.62	0.61 ± 0.7	0.02
Obese subjects			
<i>N</i> (only men)	10	18	
Age (years)	50.6 ± 14.4	57.7 ± 8	0.102
BMI (kg/m ²)	32.2 ± 2.4	33.4 ± 3.3	0.336
SBP (mmHg)	136.7 ± 13.4	143.2 ± 15.9	0.283
DBP (mmHg)	81.3 ± 12.1	82.8 ± 11	0.736
Fasting glucose (mg/dL) ^a	96.2 ± 11.5	200.6 ± 102.8	<0.0001
HbA _{1c} (%) ^b	4.81 ± 0.33	7.06 ± 2.14	<0.0001
Total cholesterol (mg/dL) ^c	223.5 ± 21.7	202.9 ± 48.6	0.136
HDL cholesterol (mg/dL) ^c	50.6 ± 11	47 ± 11.1	0.417
LDL cholesterol (mg/dL) ^c	149.2 ± 20.6	115.3 ± 38.9	0.006
Fasting triglycerides (mg/dL) ^d	119.1 ± 81.4	219.2 ± 133	0.040
Creatinine (mg/dL)	0.91 ± 0.16	1.14 ± 0.26	0.025
LBP (μg/mL)	20.1 ± 19.4	63.5 ± 32.3	0.005
CFB (μg/mL)	239.9 ± 74	310.3 ± 76.3	0.027
miR-140-5p ^e	0.47 ± 0.15	1.51 ± 1.14	<0.0001
miR-142-3p ^e	0.22 ± 0.11	1.06 ± 0.78	<0.0001
miR-222 ^e	0.58 ± 0.3	1.78 ± 1.31	0.001
miR-423-5p ^e	4.14 ± 2.52	4.54 ± 5.29	0.500
miR-125b ^e	1.32 ± 1.08	0.71 ± 0.87	0.047
miR-192 ^e	3.69 ± 6.12	1.96 ± 2.91	0.114
miR-195 ^e	0.92 ± 1.44	0.53 ± 0.74	0.102
miR-130b ^e	3.08 ± 1.88	1.57 ± 1.32	0.012
miR-532-5p ^e	1.56 ± 1.34	2.61 ± 2.27	0.121
miR-126a ^e	0.81 ± 0.62	0.42 ± 0.52	0.005

Data are means ± SD. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using the Levene test. Variables were given in a base log₂-transformation and analyzed on that log₂ scale when necessary. The Student *t* test was performed for single comparisons between assessed values in T2D and subjects with NGT. Significant data are shown in boldface. CFB, complement factor B; DBP, diastolic blood pressure; LBP, lipopolysaccharide binding protein; SBP, systolic blood pressure. ^aTo convert mg/dL glucose to mmol/L, multiply by 0.0555. ^bTo convert % HbA_{1c} to mmol/mol, multiply by 10.9 and subtract 23.5. ^cTo convert mg/dL to mmol/L, multiply by 0.0259. ^dTo convert mg/dL triglycerides to mmol/L, multiply by 0.0113. ^eThe relative quantification of miRNAs in plasma measures the presence (concentration) of a specific miRNA and is calculated as 2^(-ΔC_t) for each sample, as previously described (27). ΔC_t values are defined as the raw C_t value - average of raw C_t values for the selected reference miRNAs (miR-106a, miR-146a, miR-19b, and miR-223) in each sample.

and 5) HbA_{1c} <9% (75 mmol/mol). Exclusion criteria included 1) clinically significant major systemic disease, including malignancy; 2) clinical evidence of hemoglobinopathies or anemia; 3) history of drug or alcohol abuse, defined as >80 g/day in men and >40 g/day in women; 4) acute major cardiovascular event in the previous 6 months; 5) acute illnesses or current evidence of acute or chronic inflammatory or infective diseases; and 6) mental illness rendering the subjects unable to understand the nature, scope, and possible consequences of the study.

All participants were requested to withhold alcohol and caffeine during at least 12 h prior to the tests. During the first month, normal-weight or overweight patients were only instructed to maintain a diet containing 25 kcal · kg or 20 kcal · kg, respectively. Then, an active phase of treatment that included diet plus metformin or diet plus placebo lasted 3 months. Metformin treatment started at a 425 mg/day and increased progressively during the first week to reach 1,700 mg/day. Genomic measurements in plasma were performed at baseline and 3 months after starting treatment.

