



# High density lipoprotein-associated miRNA is increased following Roux-en-Y gastric bypass surgery for severe obesity

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**Abstract** Roux-en-Y gastric bypass (RYGB) is one of the most commonly performed weight-loss procedures, but how severe obesity and RYGB affect circulating HDL-associated microRNAs (miRNAs) remains unclear. Here, we aim to investigate how HDL-associated miRNAs are regulated in severe obesity and how weight loss after RYGB surgery affects HDL-miRNAs. Plasma HDLs were isolated from patients with severe obesity (n = 53) before and 6 and 12 months after RYGB by immunoprecipitation using goat anti-human apoA-I microbeads. HDLs were also isolated from 18 healthy participants. miRNAs were extracted from isolated HDL and levels of miR-24, miR-126, miR-222, and miR-223 were determined by TaqMan miRNA assays. We found that HDL-associated miR-126, miR-222, and miR-223 levels, but not miR-24 levels, were significantly higher in patients with severe obesity when compared with healthy controls. There were significant increases in HDL-associated miR-24, miR-222, and miR-223 at 12 months after RYGB. Additionally, cholesterol efflux capacity and paraoxonase activity were increased and intercellular adhesion molecule-1 (ICAM-1) levels decreased. The increases in HDL-associated miR-24 and miR-223 were positively correlated with an increase in cholesterol efflux capacity ( $r = 0.326$ ,  $P = 0.027$  and  $r = 0.349$ ,  $P = 0.017$ , respectively). An inverse correlation was observed between HDL-associated miR-223 and ICAM-1 at baseline. Together, these findings show that HDL-associated miRNAs are differentially regulated in healthy participants versus patients with severe obesity and are altered after RYGB. These findings provide insights into how miRNAs are regulated in obesity before and after weight reduction and may lead to the development of novel

**treatment strategies for obesity and related metabolic disorders.**

**Supplementary key words** micro-ribonucleic acid • high-density lipoprotein functionality • intercellular adhesion molecule-1 • weight loss

The global prevalence of obesity has doubled over the last three to four decades and continues to rise progressively (1, 2). Bariatric surgery results in durable reduction in weight and sustained improvement in metabolic and cardiovascular outcomes, both in a weight-dependent and weight-independent manner (3, 4). The precise molecular mechanisms driving the metabolic effects of bariatric surgery remain to be fully established.

MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate mRNAs through target transcript degradation or by inhibiting translation (5). miRNAs are involved in a diverse range of biological pathways and have been implicated in the biological processes underlying obesity and the associated cardiometabolic disease (5–9). Altered miRNA expression, including increased miR-24 (10) and decreased miR-126 in adipose tissue (11), and increased miR-222 (7) and decreased miR-223 (12) in the circulation, have all been described previously in obesity. miRNAs act at the intracellular level and are transported between cells in association with lipid-based vesicles, lipoproteins, and lipid-free protein complexes (13). We and others have recently demonstrated the involvement of HDLs in the transport of functional miRNAs within an intercellular communication network, with delivery of a specific miRNA (miR-223) to endothelial cells, contributing to the anti-inflammatory capacity of HDLs (13–15).

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HDL-C levels have been shown to correlate inversely with cardiovascular disease risk (16), and a clear link exists between excess weight and adiposity in obesity and low HDL-C levels (17). Improvements in HDL structure and function have been reported following metabolic surgery (18), although evidence for its effect on cholesterol efflux capacity has been inconsistent so far (19–21). Bariatric surgery has been shown to impact on the circulating miRNA signature of obesity (7), and significant changes have been described even prior to significant weight loss (22). The effect of bariatric surgery on HDL-associated miRNAs has not been investigated and may contribute mechanistically to improved HDL function following bariatric surgery. In this study, we assessed the changes in HDL-associated miR-24, miR-126, miR-222, and miR-223 levels following bariatric surgery.

## MATERIALS AND METHODS

### Participants

We recruited 53 patients with severe obesity (BMI 45.6–57.5 kg/m<sup>2</sup> and weight circumference 142 ± 17 cm) who underwent Roux-en-Y gastric bypass (RYGB) surgery at the Salford Royal NHS Foundation Trust tertiary weight management center (Salford, UK). Patients with acute coronary syndrome within the past 6 months, history of malignancy, anemia, active infections, HIV, and autoimmune diseases were excluded. Assessments were undertaken at baseline, 6 months, and 12 months after surgery. Eighteen healthy participants without a history of type 2 diabetes or statin therapy were recruited for comparison. This study was approved by the Greater Manchester Central Research and Ethics Committee. Written informed consent was obtained from all patients prior to participation and study assessments were conducted in accordance with the 1964 Helsinki declaration.

### Laboratory analyses

Venous blood samples were obtained from patients between 0900 and 1100 following an overnight fast of at least 12 h. Glycosylated hemoglobin (HbA<sub>1c</sub>) was measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust (Manchester, UK) on the day of collection. Isolated serum and plasma samples were stored at –80°C until use. Other laboratory measurements were performed at the end of the study.

Total cholesterol and triglyceride were measured using CHOP-PAP and GPO-PAP methods, respectively. ApoA-I and apoB were measured using immunoturbidimetry. HDL-C was assayed using a second-generation homogenous direct method (23). Serum paraoxonase (PON1) activity was measured using paraoxon [*O,O*-diethyl *O*-(4-nitrophenyl) phosphate] as a substrate (Sigma-Aldrich, St. Louis, MO) (24). All these tests were performed on a Randox Daytona+ analyzer (Randox Laboratories, Crumlin, UK). The laboratory participated in the RIQAS (Randox International Quality Assessment Scheme; Randox Laboratories, Dublin, Ireland). LDL-C was estimated using the Friedewald formula. No patients had triglyceride levels above 4.5 mmol/l.

