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Targeting the Circulating MicroRNA Signature of Obesity

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BACKGROUND: Genomic studies have yielded important insights into the pathogenesis of obesity. Circulating microRNAs (miRNAs) are valuable biomarkers of systemic diseases and potential therapeutic targets. We sought to define the circulating pattern of miRNAs in obesity and examine changes after weight loss.

METHODS: We assessed the genomewide circulating miRNA profile cross-sectionally in 32 men and after surgery-induced weight loss in 6 morbidly obese patients. The most relevant miRNAs were cross-sectionally validated in 80 men and longitudinally in 22 patients (after surgery-induced weight loss). We evaluated the effects of diet-induced weight loss in 9 obese patients. Thirty-six circulating miRNAs were associated with anthropometric variables in the initial sample.

RESULTS: In the validation study, morbidly obese patients showed a marked increase of miR-140-5p, miR-142-3p (both $P < 0.0001$), and miR-222 ($P = 0.0002$) and decreased levels of miR-532–5p, miR-125b, miR-130b, miR-221, miR-15a, miR-423-5p, and miR-520c-3p ($P < 0.0001$ for all). Interestingly, *in silico* targets leukemia inhibitory factor receptor (LIFR) and transforming growth factor receptor (TGFR) of miR-140-5p, miR-142-3p, miR-15a, and miR-520c-3p circulated in association with their corresponding miRNAs. Moreover, a discriminant function of 3 miRNAs (miR-15a, miR-520c-3p, and miR-423-5p) was specific for morbid obesity, with an accuracy of 93.5%. Surgery-induced (but not diet-induced) weight loss led to a marked decrease of miR-140-5p, miR-122, miR-193a-5p, and miR-16-1 and upregulation of miR-221 and miR-199a-3p ($P < 0.0001$ for all).

CONCLUSIONS: Circulating miRNAs are deregulated in severe obesity. Weight loss–induced changes in this profile and the study of *in silico* targets support this observation and suggest a potential mechanistic relevance.

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Obesity is a well-known epidemic health problem worldwide. Obese patients suffer from decreased life quality and expectancy, as well as increased risk of type 2 diabetes, cardiovascular disease, hepatic steatosis, and cancer *(1)*. Body composition is likely determined by genetic makeup in close relationship with behavioral and environmental factors. The intake of energy-dense foods, especially combined with reduced physical activity, contributes to the high prevalence of obesity. However, the existence of complex systems that regulate energy balance calls for a broader view of this paradigm *(2)*.

Extensive efforts are being made to identify obesity-affecting genes to better understand pathogenesis, find new targets for clinical therapy, and allow early prediction of metabolic complications. Currently, new tools such as high-throughput technologies for genomic analyses may solve common problems in clinical practice, allowing earlier and more accurate diagnosis of comorbidities and improving prediction and response to therapy *(3)*.

MicroRNAs (miRNAs)[6] are small, noncoding, highly conserved RNAs. Since the discovery of miRNAs in 1993, their expression profiles and functions have been extensively studied. Through modifying mRNA availability and protein synthesis, miRNAs regulate many cellular processes such as cell growth, prolifera-

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[6] Nonstandard abbreviations: miRNA, microRNA; BMI, body mass index; LIFR, leukemia inhibitory factor receptor; VEGFA, vascular endothelial growth factor A; RT, reverse transcription; Ct, thermal cycle; TGFBR1, transforming growth factor-β receptor; JAK-STAT, Janus kinase signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase.

tion, differentiation, and apoptosis *(4)*. Moreover, because miRNA expression is closely related to cellular behavior and, eventually, the correct development and function of body tissues, changes in miRNA profiles are being increasingly analyzed in cancer, osteoporosis, ischemic heart disease, and heart failure *(5–7)*.

Recent studies demonstrated that miRNAs are deregulated not only in tissues from tumors *(8)*, heart disease *(9)*, acute sepsis *(10)*, tissue damage *(11)*, and systemic diseases *(12)*, but also in the circulation. Indeed, mammalian cells in culture have been reported to export miRNAs into the extracellular environment *(13)*, and some circulating miRNAs have been revealed as useful biomarkers for human diseases. Although circulating miRNAs seem to be involved in intercellular communication *(14)*, it remains unclear how extracellular miRNAs are associated with cellular components *(15)*, and the main function of circulating miRNAs remains elusive. In any case, it has been described that circulating miRNAs show a high degree of reproducibility within individuals, and that miRNAs do not change during the day *(16)*. Taking into account that miRNAs are not altered by other components of plasma that can interfere with conventional tools used for diagnosis (e.g., ELISA or RIA), the availability and stability of circulating miRNAs and the possibility of detecting, amplifying (through the polymerase chain reaction), and analyzing interindividual variation make them new biomarkers for systemic diseases and interesting potential targets for therapy. To our knowledge, this technology has not been tested for targeting the potential circulating miRNA signature of obesity. The aim of this study was to describe the circulating miRNA profile for humans according to specific degree of obesity. The effects of surgery and diet-induced weight loss on circulating miRNAs were also investigated in independent cohorts.

Materials and Methods

PARTICIPANT RECRUITMENT

Plasma samples from 32 white men with a body mass index (BMI) between 20 and 60 kg/m^2, recruited and studied in the Endocrinology Service of the Hospital Universitari Dr. Josep Trueta (Girona, Spain), were used to profile circulating miRNAs. We also analyzed the most relevant circulating miRNAs in an extended sample of 80 white men using individual TaqMan hydrolysis probes (Applied Biosystems). The study protocol was approved by the Ethics Committee and the Committee for Clinical Investigation of the Hospital Universitari Dr. Josep Trueta. All participants provided written informed consent before entering the study.

ANTHROPOMETRIC AND ANALYTIC DETERMINATIONS

BMI was calculated as weight in kilograms divided by height in meters squared. We performed other measurements with the usual techniques of the clinical laboratory, as previously described *(17)*. We also analyzed 2 of the circulating factors reported as potential target genes and possible candidates for each interaction [leukemia inhibitory factor receptor (LIFR) and vascular endothelial growth factor A (VEGFA)] in plasma by ELISA (Abcam).