All subjects gave written informed consent after the purpose of the study was explained to them. The experimental protocol was approved by the Ethics Committee and the Committee for Clinical Investigation of the Hospital Universitari Dr. Josep Trueta, so we certify that all applicable institutional regulations concerning the ethical use of information and samples from human volunteers were followed during this research.

Clamp Studies and Insulin Plus Intralipid/Heparin Infusion Studies

Hyperinsulinemic-euglycemic clamp is a method for estimating insulin sensitivity, based on the assumption that, during steady-state hyperinsulinemic euglycemia, glucose infusion rate equals glucose uptake by tissues. Thus, the higher the glucose infusion rate, the higher the insulin sensitivity (18). Circulating miRNAs were analyzed in plasma samples collected from healthy young male volunteers (Table 3) before and after (360 min) insulin infusion (clamp) and when

insulin was accompanied by intralipid, a mixture of triacylglycerols and heparin, which induces insulin resistance (19).

Insulin sensitivity was measured in this independent cohort of 7 healthy men (age 28 ± 4 years, BMI 25 ± 3 kg/m², and whole-body glucose uptake [*M* value] 5.7 ± 2.3) with the hyperinsulinemic-euglycemic clamp technique, as previously described (19). Briefly, insulin (Actrapid HM; Novo Nordisk, Copenhagen, Denmark) was given as a continuous intravenous infusion for 360 min at $40 \text{ mU} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$, resulting in constant hyperinsulinemia of ~ 80 mIU/mL. Arterialized blood glucose was obtained every 5 min, and 20% dextrose (1.11 mol/L) infusion was adjusted to maintain plasma glucose levels at 5 mmol/L. The *M* value was calculated in 2 and 6 h of the clamp as the mean glucose infusion rate from 80 to 120 min and from 320 to 360 min, respectively, corrected for the glucose space and normalized for fat-free mass.

After 1 week, another clamp, with concurrent intralipid/heparin infusion, which is a mixture of triacylglycerols and heparin that acts as an activator of lipoprotein lipase to elevate circulating free fatty acids and induce insulin resistance (19), was performed. In this experiment, 20% intralipid (Fresenius Kabi, Uppsala, Sweden) was given at $0.013 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, and heparin was given at $0.2 \text{ units} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$.

No differences in the steady-state for insulin concentrations between both protocols were observed. Blood samples for the determination of circulating miRNAs were collected before the beginning of the insulin infusion and after treatment at 360 min. Plasma samples were obtained by centrifugation immediately after collection. Samples were stored into polypropylene test tubes and frozen at -80°C and were not exposed to thawing/refreezing cycles before analyses. There were no significant differences regarding the circulating miRNA concentrations between “basal” time points (i.e., before the insulin infusion and the infusion of insulin accompanied by intralipid). The protocol

was approved by the Ethics Committee of Medical University of Bialystok. All subjects gave written informed consent before entering the study.

Profile of Circulating miRNAs

Circulating RNA Extraction and Purification Plasma was obtained by standard venepuncture and centrifugation using EDTA-coated Vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ). RNA extraction was performed using the mirVana PARIS Isolation kit (Applied Biosystems) according to the manufacturer’s instructions. See the Supplementary Data for additional details.

Circulating miRNAs Retrotranscription and Preamplification

A fixed volume of 3 μL RNA solution from the 40- μL eluate of RNA isolation was used as input into the retrotranscription using the TaqMan MicroRNA Reverse Transcription kit and the TaqMan MicroRNA Multiplex RT Assays, which are required to run the TaqMan Array Human MicroRNA A+B Cards Set, version 2.0 (Life Technologies, Darmstadt, Germany). Preamplification was performed using TaqMan PreAmp Master Mix and Megaplex PreAmp Primers for either human pool set A or B.

Circulating miRNA Profiling Using TaqMan Low-Density Arrays

TaqMan miRNAs arrays covering miRNA species in plasma were applied to samples from the subcohort of 12 men (identification sample) and from the independent cohort of healthy men before and after clamp and after insulin plus intralipid/heparin infusion. RT-PCR was carried out by means of an Applied Biosystems 7900HT thermocycler. Data were analyzed with SDS Relative Quantification Software, version 2.2.2 (Applied Biosystems). In these identification samples, mean normalized values (ΔC_t) were obtained as the raw cycle threshold (C_t) value – average of raw C_t for all miRNAs with reliable results (C_t values ≤ 35) in each sample (Supplementary Tables 1 and 2).