Adiponectin, leptin, resistin, and intercellular adhesion molecule-1 (ICAM-1) were measured using DuoSet ELISA development kits (R&D Systems, Abingdon, UK), and insulin and glucose were measured using Mercodia ELISA kits (Diagenics Ltd., Milton Keynes, UK). The homeostatic model assessment of insulin resistance (HOMA-IR) was used for the assessment of insulin resistance (25).

Cholesterol efflux capacity of HDL was determined using a previously validated method (26–28). The intra- and inter-assay coefficients of variation were 3.9% and 7.3%, respectively. Briefly, J774A.1 cells were incubated with 0.2 µCi of radiolabeled <sup>3</sup>H-cholesterol in RPMI 1640 medium with 0.2% BSA at 37°C in a 5% carbon dioxide humidified atmosphere. ABCA1 expression was upregulated using 0.3 mM C-AMP [8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt] for 4 h and the cells incubated with 2.8% (v/v) apoB-depleted serum using polyethylene glycol (PEG MW8000) for 4 h. The cell medium was then collected, and the cells were dissolved in 0.5 ml 0.2 N NaOH to determine radioactivity. Cholesterol efflux was expressed as the percentage of radioactivity in the medium from the radioactivity in the cells and medium collectively:

$$\text{Cholesterol efflux (\%)} = \frac{\text{Radioactivity in medium}}{\text{Radioactivity in cell} + \text{radioactivity in medium}} \times 100$$

### HDL isolation

Isolation of HDL was performed by immunoprecipitation of serum (600 µl) as previously described (29). Serum was applied to a column containing goat anti-human apoA-I antibody covalently coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (Academy Bio-Medical Co., Inc., Houston, TX). The column was then washed 10 times with TBS to remove proteins nonspecifically bound to the beads. HDL was then eluted using stripping buffer (0.1 M acetic acid) and immediately neutralized with 1 M Tris, pH 11 (final concentration, 0.11 M). An Amicon Ultra-15 centrifuge filter unit and an Amicon Ultra-0.5 ultracel-10 membrane were used for further concentration of the samples.

### HDL-associated miRNA

HDL-miRNA levels were assessed using real-time PCR TaqMan miRNA assays as previously described (29). Total RNA was isolated from HDL using QIAzol miRNAeasy kits (Qiagen, Hilden, Germany) and total RNA was quantified by spectrophotometry. Total RNA was purified and reversed transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) and 7.5 µl of the reverse transcription product was used for detecting specific miRNAs using TaqMan miRNA assay kits (Applied Biosystems). Values were normalized to both *Caenorhabditis elegans* (Cel) miR-39 (which was spiked into the samples after the QIAzol step) and HDL total protein concentration determined by BCA assay (Thermo Scientific). Results were expressed as  $2^{-[\text{Ct}(\text{miRNA}) - \text{Ct}(\text{Cel-miR-39})]}$ .

### Statistical analysis

Data analysis was performed using SPSS 24 (IBM, Armonk, NY). Statistical significance was set at two-tailed  $P < 0.05$ . Data were examined for normality based on

TABLE 1. Baseline characteristics of patients who underwent surgery and healthy participants

Characteristics	RYGB (n = 53)	Healthy (n = 18)
Age (years)	48.9 ± 8.7	43.3 ± 11.9
Female (n, %)	40, 75	14, 78
BMI (kg/m <sup>2</sup> )	49.4 (45.8–57.4)	22.0 (20.5–23.6)
Waist circumference (cm)	137.3 (128.5–150.5)	81.0 (71.1–94.0)
SBP (mm Hg)	131 (120–146)	127 (115–140)
DBP (mm Hg)	75.4 ± 13.8	73.0 ± 12.5
Total cholesterol (mmol/l)	4.26 (3.79–5.28)	5.26 (4.77–5.87)
Triglycerides (mmol/l)	1.46 (1.12–1.93)	0.81 (0.70–1.41)
HDL-C (mmol/l)	1.03 (0.87–1.32)	1.66 (1.40–1.88)
LDL-C (mmol/l)	2.44 (1.96–3.21)	3.29 (2.61–3.60)
ApoA-I (g/l)	1.26 (1.15–1.40)	1.55 (1.39–1.84)
ApoB (g/l)	0.80 (0.68–1.02)	0.85 (0.77–0.95)

Data are presented as mean ± SD and percent (n) or median (interquartile range). SBP, systolic blood pressure; DBP, diastolic blood pressure.

skewness and kurtosis, and Shapiro-Wilk's *W* test before analysis. Non-normally distributed variables were normalized prior to analysis using nature logarithmic (ln) transformation. To evaluate the effect of time on clinical characteristics and HDL-associated miRNA levels, one-way univariate repeated measures ANOVA was performed with time (baseline before surgery, and 6 and 12 months after surgery) as the within subject factor. Participants with missing data were excluded from the analysis. As there were a substantial number of participants with missing clinical characteristics at 6 months after surgery, clinical characteristics at baseline were compared with those at 6 and 12 months after surgery using individual paired *t*-test. Multiple testing correction was performed by Bonferroni correction for the two time-points, in which the threshold of *P* value for significance was <0.025. As the miRNA levels were highly correlated with each other, multiple testing corrections for the four miRNAs was not performed. Correlations between different HDL-associated miRNAs as well as

between changes in individual miRNAs and changes in other variables were performed using bivariate Spearman correlation coefficients.