STUDY OF THE EFFECTS OF WEIGHT LOSS

We investigated the whole profile of circulating miRNAs before and after surgery-induced weight loss in 6 morbidly obese patients (3 men and 3 women). This subpopulation was randomly selected from a final cohort of 22 white morbidly obese patients [mean BMI 42.9 (SD 5.9) kg/m^2, age 44 (14) years, 5 men and 17 women] recruited at the Endocrinology Department of the University Clinic of Navarra (Navarra, Spain). We also investigated individual circulating miRNAs after conventional weight loss in 9 white obese patients [BMI 32.4 (3.8) kg/m^2, age 47 (12) years, 5 men and 4 women]. The main procedures were as previously described *(18)*. The institutional review board of the University Clinic of Navarra approved the protocol, so we certify that all applicable institutional regulations concerning the ethical use of information and samples from humans were followed during this research. Complete clinical trial registration is deposited into ClinicalTrials.gov (accession no. NCT01572090).

PROFILE OF CIRCULATING miRNAs

Circulating RNA extraction and purification. Plasma was obtained by standard venipuncture and centrifugation in EDTA-coated Vacutainer Tubes (Becton Dickinson). Separation of plasma was performed by double centrifugation with a laboratory centrifuge (Beckman J-6M Induction Drive Centrifuge, Beckman Instruments). The first spin was performed at 1000*g* for 15 min at 4 °C. The second spin further discarded the few remaining blood cells from plasma and was performed at 2000*g* for 5 min at 4 °C. We performed RNA extraction using the mirVana PARIS Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. We chose to use a fixed volume of RNA eluate (3 μL) from the given volume of starting plasma (625 μL) as input into the reverse transcription (RT) reaction. Before RNA isolation, 2 synthetic oligonucleotides corresponding to miRNAs that do not exist in the human genome were spiked in for QC, as described previously *(8)*. The spiked-in oligos were introduced into the plasma sample and measured for each sample with TaqMan quantitative RT-PCR

(qRT-PCR) miRNA hydrolysis probes from Applied Biosystems. To validate the success of each extraction, we also assessed the thermal cycle (Ct) values obtained for a serial dilution (10^{-1}) of these miRNAs. Samples with recovery values less than approximately 50% were excluded and/or remade (see Supplemental Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue5).

Circulating miRNA RT and preamplification. We used a fixed volume of 3 μL RNA solution from the 40-μL eluate of RNA isolation as input into the RT with the TaqMan miRNA Reverse Transcription Kit and the TaqMan miRNA Multiplex RT Assays, which are required to run the TaqMan Array MicroRNA Cards (Applied Biosystems). To minimize manipulation of the original sample (RNA), we conducted our original determinations by microarray and real-time PCR (cDNA) with no preamplification. Human miRNA microarrays (Agilent Technologies), containing 13 737 probes corresponding to 799 miRNAs, and 22 control probes were hybridized as previously described *(7)*. In this case, the intensities were very low and compressed. Many probes had intensities very close to background compared to previous experiments *(7)*, thus making it difficult to distinguish between background and signal. We also failed to detect a circulating microRNA profile by PCR, even when using individual hydrolysis probes. Thus, preamplification was performed with TaqMan PreAmp Master Mix and Megaplex™ PreAmp Primers (human pool sets A and B), which provided an optional amplification step before real-time analysis when analytical sensitivity was of the utmost importance and/or the sample was limiting. This step was mandatory to provide reliable results in plasma.

Circulating miRNA profiling with TaqMan low-density arrays. We applied TaqMan miRNAs arrays covering 754 miRNA species to a first cohort of 32 patients. RT-PCR was carried out on an Applied BioSystems 7900HT thermocycler. Data were analyzed with SDS Relative Quantification Software version 2.2.2 (Applied Biosystems), with an assigned minimum threshold above the baseline of all assays showing measurable amplifications above background (see online Supplemental Figs. 2 and 3).

Analysis of TaqMan low-density arrays. We performed ΔCt normalization as implemented in the HTqPCR R package, using the 4 most stable (rank-invariant) miRNAs (miR-106a, miR-146a, miR-19b, and miR-223). For the posterior validation in an extended cohort, we selected these miRNAs and 2 other miRNAs that were highly correlated with the mean expression value (miR-186 and miR-199a-3p), as previously suggested *(19)*.

Analysis of individual miRNAs with TaqMan hydrolysis probes. We used commercially available TaqMan hydrolysis probes (Applied Biosystems) to assess the presence in plasma of individual miRNAs. The preamplification product was diluted (1:100) before being combined (5 μL) with 0.25 μL TaqMan miRNA hydrolysis probes (20×) and 4.75 μL LightCycler 480 Probes master mix (2×) (Roche Diagnostics) to a final volume of 10 μL. Gene expression was assessed by real-time PCR with the LightCycler® 480 Real-Time PCR System (Roche Diagnostics), with TaqMan technology suitable for relative gene expression quantification following the manufacturer's protocol.

For the analysis by qRT-PCR in each extended sample (both cross-sectional and longitudinal studies), we first evaluated a suitable number of reference miRNAs, on the basis of increased expression stability (see online Supplemental Fig. 4) and using the GeNorm methodology implemented in the R package SL qpcrNorm (Bioconductor), as previously described *(20)*. The GeNorm analysis suggested including 6 reference miRNAs (miR-106a, miR-146a, miR-19b, miR-223, miR-186, and miR-199a-3p). The selection and addition of various endogenous controls (reference miRNAs) for measures by qRT-PCR and the use of this geometric mean have been identified as among the most accurate and robust factors for normalization *(20)*. Thus, the geometric mean of all the selected internal controls was used as a normalizing factor.

We used normalized relative $\log_2$ ratios for posterior statistical tests *(20)*. To correct for multiple testing, we considered the tests significant when *P* values were $\leq$0.05/(number of miRNA tested). The significant threshold after correcting for multiple testing was approximately 0.0002. We excluded all Ct values >35.

STATISTICAL METHODS

Before statistical analysis, normal distribution and homogeneity of the variances were evaluated with the Levene test. We performed ANOVA and/or paired *t*-tests to study differences on quantitative variables between groups. The semiquantitative concentrations for the different miRNAs were correlated (Spearman test) with clinical parameters. Data analyses were performed with the SPSS (v. 12.0) and R (http://www.r-project.org/) statistical software. The SL qPCRNorm Package (Bioconductor) was also used for the analysis and normalization of miRNA data *(20)*.

Results

COMPREHENSIVE CIRCULATING miRNA PROFILING IN THE INITIAL SAMPLE

Metabolic and clinical characteristics of the individuals included in this cross-sectional study are shown in Table 1. We detected 108 miRNAs with Ct values of <35 (see online Supplemental Table 1). Circulating miRNAs that were associated with obesity were further studied by qRT-PCR and individual TaqMan miRNA hydrolysis probes in an extended sample of 80 men.