Analysis of Individual miRNAs Using TaqMan Hydrolysis Probes

Commercially available TaqMan hydrolysis probes (Applied Biosystems) were used to assess the presence in plasma of individual miRNAs. Gene

expression was assessed by real-time PCR using the LightCycler 480 Real-Time PCR System (Roche Diagnostics, Barcelona, Spain), using TaqMan technology suitable for relative gene expression quantification following the manufacturer’s protocol.

For the analysis by quantitative RT-PCR, we first evaluated a suitable number of reference miRNAs, based on the increased expression stability (Supplementary Fig. 1). Then, the geometric mean of the most stable (or “rank invariant”) miRNAs in plasma (miR-106a, miR-146a, miR-19b, and miR-223) was used as the normalizing factor, as implemented in the HTqPCR R package (20). See in the Supplementary Data and Supplementary Fig. 2 for additional details on procedures and analytical validations.

Statistical Methods

Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using the Levene test. Variables were given in a base \log_2 -transformation, and analyzed on that \log_2 scale, when necessary. ANOVA or paired *t* tests were performed to study differences in quantitative variables between groups. The semiquantitative concentrations for the different miRNAs were correlated (Spearman test) with clinical parameters. Receiver operating characteristic analysis was run in order to measure sensitivity and specificity of circulating miRNAs in detecting diabetes at different cutoff points (21). Of note, the greater the area under the curve, the better the prediction value of circulating miRNAs for detecting diabetes, being that area under the curve of 0.5 means no prediction and 1.0 the perfect prediction value (21). Statistical analyses were performed with the SPSS statistical software (SPSS version 12.0, SPSS, Chicago, IL), and the R Statistical Software (<http://www.r-project.org/>). The SL qpcrNorm Package (Bioconductor) was also used for the analysis and normalization of the arrays (20).

RESULTS

Comprehensive Circulating miRNA Profiling

Plasma miRNA profiling was first performed on a subgroup of subjects

($n = 12$ men; 50% T2D patients). Diabetic patients and the age-matched control subjects with NGT of this identification sample only differed clinically in fasting glucose (124.6 ± 12.9 vs. 101.2 ± 12.5 mg/dL, $P = 0.009$) and HbA_{1c} (37.7 ± 4.7 vs. 31.2 ± 1.9 mmol/mol, $P = 0.048$). Concurrently with fasting glucose and HbA_{1c}, 16 circulating miRNAs were also associated with T2D. Then, all potentially miRNA candidates for T2D were cross-sectionally analyzed by quantitative RT-PCR using TaqMan miRNA hydrolysis probes in an extended validation sample of 93 men (Table 1), including the subjects selected for the identification of miRNA candidates for T2D. That validation shortlisted 10 circulating miRNAs significantly

different between men with NGT and T2D patients. Indeed, the increased circulating concentrations of miR-140-5p, miR-142-3p, and miR-222 (all $P < 0.0001$) and decreased miR-423-5p, miR-192, miR-125b ($P < 0.0001$), miR-195, miR-130b, miR-532-5p, and miR-126 ($P < 0.05$) were specifically identified in T2D subjects, especially among lean participants (Table 1 and Fig. 1). In agreement, circulating concentrations of the selected miRNA candidates were significantly associated with fasting glucose, HbA_{1c}, and fasting triglycerides, among others (Table 2).

In multiple linear regression models, combination of increased BMI (BMI,

$P = 0.03$) and circulating concentrations of miR-140-5p ($P < 0.0001$) with decreased miR-423-5p ($P < 0.0001$) in plasma contributed to explain together $\sim 51\%$ ($P < 0.0001$) of fasting glucose variance after adjustment for age (Table 2). Only decreased miR-423-5p and increased miR-140-5p concentrations in plasma (both $P < 0.0001$) explained together $\sim 49\%$ ($P < 0.0001$) of fasting glucose variance in nonobese subjects after correction for age and BMI (Table 2). Of note, discriminant analyses computed for these results revealed that four miRNAs (miR-142-3p, miR-423-5p, miR-195, and miR-126) were specific for T2D with a diagnostic accuracy of 89.2%, estimated with the “leave-one-out” method. The P value

