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PREDICTING TYPE 2 DIABETES REMISSION AFTER SLEEVE GASTRECTOMY USING CLINICAL DATA, CIRCULATING MICRORNA, AND MACHINE LEARNING APPROACH

Gladys Emmanuella Putri Wojciechowska

Supervised by **Prof. dr hab. n. med. Adam Jacek Krętowski**,
Clinical Research Center,
Medical University of Białystok

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This dissertation is prepared with thanks to tutor and supervisor

dr n. med. Łukasz Szczerbiński

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This dissertation is dedicated to my parents

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as well as my husband

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ABSTRACT

Bariatric surgery is an efficient treatment for the excess body weight in patients with severe obesity and resolving obesity-related comorbidities, including Type 2 Diabetes (T2D). Sleeve gastrectomy (SG) is the most common method among bariatric surgery procedures worldwide, including in Poland. SG is less complicated, safer, and delivers comparable weight-loss rates compared to other procedures. However, not all SG patients with T2D experience remission after surgery, so patient selection is valuable in clinic. Most prediction models were mainly built for procedures other than SG and used mainly clinical variables. There is increasing interest in using microRNAs (miRNAs) as biomarkers. Studies have shown differential changes of miRNA profile after bariatric surgery and associations between miRNA with weight loss after surgery. However, there are no studies so far on the predictive value of miRNA for T2D remission after bariatric surgery.

The aim of this doctoral dissertation is to profile pre-surgery serum miRNA from sleeve gastrectomy patients with T2D and develop prediction models using baseline clinical and miRNA data to predict T2D remission after surgery.

Before SG, clinical data and fasting serum samples were collected from 46 T2D patients. Serum miRNAs were profiled using the Serum/Plasma miRCURY LNA miRNA Focus PCR Panel (QIAGEN), and two patients were excluded due to sample hemolysis. Remission status was determined 12 months after SG. Six patients with unclear remission status were set aside for model evaluation. Model building was done with the remaining 38 patients. Variable selection was done using different approaches, including Least Absolute Shrinkage and Selection Operator (LASSO). LASSO was also used for model building. Prediction models were compared and then assessed in the validation set.

A total of 26 out of 38 patients achieved T2D remission 12 months after SG. The prediction model with only clinical variables misclassified two patients, which were correctly classified using miRNAs. Two miRNA-only models achieved an accuracy of one but performed poorly for the validation set. The best miRNA model was a mixed model (accuracy: 0.974) containing four miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) and four clinical variables (T2D medication, sex, age, and fasting blood glucose). These miRNAs are involved in pathways related to obesity and insulin resistance.

The results suggest that four serum miRNAs might be predictive biomarkers for T2D remission 12 months after SG, but further validation studies are needed.

KEYWORDS: Sleeve Gastrectomy; Type 2 Diabetes; microRNA; T2D remission; prediction

STRESZCZENIE

Chirurgia bariatryczna jest skutecznym sposobem leczenia nadmiernej masy ciała u pacjentów z otyłością olbrzymią i leczenia chorób współistniejących z nią związanych, w tym cukrzycy typu 2 (T2D). Rękawowa resekcja żołądka (SG) jest najczęstszą metodą wśród zabiegów chirurgii bariatrycznej na całym świecie, w tym w Polsce. SG jest mniej skomplikowana, bezpieczniejsza i zapewnia porównywalne wskaźniki utraty wagi, w porównaniu z innymi procedurami. Jednak nie wszyscy pacjenci chorujący na T2D, poddawani zabiegowi doświadczają remisji po operacji. Dlatego też prawidłowa selekcja pacjentów, którzy najbardziej skorzystają na zabiegu, jest tak istotna w praktyce klinicznej. Większość modeli predykcyjnych została zbudowana głównie dla procedur innych niż SG i wykorzystywała głównie zmienne kliniczne. Rośnie zainteresowanie wykorzystaniem cząsteczek mikroRNA (miRNA) jako biomarkerów. Badania wykazały zróżnicowane zmiany profilu miRNA po operacji bariatrycznej oraz związki między miRNA, a utratą masy ciała po operacji. Jednak jak dotąd nie ma badań dotyczących wartości predykcyjnej stężenia miRNA dla remisji T2D po operacji bariatrycznej.

Celem pracy doktorskiej jest opracowanie modeli predykcyjnych z wykorzystaniem wyjściowych danych klinicznych i profilu ekspresji miRNA w celu przewidywania remisji T2D po operacji.

W badaniu wykorzystano dane kliniczne i próbki surowicy pobranej na czczo od 46 pacjentów chorujących na T2D. Wykonano profilowanie cząsteczek miRNA w surowicy z wykorzystaniem panelu "Serum/Plasma miRCURY LNA miRNA Focus PCR Panel" (QIAGEN) i dwóch pacjentów wykluczono z powodu hemolizy próbki. Status remisji określono 12 miesięcy po zabiegu SG. Sześciu pacjentów z niejasnym stanem remisji zostało wykluczonych z oceny skuteczności modelu. Budowa modelu została wykonana z pozostałymi 38 pacjentami. Doboru zmiennych dokonano przy użyciu różnych podejść, w tym metody LASSO, (ang. Least Absolute Shrinkage and Selection Operator). Modele prognostyczne zostały porównane, a następnie ocenione w grupie walidacyjej.

Łącznie 26 z 38 pacjentów osiągnęło remisję T2D po 12 miesiącach od zabiegu SG. Model predykcyjny zawierający tylko zmienne kliniczne błędnie sklasyfikował dwóch pacjentów, którzy zostali prawidłowo sklasyfikowani przy użyciu modelu zawierającego miRNA. Dwa modele zawierające tylko miRNA osiągnęły dokładność równą jeden, ale wypadły słabo w zestawie walidacyjnym. Najlepszym modelem miRNA był model mieszany (dokładność: 0,974) zawierający cztery cząsteczki miRNA (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p i hsa-miR-21-5p) oraz cztery zmienne kliniczne (leki stosowane w leczeniu T2D, płeć, wiek i stężenie glukozy we krwi na czczo). Zidentyfikowane cząsteczki miRNA biorą udział w szlakach związanych z otyłością i insulinoopornością.

Wyniki sugerują, że zidentyfikowane cztery cząsteczki miRNA w surowicy mogą być biomarkerami predykcyjnymi dla remisji T2D 12 miesięcy po zabiegu SG. W celu potwierdzenia ich potencjału do zastosowania klinicznegopotrzebne są dalsze badania walidacyjne.

SŁOWA KLUCZOWE: rękawowa resekcja żołądka; cukrzyca typu 2; mikroRNA; remisja T2D; modele predykcyjne

Table of Contents

Abbreviations

Adj	Adjusted
ASMBS	American Society of Metabolic and Bariatric Surgery
BIL	Bilirubin levels
BMI	Body mass index
cDNA	Complementary DNA
CHOL	Cholesterol levels
CRP	C-reactive protein levels
Ct	Cycle threshold
CV	Cross-validation
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
F	Female sex
FDR	False discovery rate
FoxO	Forkhead box protein O
GLU_0	Fasting blood glucose levels
GLU_120	Plasma glucose levels measured at 120 minutes during OGTT
GLU_30	Plasma glucose levels measured at 30 minutes during OGTT
GLU_60	Plasma glucose levels measured at 60 minutes during OGTT
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein levels
HGB	Hemoglobin cell count
HIF-1	Hypoxia-inducible factor 1
INS_0	Fasting blood insulin levels
INS_120	Plasma insulin levels measured at 120 minutes during OGTT
INS_30	Plasma insulin levels measured at 30 minutes during OGTT
INS_60	Plasma insulin levels measured at 60 minutes during OGTT
KEGG	Kyoto Encyclopedia of Genes and Genomes
LASSO	Least absolute shrinkage and selection operator
LDL	Low-density lipoprotein levels
LNA	Locked nucleic acid
LOOCV	Leave-one-out cross-validation
M	Male sex
MAPK	Mitogen-activated protein kinase
miRNA	microRNA
mRNA	messenger RNA
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)
NA	Not available
NonRem	Non-remission group
OGTT	Oral glucose tolerance test
PBF	Percentage of body fat
PI3K-Akt	Phosphatidylinositol 3-kinase-protein kinase b
PLT	Platelet blood count
QC	Quality control
RBC	Red blood cell count
Rem	Remission group
RNA	Ribonucleic acid
RT	Reverse transcription
RYGB	Roux-en-Y gastric bypass
SG	Sleeve Gastrectomy
SNP	Single Nucleotide Polymorphism
T2D	Type 2 diabetes mellitus
TG	Triglyceride levels
WBC	White blood cell count

List of Publications

This dissertation is based on the following publications, which are referred to in the text by their Roman numerals:

Review articles:

I ***Langi G***, Szczerbinski L, Kretowski A. Meta-Analysis of Differential miRNA Expression after Bariatric Surgery. Journal of Clinical Medicine. 2019;8(8):1220. doi:10.3390/jcm8081220
IF = 4.242; MNiSW = 140

Original articles:

II ***Wojciechowska G***, Szczerbinski L, Kretowski M, Niemira M, Hady HR, Kretowski A. Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study. Obesity. 2022;30(2):435-446. doi:10.1002/oby.23342
IF = 5.002; MNiSW = 100

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PhD Candidate's academic achievements

The PhD Candidate is an author and co-author of 7 original articles and review papers, as of February 2022.

Article type	**Number**	**Impact Factor**	**MNiSW points**
Articles included in the dissertation	2	9.244	240
Articles not included in the dissertation	5	53.645	440
Conference presentations	6	NA	NA
Summary	13	62.889	680

Introduction

Obesity, defined as BMI of ≥30 kg/m^2, is a chronic disease with multiple health consequences[1]. Globally, about 604 million adults had obesity in 2015. The global prevalence of severe obesity, defined as BMI ≥40 kg/m^2 or ≥35 kg/m^2 in the presence of comorbidities[2], was 0.64% (0.46–0.86) in men and 1.6% (1.3–1.9) in women[3]. The health consequences of obesity are significant. Studies have reported over 230 comorbidities and complications of obesity, including hypertension, cardiovascular diseases, and type 2 diabetes mellitus (T2D)[4]. Obesity is currently the number one cause of preventable disease and disability, surpassing smoking[4].

Specifically for T2D, increasing body weight is strongly associated with increased T2D risk[5–9]. Obesity can be attributed to more than 80 percent of T2D cases and is related to many diabetes-related deaths[4]. A study spanning over thirty years in the US found that BMI accounted for a 50% and 100% increase in T2D prevalence in men and women, respectively[10]. BMI contributed more than age and race/ethnicity to the increase in T2D prevalence[10]. Additionally, nurses with a baseline BMI of >35 kg/m^2 had a 100-fold increased risk of incident T2D over 14 years compared to nurses with BMI <22 kg/m^2[11]. So, it is not surprising that weight loss prevents progression to T2D and even T2D remission[12]. Even modest weight loss improves glycemic control in patients with T2D[12].

There are many different approaches to obesity therapy, such as dietary changes, increased physical therapy, and pharmacologic therapy[2]. However, those with severe obesity require aggressive treatment for effective weight loss, including multi-component behavioral intervention and bariatric surgery[2].

Long-term weight loss at a great magnitude can be achieved with bariatric surgery, which is a collective term for surgical methods to treat obesity[2]. Bariatric surgery is reserved for those with a BMI ≥40 kg/m2, or a BMI of ≥ 35 kg/m2 with at least one serious comorbidity, who

have not achieved weight loss with other therapies[13]. Weight loss from bariatric surgery can be significant, reaching 40% weight loss at 12-18 months post-surgery[2]. Bariatric surgery may also alter the body's adipose tissue "set point", resulting in long-term weight loss and low recidivism rates (the regaining of lost weight)[2]. Additionally, bariatric surgery may reduce obesity-related comorbidities[14–16] and prevent obesity-related diseases[17]. The Swedish Obese Subjects study reported a reduction in incident rates of T2D, hypertension, and dyslipidemia in the bariatric surgery group compared to the conventional weight loss treatment group after 10-20 years of follow-up[16]. In the case of T2D, glucose control via surgical treatment was reported to be better than medical therapy [18–21]. Recently, bariatric surgery has been endorsed as a treatment for obese diabetic patients by the International Diabetes Federation, American Diabetes Association, and American College of Surgeons[13].

Bariatric procedures can be grouped into three methods: restriction, malabsorption, and a combination of both[22]. Restrictive procedures limit the stomach's capacity through different approaches. The most common restrictive procedure is sleeve gastrectomy (SG). This method also has an additional hormonal effect on hunger control where SG decreases ghrelin levels and increases GLP-1 and PYY levels, thus promoting less hunger[22,23]. Malabsorption methods modify the small intestine to decrease the effectiveness of nutrient absorption. Although malabsorption methods deliver superior weight loss, these methods cause significant malnutrition and micronutrient deficiencies[22]. Other methods, such as Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion with duodenal switch, are both restrictive and malabsorptive. In RYGB, the effects are delivered through a small gastric pouch and small bowel reconfiguration combination.

Globally, the top two bariatric surgery methods are RYGB and SG. In 2003, RYGB was performed at about 65% of all bariatric procedures, but the percentage decreased to 47% in 2011[22,24–26]. In 2016, SG became the most commonly performed procedure globally, including

Poland[26–29]. SG is a less complicated procedure, is less drastic than other methods[14,30–32], and delivers comparable weight loss rates[13,15,30]. However, SG has lower T2D remission rates compared to RYGB[14,31]. Long-term T2D remission for SG was relatively low in two studies: 35.3% for Taiwanese patients and 28% for American patients after five years of surgery[31,32], but another study reported a higher T2D remission rate of 66% five years after SG[33]. Therefore, identifying patients who can benefit the most from SG is valuable for effective treatment.

Different prediction models have been developed to predict T2D remission after bariatric surgery [31,34–40]. These models use clinical predictors, such as age, sex, BMI, HbA1c, T2D medication, T2D duration, and fasting glucose levels[31,34–40]. However, most models were developed using cohorts of surgery methods other than SG or a limited number of SG patients[34]. A 2019 study found that these models overestimated diabetes remission in SG patients with varying degrees[34]. Better prediction models are needed for SG patients. The inclusion of biomarkers might improve diabetes remission models, especially since molecular biology technologies are becoming more affordable and frequently used in clinics.

There is increasing interest in using biomarkers as predictive variables for bariatric surgery outcomes but still limited for T2D remission. A study demonstrated a significant weight loss difference after SG between different genotypes of the Single Nucleotide Polymorphism (SNP) rs9930506 on an obesity-associated gene (*FTO*)[41]. However, a study on another SNP on *FTO* (rs9939609) did not observe any associations with weight loss after SG[42]. Similarly, a genetic risk score of SNPs related to BMI and waist/hip ratio did not predict weight loss after obesity surgery (RYBG and SG)[43]. To the author's best knowledge, only one genetic biomarker study for T2D remission has been done: a 2016 study used structural genetic variants as predictive biomarkers for T2D remission after RYGB[44]. Epigenetic factors are yet to be evaluated but are interesting potential biomarkers. Epigenetic machinery, such as DNA methylation, histone modifications, and non-coding RNAs can respond to external

environmental cues by altering gene expression levels without changing the DNA sequence. One particular epigenetic factor that is gaining interest is microRNAs (miRNAs). These small non-coding RNAs (21–22 nucleotides) are important for regulating gene expression post-transcriptionally. Single-stranded miRNA binds to a complementary target messenger RNA (mRNA) to disrupt translational processes[45–49]. A single miRNA can have multiple targets and regulate many different biological pathways[50–52]. Studies have reported miRNAs that regulate obesity-related pathways[53–60], and miRNA dysregulation is linked to obesity and its comorbidities[61–65]. Additionally, serum miRNAs are highly stable and resistant against harsh conditions, such as ribonuclease A digestion, frequent freeze-thaw cycles, and pH changes[66], making miRNAs promising biomarkers for clinical use.