## RESULTS

### Study sample and HDL-associated miRNA levels at baseline before surgery

A total of 53 patients with severe obesity and a comparison group of 18 healthy participants were included in this study. The clinical characteristics for both groups are summarized in Table 1. Twenty-nine patients had type 2 diabetes and 26 were statin-treated, which remained unchanged following surgery. Blood samples were available for HDL-associated miRNA measurements at baseline and 12 months after surgery for all patients, and at 6 months after surgery for 42 patients. There was no significant difference in age, sex, and BMI between those with and without samples at 6 months after surgery (*P* = 0.104, 0.305, and 0.949, respectively). As shown in Fig. 1, HDL-associated miR-126, miR-222, and miR-223 levels, but not HDL-associated miR-24 levels, were significantly elevated in patients with severe obesity compared with healthy participants. The elevation of HDL-associated miR-126, miR-222, and miR-223 levels remained significant after adjusting for age and sex in multivariable linear regression analysis (*P* = 0.006, 0.034, and 0.037, respectively).

HDL-associated miR-24, miR-126, and miR-223 levels correlated positively with total cholesterol, HDL-C, LDL-C, and apoB levels (*r* = 0.318 to 0.549, all *P* < 0.05, Table 2). Positive correlations were also observed with cholesterol efflux capacity (miR-24, miR-126, and miR-

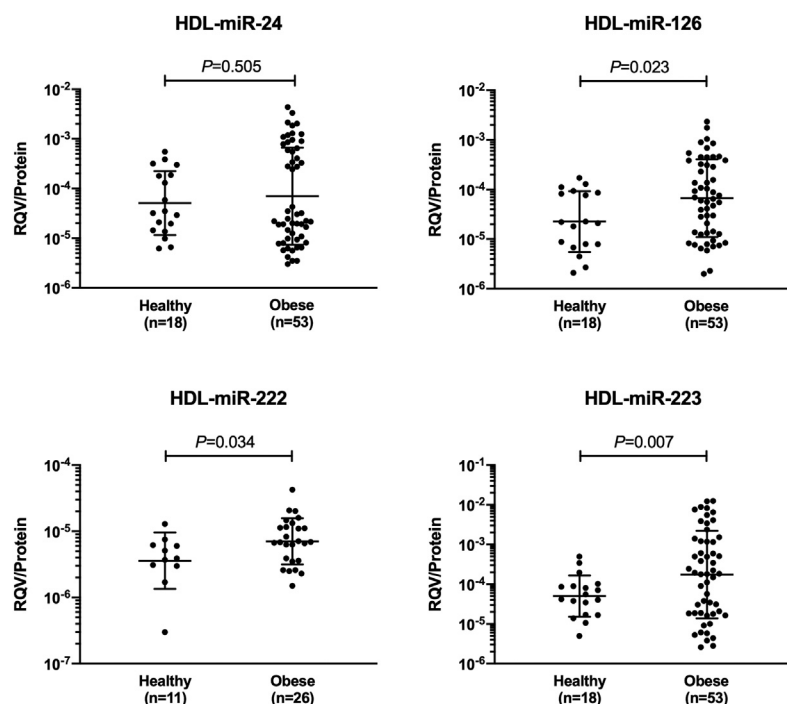


Fig. 1. Distribution of different HDL-associated miRNA levels in healthy and obese subjects. Data shown are geometric mean and SD with log scale on the y axis. Data were compared using independent *t*-test after log transformation.

TABLE 2. Spearman correlation between different HDL-associated miRNAs and clinical characteristics at baseline before surgery

Characteristics	miR-24	miR-126	miR-222	miR-223
Age (years)	-0.012	-0.127	-0.232	0.005
Height (m)	-0.019	-0.258	-0.074	-0.042
BMI (kg/m <sup>2</sup> )	-0.164	-0.122	-0.038	-0.241
Waist circumference (cm)	-0.259	-0.108	0.030	-0.256
SBP (mm Hg)	-0.115	-0.118	-0.124	-0.099
DBP (mm Hg)	-0.222	-0.272 <sup>a</sup>	-0.467 <sup>a</sup>	-0.320 <sup>a</sup>
Total cholesterol (mmol/l)	0.426 <sup>b</sup>	0.549 <sup>c</sup>	0.220	0.461 <sup>c</sup>
Triglycerides (mmol/l)	0.156	0.071	0.157	0.060
HDL-C (mmol/l)	0.373 <sup>b</sup>	0.459 <sup>c</sup>	-0.252	0.495 <sup>c</sup>
LDL-C (mmol/l)	0.318 <sup>a</sup>	0.462 <sup>c</sup>	0.288	0.350 <sup>a</sup>
ApoA-I (g/l)	0.127	0.142	-0.218	0.235
ApoB (g/l)	0.372 <sup>b</sup>	0.498 <sup>c</sup>	0.077	0.397 <sup>b</sup>
Cholesterol efflux capacity (%)	0.352 <sup>a</sup>	0.449 <sup>b</sup>	0.202	0.307 <sup>a</sup>
PON1 activity (nmol/ml/min)	0.340 <sup>a</sup>	0.427 <sup>b</sup>	-0.069	0.242
HbA <sub>1c</sub> (mmol/l)	-0.021	-0.079	-0.238	-0.085
Fasting glucose (mmol/l)	0.025	0.056	-0.213	0.113
Fasting insulin (mU/l)	0.087	0.142	-0.201	0.070
HOMA-IR, ratio	0.061	0.121	-0.336	0.094
HOMA-B, %	0.091	0.072	-0.043	-0.023
ICAM-1 (ng/ml)	-0.111	-0.382 <sup>a</sup>	-0.127	-0.281 <sup>a</sup>
Adiponectin (mg/l)	0.200	0.095	0.034	0.155
Leptin (ng/ml)	0.110	0.055	-0.021	0.002
Resistin (ng/ml)	-0.041	-0.090	0.388	-0.275 <sup>a</sup>

SBP, systolic blood pressure; DBP, diastolic blood pressure.