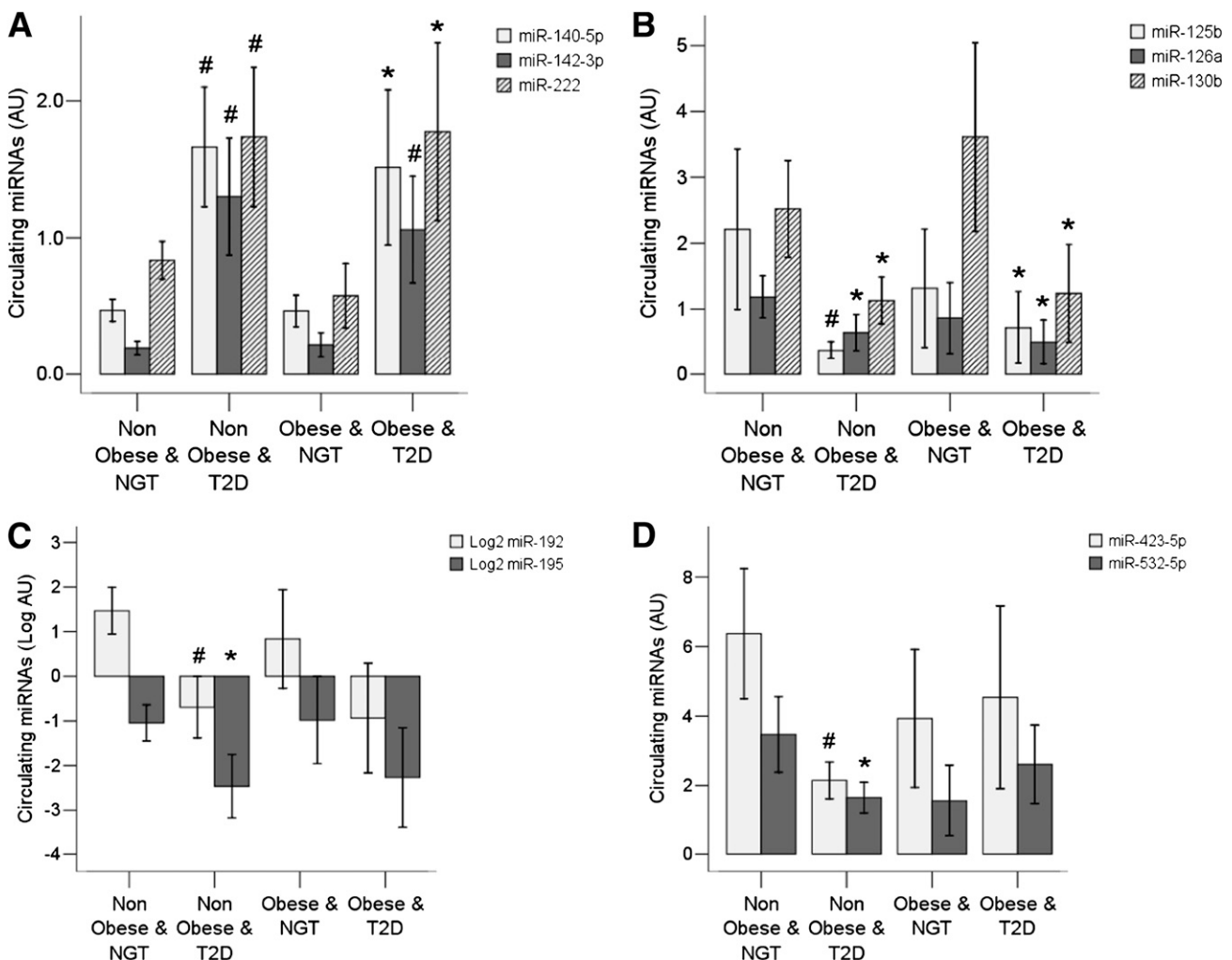


Figure 1—Mean and 95% CIs for the mean of the normalized expression values for miR-140-5p, miR-142-3p, and miR-222 (A); miR-125b, miR-126a, and miR-130b (B); miR-192 and miR-195 (C); and miR-423-5p and miR-532-5p (D) in men ($n = 93$) classified according to the diagnosis of T2D and the BMI in nonobese (BMI < 30 kg/m²) and obese (≥ 30 kg/m² BMI) subjects with NGT or T2D. * $P < 0.05$ and # $P < 0.0001$ (Student t test) for comparisons between the values assessed for subjects with or without T2D in each subgroup. AU, arbitrary units.