Online Supplemental Table 2 shows the results for the most interesting miRNAs when comparing obese (BMI $\geq$30 kg/m^2) vs nonobese individuals and morbidly obese (BMI $\geq$40 kg/m^2) vs non–morbidly obese individuals in the first cohort of 32 men. Some associations that used mean normalization did not replicate when the ΔCt normalization method was used. Thus, considering results from both methods of normalization guaranteed the identification and further consideration of all possible candidates. Of note, the most significant findings were identified when considering simple comparisons between morbidly obese (BMI $\geq$40 kg/m^2) and non–morbidly obese individuals (see online Supplemental Table 2). Regarding associations with quantitative traits (see online Supplemental Table 3), circulating miRNAs that were significantly associated with parameters of obesity [or that tended to be significant ($0.05 \leq P \leq 0.1$) after either mean or ΔCt normalization methods] were considered for study in the replication analysis. It should be noted that many of these associations remained significant after adjusting for age, and also when analyzing correlation by Spearman rank correlation method (data not shown).

REPLICATION STUDY OF THE MOST RELEVANT CIRCULATING miRNAs

According to the screening performed with microfluidic cards, many independent miRNAs were of interest for analysis by individual TaqMan miRNAs hydrolysis probes in an extended cohort of 80 men (Table 1). In this extended sample, the values in plasma for 18 specific circulating miRNAs were different ($P < 0.05$) in obese and morbidly obese men compared to the control group (Table 1). Among them, the increased expression of miR-142-3p (Fig. 1A), miR-140-5p (Fig. 1B), and miR-222 (Fig. 1C) and the decreased circulating concentrations of miR-221 (Fig. 1D), miR-15a (Fig. 1E), miR-520c-3p (Fig. 1F), miR-423-5p (Fig. 1G), and miR-130b (Fig. 1H) were differentially presented in morbidly obese patients (Table 1). Indeed, plasma concentrations of these miRNAs were significantly associated with BMI ($P < 0.0001$ for all) (see online Supplemental Fig. 5) and other measures of obesity such as fat mass and waist circumference (Table 2). Other circulating miRNAs of interest regarding their associations with BMI were miR-532-5p, miR-125b, miR-636, miR-193a-5p, miR-122, and miR-483-5p (Tables 1 and 2). Of note, the discriminant analysis computed for these results revealed that only 3 miRNAs (miR-15a, miR-520c-3p, and miR-423-5p) were specific for morbidly obese men, with a diagnostic accuracy of 93.5% (estimated with the leave-one-out method). The *P* value was <0.001 (computed by successive permutations and corresponding to the number of times that, when permuting the values, an error was found equal to or less than the real error rate divided by the number of tests). Furthermore, a discriminant function including only 4 circulating miRNAs (miR-15a, miR-520c-3p, miR-222, and miR-423-5p) was specific for morbidly obese patients, with a diagnostic accuracy of 94% ($P < 0.0001$) (see online Supplemental Fig. 6).

Interestingly, 2 major targets for the *in silico* intersection between miR-142-3p and miR-140-5p (LIFR) and between miR-15a and miR-520c-3p (VEGFA) were significantly associated with the circulating values of their specific transcriptional regulators. Indeed, LIFR concentration in plasma was negatively associated with the circulating concentrations of miR-142-3p ($P = 0.0003$) and miR-140-5p ($P = 0.009$), whereas miR-15a ($P = 0.03$) and miR-520c-3p ($P = 0.0006$) were negatively related to circulating VEGFA (Table 2).

EFFECTS OF WEIGHT LOSS ON CIRCULATING miRNAs

Surgery-induced weight loss. We investigated the profile of 754 miRNAs in 6 individuals; 44 miRNAs were deregulated in plasma from morbidly obese patients before surgery-induced weight loss (see online Supplemental Table 4). To further validate these findings, and to test whether values of miRNAs in plasma might be modified by weight loss, the previously mentioned circulating miRNAs were analyzed in an independent final cohort of 22 morbidly obese patients before and after surgically-induced weight loss.

Participants lost a mean of approximately 30% of their initial body weight at 1 year after surgery (Table 3). Interestingly, plasma concentrations of 14 circulating miRNAs changed significantly after surgery-induced weight loss, leading to a marked decrease of circulating miR-140-5p (Fig. 2A), miR-142-3p (Fig. 2B), miR-16-1 (Fig. 2C), and miR-122 (Fig. 2D) and the upregulation of miR-221 (Fig. 2E) and miR-130b (Fig. 2F). Other data of interest were the significantly decreased concentrations of miR-199a-3p and miR-125b (−91% and −86%, respectively, both $P <$ 0.0001), miR-19b-1 (−65%, $P = 0.001$), and miR-483-5p and the upregulation of miR-146a (142%, $P <$

Table 1. Clinical characteristics of men included in cross-sectional studies.[a]