<sup>a</sup>*P* < 0.05.

<sup>b</sup>*P* < 0.01.

<sup>c</sup>*P* < 0.001.

223; *r* = 0.307–0.449; all *P* < 0.05) and PON1 activity (miR-24 and miR-126; *r* = 0.340 and 0.427, respectively, both *P* < 0.05, Table 2). Both HDL-associated miR-126 and miR-223 correlated inversely with ICAM-1 (*r* = -0.382 and -0.281, respectively, both *P* < 0.05,

Table 2). HDL-associated miR-126, miR-222, and miR-223 levels inversely correlated with diastolic blood pressure (*r* = -0.272 to -0.467, all *P* < 0.05, Table 2) and HDL-associated miR-223 inversely correlated with resistin (*r* = -0.275, *P* = 0.048, Table 2).

### Change in clinical characteristics after surgery

Table 3 shows the clinical characteristics of patients with severe obesity at baseline and 6 and 12 months after RYGB. As expected, median BMI decreased significantly from 49.4 kg/m<sup>2</sup> at baseline to 37.3 kg/m<sup>2</sup> at 6 months and 35.0 kg/m<sup>2</sup> at 12 months after surgery, which was accompanied by similar reductions in waist circumference. There were significant improvements in cardiovascular risk profile, which included significant decreases in blood pressure, triglycerides, apoB, HbA<sub>1c</sub>, fasting glucose, fasting insulin, and HOMA-IR, and an increase in HDL-C at 12 months after surgery. There was also a significant increase in cholesterol efflux capacity, PON1 activity, and adiponectin levels, and significant decreases in ICAM-1, leptin, and resistin levels 12 months after surgery. Some of the changes in clinical characteristics (systolic blood pressure, triglycerides, HbA<sub>1c</sub>, fasting glucose, fasting insulin, and HOMA-IR) and biomarker levels (cholesterol efflux capacity, ICAM-1, adiponectin, and leptin) were statistically significant at 6 months after surgery.

### Change in HDL-associated miRNA levels after surgery

Table 4 shows the median and interquartile ranges of different HDL-associated miRNAs at baseline and 6

TABLE 3. Clinical characteristics before surgery and 6 and 12 months after surgery

Characteristics	Before Surgery (n = 53)		6 Months After Surgery (n = 42)			12 Months After Surgery (n = 53)			Overall <i>P</i> <sup>b</sup>
	n	Estimate	n	Estimate	<i>P</i> <sup>a</sup>	n	Estimate	<i>P</i> <sup>a</sup>	
BMI (kg/m <sup>2</sup> ) <sup>c</sup>	53	49.4 (45.6–57.5)	43	37.3 (33.4–44.0)	<0.001	53	35.0 (30.3–38.5)	<0.001	<0.001
Waist circumference (cm)	53	142 ± 17	35	118 ± 16	<0.001	53	106 ± 14	<0.001	<0.001
SBP (mm Hg) <sup>c</sup>	53	131 (120–146)	38	126 (109–137)	0.006	52	119 (110–132)	<0.001	<0.001
DBP (mm Hg)	53	75.4 ± 13.8	38	71.2 ± 14.8	0.132	52	69.6 ± 10.5	0.002	0.041
Total cholesterol (mmol/l) <sup>c</sup>	53	4.26 (3.79–5.28)	42	4.41 (3.55–5.49)	0.520	53	4.43 (3.73–5.14)	0.943	0.720
Triglycerides (mmol/l) <sup>c</sup>	53	1.46 (1.12–1.93)	41	1.29 (1.01–1.60)	0.025	53	1.08 (0.84–1.41)	0.005	0.014
HDL-C/l) <sup>c</sup>	53	1.03 (0.87–1.32)	42	1.17 (0.96–1.44)	0.070	53	1.29 (1.04–1.43)	<0.001	<0.001
LDL-C/l) <sup>c</sup>	52	2.44 (1.96–3.21)	41	2.56 (1.82–3.57)	0.817	53	2.43 (1.97–3.20)	0.547	0.468
ApoA-I (g/l) <sup>c</sup>	52	1.26 (1.15–1.40)	43	1.20 (1.10–1.42)	0.093	52	1.23 (1.11–1.38)	0.391	0.243
ApoB (g/l) <sup>c</sup>	53	0.80 (0.68–1.02)	43	0.77 (0.63–0.99)	0.139	53	0.73 (0.61–0.86)	<0.001	0.002
Cholesterol efflux capacity (%)	53	12.94 ± 3.79	43	14.28 ± 3.95	0.025	53	16.03 ± 4.38	<0.001	<0.001
PON1 activity (nmol/ml/min)	53	67.0 (36.7–172.6)	43	69.7 (43.0–162.8)	0.683	53	83.0 (46.8–162.0)	0.009	0.007
HbA <sub>1c</sub> (mmol/l) <sup>c</sup>	51	45.4 (41.0–53.0)	44	38.4 (33.5–41.0)	<0.001	52	35.0 (32.3–37.5)	<0.001	<0.001
Fasting glucose (mmol/l) <sup>c</sup>	52	5.99 (5.13–6.78)	43	5.54 (4.68–6.19)	0.006	53	5.00 (4.66–5.82)	0.002	0.001
Fasting insulin (mU/l) <sup>c</sup>	52	18.44 (13.41–32.58)	42	9.46 (6.54–17.53)	<0.001	53	6.91 (4.75–12.29)	<0.001	<0.001
HOMA-IR, ratio <sup>c</sup>	52	5.71 (3.35–8.61)	42	2.66 (1.65–4.30)	<0.001	53	1.54 (1.06–2.94)	<0.001	<0.001
HOMA-B, % <sup>c</sup>	51	153 (64–258)	42	107 (72–168)	0.047	53	103 (69–159)	0.032	0.006
ICAM-1 (ng/ml)	53	199.0 (154.7–234.2)	42	163.7 (132.9–190.3)	0.009	53	136.8 (124.5–157.1)	<0.001	<0.001
Adiponectin (mg/l)	52	3.28 ± 1.46	42	4.39 ± 1.83	<0.001	53	5.97 ± 2.67	<0.001	<0.001
Leptin (ng/ml) <sup>c</sup>	52	70.3 (48.8–98.4)	42	25.8 (13.9–44.5)	<0.001	53	17.0 (8.5–36.0)	<0.001	<0.001
Resistin (ng/ml)	52	15.1 (10.8–17.6)	42	12.6 (9.3–16.2)	0.099	53	9.4 (6.3–13.9)	<0.001	<0.001