Table 2—Single correlations and multiple linear regressions of the most significant circulating miRNAs and fasting glucose concentrations after correcting for confounders such as age and BMI

	Single correlations					
	Fasting glucose		HbA _{1c}		Triglycerides	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
All subjects						
miR-140-5p	0.572	<0.0001	0.579	<0.0001	0.35	0.001
miR-142-3p	0.57	<0.0001	0.606	<0.0001	0.349	0.001
miR-222	0.394	<0.0001	0.409	<0.0001	0.145	0.174
miR-423-5p	-0.467	<0.0001	-0.492	<0.0001	-0.462	<0.0001
miR-125b	-0.464	<0.0001	-0.476	<0.0001	-0.364	0.003
miR-192	-0.43	<0.0001	-0.513	<0.0001	-0.409	<0.0001
miR-195	-0.248	0.020	-0.206	0.054	-0.117	0.278
miR-130b	-0.269	0.009	-0.268	0.009	-0.225	0.030
miR-532-5p	-0.249	0.018	-0.255	0.015	-0.28	0.008
miR-126	-0.24	0.021	-0.255	0.014	-0.145	0.169
Fasting glucose			0.823	<0.0001	0.522	<0.0001
Nonobese						
miR-140-5p	0.534	<0.0001	0.602	<0.0001	0.353	0.005
miR-142-3p	0.533	<0.0001	0.599	<0.0001	0.351	0.004
miR-222	0.313	0.012	0.397	0.001	0.098	0.443
miR-423-5p	-0.528	<0.0001	-0.469	<0.0001	-0.519	<0.0001
miR-125b	-0.432	0.003	-0.478	0.001	-0.488	0.001
miR-192	-0.374	0.004	-0.495	<0.0001	-0.477	<0.0001
miR-195	-0.301	0.017	-0.305	0.015	-0.189	0.138
miR-130b	-0.197	0.117	-0.191	0.128	-0.257	0.039
miR-532-5p	-0.366	0.003	-0.308	0.014	-0.356	0.004
miR-126	-0.296	0.017	-0.32	0.009	-0.122	0.335
Fasting glucose			0.859	<0.0001	0.550	<0.0001
Obese subjects						
miR-140-5p	0.639	<0.0001	0.53	0.004	0.338	0.085
miR-142-3p	0.656	<0.0001	0.628	<0.0001	0.331	0.092
miR-222	0.516	0.006	0.431	0.025	0.243	0.222
miR-423-5p	-0.377	0.048	-0.544	0.003	-0.327	0.089
miR-125b	-0.536	0.015	-0.48	0.032	-0.078	0.745
miR-192	-0.504	0.010	-0.538	0.005	-0.255	0.219
miR-195	-0.16	0.446	-0.007	0.973	0.056	0.790
miR-130b	-0.496	0.007	-0.5	0.007	-0.159	0.418
miR-532-5p	-0.019	0.924	-0.133	0.509	-0.066	0.745
miR-126	-0.103	0.608	-0.093	0.645	-0.181	0.366
Fasting glucose			0.771	<0.0001	0.480	0.010
Multiple linear regression						
	All ^a		Nonobese ^b		Obese ^c	
	β	<i>P</i>	β	<i>P</i>	β	<i>P</i>
Age (years)	0.12	0.231	0.127	0.188	0.145	0.311
BMI (kg/m ²)	0.172	0.03	0.043	0.672	0.42	0.004
miR-140-5p	0.471	<0.0001	0.45	<0.0001	0.465	0.003
miR-423-5p	-0.365	<0.0001	-0.422	<0.0001	-0.243	0.081

β is the standardized regression coefficient, which allows evaluating the relative significance of each independent variable in multiple linear regression analyses. Significant associations are indicated in boldface. ^aAdjusted R^2 (the percentage of the variance explained by the independent variables in the different models [i.e., 0.50 is 50%]) = 51.3 ($P < 0.0001$) (significant). ^bAdjusted $R^2 = 49.5$ ($P < 0.0001$) (significant). ^cAdjusted $R^2 = 65.7$ ($P < 0.0001$) (significant).