	Nonobese (BMI < 30)	Obese (30 ≤ BMI < 40)	Morbidly obese (BMI ≥ 40)	*P* (ANOVA)
First population (TaqMan)				
n	12	12	8	
Type 2 diabetes, %	N/A	42	50	
Age, years	50 (11)	51 (8)	46 (5)	0.430
BMI, kg/m^2	23.7 (1.1)	33.1 (6.5)	45.3 (6.3)	<0.0001[b]
Fat mass, %	23.7 (3.4)	35.4 (4.3)	48.7 (7.0)	0.001[b]
Waist circumference, cm	83.4 (5.2)	107.8 (10.3)	130.4 (11.3)	<0.0001[b]
Systolic blood pressure, mmHg	118.1 (11.0)	139.1 (12.4)	127.0 (19.4)	0.002[b]
Diastolic blood pressure, mmHg	75.6 (10.7)	80.4 (9.8)	78.0 (11.5)	0.534
Serum glucose, mg/dL[c]	88.2 (9.3)	102.2 (14.0)	141.2 (62.3)	0.004[b]
Glycated hemoglobin, %	4.7 (0.4)	5.1 (0.5)	5.9 (1.3)	0.005[b]
Total cholesterol, mg/dL[d]	192.3 (19.6)	217.8 (28.1)	227.1 (46.7)	0.043[b]
HDL cholesterol, mg/dL	53.1 (13.2)	50.8 (12.5)	47.3 (12.5)	0.617
LDL cholesterol, mg/dL	121.5 (22.0)	142.9 (24.9)	146.5 (45.1)	0.129
Fasting triglycerides, mg/dL[e]	70 (54–88)	98 (76–169)	148 (120–318)	0.048[b]
Lipopolysaccharide-binding protein, ng/mL	8.6 (6.2–14.5)	26.5 (8.3–50.2)	82.9 (44.4–166.8)	0.005[b]
Extended sample (qRT-PCR)				
n	49	19	12	
Type 2 diabetes, %	N/A	37	33	
Age, years	49 (10)	51 (13)	42 (10)	0.069
BMI, kg/m^2	25.4 (1.9)	33.0 (7.1)	45.5 (8.9)	<0.0001[b]
Fat mass, %	25.5 (12.8)	35.3 (7.7)	48.1 (9.6)	<0.0001[b]
Waist circumference, cm	86.9 (6.1)	108.4 (14.9)	132.7 (12.8)	<0.0001[b]
Systolic blood pressure, mmHg	121.3 (11.9)	140.4 (16.0)	131.0 (19.0)	<0.0001[b]
Diastolic blood pressure, mmHg	76.9 (8.9)	83.6 (11.2)	79.4 (10.0)	0.047[b]
Serum glucose, mg/dL	94.1 (12.2)	100.1 (13.3)	127.3 (53.9)	<0.0001[b]
Glycated hemoglobin, %	4.8 (0.4)	5.0 (0.5)	5.6 (1.1)	<0.0001[b]
Total cholesterol, mg/dL	208.4 (41.4)	222.6 (26.5)	214.8 (48.3)	0.410
HDL cholesterol, mg/dL	51.8 (11.8)	49.5 (11.3)	46.7 (12.0)	0.368
LDL cholesterol, mg/dL	136.4 (38.6)	146.9 (25.2)	135.8 (41.2)	0.543
Fasting triglycerides, mg/dL	78 (61–106)	131 (75–174)	145 (117–217)	0.001[b]
Lipopolysaccharide-binding protein, ng/mL	12.3 (7.2–23.3)	26.5 (6.9–44.1)	78.2 (39.1–100.7)	<0.0001[b]
miR-423-5p	3.07 (1.82–4.61)	3.61 (1.77–9.98)	0.52 (0.44–1.04)[f]	<0.0001[b]
miR-520c-3p	45.32 (19.97–142.43)	39.87 (9.39–207.11)	1.39 (0.87–3.28)[f]	<0.0001[b]
miR-532-5p	3.51 (2.12–5.70)	6.13 (2.46–8.00)	1.60 (1.21–2.36)[f]	<0.0001[b]
miR-125b	6.09 (3.54–10.78)	5.90 (0.92–11.88)	1.84 (1.13–1.99)[f]	<0.0001[b]
miR-130b	2.04 (1.21–2.89)	3.25 (1.84–6.59)	0.65 (0.49–1.00)[f]	<0.0001[b]
miR-221	3.41 (2.31–5.82)	4.53 (2.35–8.88)	1.06 (0.88–1.96)[f]	<0.0001[b]
miR-15a	4.47 (3.13–6.45)	3.46 (2.37–5.03)	1.52 (1.16–2.26)[f]	<0.0001[b]
miR-222	0.51 (0.28–0.72)	0.52 (0.17–0.59)	0.98 (0.70–1.13)[g]	0.0034[b]
miR-140-5p	0.34 (0.21–0.69)	0.60 (0.43–1.16)	0.88 (0.74–1.78)[g]	<0.0001[b]
miR-142-3p	0.26 (0.14–0.39)	0.53 (0.33–0.91)	0.83 (0.48–1.26)[h]	<0.0001[b]

Continued on page 786

Table 1. Clinical characteristics of men included in cross-sectional studies.[a] *(Continued from page 785)*				
	Nonobese (BMI < 30)	Obese (30 ≤ BMI < 40)	Morbidly obese (BMI ≥ 40)	*P* (ANOVA)
miR-21	1.88 (1.53–2.26)	2.21 (1.62–2.73)	1.38 (0.86–1.58)[h]	0.0002[b]
miR-590-5p	1.91 (0.93–2.41)	2.06 (1.37–3.64)	0.71 (0.49–1.10)[i]	0.0003[b]
miR-193a-5p	5.00 (2.77–9.16)	1.90 (1.28–6.95)	1.09 (0.72–2.89)[i]	0.0023[b]
miR-122	1.78 (0.80–3.43)	2.41 (1.28–5.72)	0.26 (0.18–1.75)[i]	0.0063[b]
miR-483-5p	5.26 (3.04–13.93)	10.68 (3.08–51.17)	2.89 (0.57–3.62)[i]	0.0059[b]
miR-126	0.57 (0.42–0.73)	0.49 (0.42–0.66)	0.93 (0.71–0.96)[i]	0.0069[b]
miR-636	4.98 (2.85–23.46)	6.46 (1.65–41.27)	0.83 (0.68–3.40)[i]	0.0179[b]
miR-625	1.34 (0.91–2.30)	1.10 (0.79–3.65)	0.61 (0.33–1.20)[i]	0.0385[b]

[a] Data are means (SD) for Gaussian variables and medians (interquartile ranges) for non-Gaussian variables. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene test. The variables miRNAs were given in a base $\log_2$-transformation and analyzed on that $\log_2$ scale. The Student *t*-test was performed for single comparisons between circulating miRNAs from morbid obese and nonobese men.
[b] Statistically significant ($P < 0.05$).
[c] To convert mg/dL glucose to mmol/L, multiply by 0.0555.
[d] To convert mg/dL cholesterol to mmol/L, multiply by 0.0259.
[e] To convert mg/dL triglycerides to mmol/L, multiply by 0.0113.
[f] $P < 0.00001$.
[g] $P < 0.0001$.
[h] $P < 0.001$.
[i] $P < 0.01$.

0.0001), miR-423-5p, and miR-21 (Table 3). miR-15a [1.18 (0.94–1.8) vs 1.47 (1.18–2.42), $P = 0.123$] and miR-520c-3p [2.21 (1.69–9.92) vs 7.04 (1.6–32.3), $P = 0.344$] were not significantly modulated by weight loss [baseline vs after weight loss, median (interquartile range)].

Diet-induced weight loss. Of note, these results were not reproducible for studying the effects of diet-induced weight loss in 9 obese patients (−17% of the initial body weight subjects) (Table 3 and Fig. 2), and no significant associations with weight loss were found for circulating values of miR-520c-3p, miR-15a, miR-590-5p, miR-126, miR-636, or miR-625.