Data are presented as mean ± SD and percent (n) or median (interquartile range). SBP, systolic blood pressure; DBP, diastolic blood pressure.

<sup>a</sup>Data were compared with those at baseline using paired *t*-test.

<sup>b</sup>*P*s for change over time from baseline to 12 months after surgery (repeated measures ANOVA).

<sup>c</sup>*P*s were estimated using ln-transformed data.



TABLE 4. Comparison of HDL-associated miR-223, miR-24, miR-126, and miR-222 levels at baseline before surgery and 6 and 12 months after surgery

HDL-Associated miRNAs	Before Surgery (n = 53)		6 Months After Surgery (n = 42)			12 Months After Surgery (n = 53)			Overall $P^b$
	n	RQV/Protein ( $\times 10^{-5}$ )	n	RQV/Protein ( $\times 10^{-5}$ )	$P^a$	n	RQV/Protein ( $\times 10^{-5}$ )	$P^a$	
miR-24	53	2.5 (1.0–71.8)	42	11.1 (2.2–106.1)	0.033	53	4.3 (2.0–73.3)	0.004	0.023
miR-126	53	6.5 (1.3–35.4)	42	8.2 (1.2–33.7)	0.943	53	6.5 (1.9–3.9)	0.062	0.112
miR-222	26	0.7 (0.4–1.2)	17	0.8 (0.5–1.1)	0.016	26	1.3 (0.9–2.0)	0.003	0.002
miR-223	53	18.4 (1.9–131.1)	42	50.0 (3.5–232.6)	0.071	53	24.4 (3.6–325.8)	0.002	0.017

Data are presented median (interquartile range) in the unit of RQV/protein and were ln-transformed before analysis.

<sup>a</sup> $P$ s for change over time from baseline to 12 months after surgery (repeated measures ANOVA).

<sup>b</sup>Data were compared with those at baseline using paired  $t$ -test.

and 12 months after surgery, while Fig. 2 shows the corresponding geometric mean and standard deviation of these HDL-associated miRNAs.

There were significant increases in HDL-associated miR-24, miR-222, and miR-223 levels, but not the miR-126 level, at 12 months after surgery, in which the increase in HDL-associated miR-222 levels remained statistically significant at 6 months after correcting for multiple testing of two time-points (Table 4, Fig. 2). Although HDL-associated miR-24 showed a significant increase at 6 months, the increase was not statistically significant after correcting for multiple testing. For all these HDL-associated miRNAs, the change over time did not differ between groups divided based on gender, presence of type 2 diabetes, or statin therapy (all  $P$  for time interaction  $>0.05$ ).

All HDL-associated miRNAs showed strong positive correlation with each other at baseline ( $r = 0.461$ – $0.878$ , all  $P < 0.05$ ). Similar results were found at 6 and 12 months after surgery, although the correlation of HDL-

associated miR-222 with miR-126 and miR-223 was attenuated to nonsignificance at 12 months after surgery (Table 5).

Among the HDL-associated miRNA levels that showed significant increase at 12 months after surgery, increase in HDL-associated miR-24 levels correlated strongly and positively with increase in HDL-associated miR-222 and miR-223 levels ( $r = 0.732$  and  $0.577$ , respectively, both  $P < 0.05$ ). There was, however, no significant correlation between the changes in HDL-associated miR-222 and miR-223 levels (Table 6).

#### Correlation of changes in HDL-associated miRNA levels with changes in clinical characteristics

Although none of the HDL-associated miRNA levels correlated with BMI at baseline, changes in HDL-associated miR-24 levels at 12 months after surgery correlated positively with changes in BMI ( $r = 0.309$ ,  $P = 0.024$ , Table 7). In fact, a significantly larger reduction in weight was observed in patients with sub-median change in HDL-associated miR-24 levels at 12 months