(computed by successive permutations and corresponding to the number of times that, when permuting the values, an error equal or below the real error rate divided by the number of tests was found) was <0.001 , allowing the

accurate classification of subjects with NGT or T2D patients. In agreement, the area under the receiver operating characteristic curve was 0.975 using this model, indicating great sensitivity and specificity of these four circulating

miRNAs in detecting diabetes at different cutoff points.

Effects of Metformin on miRNA Candidates

For further validation of cross-sectional findings and for testing of whether values of miRNA candidates for T2D in plasma may be modified by insulin sensitizers, the previously identified circulating miRNAs were analyzed before (baseline) and 3 months after treatment in a randomized, placebo-controlled, and double-blinded validation study consisting of 18 placebo and 17 metformin-treated T2D patients (Table 3).

On average, all subjects lost 4–5% of their initial BMI at 3 months after treatment, most probably owing to the diet. However, only the metformin group showed significant improvements in insulin resistance, as demonstrated by decreased homeostasis model assessment of insulin resistance (-28% , $P = 0.02$), fasting glucose (-12.7%), and HbA_{1c} (-9.5% , both $P < 0.0001$). Interestingly, plasma concentrations of four circulating miRNA candidates for T2D were also significantly modified by metformin (but not by placebo), leading to a marked decrease of circulating miR-140-5p (-16% , $P = 0.004$) and miR-222 (-47% , $P = 0.03$), and increased miR-142-3p (38% , $P = 0.025$) and miR-192 (49.5% , $P = 0.022$) concentrations in plasma.

Effects of Clamp and of Insulin Plus Intralipid/Heparin Infusion on miRNA Candidates

The whole profile of circulating miRNAs was also investigated in seven healthy men before and after the hyperinsulinemic-euglycemic clamp with or without intralipid/heparin infusion. First, ΔC_t normalized values were obtained as the raw C_t value – average of raw C_t for all miRNAs with reliable results in each sample. This measurement disclosed the significant modification of 10 miRNAs in plasma after the clamp and/or insulin plus intralipid/heparin infusion. Among them, the circulating concentrations of miR-320 and miR-660 significantly decreased after clamp (-2.0 and -1.8 -fold, $P < 0.02$) but were enhanced by insulin plus intralipid (1.6 - and 2.7 -fold, respectively, $P < 0.05$).

Table 3—Clinical characteristics of the diabetic patients treated with metformin ($n = 17$) or placebo ($n = 18$) and healthy volunteers ($n = 7$) recruited for treatments with hyperinsulinemic-euglycemic clamp without or with intralipid/heparin infusion in the longitudinal assessment of changes in circulating miRNA concentrations

	Placebo	Metformin	Clamp and clamp + intralipid
n (men/women)	18 (8/10)	17 (8/9)	7 (7/0)
Age (years)	54 \pm 8	52 \pm 9	25 \pm 4
BMI (kg/m^2)	34.0 \pm 7.7	35.7 \pm 6.2	25.7 \pm 3.2
Fasting glucose (mg/dL) ^a	136.8 \pm 23	125.2 \pm 18.5	89.1 \pm 3.2
Fasting insulin ($\mu\text{IU}/\text{mL}$)	23.2 \pm 17.4	27.0 \pm 15.7	12.8 \pm 7.2
HbA _{1c} (%) ^b	8.3 \pm 6.6	8.3 \pm 4.6	N/A
HOMA-IR	6.6 \pm 0.8	6.0 \pm 0.4	2.8 \pm 1.6
Total cholesterol (mg/dL) ^c	205.7 \pm 37.3	204.8 \pm 34	181.9 \pm 23.7
HDL cholesterol (mg/dL) ^c	46.1 \pm 14.5	47.9 \pm 12.1	50.1 \pm 12.5
LDL cholesterol (mg/dL) ^c	128.1 \pm 26.4	126.3 \pm 26.4	110.6 \pm 28.6
Fasting triglycerides (mg/dL) ^d	149.3 \pm 79.7	135.8 \pm 92.0	107.3 \pm 75.1

Clamp + intralipid, insulin with intralipid/heparin infusion; HOMA-IR, homeostasis model assessment of insulin resistance. ^aTo convert mg/dL glucose to mmol/L , multiply by 0.0555. ^bTo convert % HbA_{1c} to mmol/mol , multiply by 10.9 and subtract 23.5. ^cTo convert mg/dL to mmol/L , multiply by 0.0259. ^dTo convert mg/dL triglycerides to mmol/L , multiply by 0.0113.