Discussion

Plasma miRNAs are useful biomarkers for the diagnosis, prognosis, and therapeutic value of systemic diseases. We provide here the first evidence, to our knowledge, of a plasma miRNA signature in patients with severe obesity. We identified at least 9 circulating miRNAs in morbidly obese patients, including increased concentrations of miR-140-5p and miR-142-3p and decreased concentrations of miR-532-5p, miR-125b, miR-130b, miR-221, miR-15a, miR-520c-3p, and miR-423-5p, which were strongly linked to measures of fat mass. Indeed, discriminant analyses showed that 3 of them (miR-15a, miR-520c-3p, and miR-423-5p) were highly specific for morbidly obese men. Moreover, our data clearly demonstrate a significant modulation of 14 circulating miRNAs upon surgery-induced weight loss, as evidenced by the marked downregulation of miR-140-5p, miR-122, miR-193a-5p, and miR-16-1 and the upregulation of miR-221 and miR-199a-3p. Gastric bypass surgery is the most commonly performed bariatric surgical procedure and is an effective approach for achieving weight loss in obese patients. Indeed, surgery-induced weight loss is known to improve and even completely solve most obesity-associated complications and leads to increased survival *(21, 22)*, whereas in most cases conventional diet-induced weight loss does not *(23)*. Thus, miRNAs deregulated in morbid obesity might also be attractive candidates in the study of the regulation of cell fate decisions and complex obesity-related complications, as shown by the intersection for the target genes identified *in silico* for the miRNAs of interest in this field with DIANA-mirPath, a free web-based computational tool that identifies potentially altered molecular pathways by the expression of 1 or multiple microRNAs *(24)*.

INCREASED CIRCULATING miR-142-3p AND miR-140-5p IN MORBID OBESITY

The expression of miR-142-3p is related to the correct development of hematopoietic lineage–specific cells *(25)*, and its expression in tissues is being considered as a marker of acute and chronic inflammation *(26)*.

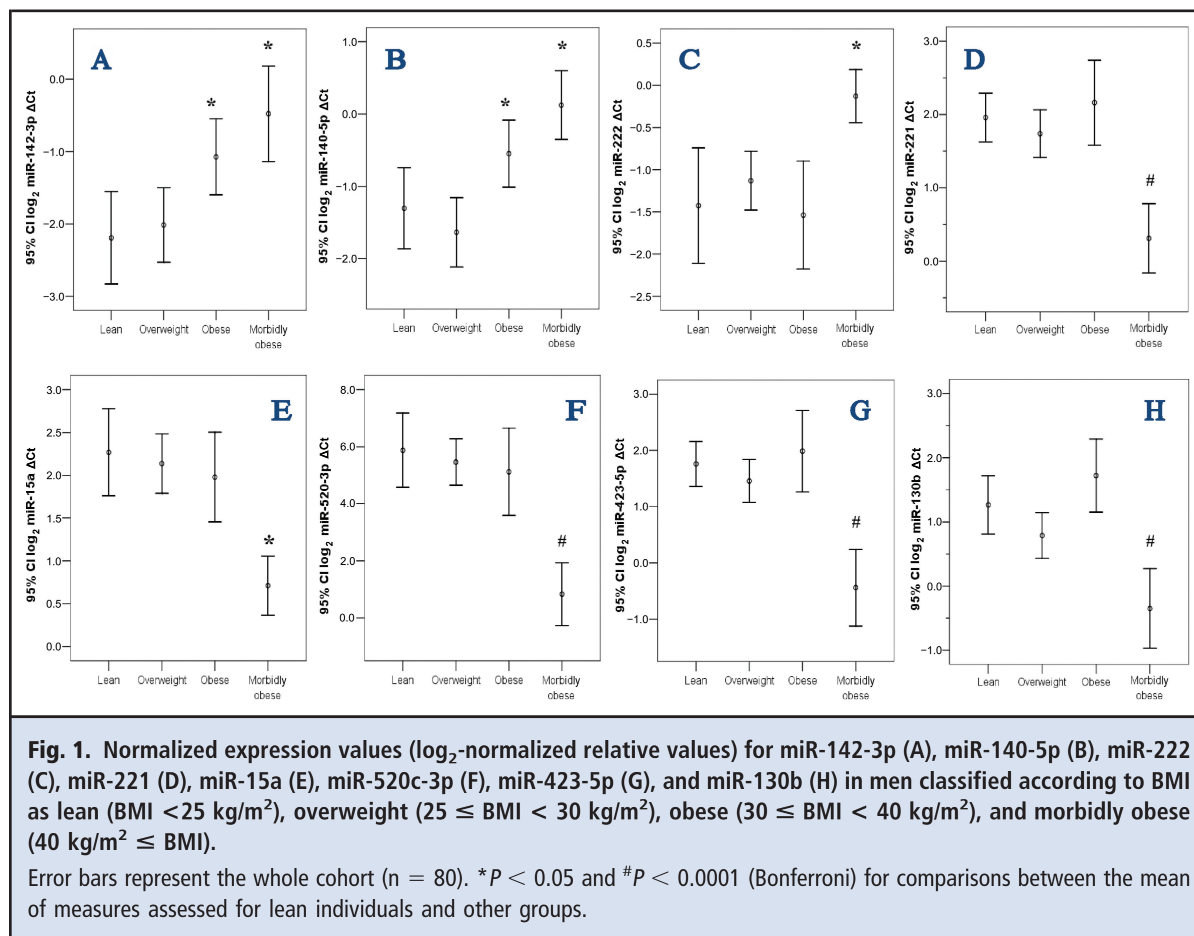

Fig. 1. Normalized expression values ($\log_2$-normalized relative values) for miR-142-3p (A), miR-140-5p (B), miR-222 (C), miR-221 (D), miR-15a (E), miR-520c-3p (F), miR-423-5p (G), and miR-130b (H) in men classified according to BMI as lean (BMI <25 kg/m^2), overweight ($25 \leq$ BMI < 30 kg/m^2), obese ($30 \leq$ BMI < 40 kg/m^2), and morbidly obese (40 kg/m^2 $\leq$ BMI).

Error bars represent the whole cohort (n = 80). $^*P < 0.05$ and $^\# P < 0.0001$ (Bonferroni) for comparisons between the mean of measures assessed for lean individuals and other groups.