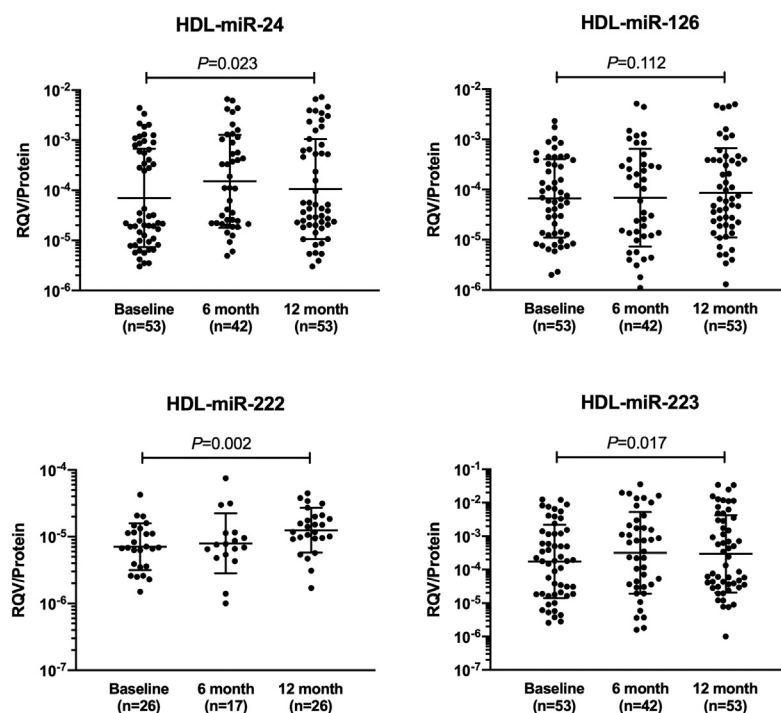


Fig. 2. Distribution of different HDL-associated miRNA levels in obese subjects at baseline before surgery and 6 and 12 months after surgery. Data shown are geometric mean and SD with log scale on the y axis. Data were compared using one-way univariate repeated measures ANOVA and participants with missing data at any time-points were excluded from the analysis.

TABLE 5. Cross-sectional bivariate Spearman correlation among different HDL-associated miRNAs at baseline and 6 and 12 months after surgery

HDL-Associated miRNA	miR-24	miR-126	miR-222	miR-223
Before surgery				
miR-24	—			
miR-126	0.824 <sup>a</sup>	—		
miR-222	0.610 <sup>a</sup>	0.837 <sup>a</sup>	—	
miR-223	0.762 <sup>a</sup>	0.878 <sup>a</sup>	0.461 <sup>b</sup>	—
6 months				
miR-24	—			
miR-126	0.875 <sup>a</sup>	—		
miR-222	0.709 <sup>c</sup>	0.387	—	
miR-223	0.824 <sup>a</sup>	0.859 <sup>a</sup>	0.700 <sup>c</sup>	—
12 months				
miR-24	—			
miR-126	0.800 <sup>a</sup>	—		
miR-222	0.646 <sup>a</sup>	0.354	—	
miR-223	0.810 <sup>a</sup>	0.817 <sup>a</sup>	0.363	—

<sup>a</sup>*P* < 0.001.

<sup>b</sup>*P* < 0.05.

<sup>c</sup>*P* < 0.01.

after surgery (Table 8). A larger weight reduction was also observed among patients with decreased miR-24 (*n* = 17) compared with patients with increased miR-24 (*n* = 36) at 12 months after RYGB ( $-17.9 \pm 5.7$  kg/m<sup>2</sup> vs.  $-15.9 \pm 5.8$  kg/m<sup>2</sup>, *P* = 0.248) although statistical significance was not achieved. Weight reduction did not differ between groups divided using median baseline HDL-associated miRNA levels (Table 8).

There were significant positive correlations between changes in HDL-associated miR-24 and miR-223 levels with cholesterol efflux capacity (*r* = 0.326, *P* = 0.027 and *r* = 0.349, *P* = 0.017, respectively, Table 7). The correlation between HDL-associated miR-222 and cholesterol efflux capacity did not achieve statistical significance (*r* = 0.453, *P* = 0.052, Table 7). There were also trends for positive correlations between HDL-associated miR-24, miR-222, and miR-223 with PON1 activity, which did not achieve statistical significance (*r* = 0.248, *P* = 0.074; *r* = 0.333, *P* = 0.097 and *r* = 0.242, *P* = 0.081, respectively). Similarly, a trend for negative correlation was observed between changes in ICAM-1 and changes in HDL-associated miR-223 (*r* =  $-0.265$ , *P* = 0.055) but not the other miRNAs.

Furthermore, HDL-associated miR-24 levels positively correlated with changes in triglyceride levels, and inversely with changes in adiponectin (*r* = 0.331, *P* =

TABLE 6. Bivariate Spearman correlation among changes in HDL-associated miR-24, miR-222 and miR-223 at 12 months after surgery

HDL-Associated miRNA	miR-24	miR-222	miR-223
6 months			
miR-24	—		
miR-222	0.732 <sup>a</sup>	—	
miR-223	0.577 <sup>a</sup>	0.354	—
12 months			
miR-24	—		
miR-222	0.744 <sup>a</sup>	—	
miR-223	0.757 <sup>a</sup>	0.342	—

<sup>a</sup>*P* < 0.001.

TABLE 7. Spearman correlation between absolute changes in HDL-associated miRNAs and absolute changes in clinical characteristics at 12 months after surgery