In recent years, good associations between microRNAs (miRNAs) and bariatric surgery outcomes were reported[67,68]. Recent studies demonstrated a significant change in serum miRNA expression before and after RYGB in T2D patients[69–71]. A total of 17 animal model and human studies have been done on miRNA and bariatric surgery by 2019[68]. There are some common findings despite differences in study design, surgery procedures, and profiling methods. Fourteen miRNAs had the same direction of modulation after surgery in at least two studies (downregulated: hsa-miR-93-5p, hsa-miR-106b-5p, hsa-let-7b-5p, hsa-let-7i-5p, hsa-miR-16-5p, hsa-miR-19b-3p, hsa-miR-92a-3p, hsa-miR-222-3p, hsa-miR-142-3p, hsa-miR-140-5p, hsa-miR-155-5p, rno-miR-320-3p; upregulated: hsa-miR-7-5p, hsa-miR-320c)[68]. A recent publication in 2022 reported good predictive performance on preoperative serum ratios of hsa-miR-328-3p/hsa-miR-31-5p or hsa-miR-181a-5p/hsa-miR-31-5p and BMI on excess weight loss > 55% at six months after SG or gastric bypass surgery[72]. However, the predictive value of miRNAs for T2D remission has not been explored before.

High-throughput profiling methods have made detecting hundreds of biomarkers from a biological sample easier, but the number of study participants is often small. This high-

dimensional situation is problematic for traditional statistical models[73]. One approach to handling such situations is regularization, such as LASSO (least absolute shrinkage and selection operator)[73–75]. LASSO adds a shrinkage penalty to least square estimates, and the shrinkage penalty is tuned using the parameter λ[74]. When $\lambda = 0$, the penalty term has no effect, and the regression will produce the least squares estimates[74]. When λ is large enough, some coefficient estimates will be equal to zero, thus performing variable selection[74].

Selecting a good value for LASSO's tuning parameter is important and is done using cross-validation. Cross-validation is also used to estimate test set error when the number of samples is too small for the typical validation set approach[74]. Typically, samples are separated randomly into a training set and a test or validation set. However, this creates a situation where only a subset of observations (the training set) is used to build the model. Statistical methods tend to perform worse when trained on fewer observations[74].

Additionally, the test error rate can be highly variable, depending on the training-test split process[74]. Cross-validation addresses these two issues, and there are two types of cross-validation: leave-one-out cross-validation (LOOCV) and k-fold cross-validation (k-fold CV). LOOCV sets aside a single observation as the validation set, and the remaining observations are used to train the model. The procedure is repeated for n times (n = number of observations or patients), and the final error rate estimate is the average error rate from each round[74]. So, LOOCV uses as many observations as possible for building the model, but the process can be computationally expensive.

An alternative to LOOCV is k-fold CV. This approach randomly divides the observations into k groups equally and randomly. One group, or fold, is set aside for validation, while the remaining observations are used to build the model. The method is repeated k times (k = number of folds or groups) then the error rates are averaged to get the final error rate estimate[74].

Aims

This doctoral dissertation studies the predictive value of pre-surgery clinical variables and serum miRNAs for predicting T2D remission 12-months after SG. A machine learning approach was used to select variables, build, and evaluate prediction models.

A meta-analysis of microRNA profiling studies for bariatric surgery has been described in the review article (I). At the time of publication, there were no miRNA studies for predicting bariatric surgery outcomes. Thus, the presented articles are focused on describing miRNA profile changes after bariatric surgery. Despite differences in study design, some common findings have been described in the Introduction section.

Prediction modeling using microRNAs and clinical data was reported previously by the PhD candidate in the original article (II). The authors aimed (1) to profile pre-surgery serum miRNA from sleeve gastrectomy patients with T2D and (2) to develop prediction models using baseline clinical and miRNA data to predict T2D remission after surgery. Clinical variables and miRNAs' predictive value were described, compared, and presented in the article (II).

Materials and methods

Study participants

Figure 1 illustrates an overview of the study design. Initially, between 2016 to 2019, 321 Polish patients with obesity were recruited to the Bialystok Bariatric Surgery Study (BBSS; ClinicalTrials.gov Identifier: NCT04634591). A subset of 46 patients with T2D (based on the American Diabetes Association criteria[76], which matches the Diabetes Poland criteria[77]) underwent Sleeve Gastrectomy and had follow-up data 12 months post-surgery. The inclusion criteria for surgery was BMI >= 40 kg/m2 or BMI >= 35 kg/m2 with comorbidities. The exclusion criteria include prior bariatric surgery, substance abuse, uncontrolled psychiatric illness, expected lack of compliance, or advanced cancer. Baseline clinical data and fasted serum samples were collected two to four weeks prior to surgery. Two patients were then excluded due to hemolysis observed in serum sample. T2D remission status was determined using the American Society of Metabolic and Bariatric Surgery (ASMBS) Criteria[78], based on T2D medication status, HbA1c, and fasting glucose 12 months after surgery. Binary remission status was created: patients with complete and partial remission were grouped into Remission, while patients with improvement, unchanged, and recurrence were grouped into Non-remission. Six patients had unclear remission status due to missing information post-surgery. These six patients were held out for model evaluation. The remaining 38 patients with clear remission status were used for variable selection and building classifiers. All participants provided informed consent before the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical University of Białystok (project identification code: R-I-002/546/2015)[79]

Sample preparation and miRNA extraction

Serum miRNA profiling was done from baseline (before the surgery) serum samples. Blood samples were collected in Sarstedt S-Monovette tubes (Sarstedt, Inc., Nümbrecht, North Rhine-Westphalia, Germany) with separator gel. The samples were allowed to clot for at least 30 minutes and were then centrifuged for 10 minutes at 2,500 rpm. Serum samples were immediately stored at −80°C until use.

RNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (QIAGEN, Hilden, Germany). Three RNA spike-ins (UniSP2, UniSP4, and UniSP5) were added to the kit's "RPL buffer" as RNA isolation controls. Serum volumes of 200 μL were used for isolation, and 20 μL of nuclease-free water was used for elution. A no-template sample (nuclease-free water) was also included to evaluate RNA isolation quality.

Quality control of miRNA extraction

The miRCURY locked nucleic acid (LNA) miRNA QC PCR Panel (QIAGEN) was used to assess miRNA quality, monitor complementary DNA (cDNA) synthesis, evaluate hemolysis, and assess polymerase chain reaction (PCR) efficiency. For this quality control (QC) panel, two μL of miRNA elute was used for ten μL of reverse transcription (RT) reaction using the miRCURY LNA RT Kit (QIAGEN). Two spike-ins were used for cDNA synthesis (UniSp6 and cel- miR-39). A total of 1.5 μL of cDNA was used for the QC panel. Two samples were later excluded because of hemolysis (final n = 44), as indicated by a difference in cycle threshold (Ct) values between hsa-miR-23a-3p and hsa-miR-451a of more than five[80]. PCR was done using the Roche LightCycler 480 Instrument (Roche, Basel, Switzerland) with SYBR Green dye.

miRNA profiling

The Serum/Plasma miRCURY LNA miRNA Focus PCR panel (Qiagen, CA, USA) was used for profiling, and PCR was done using the Roche LightCycler 480 Instrument with SYBR

Green dye. Profiling was done with a 96-well plate format. For this panel, four μL of miRNA elute was used for 20-μL cDNA synthesis, along with the two spike-ins for cDNA synthesis. The whole cDNA reaction was used for profiling. No-template controls were also used to evaluate background miRNA levels.

Data pre-processing

Raw miRNA data was pre-processed using the GeneGlobe Data Analysis Center (Qiagen; geneglobe.qiagen.com) to remove miRNAs below a Ct cut-off (Ct = 35) and apply interplate calibration. The processed data was then normalized using a global mean normalization, and there were no missing values for miRNAs.

A total of 43 baseline clinical variables were collected from patients, including blood biochemical parameters, blood morphology, and anthropometric measurements. A total of 26 clinical variables with missingness less than 10% were selected, and median imputation was used for missing values. The total number of clinical and miRNA variables was 205.

Variable selection

Ten unique variable sets were created: six sets with only miRNA variables, two with only clinical variables, and two sets with miRNA and clinical variables. Variables were normalized to obtain z scores.

Out of the 44 patients with miRNA data, six patients with unclear remission status were set aside for model evaluation. Therefore, 38 patients with clear remission status were used for variable selection and building classifiers.

Selecting serum miRNA variables

Six miRNA-only variable sets were created using different methods. One set contains all 179 miRNAs, another includes miRNAs from statistical testing, and four other sets contain LASSO-selected miRNAs.

miRNA selection using statistical significance and fold change

Fold change is the ratio of relative normalized miRNA expression between remission groups. Unpaired *t* tests were used to calculate *p* values. Four miRNAs with $p < 0.05$ and fold regulations of at least 1.5 were selected in this variable set.

Variable selection with LASSO

LASSO[75] with repeated 10-fold cross-validation (500 repeats) was built using all 179 miRNAs. A total of 20 miRNAs had nonzero coefficients, and they were ranked based on their importance. The top five, ten, fifteen, and all nonzero miRNAs were selected as four sets of LASSO-selected miRNAs.

Selecting pre-surgery clinical variables

Two sets of clinical variables were created: one set contains all 26 variables, and another has LASSO-selected variables. The LASSO selection process is the same as that for miRNAs. Repeated cross-validation with ten folds and 500 repeats was done using all 26 clinical variables, and then the resulting nonzero variables were selected.

Selecting serum miRNA and clinical variables

Two sets of miRNA and clinical variables were created: one set contains all available variables (205 variables), and another has LASSO-selected variables. The LASSO selection process was done using all variables with the same repeated cross-validation approach, and the nonzero variables were selected.

Prediction models

Ten LASSO models were built with each variable set. A leave-one-out cross-validation approach was used. Model performances were obtained using caret and epiR in R (R Foundation, Vienna, Austria), and models were compared based on their accuracy.

Model evaluation using six patients with unclear remission status

Remission labels were determined using available post-surgery clinical measures. Label decision was first made based on the discontinuation of T2D medicines. Then, HbA1c and fasting glucose information were considered. For prediction, we first applied the same median imputation and Z score scalar used for the model-building data. Then prediction was made using four models: one clinical-only model, one clinical and miRNA model, and two miRNA models. We then compared the prediction with their remission labels.

Statistical testing

Statistical testing was done to compare remission groups: chi-squared test and Kruskal-Wallis for categorical and continuous clinical variables, respectively, and unpaired Student's *t*-tests for miRNA profiles. Pearson correlation was calculated between clinical variables and miRNAs hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p. False discovery rate (FDR) was done for multiple testing correction for all statistical testing. For the correlation analysis, two plots for unadjusted and adjusted p values were made using ggcorrplot package in R (R Foundation).

Pathway analysis

Pathway analysis was done for miRNAs hsa-miR-32-5p, hsa-miR- 382-5p, hsa-miR-1-3p, and hsa-miR-21-5p. The DIANA miRPath version 3 software (http://www.microrna.gr/miRPathv3) was used to identify experimentally reported target genes and evaluate the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Results

Patient demographics and miRNA profiles

Six clinical variables were significantly associated with remission after SG: T2D medication; age; HbA1c; and fasting plasma glucose, as well as plasma glucose 30 and 60 minutes after oral glucose tolerance test (OGTT; Table 1). The remission group had a much lower proportion of patients taking diabetes medication before surgery (remission vs. non-remission: 12% vs. 83.3%, adjusted $p = 0.003$). The remission group was also significantly younger and had lower plasma glucose and HbA1c. Additionally, the remission group had higher plasma insulin and took fewer medications for chronic diseases, but the relationships were not significant after FDR (Table 1). A total of 179 circulating miRNAs were profiled from serum samples collected before surgery. None of the miRNAs was significant between remission and non-remission groups after multiple testing correction using FDR (Table 2). However, eight miRNAs had unadjusted $p \leq 0.05$, and four of them had a fold regulation of at least 1.5 (remission vs. non-remission group: upregulation = hsa-miR-382-5p, hsa-miR-409-3p; downregulation = hsa-miR-375, hsa-miR-1-3p, respectively).

Variable selection and modeling results

Ten variable sets were created based on different variable selection processes (Table 3). One set for miRNAs contained the four significantly differentially expressed miRNAs (GeneGlobe miRNAs: hsa-miR-382-5p, hsa-miR-409-3p, hsa-miR-375, and hsa-miR-1-3p). LASSO selected 20 out of 179 miRNAs after repeated cross-validation, including three out of 4 significant miRNAs (hsa-miR-382-5p, hsa-miR-375, and hsa-miR-1-3p). LASSO selected four out of twenty-six clinical variables: T2D medication, age, fasting plasma glucose, and sex. When all variables were provided, LASSO chose the same four clinical variables (T2D

medication, sex, age, and fasting plasma glucose) and four miRNAs (hsa-miR-1-3p, hsa-miR-21-5p, hsa-miR-32-5p, and hsa-miR-382-5p; Table 3, set 4).

Among the ten prediction models, classifiers with miRNA variables performed best. Models with 10 or 15 miRNAs achieved an accuracy of 1 (95% CI: 0.91-1; Table 3). Models with only clinical variables misclassified two non-remission patients, with an accuracy of 0.947 (95% CI: 0.82-0.99; Tables 3 and 4). When four miRNAs were added into the clinical model, patient 1 was correctly predicted but not patient 2 (Figure 2A; Table 4). Patient 2 was later correctly classified in the miRNA-only models, and no other misclassifications were found (Figure 2B; Table 4).

Evaluating prediction models using six patients with unclear remission status

Four classifiers were selected for evaluation: a clinical-only model, a mixed model with miRNA and clinical variables, and two miRNA-only models (Table 5). Models with clinical variables agreed the most with post-surgery data (Table 5). All models predicted patient A as non-remission, but post-surgery data suggested remission. All miRNA models predicted non-remission for patient C. Post-surgery values were within the remission group, but this patient had missing medication information. The miRNA-only models had an increasing disagreement with post-surgery data, indicating overfitting with the training data.

Evaluating the four predictive miRNAs (hsa-miR-32- 5p, hsa-miR-382-5p, hsa-miR-1-3p, hsa-miR-21-5p)

Four miRNAs that improved prediction for clinical models had significant correlations with glucose measures and HbA1c, but not with other clinical measures (Figure 3). The miRNA hsa-miR-382-5p was significantly positively correlated with HbA1c (r = 0.432) and plasma glucose (r = 0.485 for fasting and r = 0.359 for 30 minutes during OGTT). The relationship with fasting plasma glucose was maintained after FDR (Figure 3). There were other significant correlations between miRNA and clinical variables, but they were not significant after FDR; for example,

fasting plasma glucose with hsa-miR-32-5p ($r = -0.354$) and hsa-miR-21-5p ($r = -0.346$), as well as hemoglobin cell count with hsa-miR-21-5p ($r = -0.456$). The miRNA hsa-miR-1-3p was not significantly correlated with any of the selected clinical variables. The miRNA hsa-miR-32-5p was positively correlated with hsa-miR-1-3p ($r = 0.393$) and hsa-miR-21-5p ($r = 0.362$) but was no longer significant after FDR.

Pathway analysis was done using the DIANA miRPath version 3 software for these miRNAs. Three out of four miRNAs regulated 39 KEGG pathways, including 19 signaling pathways related to obesity and insulin resistance (Table 6). There was no information for hsa-miR-1-3p in this database. Within these 19 pathways, hsa-miR-32-5p regulated 253 genes, hsa-miR-21-5p regulated 330 genes, and hsa-miR-382-5p regulated 73 genes.