Increased expression measures of miR-142-3p have been reported in serum from patients with chronic inflammation, autoimmune attack, and vascular damage *(27)*. Our findings revealed a close relationship between miR-142-3p concentrations in plasma and obesity measures, further demonstrated by decreased circulating miR-142-3p upon weight loss. Although no associations with conventional markers of obesity-associated inflammation (e.g., tumor necrosis factor-α, interleukin-6, or C-reactive protein) were found, circulating miR-142-3p concentrations were positively associated with the liver marker of inflammation, lipopolysaccharide-binding protein (Table 2).

The available literature suggests that miR-140 species may be involved in chondrocyte proliferation and differentiation, as well as cartilage development *(28)*, together with miR-142-3p *(29)*. However, the experimental evidence for miR-140-5p in other tissues remains scarce, as do associations of circulating miR-140-5p with other diseases. The marked decrease of miR-140-5p in plasma from obese patients after surgery-induced weight loss, in agreement with cross-sectional associations, suggests the influence of body fat on the presence of this circulating miRNA, as well as on miR-142-3p.

The close association shown between miR-142-3p and miR-140-5p may point out a similar function and/or origin for these 2 circulating miRNAs that are significantly increased in morbid obesity. Indeed, the intersection for these miRNAs identified *in silico (24)* some target genes (see online Supplemental Table 5), indicating that both miRNAs working together might influence the expression of the receptor for transforming growth factor-β (TGFBR1) and LIFR, among others (see online Supplemental Table 5). The ELISA analyses in vivo of circulating LIFR further confirmed this association. TGFRB1, as well as LIFR, is involved in the interaction between cytokines and cytokine receptors *(30)* and the Janus kinase signal transducer and activator of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) signaling pathway *(31)*. On the other hand, it is known that members of the TGF-β superfamily regulate many aspects of adipocyte development, adiposity, and energy expenditure *(32)*, as LIF does *(33)*. Therefore, miRNAs regu-

Table 2. Correlation between log_2-transformed miRNAs concentrations and studied variables in the cross-sectional study.[a]

	R (Spearman p)									
Whole cohort (qRT-PCR)	Age, years	BMI, kg/m²	Fat mass, %	Waist circumerence, cm	Fasting glucose, mg/dL	Glycated hemoglobin, %	Blood lymphocyte count, U/μL	Lipopolysaccharide-binding protein, μg/mL	LIFR, ng/mL	VEGFA, pg/mL
miR-423-5p	0.10 (ns)	−0.44 (<0.0001)	−0.42 (0.0001)	−0.41 (0.0002)	−0.22 (0.05)	−0.31 (0.006)	−0.40 (0.0002)	−0.53 (0.0005)	−0.05 (NS)	−0.22 (NS)
miR-520c-3p	0.14 (ns)	−0.57 (<0.0001)	−0.54 (<0.0001)	−0.52 (<0.0001)	−0.27 (0.03)	−0.32 (0.01)	−0.46 (0.0002)	−0.64 (<0.0001)	−0.18 (NS)	−0.47 (0.0006)
miR-532-5p	−0.03 (ns)	−0.35 (0.002)	−0.12 (NS)	−0.20 (0.04)	−0.12 (NS)	−0.25 (0.03)	−0.29 (0.009)	−0.41 (0.01)	−0.05 (NS)	−0.22 (NS)
miR-125b	−0.09 (ns)	−0.42 (0.001)	−0.24 (0.05)	−0.36 (0.005)	−0.15 (NS)	−0.35 (0.005)	−0.32 (0.01)	−0.34 (0.05)	0.02 (NS)	−0.28 (0.05)
miR-130b	0.11 (ns)	−0.32 (0.004)	−0.16 (NS)	−0.16 (NS)	−0.18 (NS)	−0.19 (NS)	−0.36 (0.001)	−0.38 (0.01)	−0.07 (NS)	−0.11 (NS)
miR-221	0.08 (ns)	−0.42 (<0.0001)	−0.25 (0.03)	−0.24 (0.03)	−0.14 (NS)	−0.19 (NS)	−0.41 (<0.0001)	−0.39 (0.01)	−0.11 (NS)	−0.22 (NS)
miR-15a	0.05 (ns)	−0.5 (<0.0001)	−0.49 (<0.0001)	−0.48 (<0.0001)	−0.25 (0.04)	−0.37 (0.001)	−0.18 (NS)	−0.53 (0.001)	0.12 (NS)	−0.3 (0.03)
miR-222	−0.02 (ns)	0.27 (0.01)	0.27 (0.02)	0.21 (NS)	0.12 (NS)	0.13 (NS)	0.29 (0.01)	0.17 (NS)	0.21 (NS)	0.28 (0.04)
miR-140-5p	−0.25 (0.03)	0.43 (<0.0001)	0.37 (0.001)	0.41 (0.0004)	0.25 (0.03)	0.15 (NS)	0.31 (0.006)	0.39 (0.01)	−0.48 (0.0003)	0.36 (0.008)
miR-142-3p	−0.24 (0.03)	0.43 (<0.0001)	0.37 (0.0008)	0.39 (0.0004)	0.22 (0.05)	0.16 (NS)	0.22 (0.05)	0.5 (0.001)	−0.36 (0.009)	0.25 (NS)
miR-21	0.13 (ns)	−0.24 (0.03)	−0.21 (NS)	−0.25 (0.03)	−0.36 (0.001)	−0.39 (0.0005)	−0.14 (NS)	−0.33 (0.04)	0.11 (NS)	−0.09 (NS)
miR-590-5p	0.06 (ns)	−0.33 (0.01)	−0.33 (0.01)	−0.19 (NS)	−0.12 (NS)	−0.19 (NS)	−0.42 (0.001)	−0.34 (NS)	−0.26 (NS)	−0.22 (NS)
miR-193a-5p	0.09 (ns)	−0.44 (0.0006)	−0.43 (0.001)	−0.46 (0.0008)	−0.16 (NS)	−0.32 (0.02)	−0.25 (0.05)	−0.58 (0.001)	0.44 (0.004)	−0.46 (0.002)
miR-122	−0.12 (ns)	−0.29 (0.009)	−0.33 (0.003)	−0.25 (0.03)	−0.02 (NS)	−0.28 (0.01)	−0.17 (NS)	−0.4 (0.01)	0.06 (NS)	−0.38 (0.004)
miR-483-5p	0.01 (ns)	−0.31 (0.009)	−0.32 (0.008)	−0.24 (0.05	−0.11 (NS)	−0.21 (NS)	−0.35 (0.003)	−0.43 (0.01)	0.11 (NS)	−0.38 (0.007)
miR-126	−0.19 (ns)	0.36 (0.001)	0.32 (0.005)	0.34 (0.003)	0.19 (NS)	0.24 (0.04)	0.33 (0.003)	0.57 (0.0002)	−0.16 (NS)	0.28 (0.04)
miR-636	0.16 (ns)	−0.64 (0.0007)	−0.64 (0.0008)	−0.7 (0.0005)	−0.4 (0.05)	−0.49 (0.02)	−0.36 (NS)	−0.6 (0.02)	−0.10 (NS)	−0.34 (NS)
miR-625	−0.01 (ns)	−0.33 (0.02)	−0.35 (0.01)	−0.33 (0.02)	−0.06 (NS)	−0.19 (NS)	−0.31 (0.02)	−0.33 (NS)	−0.10 (NS)	−0.02 (NS)

[a] Data are *R* (Spearman *P*). NS, not significant.