Characteristics	ΔmiR-24	ΔmiR-222	ΔmiR-223
ΔBMI (kg/m <sup>2</sup> )	0.309 <sup>a</sup>	0.238	0.259
ΔWaist circumference (cm)	0.175	0.146	0.206
ΔSBP (mm Hg)	0.285 <sup>a</sup>	0.232	0.089
ΔDBP (mm Hg)	0.223	$-0.030$	0.153
ΔTotal cholesterol (mmol/l)	0.034	0.142	$-0.126$
ΔTriglycerides (mmol/l)	0.331 <sup>a</sup>	$-0.069$	0.112
ΔHDL-C (mmol/l)	$-0.230$	$-0.025$	$-0.117$
ΔLDL-C (mmol/l)	$-0.023$	0.244	$-0.115$
ΔApoA-I (g/l)	0.003	0.077	0.060
ΔApoB (g/l)	0.085	$-0.111$	$-0.060$
ΔCholesterol efflux capacity (%)	0.326 <sup>a</sup>	0.453	0.349 <sup>a</sup>
ΔPON1 activity (nmol/ml/min)	0.248	0.333	0.242
ΔHbA <sub>1c</sub> (mmol/l)	0.230	$-0.123$	0.187
ΔFasting glucose (mmol/l)	0.023	$-0.215$	0.048
ΔFasting insulin (mU/l)	$-0.089$	0.022	$-0.057$
ΔHOMA-IR, ratio	$-0.060$	$-0.025$	$-0.044$
ΔHOMA-B, %	$-0.179$	0.433 <sup>a</sup>	$-0.185$
ΔICAM-1 (ng/ml)	$-0.147$	0.109	$-0.265$
ΔAdiponectin (mg/l)	$-0.336$ <sup>a</sup>	$-0.245$	$-0.246$
ΔLeptin (ng/ml)	0.033	0.099	$-0.089$
ΔResistin (ng/ml)	0.082	0.073	0.029

<sup>a</sup>*P* < 0.05.

0.015 and *r* =  $-0.336$ , *P* = 0.015, Table 7), while changes in HDL-associated miR-222 positively correlated with changes in homeostatic model assessment for beta cell function (HOMA-B) (*r* = 0.433, *P* = 0.035, Table 7).

## DISCUSSION

This is the first study to assess the changes in HDL-associated miRNAs following RYGB in patients with severe obesity. Here, we demonstrate an alteration in miRNA signature in patients with severe obesity following bariatric surgery in tandem with the

TABLE 8. Reduction in BMI according to HDL-associated miRNAs at baseline before surgery and their changes from baseline to 12 months after surgery

Subgroups	Reduction in BMI (kg/m <sup>2</sup> )	<i>P</i>
Before surgery		
miR-24		
<Median ( <i>n</i> = 26)	15.2 (12.4–20.1)	0.803
≥Median ( <i>n</i> = 27)	15.7 (13.2–19.7)	
miR-222		
<Median ( <i>n</i> = 26)	14.1 (11.6–19.6)	0.300
≥Median ( <i>n</i> = 27)	18.3 (13.1–20.2)	
miR-223		
<Median ( <i>n</i> = 26)	15.7 (12.8–20.5)	0.669
≥Median ( <i>n</i> = 27)	15.5 (13.0–19.5)	
Change at 12 months		
ΔmiR-24		
<Median ( <i>n</i> = 26)	18.2 (14.0–20.7)	0.015
≥Median ( <i>n</i> = 27)	14.0 (10.9–17.9)	
ΔmiR-222		
<Median ( <i>n</i> = 26)	16.1 (13.3–19.3)	0.762
≥Median ( <i>n</i> = 27)	13.4 (11.4–20.8)	
ΔmiR-223		
<Median ( <i>n</i> = 26)	15.7 (13.3–20.1)	0.355
≥Median ( <i>n</i> = 27)	14.5 (11.8–19.0)	

Data are presented median (interquartile range). *P* was estimated using Mann-Whitney U test.

expected reduction in BMI and improvements in metabolic and glycemic markers.

Following RYGB, there were significant increases in HDL-associated miR-24, miR-222, and miR-223, with positive correlations between miRNAs at baseline maintained at 12 months after surgery. The increase in HDL-associated miRNAs appears to indicate an overall increase in HDL function following surgery and this is supported by the positive correlations and trends observed with improvements in markers of HDL functionality such as cholesterol efflux capacity and PON1 activity. Both miR-222 and miR-223 are modulators of key components within the pathophysiology of cardiometabolic disease in obesity, and increases in these HDL-associated miRNAs could reflect enhancement of HDL's cardioprotective functions and explain at least some of the metabolic improvements that are observed following RYGB.

Multiple studies have suggested a role for miR-222 within the pathophysiological process underlying obesity and cardiometabolic disease. miR-222 is closely related to glucose metabolism and is thought to negatively regulate adipose tissue insulin sensitivity (5) with increased expression of miR-222 in circulation being described in obesity (7, 30), and within the adipose tissue of patients with diabetes and insulin resistance (5). Higher levels of HDL-associated miR-222 have also been previously reported in patients with familial hypercholesterolemia (14). In our study, we found a positive correlation between changes in HDL-miR-222 and HOMA-B, suggesting that it may have a role in improving glycemia after bariatric surgery. The higher levels of HDL-miR-222 in obesity and diabetes shown in previous studies may reflect a compensatory rise in response to the underlying metabolic derangement rather than the cause. This hypothesis is consistent with miR-222 being shown to improve hyperglycemia through proliferation of pancreatic  $\beta$  cells in a previous study using murine models (31). It is, however, important to point out that assessment of  $\beta$  cell function using static measures of insulin and glucose is suboptimal and is influenced by factors such as insulin action, alterations in energy balance, and marker alterations in glucose and insulin before and after surgery (32). Indeed, a reduction in HOMA-B was observed in our cohort despite improvements in all markers of glycemia and, therefore, require further study with dynamic testing. Also, interestingly, in contrast with our findings, a previous study of patients who underwent RYGB found a significant postsurgical reduction of circulating plasma miR-222 (7). One potential explanation for these discrepant results may be that, despite a reduction in overall circulating miR-222, the amount transported by HDL is increased due to the enhanced uptake capacity. Further studies will be required to further assess this.

miR-223 has an important role in the development and regulation of the immune system and is established

as a potent regulator of inflammatory processes (33). It has been previously associated with obesity with increased levels of visceral adipose tissue miR-223 being demonstrated (34). Increased adipose tissue inflammation and marked systemic insulin resistance have been shown in miR-223 knockout mice on a high-fat diet (35). Furthermore, the transfer of miR-223 from HDL has been shown to decrease ICAM-1 expression in endothelial cells (15). Indeed, a negative correlation between HDL-associated miR-223 and serum ICAM-1 levels at baseline and a negative trend between postsurgical changes were observed following RYGB. This provides support for enhanced HDL anti-inflammatory function following surgery, conferred in part through the transfer of miR-223. Furthermore, we have also demonstrated a positive correlation between HDL-associated miR-223 and cholesterol efflux capacity and a trend with PON1 activity, contributing further to an overall picture of enhanced HDL function after RYGB.