Table 1 – Baseline clinical data from patients measured before surgery

Variable	Remission	Non-remission	*p* value	*p* value (adj)
No. of patients	26	12		
Age at time of SG (years)	45.5 (38.25;54)	58 (56.25;65.25)	**0**	**0.004**
Diabetes medication before SG (n = 37)	3 (12%)	10 (83.3%)	**0**	**0.003**
Fasting blood glucose levels before SG (mg/dl)	132.5 (123.25;143.5)	154.5 (146.75;178.75)	**0**	**0.004**
Plasma glucose levels measured at 60 minutes during OGTT (n = 35) (mg/dl)	248 (224.75;282.25)	298 (283;315)	**0.002**	**0.012**
Haemoglobin A1c before SG (%)	6.4 (5.9;6.88)	7.1 (6.65;8.25)	**0.005**	**0.021**
Plasma glucose levels measured at 30 minutes during OGTT (n = 35) (mg/dl)	232.5 (194.5;239)	248 (235;271)	**0.011**	**0.042**
Plasma insulin levels measured at 30 minutes during OGTT (n = 35) (IU/ml)	128.08 (109.6;173.73)	74.08 (61.61;126.53)	**0.031**	0.091
Plasma insulin levels measured at 120 minutes during OGTT (n = 35) (IU/ml)	121.86 (82.54;243.84)	90.56 (52.09;105.67)	**0.045**	0.118
Number of chronic disease medications before SG (two or more)	12 (46%)	12 (100%)	**0.017**	0.216
Plasma insulin levels measured at 60 minutes during OGTT (n = 35) (IU/ml)	159.18 (146.12;231.63)	123.27 (73.27;168.24)	0.213	0.395
Plasma glucose levels measured at 120 minutes during OGTT (n = 35) (mg/dl)	194.5 (159.75;218.25)	225 (183;243)	0.186	0.395
High-density Lipoprotein levels before SG (mg/dl)	39.5 (35;45)	44.5 (37.75;53.5)	0.209	0.395
Low-density Lipoprotein levels before SG (mg/dl)	118.5 (97.12;146)	103.95 (82.83;133.75)	0.272	0.471
Bilirubin levels before SG (mg/dl)	0.47 (0.36;0.59)	0.38 (0.3;0.57)	0.307	0.499
Number of chronic diseases before SG (one or more)	19 (73%)	12 (100%)	0.084	0.546
Male sex	16 (61.5%)	10 (83.3%)	0.333	0.546
Fasting blood insulin levels before SG (IU/ml)	34.55 (29.53;53.57)	34.72 (27.55;43.52)	0.396	0.567
Cholesterol levels before SG (mg/dl)	190 (165.5;214)	184 (152.5;203.25)	0.387	0.567
Platelet blood count before SG (10^3/ul)	224 (203.75;263)	209.5 (190;283.75)	0.414	0.567
Percent body fat before SG (%)	47.1 (44.77;50.58)	49.4 (44.83;51.4)	0.46	0.594
C-reactive protein levels before SG (mg/l)	5.89 (2.62;10.53)	3.92 (1.69;10.24)	0.48	0.594
White blood cell count before SG (10^3/ul)	7.95 (6.65;9.07)	8.2 (7.5;8.62)	0.753	0.879
Red blood cell count before SG (10^6/ul)	4.98 (4.7;5.26)	5.07 (4.82;5.29)	0.777	0.879
Hemoglobin cell count before SG (g/dl)	14.35 (13.25;15.05)	14.45 (13.3;15.05)	0.826	0.895
Triglyceride levels before SG (mg/dl)	146 (131.25;231)	163 (126;225)	0.888	0.923
BMI before SG (kg/m2)	46.87 (43.33;50.77)	45.87 (43.65;52.75)	0.975	0.975

Note: Values show the median (first;third quartiles) or the number of patients and percentages. *P* values are shown for the χ^2 test (categorical variables) and Kruskal-Wallis test (continuous variables). Rows with $p < 0.05$ are shown in bold. Multiple testing correction was done using the false discovery method. If not otherwise stated, $n = 38$.

Table 2 – Comparing miRNA profiles between the Remission and Non-remission groups.

miRNA	Fold change	Fold regulation	*p* value	*p* value (adj)
hsa-miR-382-5p	1.800	**1.800**	**0.002**	0.420
hsa-miR-1-3p	0.620	**-1.610**	**0.015**	0.819
hsa-miR-375	0.630	**-1.580**	**0.017**	0.819
hsa-miR-409-3p	1.530	**1.530**	**0.037**	0.824
hsa-miR-28-5p	1.400	1.400	0.024	0.819
hsa-miR-28-3p	1.330	1.330	0.027	0.819
hsa-miR-27a-3p	1.300	1.300	0.027	0.819
hsa-miR-27b-3p	1.260	1.260	0.032	0.823
hsa-miR-376c-3p	1.540	1.540	0.054	0.851
hsa-miR-584-5p	1.410	1.410	0.070	0.851

Note: Top ten miRNAs with the smallest p-values are shown. The miRNAs with fold regulation of at least 1.5 and significant p-values are bolded.

Table 3 – Prediction models using ten different variable sets

	Variable set	Variables	Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
1	Top 10 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a	1 (0.91-1)	1 (0.74-1)	1 (0.87-1)
2	Top 15 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a, hsa-miR-140-5p, hsa-miR-543, hsa-miR-26a-5p, hsa-miR-27b-3p, hsa-miR-423-3p	1 (0.91-1)	1 (0.74-1)	1 (0.87-1)
3	Top 20 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a, hsa-miR-140-5p, hsa-miR-543, hsa-miR-26a-5p, hsa-miR-27b-3p, hsa-miR-423-3p, hsa-miR-151a-5p, hsa-miR-29b-3p, hsa-miR-1-3p, hsa-miR-30e-5p, hsa-miR-125a-5p	0.974 (0.86-1)	0.917 (0.62-1)	1 (0.87-1)
4	8 LASSO-selected miRNAs and clinical variables	T2D medication, age, hsa-miR-382-5p, hsa-miR-32-5p, fasting blood glucose, sex, hsa-miR-1-3p, hsa-miR-21-5p	0.974 (0.86-1)	0.917 (0.62-1)	1 (0.87-1)
5	All clinical variables	All clinical variables (26)	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
6	4 LASSO-selected clinical variables	T2D medication, age, fasting blood glucose, sex	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
7	All available variables	All miRNAs and clinical variables (205)	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
8	Top 5 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p	0.921 (0.79-0.98)	0.917 (0.62-1)	0.923 (0.75-0.99)
9	All miRNAs	All miRNAs (179)	0.842 (0.69-0.94)	0.583 (0.28-0.85)	0.962 (0.8-1)
10	GeneGlobe miRNAs	hsa-miR-409-3p, hsa-miR-382-5p, hsa-miR-375, hsa-miR-1-3p	0.789 (0.63-0.9)	0.5 (0.21-0.79)	0.923 (0.75-0.99)

Note: LASSO models ranked based on accuracy.

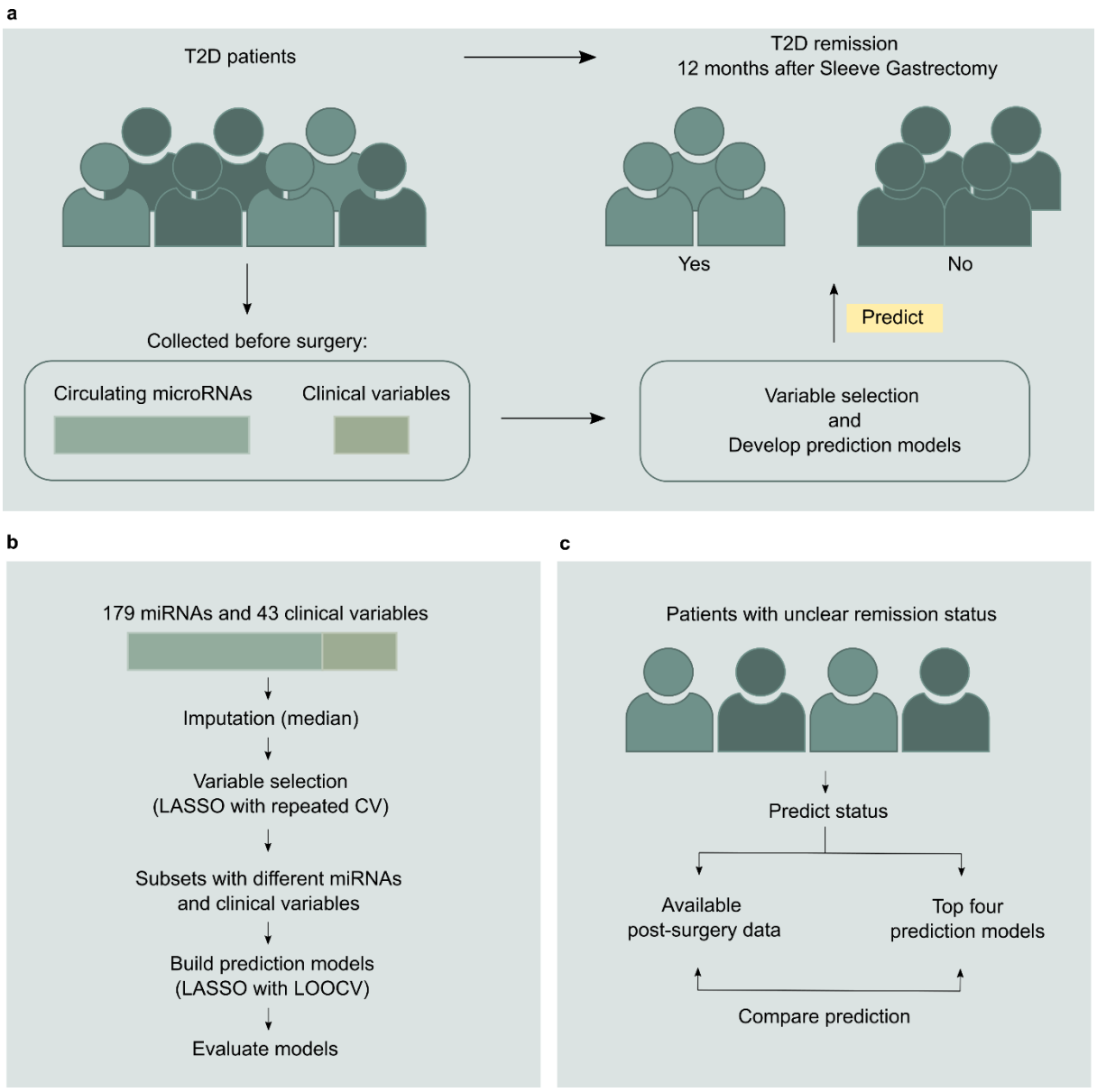

Figure 1 – Overview of study design. (A) General framework of patient stratification based on miRNAs and clinical variables. (B) The study's approach for variable selection and building prediction models with miRNAs and clinical variables. (C) The approach for evaluating the prediction models using patients with unclear remission status.

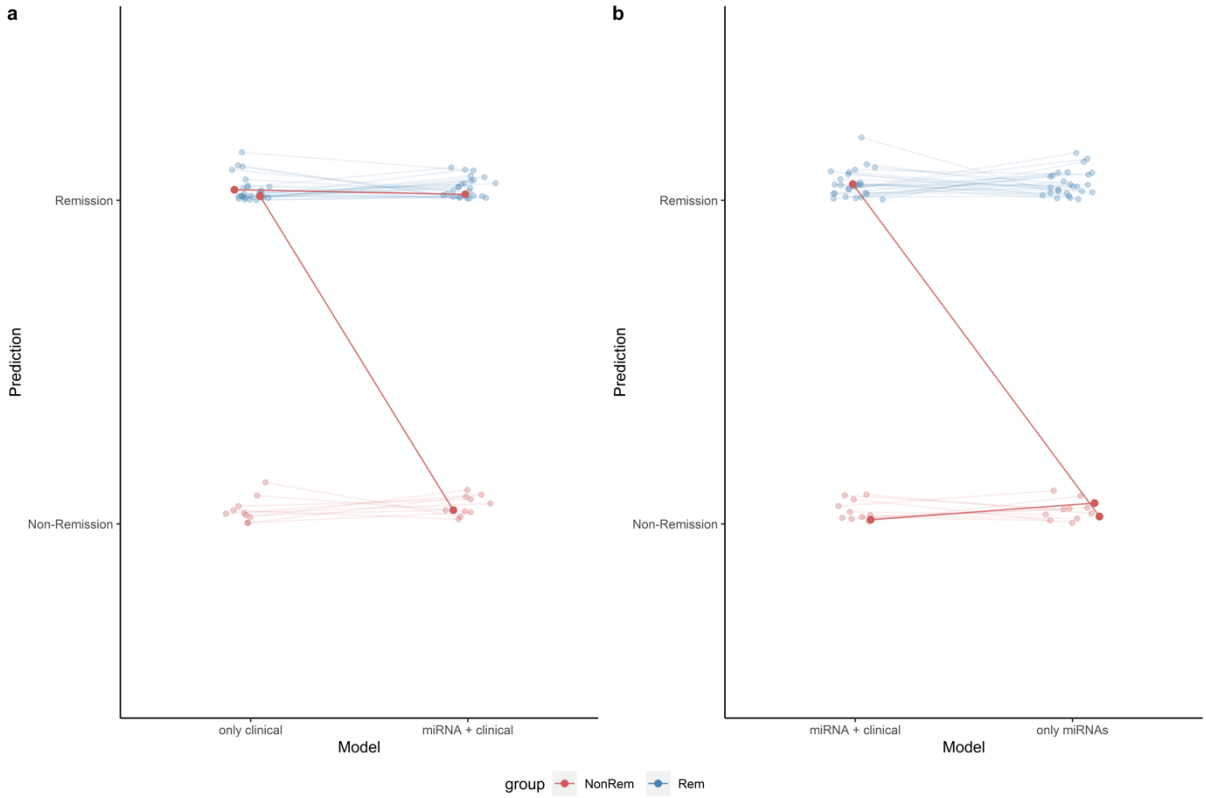

Figure 2 – Adding miRNA information increases model accuracy. (A) Two non-remission patients (highlighted as dark red) were misclassified in a model with four clinical variables (accuracy = 0.947). One patient was correctly classified when four miRNAs were added (accuracy = 0.974). (B) The second patient was correctly classified in a miRNA-only model (10 miRNAs, accuracy = 1). Other patients remained correctly classified.