In this longitudinal study, we also evaluated the specific modulation of the identified miRNA candidates for T2D using the geometric mean of the selected reference miRNAs (miR-106a, miR-146a, miR-19b, and miR-223) as endogenous control. In this case, only miR-222 was significantly modified by clamp (-62% , $P = 0.002$), while insulin plus intralipid/heparin infusion significantly increased circulating concentrations of miR-140-5p (67.5% , $P = 0.05$), miR-222 (163% , $P = 0.015$), and miR-195 (165% , $P = 0.003$).

CONCLUSIONS

The proper identification and characterization of T2D will gain importance if researchers succeed in developing treatments able to delay or even stop the progression of this disease. Such treatments may be most effective if used as early as possible in the course of the illness, targeting the basis of the glucose homeostasis deregulation. Also, during the follow-up, this goal can only be achieved by identifying biomarkers for predicting and monitoring the progression of insulin resistance and further complications. The weaknesses of the blood parameters thus far used in this context highlights the importance of research targeting the potential use of

new biomarkers such as miRNAs in this field (14).

The discovery and characterization of miRNAs has been considered a major scientific breakthrough in the last years (4). Many studies have demonstrated the important role of miRNAs in the knowledge regarding several aspects of health and disease (22). In the current study, we postulated the identification of distinct circulating miRNA profiles, which may also be of relevance in T2D. Indeed, we provide here the cross-sectional identification of at least 10 circulating miRNAs associated with T2D. Thus, beyond fasting glucose and HbA_{1c}, circulating differences between subjects with NGT and T2D patients included increased concentrations of miR-140-5p, miR-142-3p, and miR-222 and decreased miR-423-5p, miR-125b, miR-130b, miR-192, and miR-126, among others. Noteworthy, discriminant analyses revealed that the circulating concentration of four of them (miR-142-3p, miR-423-5p, miR-195, and miR-126) was specific for T2D, with a diagnostic accuracy of 89.2%, and that only two (miR-140-5p and miR-423-5p) contributed to explain together $\sim 49\%$ of fasting glucose variance after correction for confounders. However, the most striking aspects in this study include longitudinal validations of

transversal findings, which imply the possibility of modulating circulating miRNAs through the application of insulin sensitizers such as metformin.

Decreased Circulating miRNAs in T2D

In 2010, Zampetaki et al. (13) defined a miRNA profile in pools of plasma from subjects with T2D, identifying low miR-15a, miR-126, miR-223, miR-29b, and slightly increased circulating miR-28-3p in patients compared with control subjects. Indeed, except for miR-28-3p, all differentially expressed species miRNA in plasma were less abundant in T2D (13). More recently, Karolina et al. (23) identified miR-197, miR-23a, miR-509-5p, miR-130a, miR-195, miR-27a, and miR-320a as potential contributors in metabolic syndrome and T2D, and Kong et al. (24) validated the clinical relevance of seven hypothetically diabetes-related miRNAs (miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375) in serum samples from 18 T2D cases, 19 individuals with impaired glucose tolerance, and 19 subjects with NGT, disclosing elevated expressions of these circulating miRNAs in T2D patients. Differences in methodology management and statistical evaluation of results, such as the starting sample (e.g., serum vs. plasma, utilization of pools of samples, etc.), the proper selection of endogenous controls and normalization during experimental procedures, and clinical differences between case and selected and/or available control subjects, may explain most part of these discrepancies. The present work corroborates the decreased concentrations of miR-126 in plasma from T2D patients and identifies decreased miR-192, miR-125b, miR-130b, and miR-423-5p in T2D subjects.

Decrease of miR-192 has been associated with the progression of diabetic nephropathy (25). In agreement with this, current findings showed the inverse relationship between circulating concentrations of miR-192, urea ($r = -0.47$, $P = 0.002$), and creatinine ($r = -0.26$, $P = 0.03$) in a subsample of 71 healthy subjects (data not shown). Current results also reveal decreased miR-192 in plasma from T2D patients and its upregulation after treatments with metformin in parallel to

the improved metabolic control. Decreased miR-125b expressions have been identified in different pancreatic cell types, being specific to β -cells (26). Despite the close association with measures of insulin resistance, neither metformin nor insulin or insulin/intralipid modified miR-125b concentrations in plasma. Decreased circulating miR-130b and miR-423-5p concentrations were identified in morbid obese subjects (27), as well as in T2D patients (current results), depicting the close correspondence between circulating miRNA profiles for severe obesity and T2D. No significant changes in response to metformin or insulin were observed, despite the relatively strong and consistent inverse association of miR-423-5p with parameters of insulin resistance. Less information is available about circulating levels of miR-130b. Of note, decreased miR-130b has been described in subcutaneous adipose tissue from T2D subjects (28).