Although miR-223 has previously been shown to predict the response to a nonsurgical weight loss intervention with an 800–880 kcal/day hypocaloric diet (36), miR-223 expression did not differ between patients who achieved supra- and sub-median reductions in BMI following RYGB. Furthermore, in contrast to our post-RYGB observation, a reduction in HDL-associated miR-223 had been demonstrated following high-protein diet-induced weight loss in patients with obesity (29). This observed difference in impact on circulating miRNA can be explained by the difference in magnitude of reduction in weight and, therefore, adiposity following the dietary weight loss study (37) and this study. This is supported by both subcutaneous and omental adipose tissue being established sites of altered miRNA expression, including miR-223, following weight loss intervention (38, 39). There is likely also a difference in the impact on HDL functionality, particularly its transporting capacity between dietary and surgical weight loss. While cholesterol efflux capacity is shown to be increased post-RYGB in our study, studies on dietary weight loss are limited with no significant increase in cholesterol efflux capacity being noted following hypocaloric diet in the absence of exercise training (40).

Elevated levels of miR-24 have been reported in abdominal adipose tissue of patients with obesity, and are positively correlated with percentage body fat (10). Somewhat contrastingly, it has also been demonstrated that miR-24 has a role in modulating the expression of von Willibrand factor, where its levels are increased in type 2 diabetes when miR-24 levels are reduced through application of anti-miR-24, implicating a potential role in the risk of thrombotic cardiovascular events (41). In the same study, miR-24 was also shown to be downregulated in endothelial cells in response to hyperglycemia. It is therefore possible that the postsurgical increase in HDL-associated miR-24 observed in our cohort may


result from glycaemic improvement as well as enhancement of HDL function, and may contribute to a reduction in cardiovascular risk following RYGB. Interestingly, although both HDL-associated miR-24 and adiponectin levels increased after RYGB, a negative correlation was seen between the two. Similarly, despite contrasting post-RYGB changes, positive correlations were observed between changes in miR-24 and changes in BMI, triglyceride levels, and systolic blood pressure. In keeping with this, a significantly larger reduction in BMI was also observed in patients with decreased or smaller increase in miR-24 at 12 months. This may reflect the complex pathophysiological changes following RYGB, with differing relationships between miR-24 and changes in weight, adiposity, and glycaemia.

Despite increases after RYGB, both HDL-associated miR-222 and miR-223 were higher at baseline compared with healthy participants, with similar observations also noted with HDL-associated miR-126. Although the lower miRNA expression in healthy participants may seem unexpected given the post-surgical upregulation, there are two potential explanations for this observation. First, the higher miR-126 (7), miR-222 (30), and miR-223 (34) expressions in obesity are in keeping with previous studies, and we postulate that this is likely triggered by underlying metabolic derangement, which would be in line with the effect of miR-222 on glycaemia (31) and miR-223 on ICAM-1 (15) shown in previous studies. The increase in HDL-associated miRNA after surgery is in keeping with the improvement in HDL function, which may represent a dynamic process that drives metabolic improvements. It would be of great interest to see whether this upregulation of HDL-associated miRNA then reverts to the levels observed in healthy participants once the process of metabolic correction is completed. Second, statin therapy had been shown to upregulate both miR-222 and miR-223 expression (42), and a significant proportion of statin-use within our cohort with severe obesity is therefore likely to have contributed to the difference in miRNA expression.

Limitations to our study include the observational design and the small sample size, particularly within the control group. As only patients who underwent RYGB were included in our study, these findings may therefore not be extended to other weight loss procedures. Further studies with a larger study population including other metabolic surgical procedures would allow for comparison of surgical procedures and confirm the findings in our study.

In conclusion, severe obesity is associated with altered HDL-associated miRNAs, which is significantly changed following RYGB. The increase in expression of HDL-associated miRNAs following surgery may reflect an improvement in HDL function and may explain some of the cardiometabolic benefits observed following RYGB in severe obesity.

## Data availability statement

The data supporting the findings of this study are available in the article and from the corresponding author upon reasonable request. 

## Author contributions

Study concept and design were by F. T. and H. S. J. H. H., S. A., Z. I., and S. D. undertook patient recruitment and clinical assessments. K. L. O., J. H. H., L. F. C. T., and Y. L. performed laboratory analyses. J. H. H. and K. L. O. performed the data analyses and undertook interpretation of findings with F. T. and H. S. J. H. H. produced the first draft and the final version with F. T. and H. S. S. A., Z. I., S. D., B. J. A., A. A. S., P. N. D., and K.-A. R. provided critical review for important intellectual content.

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## Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

## Abbreviations

DBP, diastolic blood pressure; HbA<sub>1c</sub>, glycosylated hemoglobin; HOMA-B, homeostatic model assessment for beta cell function; HOMA-IR, homeostatic model assessment of insulin resistance; ICAM-1, intercellular adhesion molecule-1; miRNA, microRNA; PON1, paraoxonase-I; RYGB, Roux-en-Y gastric bypass; SBP, systolic blood pressure.

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