Table 4 – Pre- and post-surgery characteristics of the two misclassified patients and predictions shown from LASSO models: with only clinical variables, with clinical and miRNA variables, and with ten miRNAs

		Pre-surgery				12-months post-surgery				Remission prediction		
Patient	**Sex**	**Age**	**T2D medication**	**Fasting plasma glucose**	**HbA1c**	**T2D medication**	**Fasting plasma glucose**	**HbA1c**	**Remission**	**Only clinical variables**	**Clinical and miRNAs**	**Only miRNAs**
1	M	63	No	193	8.1	Yes	117	6.3	No	Yes	No	No
2	M	66	No	135	6	No	128	5.9	No	Yes	Yes	No

Table 5 – Pre- and post-surgery characteristics of six unclear patients and predictions shown from post-surgery data and LASSO models: with only clinical variables, with clinical and miRNA variables, with ten miRNAs, and with 15 miRNAs

		Pre-surgery				12-months post-surgery			Remission prediction				
Patient	**Sex**	**Age**	**T2D medication**	**Fasting plasma glucose**	**HbA1c**	**T2D medication**	**Fasting plasma glucose**	**HbA1c**	**Based on post-surgery data**	**Only clinical variables**	**Clinical and miRNAs**	**Only 10 miRNAs**	**Only 15 miRNAs**
A	F	63	Yes	137	NA	No	NA	6.1	Yes	No	No	No	No
B	F	41	Yes	118	6.4	No	NA	5.7	Yes	Yes	Yes	Yes	No
C	M	43	Yes	127	6	NA	102	5.4	Yes	Yes	No	No	No
D	F	49	Yes	NA	7.6	No	NA	NA	Yes	No	Yes	Yes	Yes
E	F	54	Yes	135	7.5	Yes	NA	5.7	No	No	No	No	No
F	M	37	No	110	6.6	NA	95	4.9	Yes	Yes	Yes	No	No

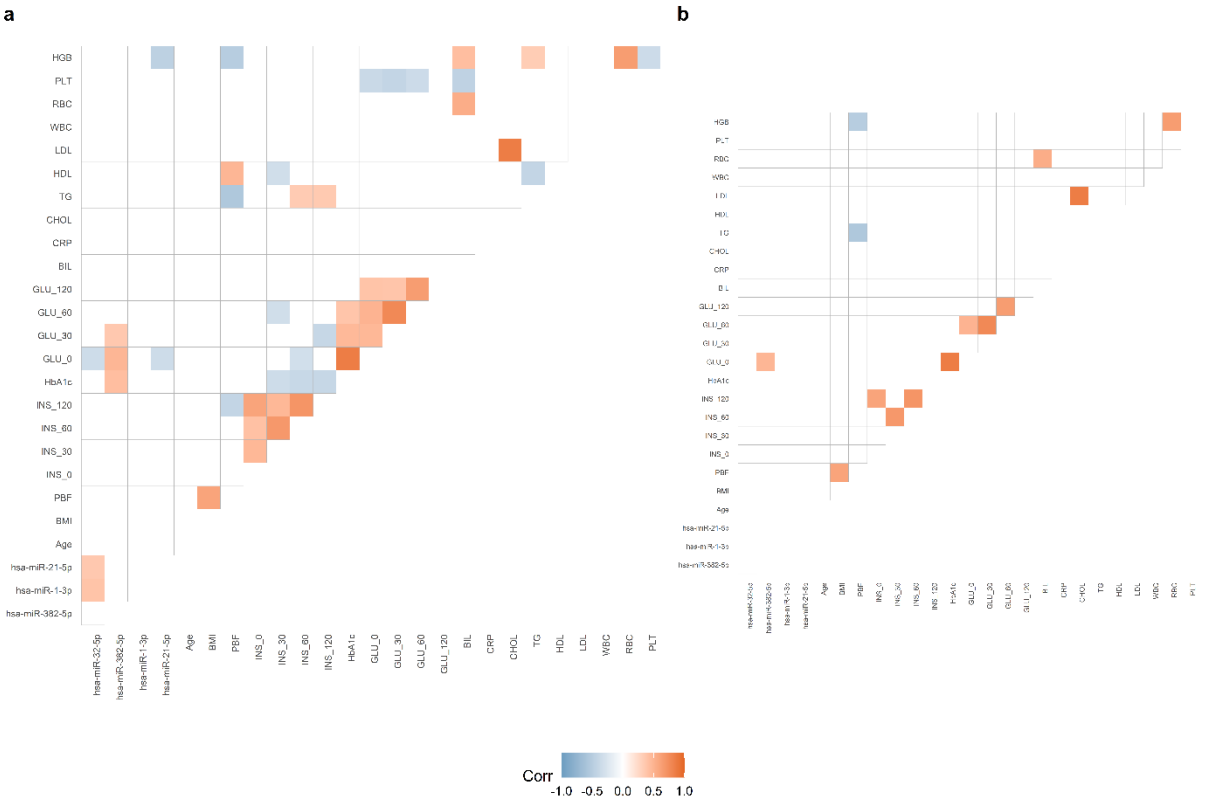

Figure 3 – Significant Pearson correlations between selected miRNA and clinical variables. The analysis was done using R packages Hmisc and ggcorrplot. Nonsignificant correlations based on (A) $p < 0.05$ and (B) adjusted $p < 0.05$ are set to blank. Red boxes indicate positive correlations, whereas blue boxes represent negative correlations.

Table 6 – Obesity- and insulin resistance-related pathways regulated by the four predictive miRNAs

No.	KEGG pathway	*p* value	No. of genes	No. of miRNAs
1	Thyroid hormone signaling pathway	9.22E-05	33	3
2	Lysine degradation	2.04E-04	15	2
3	FoxO signaling pathway	2.34E-04	41	3
4	Fatty acid elongation	0.0012	7	3
5	Prolactin signaling pathway	0.0014	21	3
6	Focal adhesion	0.0021	52	3
7	Adherens junction	0.0024	20	2
8	ECM-receptor interaction	0.0025	19	3
9	Valine, leucine, and isoleucine biosynthesis	0.0036	2	2
10	Regulation of actin cytoskeleton	0.0061	50	3
11	MAPK signaling pathway	0.0102	54	3
12	p53 signaling pathway	0.0102	21	3
13	mTOR signaling pathway	0.0133	18	3
14	Protein processing in endoplasmic reticulum	0.0140	39	3
15	Hippo signaling pathway	0.0157	32	3
16	Fatty acid degradation	0.0241	7	2
17	Endocytosis	0.0263	41	3
18	PI3K-Akt signaling pathway	0.0370	68	3
19	HIF-1 signaling pathway	0.0478	26	3

Discussion

This study evaluated miRNAs as predictive biomarkers and used machine learning approaches to select the most potential miRNAs and model building. We found that miRNAs might improve T2D remission prediction and are best used with clinical variables. We considered all miRNAs because statistically significant variables are not always good predictive variables[81].

Our clinical model, based on T2D medication, age, sex, and fasting plasma glucose, misclassified two non-remission patients. Both patients had similar pre-surgery conditions: they did not take any T2D medications before surgery and were in their 60s. Patient 1 needed T2D medicines after surgery; therefore, this patient had a non-remission status. In contrast, patient 2 seemed to be borderline partial remission after surgery. The second patient's fasting blood glucose was only three points above the upper limit for partial remission (≤125 mg/dL). Therefore, the clinical models correctly predicted that patient 2 could achieve remission after surgery.

Adding miRNA information improved prediction for patient 1. When the miRNAs hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p were added into the clinical model, patient 1 was correctly predicted to have non-remission, and patient 2 was still predicted as remission. When 10 or 15 miRNAs were used instead of clinical variables, both patients were classified as non-remission. Considering that patient 2 seemed to be borderline remission, the model with both clinical variables and miRNAs appears to be the most accurate.

Data from the six patients with unclear remission status also agree that clinical variables are essential in the prediction model. Models with clinical predictors matched the most with post-surgery information. Using only miRNAs increased the disagreement between prediction and post-surgery data. Although more samples are needed to confirm, this suggests that our

miRNA-only models are likely to be an overfit, and clinical variables should be kept in prediction models.

When available, miRNA information can help improve prediction for difficult patients and provide additional information to potentially imprecise clinical measures. Two out of four variables can be inaccurate in our clinical model: fasting plasma glucose and T2D medication information. We requested our patients to fast before the OGTT, but we could not guarantee that they genuinely fasted. T2D medication was obtained through the patient questionnaire, which is subject to recall bias.

Our prediction models can help decision-making for newly diagnosed T2D patients who qualify for SG. Some of our patients were unaware of their T2D status and were diagnosed during their pre-surgery visit, which might explain the relatively low percentageof patients taking T2D medication. We found that most patients who did not report taking T2D medication achieved remission after SG, but not everyone. SG is a simpler surgery procedure, but it has a lower T2D remission rate than Roux-en-Y gastric bypass (RYGB)[14,31]. Therefore, deciding on bariatric surgery for new T2D patients is not straightforward. Our prediction models might help predict whether SG would result in rapid T2D remission or not for these patients.

Previous prediction models, which used similar clinical variables, predicted remission in SG patients with sensitivity and specificity up to 0.92 and 0.83, respectively[34]. Our clinical model with four variables achieved sensitivity and specificity of 0.83 and 1, respectively, and adding four miRNAs increased the sensitivity to 0.917. Confirmation in external cohorts is vital to confirm the usefulness of our models.

To our knowledge, these four serum miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) have not been studied as predictive biomarkers for T2D remission after bariatric surgery. However, studies have reported associations between these miRNAs with obesity and T2D. The miRNA hsa-miR-382-5p is involved in cholesterol

homeostasis[82]. Plasma and serum levels of hsa-miR-21-5p are associated with T2D[83–85], as well as with obesity[61,86]. The miRNA hsa-miR-32-5p is also associated with T2D[87] and obesity[87,88]. Our pathway analysis identified 19 obesity- and T2D-related pathways regulated by these miRNAs, including the mechanistic target of rapamycin (serine/threonine kinase) (mTOR), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase-protein kinase b (PI3K-Akt), fatty acid elongation, and degradation pathways. The miRNA hsa-miR-1-3p has regulatory roles in cardiac muscle tissues and tumor suppressors in various cancers[89]. It is also dysregulated in pancreatic cancer patients[90].

These miRNAs have been studied in bariatric surgery patients to measure differential expression before and after surgery[68]. An RYGB study reported that plasma hsa-miR-32-5p and hsa-miR-21-5p were significantly reduced 9 and 12 months after surgery[71]. However, another RYGB study reported an increase of plasma hsa-miR-21-5p 12 months after surgery[61]. The miRNAs hsa-miR-1-3p and hsa-miR-382-5p were not significantly differentially expressed after RYGB[71]. It appears that predictive miRNAs do not need to be differentially expressed after surgery. However, these studies were primarily done in RYGB patients, and more studies with SG patients are needed.

Our study suggests that miRNAs could potentially predict T2D remission after the intervention. Our findings agree with a recent study identifying predictive miRNAs for T2D remission after diet intervention[91]. A recent study also reported predictive serum miRNAs for weight loss after bariatric surgery[72]. The set of miRNAs is different from these studies, which might reflect the study population. Our study focused on patients with T2D and obesity, whereas the other study's patients had BMI around 30 as well as coronary heart disease. Nevertheless, our study has limitations, including the small number of participants and limited external validation. Owing to sample size limitations, we simplified T2D and remission groups as dichotomous traits. Future studies could also investigate T2D subtypes based on β-cell

function and insulin resistance measures[92] and include other diabetes-related variables such as C-peptide and T2D duration. Some of the patients were unaware of their T2D status, so we could not obtain an accurate T2D duration for these patients. Patients with differing risk profiles might have different remission rates after surgery. Another limitation is thatwe focused on SG without comparing other surgery types like RYGB. RYGB has better long-term T2D remission rates[14,31], but only 8% of our BBSS patients underwent RYGB. Due to study size limitations, we could not compare miRNA's predictive value between these two surgery types adequately. It would also be interesting to see whether miRNAs can differentiate between the original ASMBS remission groups ("complete remission," "partial remission," "improvement," "unchanged," and "recurrence"). Additionally, we considered only 179 miRNAs that were included in the quantitative PCR profiling platformfor serum samples. Using larger profiling platforms such as small RNAsequencing might uncover more or better predictive miRNAs.

In conclusion, we identified four miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) that might complement clinical models in predicting T2D remission after SG. Further studies in much larger data are needed to confirm the utility of these serum miRNAs as predictive biomarkers. Due to the sample size, our study might be considered a pilot study. However, our results provide insights for future research. For example, the four serum miRNAs could be studied further to understand molecular subtypes of T2D that separate remission and non-remission patients.

Conclusions

1. Four serum miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) that might predict T2D remission 12 months after SG were identified.
2. These miRNAs are involved in pathways related to obesity and insulin resistance.
3. Biomarker research could focus on these miRNAs and validate them in larger cohorts to evaluate their predictive value.
4. The miRNAs could also be studied further to understand molecular subtypes of T2D patients with obesity.

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Review Article

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Meta-Analysis of Differential miRNA Expression after Bariatric Surgery.

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Review

Meta-Analysis of Differential miRNA Expression after Bariatric Surgery

Gladys Langi [1,*], Lukasz Szczerbinski [2] and Adam Kretowski [1,2]

[1] Clinical Research Centre, Medical University of Bialystok, 15-276 Bialystok, Poland
[2] Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Bialystok, 15-276 Bialystok, Poland
* Correspondence: gladys.langi@umb.edu.pl; Tel.: +48-8583-18000

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Abstract: Bariatric surgery is an efficient treatment for weight loss in obese patients and for resolving obesity comorbidities. However, the mechanisms behind these outcomes are unclear. Recent studies have indicated significant alterations in the transcriptome after surgery, specifically in the differential expression of microRNAs. In order to summarize the recent findings, we conducted a systematic summary of studies comparing microRNA expression levels before and after surgery. We identified 17 animal model and human studies from four databases (Ovid, Scopus, Web of Science, and PubMed) to be enrolled in this meta-analysis. From these studies, we identified 14 miRNAs which had the same direction of modulation of their expression after surgery in at least two studies (downregulated: hsa-miR-93-5p, hsa-miR-106b-5p, hsa-let-7b-5p, hsa-let-7i-5p, hsa-miR-16-5p, hsa-miR-19b-3p, hsa-miR-92a-3p, hsa-miR-222-3p, hsa-miR-142-3p, hsa-miR-140-5p, hsa-miR-155-5p, rno-miR-320-3p; upregulated: hsa-miR-7-5p, hsa-miR-320c). Pathway analysis for these miRNAs was done using database resources (DIANA-TarBase and KEGG pathway database) and their predicted target genes were discussed in relation with obesity and its comorbidities. Discrepancies in study design, such as miRNA source, bariatric surgery type, time of observation after surgery, and miRNA profiling methods, were also discussed.

Keywords: microRNA; bariatric surgery; Type 2 Diabetes; obesity

1. Introduction

Bariatric surgery was first performed in 1963 to help obese patients lose excess weight permanently [1]. Since then, numerous surgery procedures were developed with varying gastrointestinal effects [1,2]. For example, Sleeve Gastrectomy (SG) and Gastric Band are primarily restrictive to limit food intake and induce early satiety, while Roux-en-Y (RYGB) is both restrictive and malabsorptive [1,2]. All procedures result in significant weight loss (14.9%–28.4%) and minimal weight regain (1.4%–3.9%) years after surgery [3–5]. SG is more popular in Poland and the US [6–8] as it is a relatively less complicated procedure and has less surgery complications and reoperation compared to other procedures [4,9–14].

In addition to weight loss, many bariatric surgery patients demonstrate improvement in comorbidities of obesity post-operation. This includes recovery from Type 2 Diabetes Mellitus (T2DM) and achieving long-term favorable levels of cardiovascular risk factors, such as high-density lipoprotein cholesterol and hypertension [4,15,16]. Bariatric surgery is also associated with reduced risk of obesity-related cancers, such as colon and endometrial cancer [17]. Although this surgery is mainly reserved for class III obese patients (BMI > 40 kg/m^2), it is also recommended for less obese patients (BMI > 35 kg/m^2) with obesity comorbidities due to these beneficial outcomes [16,18].

In the case of T2DM, glucose control via surgical treatment was reported to be better than medical therapy [19–22]. Recently, bariatric surgery is endorsed as a treatment for obese diabetic patients by the International Diabetes Federation, American Diabetes Association (ADA), and American College of Surgeons [23]. However, T2DM remission rates appear to differ between surgery procedures, where RYGB has higher rates compared to SG and Gastric Band [4,24]. A study reported 60.2% of RYGB patients achieved diabetes remission after 7 years of surgery, compared to 20.3% for Gastric Banding [4]. Long-term diabetes remission for SG was quite low in two studies: 35.3% for Taiwanese patients and 28% for American patients after 5 years of surgery [13,24], but another study reported a remission rate of 66% five years after SG [25].

The mechanisms of these long-term beneficial effects after bariatric surgery is poorly understood. The acronym "BRAVE" is often used to describe RYGB physiological effects, which are to alter bile flow, restrict stomach size, alter anatomy/flow of nutrients, manipulate vagal, and modulate enteric gut and adipose hormones [26]. However, these effects cannot explain all the observed metabolic changes associated with RYGB [27]. Thus, researchers are looking into the molecular biological explanations for these metabolic effects after surgery. Novel biomarkers from these studies would not only help us understand the mechanisms behind bariatric surgery outcomes, but also serve as patient-level factors for predicting these outcomes [2].