Increased Circulating miRNAs in T2D

One of the most interesting aspects of this study is the positive association of circulating miRNAs that have previously been identified in morbid obesity, namely, miR-142-3p, miR-140-5p, and miR-222 (27), with T2D. This fully agrees with the well-recognized incidence and prevalence of T2D among severe obese subjects (29). It is possible that common underlying mechanisms leading to T2D are overrepresented in these subjects, and miRNA may constitute one of them.

The expression of miR-142-3p has been postulated as a biomarker for acute and chronic inflammation (30). Increased levels have been reported in patients with vascular damage (31). miR-142-3p (32) and miR-140 (33) species are consistently increased in plasma from obese subjects (27) in addition to T2D patients (current results). Although circulating miR-142-3p showed no significant response to metformin or insulin infusion, miR-140-5p concentrations decreased after the metformin-induced improvement of insulin resistance while increasing with worsening of insulin resistance using insulin infusion plus intralipid in healthy subjects (34). Thus, both insulin

sensitizations in T2D patients and insulin resistance in healthy volunteers significantly modified circulating levels of miR-140-5p.

miR-222 also showed increased expression in response to stress (35), increased circulating concentrations in T2D patients, and a concordant downregulation after treatment with insulin sensitizers. One previous work identified increased expression of miR-222 and its paralogue, miR-221, which are known to promote intimal thickening in internal mammary artery segments from T2D patients (35). Interestingly, miR-222 levels inversely correlated with the metformin dose in T2D patients who were treated with this drug (36), suggesting the negative modulation of this miRNA by metformin. Current findings further demonstrate the negative effect of metformin on the circulating concentrations of this miRNA in a double-blinded fashion. Moreover, the circulating concentrations of miR-222 decreased during insulin infusion, while increasing after insulin plus intralipid/heparin. Of note, increased circulating miR-222 concentrations were reported in response to acute exhausting exercise training (37) and in healthy subjects with low fitness (38), which also highlights the association of this miRNA in plasma with insulin action.

Summary

This study provides a comparative profiling of circulating miRNAs in T2D case subjects and age- and sex-matched control subjects and the identification of specific miRNAs in the circulation that are associated with insulin action. To our knowledge, this is the first study to investigate the effect of metformin treatment and intralipid/heparin infusion on circulating miRNAs. Noteworthy, the modifications observed after metabolic changes induced by metformin, insulin, and insulin plus intralipid infusions demonstrate that components of this profile are regulated in parallel to insulin action. However, the current study has some limitations that may impact on the interpretation of the results. 1) The relatively small size of the cohorts analyzed, which call for further validations in extended samples. 2) Since changes in the levels of

circulating miRNAs as biomarkers do not necessarily reflect deregulation of miRNAs expression inside cells, the functional roles and significance of miRNAs deregulated in plasma from T2D patients still need to be determined. However, from a clinical point of view, the results obtained in the current study give promising evidence for future analyses using circulating miRNAs to examine the pathophysiology of T2D. Obviously, such studies are needed before any more definite conclusion can be drawn. Nevertheless, the data presented here might have direct implications for diabetologists, since genomic analyses in plasma may be a promising strategy for predicting metabolic disturbances and can be extrapolated to quantifying the severity of metabolic diseases.

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participated in the execution of the study. S.R. helped with the study design and the statistical analysis, contributed to the conception and design of the study, provided important intellectual content, and directly participated in the execution of the study. M.K.-K. and M.S. performed the treatments and provided samples before and after insulin (clamp) and insulin plus intralipid/heparin infusion and directly participated in the execution of the study. M.S. contributed to the conception and design of the study, provided important intellectual content, and directly participated in the execution of the study. J.M.F.-R. carried out the conception and coordination of the study, contributed to writing the manuscript, and directly participated in the execution of the study. J.M.F.-R. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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