Table 3. Clinical characteristics of subjects included in longitudinal studies.[a]

	Baseline	After weight loss	Variation, %	*P* (Student *t*-test)
Surgery-induced weight loss[b]				
Weight, kg	115.1 (22.4)	77.7 (16.3)	−32.5	<0.0001
BMI, kg/m^2	42.9 (5.9)	28.9 (4.3)	−32.6	<0.0001
Fat mass, kg	51.2 (6.5)	35 (8.2)	−31.6	<0.0001
Waist circumference, cm	124.7 (13.4)	97.1 (10.9)	−22.1	<0.0001
Waist-to-hip ratio	0.98 (0.09)	0.95 (0.08)	−4.1	0.024
Systolic blood pressure, mmHg	134.4 (16.7)	112.5 (16.5)	−16.3	<0.0001
Diastolic blood pressure, mmHg	82 (10.3)	67.9 (9.6)	−17.2	<0.0001
Fasting glucose, mg/dL[c]	114.4 (50.5)	94.3 (26)	−17.6	0.002
Fasting insulin, μUI/mL[d]	29 (32.4)	7.5 (7.5)	−73.4	0.004
Homeostasis model assessment for insulin resistance	8.2 (9.1)	2.1 (3.4)	−73.8	0.004
Total cholesterol, mg/dL[e]	178.7 (32.6)	170.3 (40.5)	−4.7	0.210
LDL cholesterol, mg/dL	103.6 (31)	91.4 (36.1)	−11.8	0.071
HDL cholesterol, mg/dL	45.3 (11.9)	61.5 (14.2)	35.8	<0.0001
Fasting triglycerides, mg/dL[f]	123 (87–173)	79 (65–101)	−41.6	0.004
Leptin, μg/L	40.3 (29.7–54.2)	8.5 (5.3–12.6)	−77.3	<0.0001
miR-423-5p	0.97 (0.67–1.36)	1.10 (0.88–2.27)	17.6	0.049
miR-130b	0.44 (0.36–1.17)	1.11 (0.56–2.94)	128.2	0.018
miR-221	0.32 (0.26–0.52)	0.72 (0.53–1.20)	80.4	0.005
miR-222	1.85 (1.47–2.59)	1.34 (1.15–2.02)	−33.3	0.047
miR-140-5p	1.80 (1.33–3.09)	0.72 (0.46–0.88)	−66.6	<0.0001
miR-142-3p	1.68 (0.51–2.64)	0.70 (0.50–1.29)	−52.1	0.026
miR-21	0.79 (0.43–1.51)	1.23 (0.90–2.00)	28.5	0.049
miR-193a-5p	4.62 (1.66–11.36)	0.40 (0.13–0.67)	−91.2	<0.0001
miR-122	5.39 (1.88–13.98)	0.28 (0.14–0.47)	−94.7	<0.0001
miR-483-5p	8.90 (4.30–27.54)	1.25 (0.73–10.29)	−78.9	0.001
Diet-induced weight loss[g]				
BMI, kg/m^2	34.4 (6.1)	28.7 (4.9)	−16.6	0.006
Leptin, μg/L	20.3 (16.1–30.3)	10.5 (5.4–19.2)	−57.1	NS
miR-423-5p	2.07 (1.27–4.29)	2.09 (1.12–3.11)	−17.4	NS
miR-130b	1.43 (1–11–2.00)	1.48 (0.83–2.29)	−8.3	NS
miR-221	0.85 (0.72–1.07)	0.98 (0.80–1.39)	14.7	NS
miR-222	0.94 (0.32–1.06)	0.59 (0.29–1.20)	1.3	NS
miR-140-5p	0.66 (0.41–1.14)	0.93 (0.64–1.73)	32.9	NS
miR-142-3p	0.82 (0.51–1.45)	0.60 (0.15–1.21)	−12.5	NS
miR-21	2.33 (1.94–2.84)	2.08 (1.89–2.61)	−9.8	NS
miR-193a-5p	0.27 (0.23–1.09)	0.54 (0.18–2.17)	167.2	NS
miR-122	0.30 (0.18–0.69)	0.18 (0.02–0.65)	−18.5	NS
miR-483-5p	3.64 (0.73–7.02)	1.56 (0.92–2.93)	26.8	NS

[a] Data are means (SD) for Gaussian variables and medians (interquartile ranges) for non-Gaussian variables. NS, not significant. The variables miRNAs were given in a base $\log_2$-transformation and analyzed on that $\log_2$ scale. The Student *t*-test was performed for single comparisons between circulating miRNAs at baseline and after weight loss.

[b] n = 22, 5 men and 17 women, age 44 (14) years.

[c] To convert mg/dL glucose to mmol/L, multiply by 0.0555.

[d] To convert μUI/mL insulin to pmol/L, multiply by 6.95.

[e] To convert mg/dL cholesterol to mmol/L, multiply by 0.0259.

[f] To convert mg/dL triglycerides to mmol/L, multiply by 0.0113.

[g] n = 9, 5 men and 4 women, age 47 (12) years.