Epigenetic changes due to surgery could give insight into these mechanisms. Epigenetic machinery, such as DNA methylation, histone modifications, and non-coding RNAs, can respond to external environmental cues by altering gene expression levels without changing DNA sequence. In recent years, there is an increasing interest in studying the relationship between epigenetic changes and bariatric surgery outcomes [28]. Among them are studies on microRNAs (miRNA) [29–45]. These small non-coding RNAs (21–22 nucleotides) are important for regulating gene expression post-transcriptionally. Single-stranded miRNA binds to a complementary target messenger RNA (mRNA) to disrupt translational processes [46–49]. A single miRNA can have multiple targets and regulate many different biological pathways [50–52]. Studies have reported miRNAs that regulate obesity-related pathways [53–60] and miRNA dysregulation is linked to obesity and its comorbidities [29,61–64].

Several miRNA studies have reported short- and long-term miRNA profile changes after bariatric surgery in various tissues of animal models and humans [29–45]. However, these studies typically have small sample sizes, use different profiling strategies, and study different types of bariatric surgery. There are no literature reviews so far on these surgery-related miRNAs. Thus, this study aims to identify consistently modulated miRNAs after bariatric surgery and report biological pathways that are predicted to be regulated by these miRNAs. These pathways may give insight into the molecular mechanisms behind weight loss and remission of obesity comorbidities after bariatric surgery.

2. Methods

2.1. Search Strategies

The databases for the literature search were chosen based on a recommendation of the optimal database combinations [65] and database accessibility in our institution. The four databases chosen were Ovid, Scopus, Web of Science, and PubMed. The databases were searched for studies profiling modulation of miRNA expression in bariatric surgery patients published up until 10 February 2019. The search terms were: (miRNA AND Bariatric surgery) OR (microRNA AND Bariatric surgery). For Ovid, an advanced search was used with the search terms. A basic search was used for the other databases.

2.2. Study Selection

During the screening stage, the exclusion criteria were: (1) non-English publications, (2) abstracts-only publications, case reports, comments, or reviews, (3) no report or comparison

of miRNA profiles before and after surgery or between bariatric surgery-operated animals and sham-operated animals, or (4) added another intervention post-surgery before miRNA assessment. Inclusion criteria were (1) animal and human studies, (2) any profiling method, (3) any bariatric surgery method, (4) any biological sample type, and (5) reported cut-off criteria for differentially expressed miRNAs. One full-text study was later excluded due to inconsistent reporting of the direction of miRNA expression.

2.3. Data Collection Process

The items collected from the full text and Supplementary Information followed a recent methods paper for meta-analysis of miRNAs studies [66]. The items were: first author, year of publication, digital object identifier (DOI) when available, study location, species of the samples, tissue types, bariatric surgery type, sample sizes, body mass index (BMI) before and after surgery, comparison groups, number of follow-up visits and their time after surgery, miRNA expression profiling platform, cut-off criteria of dysregulated miRNAs, and the list of differentially expressed miRNAs. Study authors were contacted to identify missing information on bariatric surgery type.

2.4. Synthesis of Results

Only miRNAs reported in at least two independent studies were retained for analysis. The selected miRNAs were grouped into three categories based on their consistency. The first group included miRNAs with consistent report of expression direction in two or more studies. The second group included miRNAs with some discrepancies in the direction, but two or more studies agreed on a direction. The third group included miRNAs with no consistent reports of expression direction. Pathway analysis was done only for the first two miRNA groups. Pathway analysis was done using DIANA miRPath v.3 to predict their target genes and KEGG pathways (http://www.microrna.gr/miRPathv3) [67].

3. Results

3.1. Selected Studies for the Meta-Analysis

A total of 164 articles were retrieved from Pubmed, OVID, Scopus, and Web of Science. After screening and assessment, 17 studies were selected for the meta-analysis (Figure 1). These studies have varying sources of miRNA, surgery type, and profiling strategies.

Most reported studies profiled miRNA levels before and after bariatric surgery in human patients (n = 13) (Table 1) [29–33,36–38,40–44], but some studied animal models (n = 4) [34,35,39,45]. The human studies were conducted in Caucasian [29–31,33,36–38,41–43] and Asian populations [32,40,44]. Most of these studies have small sample sizes (less than 30 participants; n = 15). However, a recent study in Austria profiled 58 patients [36] and a study in China profiled 124 patients [44]. The human studies mostly had more female patients, with the exception of one study [33], while animal studies investigated exclusively male animals.

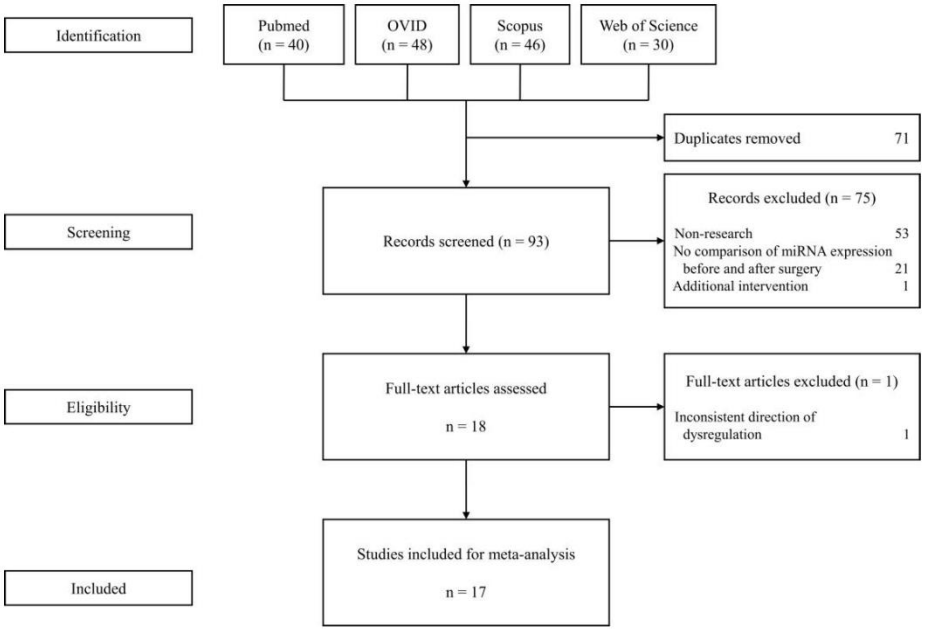

Figure 1. Flow diagram for study selection.

Table 1. The studies selected for meta-analysis.

Study	Year	Country	Sample Size	Sex (Males/Females)
Human studies (comparing before vs. after bariatric surgery)				
Ortega et al. [29]	2013	Spain	22	5/17
Alkandari et al. [30]	2018	UK	9	4/5
Atkin et al. [31]	2019	USA	29	9/20
Bae et al. [32]	2019	South Korea	12	Unspecified
Blum et al. [33]	2017	Israel	21	14/7
Hohensinner et al. [36]	2018	Austria	58	17/41
Hubal et al. [37]	2017	USA	6	0/6
Hulsmans et al. [38]	2012	Belgium	21	7/14
Lirun et al. [40]	2015	China	18	4/11
Mysore et al. [41]	2017	Spain	22	0/22
Ortega et al. [42]	2015	Spain	25	0/25
Ortega et al. [43]	2015	Spain	9	0/9
Wang et al. [44]	2018	China	124	46/78
Animal studies (comparing bariatric vs sham surgery)				
Guo et al. [34]	2016	China	35	35/0
Wei et al. [35]	2018	China	45	45/0
Kwon et al. [39]	2015	South Korea	25	25/0
Wu et al. [45]	2015	UK	12	12/0

The studies isolated miRNAs from different tissues: blood (plasma and serum) (n = 7) [29–31,33,36,40,45], circulating exosomes [32,37], monocytes [38], circulating endothelial progenitor cells [44], adipose tissue [41–43], liver [34,35,45], and hypothalamus [39] (Table 2).

Table 2. Tissue source and miRNA profiling strategies.

Study	Tissue	Isolation	Platform	Normalization
Human studies				
Ortega et al. [29]	Plasma	mirVana PARIS Isolation Kit	TaqMan array miRNA cards in a subset and qPCR in the final sample	Geometric mean of six miRNAs (hsa-miR-106a-5p, hsa-miR-146a-5p, hsa-miR-19b-3p, hsa-miR-223-3p, hsa-miR-186-5p, hsa-miR-199a-3p)
Alkandari et al. [30]	Plasma	mirVana PARIS Isolation Kit	miRCURY qPCR panel	Four miRNAs (hsa-miR-223-3p, hsa-miR-26a-5p, hsa-miR-101-3p, and hsa-miR-19a-3p)
Atkin et al. [31]	Plasma	miRCURY RNA Isolation kit	qPCR and a FANTOM miRNA atlas [68]	Global mean
Bae et al. [32]	Exosome	miRNeasy Mini Kit	Small RNA sequencing	Relative log expression using DESeq2
Blum et al. [33]	Serum	miRNeasy serum/plasma kit	RNA sequencing in a subset and qPCR in the final sample	hsa-miR-451a
Hohensinner et al. [36]	Plasma	miRNA tissue lysis kit	qPCR	RNA spike-in
Hubal et al. [37]	Exosome	mirVANA miRNA Isolation Kit	GeneChip miRNA 4.0 Array	RMA algorithm
Hulsmans et al. [38]	Monocytes	TRIzol reagent	qPCR	RNU5G
Lirun et al. [40]	Plasma	mirVana RNA Isolation Kit	GeneChip miRNA 3.0 Array	RMA algorithm
Mysore et al. [41]	Subcutaneous Adipose Tissue (SAT)	miRNeasy Mini kit	qPCR	RNU44
Ortega et al. [42]	SAT	miRNeasy Mini Kit	GeneChip miRNA 3.0 array in a subset and qPCR in the final sample	RMA algorithm and RNU48
Ortega et al. [43]	SAT	miRNeasy Mini Kit	qPCR	RNU6B
Wang et al. [44]	Circulating Endothelial Progenitor Cells	High Pure RNA kit	qPCR	RNU6
Animal studies				
Guo et al. [34]	Liver	TRIzol reagent	miProfile Customized Rat qPCR arrays	5S rRNA and RsnRNA U6
Wei et al. [35]	Liver	TRIzol reagent	miProfile Customized Rat qPCR arrays	5S rRNA, RsnRNA U6, rno-miR-25, and rno-miR-186
Kwon et al. [39]	Hypothalamus, Heart, and Liver	Unspecified	Agilent Rat miRNA 8x15k microarray for hypothalamus and heart samples, then qPCR for liver and validation	Whole-array and RNU6
Wu et al. [45]	Plasma and Liver	mirVANA PARIS RNA Isolation kit	TaqMan Array Rodent Card	RNU6-1, RNU6-2, rno-miR-16-5p, rno-miR-223-3p, mmu-miR-1937b

The studies also differ in the miRNA profiling strategies (Table 2). For isolation methods, the studies used either mirVANA isolation kits (n = 5), miRNeasy kits (n = 5), TRIzol reagent (n = 3) or other kits. Most of the studies then used qPCR (n = 7) or microarrays (n = 5) as their main profiling

method. RNA sequencing was used as the main analysis in one study [32]. Other studies (n = 4) used a screening step using high-throughput profiling methods, such as microarrays and RNA sequencing, in a subset of their patients, then qPCR as validation in the final sample. Human studies using microarrays used the Robust MultiArray Average (RMA) method for normalization (n = 3). Studies with cells and tissue samples normalized their data using small non-coding RNAs with RNU6 and RNU6B being most commonly used (n = 6). Studies with plasma and serum samples used a number of stable miRNAs, which were unique for each study. One plasma study used RNA spike-in levels for normalization [36] and the RNA sequencing study used DESeq2 package for normalization [32].

The surgery type most commonly assessed is RYGB (n = 13) (Table 3) [29–32,36–43,45], but one study collected two SG patients in addition to RYGB [32] and one study profiled only SG patients [33]. In rats, the studies compared a duodeno–jejunal bypass (DJB) [34,35] or RYGB [39,45] with sham surgery. One study also performed SG in rats to compare with DJB results [34].

Lastly, the studies differ in the duration of study and number of observations after bariatric surgery (Table 3). One study of RYGB patients profiled miRNAs in five time points (1-, 3-, 6-, 9-, and 12-months post-surgery) [30]. Two studies in rats also studied miRNA levels two-, four-, and eight-weeks post-surgery [34,35]. Other studies only profiled miRNA once after surgery. Time of observation also differs between studies. Some studies looked into short-term expression changes (less than or equal to 3 months; n = 9), while others looked at long-term response (n = 9; maximum 2-years post-surgery).

Table 3. Bariatric surgery type and time of observation after surgery.

Study	Year	Bariatric Surgery Type	Time of Observation after Surgery
Human studies			
Ortega et al. [29]	2013	RYGB	12 months
Alkandari et al. [30]	2018	RYGB	1, 3, 6, 9, and 12 months
Atkin et al. [31]	2019	RYGB	21 days
Bae et al. [32]	2019	RYGB and SG	6 months
Blum et al. [33]	2017	SG	3 months
Hohensinner et al. [36]	2018	RYGB	24 months
Hubal et al. [37]	2017	RYGB	12 months
Hulsmans et al. [38]	2012	RYGB	3 months
Lirun et al. [40]	2015	RYGB	3 months
Mysore et al. [41]	2017	RYGB	24 months
Ortega et al. [42]	2015	RYGB	24 months
Ortega et al. [43]	2015	RYGB	24 months
Wang et al. [44]	2018	Not specified	3 months
Animal studies			
Guo et al. [34]	2016	DJB and SG	2, 4, 8 weeks
Wei et al. [35]	2018	DJB	2, 4, 8 weeks
Kwon et al. [39]	2015	RYGB	25 days
Wu et al. [45]	2015	RYGB	53 days

3.2. Differential Expression of miRNA before and after Surgery

According to the selected studies, a total of 50 miRNA families and 205 unique miRNAs were significantly differentially expressed after surgery compared to baseline. Among these, 32 differentially expressed miRNAs were identified in at least two different studies. The 32 miRNAs can be grouped based on the consistency of findings and reasons for discrepancies (Table 4).

Table 4. Differentially expressed miRNA before vs after surgery reported in at least two studies.

	miRNA	miRBase	References	Direction of Expression	No. of Subjects	Tissue	Time of Observation
Group 1 miRNAs (same direction of expression after surgery in two or more studies)							
1	hsa-miR-93-5p	MIMAT0000093	Lirun [40]	–	15	Plasma	3 months
			Alkandari [30]		9	Plasma	3 months
2	hsa-miR-106b-5p	MIMAT0000680	Lirun [40]	–	15	Plasma	3 months
			Alkandari [30]		9	Plasma	3, 12 months
3	hsa-let-7b-5p	MIMAT0000063	Lirun [40]	–	15	Plasma	3 months
			Alkandari [30]		9	Plasma	3 months
4	hsa-let-7i-5p	MIMAT0000415	Lirun [40]	–	15	Plasma	3 months
			Alkandari [30]		9	Plasma	6, 9 months
			Atkin [31]		29	Plasma	21 days
5	hsa-miR-16-5p	MIMAT0000069	Lirun [40]	–	15	Plasma	3 months
			Hubal [37]		6	Exosomes	12 months
6	hsa-miR-19b-3p	MIMAT0000074	Ortega [43]	–	9	SAT	24 months
			Lirun [40]		15	Plasma	3 months
			Ortega [29]		22	Plasma	12 months
7	hsa-miR-92a-3p	MIMAT0000092	Lirun [40]	–	15	Plasma	3 months
			Alkandari [30]		9	Plasma	9, 12 months
8	hsa-miR-222-3p	MIMAT0000279	Ortega [29]	–	22	Plasma	12 months
			Ortega [43]		9	SAT	24 months
9	hsa-miR-142-3p	MIMAT0000434	Bae [32]	–	12	Exosome	6 months
			Ortega [29]		22	Plasma	12 months
10	hsa-miR-140-5p	MIMAT0000431	Bae [32]	–	12	Exosome	6 months
			Ortega [29]		22	Plasma	12 months
11	hsa-miR-155-5p	MIMAT0000646	Ortega [43]	–	9	SAT	24 months
			Ortega [42]		25	SAT	24 months
12	rno-miR-320-3p	MIMAT0000903	Wu [45]	–	4	Plasma	53 days
			Wei [35]		5	liver	2 months

Table 4. *Cont.*

	miRNA	miRBase	References	Direction of Expression	No. of Subjects	Tissue	Time of Observation
Group 1 miRNAs (same direction of expression after surgery in two or more studies)							
13	hsa-miR-320c	MIMAT0005793	Atkin [31]	+	29	Plasma	21 days
			Lirun [40]		15	Plasma	3 months
14	hsa-miR-7-5p	MIMAT0000252	Atkin [31]	+	29	Plasma	21 days
			Bae [32]		12	Exosome	6 months
Group 2 miRNAs (overall same direction of expression after surgery in two or more studies)							
1	hsa-miR-125b-5p	MIMAT0000423	Ortega [29]	–	22	Plasma	12 months
			Alkandari [30]	–	9	Plasma	6, 9, 12 months
			Hubal [37]	+	6	Exosomes	12 months
2	hsa-miR-130b-3p	MIMAT0000691	Ortega [42] Alkandari [30]	– –	25 9	SAT Plasma	24 months 12 months
			Ortega [29]	+	22	Plasma	12 months
3	hsa-miR-221-3p	MIMAT0000278	Ortega [43]	–	9	SAT	24 months
			Ortega [42]	–	25	SAT	24 months
			Mysore [41]	–	22	SAT	24 months
			Lirun [40]	–	15	Plasma	3 months
			Ortega [29]	+	22	Plasma	12 months
4	rno-miR-122-5p	MIMAT0000827	Kwon [39]	–	25	heart	25 days
			Kwon [39]	–	25	liver	25 days
			Wu [45]	–	4	Plasma	53 days
			Wu [45]	–	8	Liver	53 days
			Kwon [39]	+	25	hypothalamus	25 days
5	hsa-miR-146a-5p	MIMAT0000449	Lirun [40]	–	15	Plasma	3 months
			Ortega [43]	–	9	SAT	24 months
			Ortega [29]	+	22	Plasma	12 months
6	rno-miR-503-5p	MIMAT0003213	Kwon [39]	+	25	hypothalamus	25 days
			Kwon [39]	+	25	heart	25 days
			Wei [35]	–	4	liver	2 months

Table 4. *Cont.*

	miRNA	miRBase	References	Direction of Expression	No. of Subjects	Tissue	Time of Observation
Group 3 miRNAs (reported in at least two studies, but with no agreement in direction of expression)							
1	hsa-miR-21-5p	MIMAT0000076	Alkandari [30]	−	9	Plasma	9, 12 months
			Ortega [29]	+	22	Plasma	12 months
2	hsa-miR-33a-5p	MIMAT0000091	Bae [32]	−	12	Exosome	6 months
			Alkandari [30]	+	9	Plasma	6 months
3	hsa-miR-320a-3p	MIMAT0000510	Alkandari [30]	−	9	Plasma	6, 9, 12 months
			Lirun [40]	+	15	Plasma	3 months
4	hsa-miR-320b	MIMAT0005792	Alkandari [30]	−	9	Plasma	9 months
			Lirun [40]	+	15	Plasma	3 months
5	hsa-miR-378a-3p	MIMAT0000732	Alkandari [30]	−	9	Plasma	6, 9, 12 months
			Lirun [40]	+	15	Plasma	3 months
6	hsa-miR-103-3p	MIMAT0000101	Lirun [40]	−	15	Plasma	3 months
			Hubal [37]	+	6	Exosomes	12 months
7	rno-miR-133b-3p	MIMAT0003126	Wei [35]	−	4	liver	2 months
			Kwon [39]	+	25	hypothalamus	25 days
8	rno-miR-194-5p	MIMAT0000869	Kwon [39]	−	25	heart	25 days
			Guo [34]	+	4	liver	2 months
9	hsa-miR-122-5p	MIMAT0000421	Ortega [29]	−	22	Plasma	12 months
			Blum [33]	+	21	Serum	3 months
10	rno-miR-146a-5p	MIMAT0000852	Wu [45]	−	4	Plasma	53 days
			Kwon [39]	+	25	hypothalamus	25 days
11	rno-miR-542-3p	MIMAT0003179	Wei [35]	−	4	liver	2 months
			Kwon [39]	+	25	hypothalamus	25 days
12	hsa-miR-191-5p	MIMAT0000440	Lirun [40]	−	15	Plasma	3 months
			Bae [32]	+	12	Exosome	6 months

Group 1 includes 14 miRNAs that changed in the same direction of expression, regardless of sample type and time of observation (downregulated: hsa-miR-93-5p, hsa-miR-106b-5p, hsa-let-7b-5p, hsa-let-7i-5p, hsa-miR-16-5p, hsa-miR-19b-3p, hsa-miR-92a-3p, hsa-miR-222-3p, hsa-miR-142-3p, hsa-miR-140-5p, hsa-miR-155-5p, rno-miR-320-3p; upregulated: hsa-miR-7-5p, hsa-miR-320c). Group 2 includes six miRNAs with inconsistent findings, but at least two studies agreed on a direction of expression (overall downregulated: hsa-miR-125b-5p, hsa-miR-130-3p, hsa-miR-221-3p, hsa-miR-146a-5p, rno-miR-122-5p; overall upregulated: rno-miR-503-5p). For example, hsa-miR-125b-5p was found to be downregulated in two studies profiling miRNA from plasma samples, but was upregulated in an exosome study. Lastly, group 3 includes 12 miRNAs reported in two studies but with no agreement in direction (hsa-miR-21-5p, hsa-miR-33a-5p, hsa-miR-320a-3p, hsa-miR-320b, hsa-miR-378a-3p, hsa-miR-103-3p, rno-miR-133b-3p, rno-miR-194-5p, hsa-miR-122-5p, rno-miR-146a-5p, rno-miR-542-3p, hsa-miR-191-5p).

3.3. *Pathway Analysis*

DIANA-miRPath was used to identify pathways regulated by miRNAs in Group 1 and 2. The first analysis was done with only Group 1, and a total of 74 KEGG pathways were significantly predicted to be regulated by these miRNAs. The miRNAs were predicted to target genes involved in cancer, cell cycle, fatty acid metabolism, signaling pathways, infectious diseases, and RNA processes in cells (Figure 2). The inclusion of Group 2 miRNAs resulted in a slightly different pathway profile. This secondary analysis retained most pathways from the first analysis (69 out of 74) and added eight different significant pathways. The additional pathways were related to signaling pathways, metabolism, and biosynthesis processes (not shown).

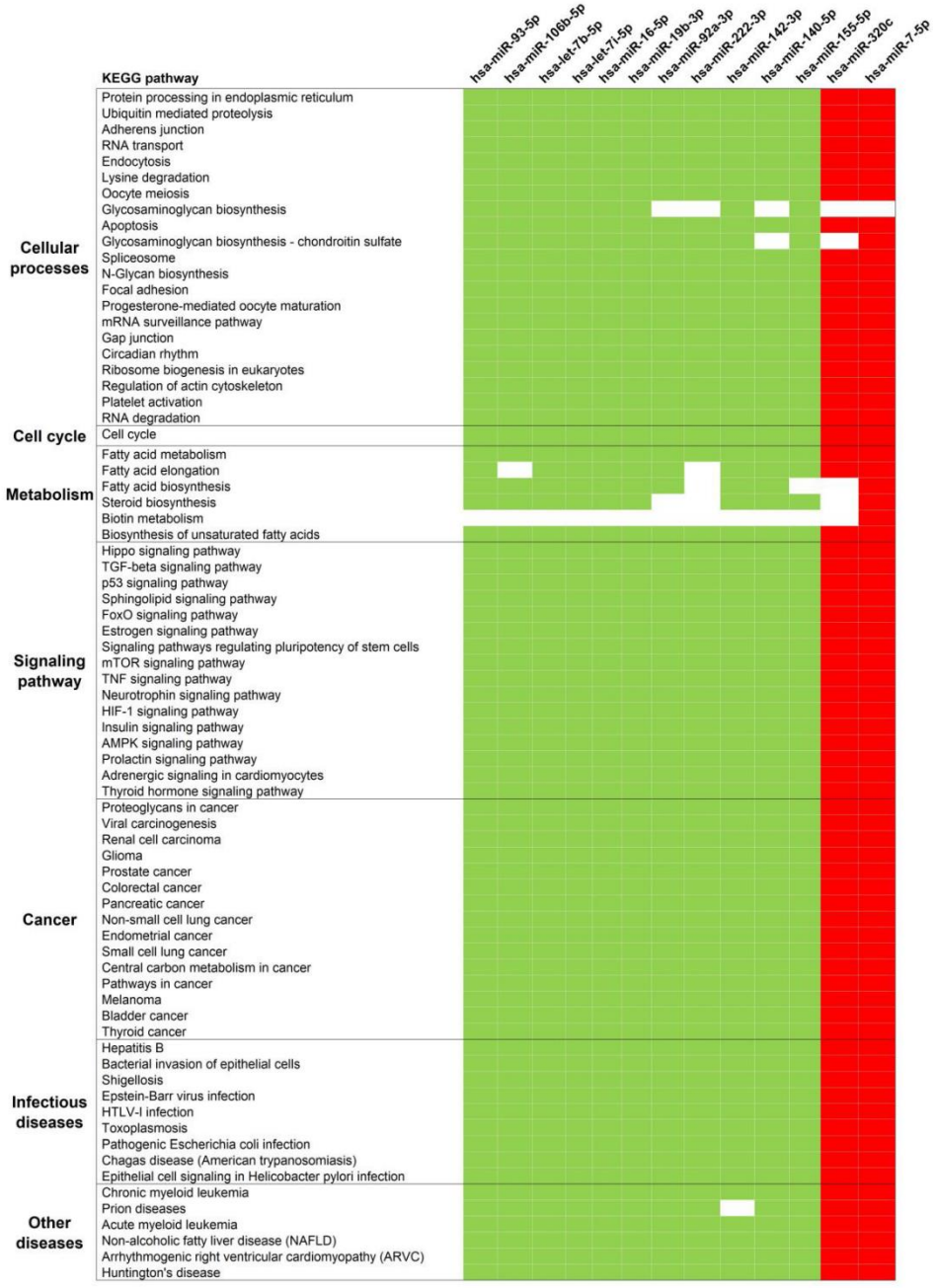

Figure 2. Significantly enriched KEGG pathways of surgery-responsive miRNAs. The miRNAs reported to be involved in a particular pathway are indicated in colors green or red; otherwise, they are indicated as white. Green indicates pathways targeted by down-regulated miRNAs. Red indicates pathways targeted by up-regulated miRNAs.

4. Discussion

The benefits of bariatric surgery beyond weight loss, such as T2DM remission, have been reported extensively [15,16,19–22]. However, the mechanisms behind successful weight loss and improvement of obesity comorbidities are poorly understood. In recent years, more and more studies are looking into a patient's miRNAome before and after bariatric surgery. The miRNA profile changes as a response to environmental changes, including bariatric surgery. Understanding how miRNA profile changes due to bariatric surgery might uncover important pathways behind its outcomes.

We found that through February 2019, there were 17 studies on miRNA profiles of patients before and after bariatric surgery. Although a relatively small number, there is a sharp increase in publications in the last five years. The first study among them was published in 2012 [38] and 15 studies were published in and after 2015. This indicates a rapid increase in interest of miRNAs related to bariatric surgery. These studies consistently found differential expression of miRNAs after surgery in various tissues with a total of 205 unique miRNAs reported so far. This is in contrast to other genetic studies that found inconsistent findings of the influence of bariatric surgery on DNA methylation [28,69] and no associations between Single-Nucleotide Polymorphisms with weight loss success after bariatric surgery [70,71].

However, these recent miRNA studies were highly variable in study design. Studies on rats looked into a wide range of tissues and included tissues inaccessible in human studies, such as the hypothalamus and liver. Most human studies profiled easily accessible tissues, including circulating miRNA in plasma, serum, exosomes, and monocytes. Some studies had access to adipose tissue biopsies which were collected from patients a few years after surgery. In contrast, human blood samples were able to be collected earlier and at more time points. The earliest time point was 21 days after surgery [31] and one study had five time points after surgery [30]. The sample type and time of observation appeared to be the main reasons for the discrepancy in miRNA expression direction, especially in Group 3's miRNAs. For example, hsa-mir-21-5p, hsa-miR-320a-3p, hsa-miR-320b, and hsa-miR-378a-3p expressions appear to be time-dependent. Whereas, hsa-miR-33a-5p appears to have sample-specific expression, where its expression was increased in plasma samples, but reduced in exosomes. The other seven miRNAs in Group 3 had both sample type and time differences between the studies that reported them. Studies with more participants on the same sample type and time points are needed to confirm the time and tissue specificity of these miRNAs.

Despite the high variability between studies, there were 14 human and rat miRNAs with consistent direction of differential expression after surgery. In at least two studies, hsa-miR-93-5p, hsa-miR-106b-5p, hsa-let-7b-5p, hsa-let-7i-5p, hsa-miR-16-5p, hsa-miR-19b-3p, hsa-miR-92a-3p, hsa-miR-222-3p, hsa-miR-142-3p, hsa-miR-140-5p, hsa-miR-155-5p, and rno-miR-320-3p were reported to have lower expression levels, while hsa-miR-7-5p and hsa-miR-320c had increased expression levels after surgery. These miRNAs are predicted to be important in various cellular pathways, including those related to lipid metabolism, insulin signaling pathway, and cardiac function. The genes within these pathways are interesting targets for functional studies to understand the mechanisms behind weight loss and remission of obesity-related comorbidity after surgery.

For instance, the most significant pathway is the "proteoglycans in cancer" (hsa05205) and the 13 human miRNAs were predicted to target 140 genes in this pathway. One of them is FZD7, which is one of the Frizzled (Fzd) transmembrane receptors for Wnt proteins [72]. Reduced expression of Wnt proteins is associated with obesity [73]. The hsa-miR-142-3p, which was reported to be downregulated after surgery, is predicted to interact with FZD7. This might lead to an increase in FZD7 expression, activation of the Wnt/Fzd signaling, and thus attenuation of obesity.

These miRNAs were also predicted to target 30 genes in the fatty acid metabolism pathway. The upregulated hsa-miR-7-5p was predicted to target FASN, which is inversely correlated with parameters of glycemic status [74] and its expression is elevated in numerous obesity-related cancers [75]. The downregulation of FASN would result in lower risks for these comorbidities. In addition, Ortega et al. focused on inflammation-responsive miRNAs in adipose tissues [43] and among them,

hsa-miR-155-5p and hsa-miR-222-3p were included in the Group 1 miRNAs. The hsa-miR-155-5p has been reported to be elevated in numerous inflammatory conditions [76]. Transfection of an hsa-miR-155 inhibitor in myeloid cells was found to decrease proinflammatory cytokine expression [77]. Deregulation of hsa-miR-155-5p and hsa-miR-222 was also found to be associated with cardiovascular diseases [78,79]. These reports indicate that these miRNAs might be involved in the mechanisms behind reduced inflammation and cardiovascular risks after bariatric surgery. Functional studies are needed to determine the role of these surgery-responsive miRNAs in promoting bariatric surgery outcomes.

Although limited in sample size and the number of miRNAs analyzed, studies on SG patients and animal models suggest different miRNA profiles compared to other surgery types. A study in rats compared rno-miR-200a-3p expression levels between DJB and SG [34]. In this study, rno-miR-200a-3p expression was significantly higher in DJB compared to sham-operated animals. In contrast, this miRNA expression was unchanged after SG and comparable to the sham-operated group [34]. In humans, a study of SG patients reported significant increase in hsa-miR-122-5p levels in serum after surgery [33], but another study reported decreased levels of hsa-miR-122-5p in plasma after RYGB [29]. The discrepancy might explain the apparent differences in bariatric success rates between RYGB and SG, especially concerning the remission of comorbidities such as diabetes. More comparative studies between RYGB and SG patients are needed to confirm these observations.