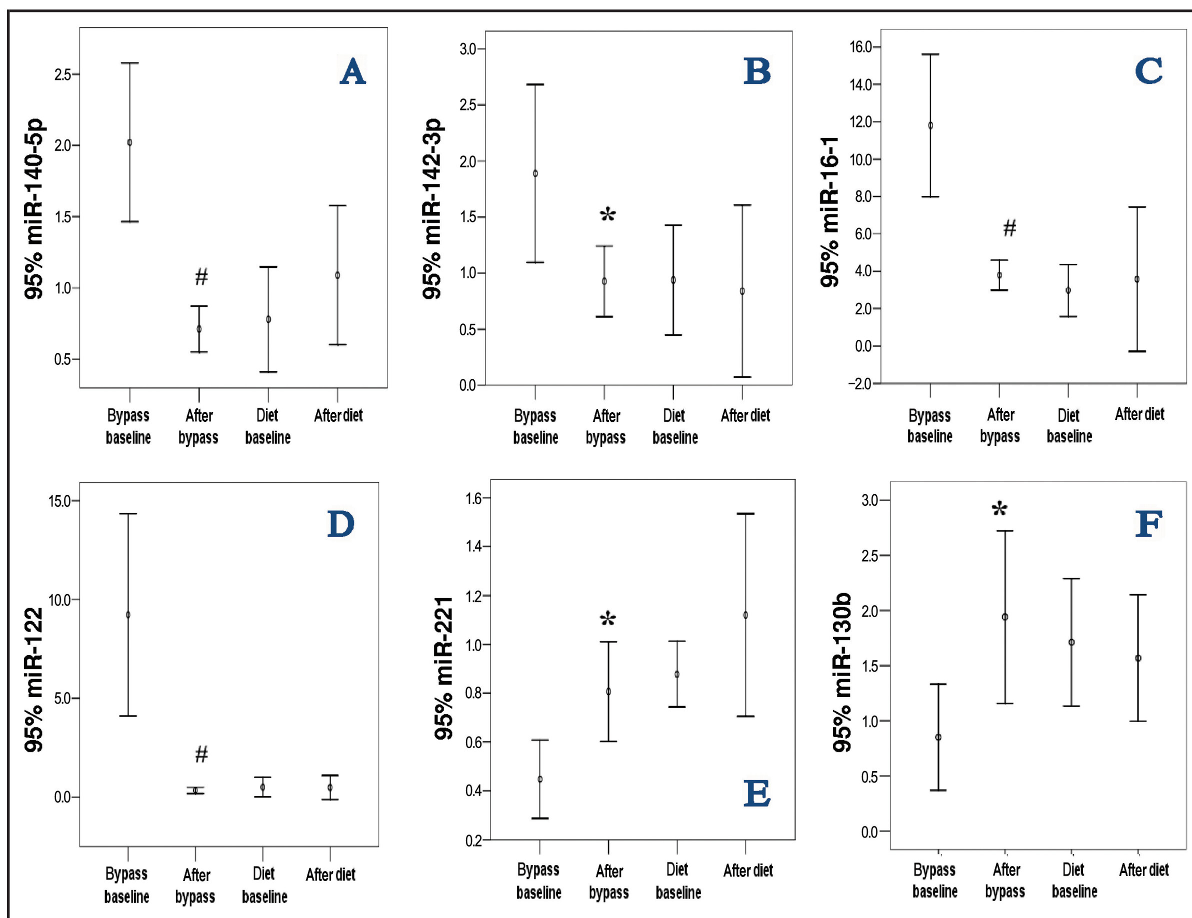

Fig. 2. Baseline and post–gastric bypass bariatric surgery and diet-induced weight loss levels of miR-140-5p (A), miR-142-3p (B), miR-16-1 (C), miR-122 (D), miR-221 (E), and miR-130b (F).
$^*P < 0.05$ and $^\#P < 0.0001$ (Student *t*-test) for single comparisons between circulating miRNAs at baseline and after weight loss.

lating *LIFR* (leukemia inhibitory factor-α receptor)[7] and *TGFRB1* (transforming growth factor-β receptor 1) gene expressions are potential targets for obesity.

DECREASED CIRCULATING miR-520C-3p, miR-15a, AND miR-423-5p IN MORBID OBESITY

In agreement with cross-sectional findings, weight loss induced a slight upregulation of certain circulating miRNAs (Table 3). The miR-520 cluster target genes have overlapping functions involved in the cell cycle and have been reported to be highly expressed in undifferentiated human embryonic stem cells *(34)*. The cluster of miR-15a and miR-16-1 (miR-15a/16-1) is implicated in apoptosis and the cell-cycle regulation of tumor cells *(35)*. The interaction of target genes *(24)* for these 2 miRNAs (see online Supplemental Table 5) includes VEGFA, a well-known mitogen produced by the adipocyte that acts on endothelial cells mediating increased vascular permeability, angiogenesis, vasculogenesis, and endothelial cell growth, which is upregulated in obesity *(36)*. Interestingly, the analysis in vivo of circulating plasma VEGFA concentrations showed significant associations of VEGFA with the concentrations of miRNAs involved in VEGFA regulation (Table 2).

Circulating miR-15a concentrations were inversely associated with measures of obesity, a finding consistent with the results reported by Zampetaki et al. *(12)* (decreased concentrations of miR-15a in patients with type 2 diabetes). In addition, increased miR-15a has been described in mouse β-cells after high glucose *(37)*. On the other hand, Liu et al. *(38)* identified by genomewide miRNA expression profiling 5 miRNAs (including miR-423-5p) of utility as biomarkers for gastric cancer and tumor progression stages. Also with

[7] Human genes: *LIFR*, leukemia inhibitory factor-α receptor; *TGFRB1*, transforming growth factor-β receptor 1.

microarray techniques, Tijsen et al. *(39)* identified miR-423-5p as a predictor of heart failure in a multivariate logistic regression model. Current findings revealed the inverse associations of miR-423-5p with obesity measures and markers of inflammation such as blood lymphocyte count and the weight loss–induced upregulation of miR-423-5p in obese patients.

Conclusion

To our knowledge, this study provides the first evidence that miRNAs in plasma are deregulated in morbidly obese men. Moreover, the signature of circulating miRNAs changed concomitantly with extensive weight loss. Interestingly, recent studies have demonstrated that adipocytes secrete microvesicles that may contain oligonucleotides *(40)*. Indeed, adipocyte/adipose tissue–specific gene transcripts and microRNAs were found in microvesicles isolated from rat serum *(13)*. Thus, these microvesicles might play a role as novel intercellular communication tools by transporting miRNA in paracrine and possibly endocrine manners and participating in the pathophysiology of adipose tissue in obesity. miR-142-3p, miR-140-5p, miR-15a, miR-520c-3p, and miR-423-5p may constitute novel biomarkers for risk estimation and classification of morbidly obese patients. Studies targeting the effects of these circulating miRNAs deregulated in obesity would provide further insight into the specific role of miRNAs in obesity-associated diseases.

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