However, it is interesting that although many studies used high-throughput methods, only 32 miRNAs were reported in at least two studies. This might be due to the differences in miRNA isolation, profiling, and normalization strategies between studies. For isolation methods, some studies showed that miRNeasy isolation kits produce higher RNA quantity and better quality compared to miRVana [80,81]. Maximizing the isolated miRNA yield is particularly important for plasma and serum samples as their miRNA abundance is significantly lower than tissues [80]. Low yield might result in failure of detecting low-abundance miRNAs [80] and this may contribute to the poor agreement in miRNA profiles between plasma and serum studies [82].

The highly different profiling methods between studies could also be the source of this limited agreement in their findings. Comparative studies have found low correlation between different profiling methods when used to analyze the same samples [82]. Different microarray platforms were found to share a large number of common miRNAs, but the vast majority of the differentially expressed calls were not unanimous across platforms [83]. The median rank correlation between microarray platforms in a different study was only 0.55, while the median correlation between microarray and qPCR was 0.7 [84]. However, one microarray platform had a correlation of lower than 0.5 with qPCR [84]. The cause of this disagreement is unclear [83]. For different qPCR-based platforms, a study found good correlation of CT data between two platforms, but gel electrophoresis suggests a large number of false positive results for an assay [82]. Although these comparative studies did not compare the exact arrays used in our analysis, they suggest there might also be little agreement between profiling methods in our selected studies, leading to a limited number of miRNAs reported in two or more studies.

Finally, normalization is crucial for providing robust expression data, but there is no consensus regarding normalization methods for miRNA results [85]. Several studies have discussed commonly used normalization methods and found that small nuclear RNAs such as U6 are not good normalizers for miRNA expression [82,85]. This is because RNU6 and other small nuclear RNAs do not reflect the biochemical character of miRNAs and their efficiency throughout the profiling experiments may differ from miRNAs [82,85]. However, many studies profiling miRNA from bariatric surgery patients used RNU6 as their normalization method. Several authors recommend a global mean normalization of a set of reference genes, which may be tissue-specific, with a minimum of three stable housekeeping genes [82,86]. Some of the studies in our analysis used this method, particularly studies with plasma and serum samples.

In addition to these study design limitations, our analysis has not considered population and sex differences, as well as analyzing miRNA results by sample type due to the limited number of studies

published so far. Some studies have demonstrated population-specific miRNA expression between populations [87,88]. For example, a study found 16% of the evaluated miRNAs differ significantly between these Caucasians and Africans [87]. There were three studies in Asians in this analysis and their miRNA modulation patterns might not be the same as those of Caucasians. More studies with Asians and other populations should be done to investigate population-specific patterns in miRNA modulation after surgery. Sex differences were also not explored in the current analysis as most human studies were carried out in female patients, while animal studies were performed in male rats. Recent studies in patients and healthy participants have reported sex-biased miRNA expression [89,90]. More studies with male patients are needed to investigate sex-biased miRNA patterns after bariatric surgery. Lastly, our analysis combined findings from different tissue types, but this global approach might mask tissue-specific miRNA patterns after surgery. Unfortunately, there are limited human studies comparing miRNA profiles in tissue samples before and after surgery. As mentioned before, this is likely because of the difficulty in obtaining tissues after surgery. The three studies using SAT samples collected the tissues from the same hospital in Spain [41–43]. Only two miRNAs (hsa-miR-155-5p and hsa-miR-221-3p) were reported in at least two of these studies. This is because two SAT studies had targeted miRNA profiling, where Mysore et al. profiled only hsa-miR-221-3p and Ortega et al. profiled only inflammation-induced miRNAs in one study [43]. More untargeted miRNA studies from SAT samples are needed to explore tissue-specific miRNA patterns after surgery.

5. Conclusions

We have identified 14 miRNAs with consistently altered expression after bariatric surgery, regardless of sample type, surgery type, and time of observation after surgery. However, these findings should be taken with caution. These miRNAs were identified from 13 studies with highly variable study design and small sample sizes. A consensus in miRNA profiling methods is crucial for a better comparative study of profiling studies. Until then, a better analysis would be to compare findings of studies with similar strategies. Future studies should also aim to profile a larger number of participants and untargeted profiling of SAT samples. Additionally, more profiling studies in different populations and in males are needed to investigate the generalizability of miRNA modulation after surgery. Studies investigating SG patients are also needed as this surgery type is becoming the most commonly used technique in many countries. Finally, functional studies are needed to understand the role of these miRNAs in promoting weight-loss and remission of obesity-related comorbidities after bariatric surgery. This may lead to novel targets for non-surgical treatment of obesity and its comorbidities and provide novel biomarkers for predicting bariatric surgery outcomes.

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Original Article

II

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Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study

Gladys Wojciechowska[1] | Lukasz Szczerbinski[1,2] | Marek Kretowski[3] | Magdalena Niemira[1] | Hady Razak Hady[4] | Adam Kretowski[1,2]

[1]Clinical Research Centre, Medical University of Białystok, Białystok, Poland

[2]Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Białystok, Białystok, Poland

[3]Faculty of Computer Science, Białystok University of Technology, Białystok, Poland

[4]1st Clinical Department of General and Endocrine Surgery, Medical University of Białystok, Białystok, Poland

Correspondence
Gladys Wojciechowska, Clinical Research Centre, Medical University of Bialystok, M. Skłodowskiej-Curie 24a, 15-276 Białystok, Poland.
Email: gladys.wojciechowska@umb.edu.pl

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Abstract

Objective: This study aimed to evaluate microRNAs (miRNAs) as predictive biomarkers for type 2 diabetes (T2D) remission 12 months after sleeve gastrectomy (SG).

Methods: A total of 179 serum miRNAs were profiled, and 26 clinical variables were collected from 46 patients. Two patients were later excluded because of hemolysis, and six patients with unclear remission status were set aside to evaluate the prediction models. The remaining 38 patients were included for model building. Variable selection was done using different approaches, including Least Absolute Shrinkage and Selection Operator (LASSO). Prediction models were then developed using LASSO and assessed in the validation set.

Results: A total of 26 out of 38 patients achieved T2D remission 12 months after SG. The prediction model with only clinical variables misclassified two patients, which were correctly classified using miRNAs. Two miRNA-only models achieved an accuracy of one but performed poorly for the validation set. The best miRNA model was a mixed model (accuracy: 0.974) containing four miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) and four clinical variables (T2D medication, sex, age, and fasting blood glucose). These miRNAs are involved in pathways related to obesity and insulin resistance.

Conclusions: This study suggests that four serum miRNAs might be predictive biomarkers for T2D remission 12 months after SG, but further validation studies are needed.

INTRODUCTION

Sleeve gastrectomy (SG) is the most common bariatric surgery procedure in Poland and other countries, including the United States (1-3). It is a less complicated procedure and it has fewer surgery complications compared with other methods (4-7). Although SG is comparable to other methods for weight loss and weight regain (8-10), SG has a lower success rate for type 2 diabetes (T2D) remission

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(4,11). Therefore, identifying patients who can benefit the most from SG is valuable for effective treatment.

Different prediction models have been developed to predict T2D remission after bariatric surgery (12). However, most models were developed using cohorts of surgery methods other than SG or a limited number of SG patients (12). A 2019 study found that these models overestimated diabetes remission in SG patients with varying degrees (12). Better prediction models are needed for SG patients.

There is increasing interest in using biomarkers as predictive variables. A 2016 study used structural genetic variants as predictive biomarkers for T2D remission (13). We are interested in studying microRNA (miRNA), an epigenetic factor that regulates protein expression through destabilization of target mRNA (14). Epigenetic factors were reported to have a relationship with bariatric surgery outcomes (15,16). However, the predictive value of miRNAs for surgery outcomes has not been explored before, to our knowledge.

We selected patients with diabetes and obesity from a larger cohort of the Białystok Bariatric Surgery Study (BBSS), an ongoing longitudinal study of Eastern Polish SG patients. Presurgery serum miRNA levels were collected for 46 diabetic patients with T2D remission status 12 months post surgery. We aim to explore whether miRNA information gives any added value to clinical data for predicting T2D remission after SG.

Additionally, we implemented machine learning approaches for variable selection and developed the prediction models. We profiled 179 serum miRNAs and collected 26 clinical variables, including those used in other T2D prediction models. To reduce data dimensionality, we chose Least Absolute Shrinkage and Selection Operator (LASSO) for variable selection and to develop classifiers.

Therefore, this pilot study aims to evaluate the added value of including miRNAs as predictive biomarkers for T2D remission after SG through machine learning approaches (Figure 1).

Study Importance

What is already known?

- Sleeve gastrectomy (SG) is an effective weight loss surgery that may result in type 2 diabetes (T2D) remission.
- Prediction models for T2D remission have been built for surgery types other than SG and have not included microRNAs (miRNAs).

What does this study add?

- Four serum miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) that might predict T2D remission 12 months after SG were identified.
- These miRNAs are involved in pathways related to obesity and insulin resistance.

How might these results change the direction of research or the focus of clinical practice?

- Biomarker research could focus on these miRNAs and validate them in larger cohorts to evaluate their predictive value.
- The miRNAs could also be studied further to understand molecular subtypes of T2D patients with obesity.

METHODS

Study participants

Patients were recruited from the BBSS (17), in which 321 Polish patients with obesity had undergone bariatric surgery, including SG, from 2016 to 2019. The inclusion criteria for surgery were BMI ≥ 40 kg/m^2 or BMI ≥ 35 with comorbidities. The exclusion criteria included prior bariatric surgery, substance abuse, uncontrolled psychiatric illness, expected lack of compliance, or advanced cancer (17). A subset of the SG cohort had T2D based on the American Diabetes Association criteria and had T2D remission status 12 months post surgery (n = 46). Remission status was determined using the American Society of Metabolic and Bariatric Surgery (ASMBS) criteria, based on T2D medication status, hemoglobin A1c (HbA1c), and fasting glucose 12 months after surgery (18). The ASMBS criteria creates five remission groups (18). However, we regrouped patients into a binary remission status because of the sample size: patients with "complete" and "partial" remission were grouped into "remission." "Improvement," "unchanged," and "recurrence" were grouped into "nonremission." Six patients had unclear remission status due to missing information post surgery. We held out these six patients for model evaluation. All participants provided informed consent before the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical University of Białystok (project identification code: R-I-002/546/2015) (17).

Sample preparation and miRNA extraction

Serum samples were obtained between 2 and 4 weeks before surgery from patients in the overnight fasting state. Blood samples were collected in Sarstedt S-Monovette tubes (Sarstedt, Inc., Nümbrecht, North Rhine-Westphalia, Germany) with separator gel. The samples were allowed to clot for at least 30 minutes and were then centrifuged for 10 minutes at 2,500 rpm. Serum samples were immediately stored at −80°C until use.

RNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (QIAGEN, Hilden, Germany). Three RNA spike-ins (UniSP2, UniSP4, and UniSP5) were added to the kit's "RPL buffer" as RNA isolation controls. Serum volumes of 200 uL were used for isolation,

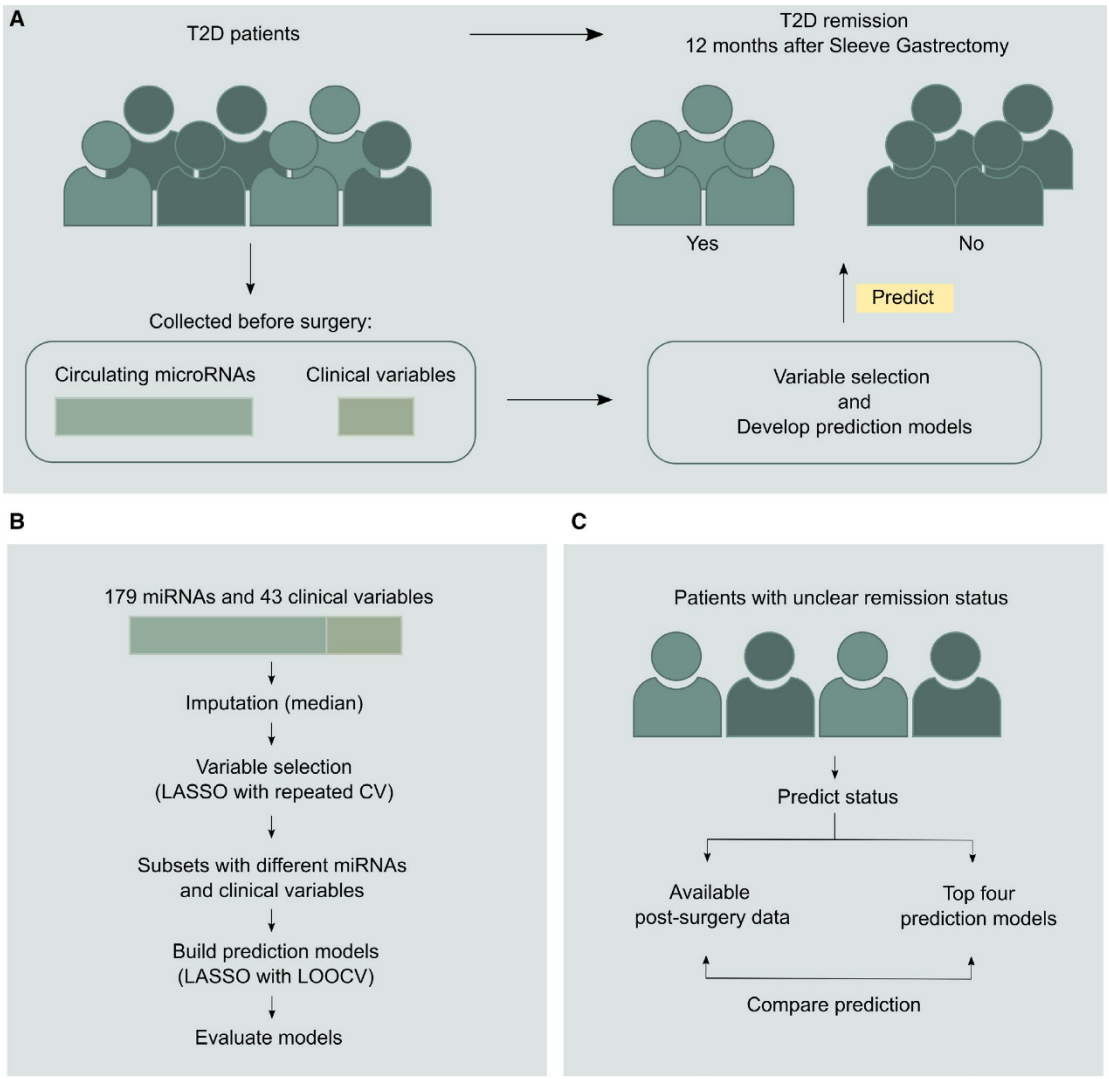

FIGURE 1 Overview of study design. (A) General framework of patient stratification based on miRNAs and clinical variables. (B) The study's approach for variable selection and building prediction models with miRNAs and clinical variables. (C) The approach for evaluating the prediction models using patients with unclear remission status. CV, cross validation; LASSO, Least Absolute Shrinkage and Selection Operator; LOOCV, leave-one-out cross validation; miRNA, microRNA; T2D, type 2 diabetes [Color figure can be viewed at wileyonlinelibrary.com]

and 20 uL of nuclease-free water was used for elution. A no-template sample (nuclease-free water) was also included to evaluate RNA isolation quality.

Quality control of miRNA extraction

The miRCURY locked nucleic acid (LNA) miRNA QC PCR Panel (QIAGEN) was used to assess miRNA quality, monitor complementary DNA (cDNA) synthesis, evaluate hemolysis, and assess polymerase chain reaction (PCR) efficiency. For this quality control (QC) panel, 2 uL of miRNA elute was used for 10 uL of reverse transcription (RT) reaction using the miRCURY LNA RT Kit (QIAGEN). Two spike-ins were used for cDNA synthesis (UniSp6 and cel-miR-39). A total of 1.5 uL of cDNA was used for the QC panel. Two samples were later excluded because of hemolysis (final $n = 44$), as indicated by a difference in cycle threshold (Ct) values between miR-23a-3p and miR-451a of more than five (19). PCR was done using the Roche LightCycler 480 Instrument (Roche, Basel, Switzerland) with SYBR Green dye.

miRNA profiling

Using the Serum/Plasma miRCURY LNA miRNA Focus PCR Panel (QIAGEN), profiling was done with a 96-well plate format (20) (Supporting Information Table S1). For this panel, 4 uL of miRNA elute was used for 20-uL cDNA synthesis, along with the two spike-ins for cDNA synthesis. The whole cDNA reaction was used for profiling. No-template controls were also used to evaluate background miRNA levels. PCR was done using the Roche LightCycler 480 Instrument with SYBR Green dye.

Data preprocessing

Raw miRNA data were preprocessed using the GeneGlobe Data Analysis Center (QIAGEN; geneglobe.qiagen.com) to remove miRNAs below a Ct cutoff (Ct = 35) and to apply interplate calibration. The processed data were then normalized using a global mean normalization. There were no missing values for miRNAs.

A total of 43 baseline clinical variables were collected from patients, including blood biochemical parameters, blood morphology measures, and anthropometric measurements. We selected 26 clinical variables with missingness less than 10%. Median imputation was used for missing values. The total number of clinical and miRNA variables was 205.

Variable selection

Ten unique variable sets were created: six sets with only miRNA variables, two with only clinical variables, and two sets with miRNA and clinical variables. Variables were normalized to obtain z scores.

Out of the 44 patients with miRNA data, six patients with unclear remission status were set aside for model evaluation. Therefore, 38 patients with clear remission status were used for variable selection and building classifiers.

Selecting serum miRNA variables

Six miRNA-only variable sets were created using different methods. One set contains all 179 miRNAs, another includes miRNAs from statistical testing, and four other sets contain LASSO-selected miRNAs.

miRNA selection using statistical significance and fold change

Fold change is the ratio of relative normalized miRNA expression between remission groups. Unpaired t tests were used to calculate p values. Four miRNAs with $p < 0.05$ and fold regulations of at least 1.5 were selected in this variable set.

Variable selection with LASSO

LASSO (21) with repeated 10-fold cross validation (500 repeats) was built using all 179 miRNAs. A total of 20 miRNAs had nonzero coefficients and they were ranked based on their importance. The top five, ten, fifteen, and all nonzero miRNAs were selected as four sets of LASSO-selected miRNAs.

Selecting pre-surgery clinical variables

Two sets of clinical variables were created: one set contains all 26 variables, and another has LASSO-selected variables. The LASSO selection process is the same as that for miRNAs. Repeated cross validation with 10 folds and 500 repeats was done using all 26 clinical variables, and then the resulting nonzero variables were selected.

Selecting serum miRNA and clinical variables

Two sets of miRNA and clinical variables were created: one set contains all available variables (205 variables), and another has LASSO-selected variables. The LASSO selection process was done using all variables with the same repeated cross-validation approach. The nonzero variables were selected.

Prediction models

Ten LASSO models were built with each variable set. A leave-one-out cross-validation approach was used. Model performances were obtained using caret and epiR in R (R Foundation, Vienna, Austria), and models were compared based on their accuracy.

Model evaluation using six patients with unclear remission status

Remission labels were determined using available postsurgery clinical measures. The label decision was first made based on the discontinuation of T2D medicines. Then HbA1c and fasting glucose information was considered. For prediction, we first applied the same median imputation and z score scalar used for the model-building data. Then prediction was made using four models: one clinical-only model, one clinical and miRNA model, and two miRNA models (Supporting Information Table S2). We then compared the prediction with their remission labels.

Correlation analysis

Pearson correlation was done to evaluate the relationship between clinical variables and miRNAs hsa-miR-32-5p, hsa-miR-382-5p,

hsa-miR-1-3p, and hsa-miR-21-5p. Multiple testing correction was done using false discovery rate (FDR). Two plots for unadjusted and adjusted *p* values were made using ggcorrplot package in R (R Foundation).

Pathway analysis

Pathway analysis was done for miRNAs hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p. The DIANA miRPath version 3 software (http://www.microrna.gr/miRPathv3) was used to identify experimentally reported target genes and evaluate the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

RESULTS

Patient demographics and miRNA profiles

Six clinical variables were significantly associated with remission after SG: T2D medication; age; HbA1c; and fasting plasma glucose, as well as plasma glucose 30 and 60 minutes after oral glucose tolerance test (OGTT; Table 1). The remission group had a much lower proportion of patients taking diabetes medication before surgery (remission vs. nonremission: 12% vs. 83.3%, adjusted $p = 0.003$). The remission group was also significantly younger and had lower plasma glucose and lower HbA1c. Additionally, the remission group had higher plasma insulin and took fewer medications for chronic diseases, but the relationships were not significant after FDR (Table 1).

A total of 179 circulating miRNAs were profiled from serum samples collected before surgery. None of the miRNAs was significant between remission and nonremission groups after multiple testing correction using FDR (Supporting Information Table S1). However, eight miRNAs had unadjusted $p \leq 0.05$, and four of them had a fold regulation of at least 1.5 (remission vs. nonremission group: upregulation = hsa-miR-382-5p, hsa-miR-409-3p; downregulation = hsa-miR-375, hsa-miR-1-3p, respectively).

Variable selection and modeling results

Ten variable sets were created based on different variable selection processes (Table 2). One set for miRNAs contained the four significantly differentially expressed miRNAs (GeneGlobe miRNAs: hsa-miR-382-5p, hsa-miR-409-3p, hsa-miR-375, and hsa-miR-1-3p). LASSO selected 20 out of 179 miRNAs after repeated cross validation, including three out of 4 significant miRNAs (hsa-miR-382-5p, hsa-miR-375, and hsa-miR-1-3p). For clinical variables, LASSO selected four out of twenty-six variables: T2D medication, age, fasting plasma glucose, and sex. When all variables were provided, LASSO chose the same four clinical variables (T2D medication, sex, age, and fasting plasma glucose) and four miRNAs (hsa-miR-1-3p, hsa-miR-21-5p, hsa-miR-32-5p, and hsa-miR-382-5p; Table 2, set 4).

Among the 10 prediction models, classifiers with miRNA variables performed best. Models with 10 or 15 miRNAs achieved an accuracy of 1 (95% CI: 0.91-1; Table 2). Models with only clinical variables misclassified two nonremission patients, with an accuracy of 0.947 (95% CI: 0.82-0.99; Tables 2 and 3). When four miRNAs were added into the clinical model, patient 1 was correctly predicted, but not patient 2 (Figure 2A; Table 3). Patient 2 was later correctly classified in the miRNA-only models, and no other misclassifications were found (Figure 2B; Table 3).

Evaluating prediction models using six patients with unclear remission status

Four classifiers were selected for evaluation: a clinical-only model, a mixed model with miRNA and clinical variables, and two miRNA-only models (Table 4; Supporting Information Table S2). Models with clinical variables agreed the most with postsurgery data (Table 4). All models predicted patient A as nonremission, but postsurgery data suggested remission. All miRNA models predicted nonremission for patient C. Postsurgery values were within the remission group, but this patient had missing medication information. The miRNA-only models had increasing disagreement with postsurgery data, indicating overfitting with the training data.

Evaluating the four predictive miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, hsa-miR-21-5p)

Four miRNAs that improved prediction for clinical models had significant correlations with glucose measures and HbA1c, but not with other clinical measures (Figure 3; Supporting Information Figure S1). The miRNA hsa-miR-382-5p was significantly positively correlated with HbA1c ($r = 0.432$) and plasma glucose ($r = 0.485$ for fasting and $r = 0.359$ for 30 minutes during OGTT). The relationship with fasting plasma glucose was maintained after FDR (Figure 3). There were other significant correlations between miRNA and clinical variables, but they were not significant after FDR; for example, fasting plasma glucose with hsa-miR-32-5p ($r = -0.354$) and hsa-miR-21-5p ($r = -0.346$), as well as hemoglobin cell count with hsa-miR-21-5p ($r = -0.456$). The miRNA hsa-miR-1-3p was not significantly correlated with any of the selected clinical variables. The miRNA hsa-miR-32-5p was positively correlated with hsa-miR-1-3p ($r = 0.393$) and hsa-miR-21-5p ($r = 0.362$) but was no longer significant after FDR.

Pathway analysis was done for these miRNAs using the DIANA miRPath version 3 software. Three out of four miRNAs regulated 39 KEGG pathways, including 19 signaling pathways related to obesity and insulin resistance (Table 5). There was no information for hsa-miR-1-3p in this database. Within these 19 pathways, hsa-miR-32-5p regulated 253 genes, hsa-miR-21-5p regulated 330 genes, and hsa-miR-382-5p regulated 73 genes.

TABLE 1 Baseline clinical data from patients measured before surgery

Variable	Remission	Nonremission	*p* value	*p* value (adj)
No. of patients	26	12		
Age at time of SG (y)	45.5 (38.25;54)	58 (56.25;65.25)	**0**	**0.004**
BMI before SG (kg/m^2)	46.87 (43.33;50.77)	45.87 (43.65;52.75)	0.975	0.975
Percentage of body fat before SG (%)	47.1 (44.77;50.58)	49.4 (44.83;51.4)	0.46	0.594
Fasting blood insulin before SG (IU/mL)	34.55 (29.53;53.57)	34.72 (27.55;43.52)	0.396	0.567
Plasma insulin measured at 30 min during OGTT (n = 35; IU/ml)	128.08 (109.6;173.73)	74.08 (61.61;126.53)	**0.031**	0.091
Plasma insulin measured at 60 min during OGTT (n = 35; IU/mL)	159.18 (146.12;231.63)	123.27 (73.27;168.24)	0.213	0.395
Plasma insulin measured at 120 min during OGTT (n = 35; IU/mL)	121.86 (82.54;243.84)	90.56 (52.09;105.67)	**0.045**	0.118
Number of chronic diseases before SG (1 or more)	19 (73%)	12 (100%)	0.084	0.546
Number of chronic disease medications before SG (2 or more)	12 (46%)	12 (100%)	**0.017**	0.216
HbA1c before SG (%)	6.4 (5.9;6.88)	7.1 (6.65;8.25)	**0.005**	**0.021**
Fasting blood glucose before SG (mg/dL)	132.5 (123.25;143.5)	154.5 (146.75;178.75)	**0**	**0.004**
Plasma glucose measured at 30 min during OGTT (n = 35; mg/dL)	232.5 (194.5;239)	248 (235;271)	**0.011**	**0.042**
Plasma glucose measured at 60 min during OGTT (n = 35; mg/dL)	248 (224.75;282.25)	298 (283;315)	**0.002**	**0.012**
Plasma glucose measured at 120 min during OGTT (n = 35; mg/dL)	194.5 (159.75;218.25)	225 (183;243)	0.186	0.395
Bilirubin before SG (mg/dL)	0.47 (0.36;0.59)	0.38 (0.3;0.57)	0.307	0.499
C-reactive protein before SG (mg/L)	5.89 (2.62;10.53)	3.92 (1.69;10.24)	0.48	0.594
Cholesterol before SG (mg/dL)	190 (165.5;214)	184 (152.5;203.25)	0.387	0.567
Triglyceride before SG (mg/dL)	146 (131.25;231)	163 (126;225)	0.888	0.923
High-density lipoprotein before SG (mg/dL)	39.5 (35;45)	44.5 (37.75;53.5)	0.209	0.395
Low-density lipoprotein before SG (mg/dL)	118.5 (97.12;146)	103.95 (82.83;133.75)	0.272	0.471
White blood cell count before SG (10^3/uL)	7.95 (6.65;9.07)	8.2 (7.5;8.62)	0.753	0.879
Red blood cell count before SG (10^6/uL)	4.98 (4.7;5.26)	5.07 (4.82;5.29)	0.777	0.879
Platelet blood count before SG (10^3/uL)	224 (203.75;263)	209.5 (190;283.75)	0.414	0.567
Hemoglobin cell count before SG (g/dL)	14.35 (13.25;15.05)	14.45 (13.3;15.05)	0.826	0.895
Male sex	16 (61.5%)	10 (83.3%)	0.333	0.546
Diabetes medication before SG (n = 37)	3 (12%)	10 (83.3%)	**0**	**0.003**

Note: Values show the median (first;third quartiles) or the number of patients and percentages. *p* values are shown for the χ^2 test (categorical variables) and Kruskal-Wallis test (continuous variables). Rows with $p < 0.05$ are shown in bold. Multiple testing correction was done using the false discovery method. If not otherwise stated, $n = 38$.

Abbreviations: adj, adjusted; OGTT, oral glucose tolerance test; sleeve gastrectomy.

DISCUSSION

This pilot study evaluated miRNAs as predictive biomarkers and used machine learning approaches to select the most potential miRNAs and for model building. We found that miRNAs might improve T2D remission prediction and that they are best used with clinical variables. We considered all miRNAs because statistically significant variables are not always good predictive variables (22).

Our clinical model, based on T2D medication, age, sex, and fasting plasma glucose, misclassified two nonremission patients. Both patients had similar presurgery conditions: they did not take any T2D medications before surgery, and they were in their 60s. Patient 1 needed T2D medicines after surgery; therefore, this patient had a nonremission status. In contrast, patient 2 seemed to be borderline partial remission after surgery. The second patient's fasting blood glucose was only three points above the upper limit for partial remission (≤125 mg/dL). Therefore, the clinical models well predicted that patient 2 could achieve remission after surgery.

Adding miRNA information improved prediction for patient 1. When the miRNAs hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p,

TABLE 2 Prediction models using 10 different variable sets

	Variable set	Variables	Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
1	Top 10 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a	1 (0.91-1)	1 (0.74-1)	1 (0.87-1)
2	Top 15 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a, hsa-miR-140-5p, hsa-miR-543, hsa-miR-26a-5p, hsa-miR-27b-3p, hsa-miR-423-3p	1 (0.91-1)	1 (0.74-1)	1 (0.87-1)
3	Top 20 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p, hsa-miR-141-3p, hsa-miR-375, hsa-miR-32-5p, hsa-miR-2110, hsa-miR-1260a, hsa-miR-140-5p, hsa-miR-543, hsa-miR-26a-5p, hsa-miR-27b-3p, hsa-miR-423-3p, hsa-miR-151a-5p, hsa-miR-29b-3p, hsa-miR-1-3p, hsa-miR-30e-5p, hsa-miR-125a-5p	0.974 (0.86-1)	0.917 (0.62-1)	1 (0.87-1)
4	8 LASSO-selected miRNAs and clinical variables	T2D medication, age, hsa-miR-382-5p, hsa-miR-32-5p, fasting blood glucose, sex, hsa-miR-1-3p, hsa-miR-21-5p	0.974 (0.86-1)	0.917 (0.62-1)	1 (0.87-1)
5	All clinical variables	All clinical variables (26)	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
6	4 LASSO-selected clinical variables	T2D medication, age, fasting blood glucose, sex	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
7	All available variables	All miRNAs and clinical variables (205)	0.947 (0.82-0.99)	0.833 (0.52-0.98)	1 (0.87-1)
8	Top 5 LASSO-selected miRNAs	hsa-miR-382-5p, hsa-miR-193a-5p, hsa-miR-501-3p, hsa-miR-21-5p, hsa-miR-877-5p	0.921 (0.79-0.98)	0.917 (0.62-1)	0.923 (0.75-0.99)
9	All miRNAs	All miRNAs (179)	0.842 (0.69-0.94)	0.583 (0.28-0.85)	0.962 (0.8-1)
10	GeneGlobe miRNAs	hsa-miR-409-3p, hsa-miR-382-5p, hsa-miR-375, hsa-miR-1-3p	0.789 (0.63-0.9)	0.5 (0.21-0.79)	0.923 (0.75-0.99)

Note: LASSO models ranked based on accuracy.

Abbreviations: LASSO, Least Absolute Shrinkage and Selection Operator; miRNA, microRNA; T2D, type 2 diabetes.

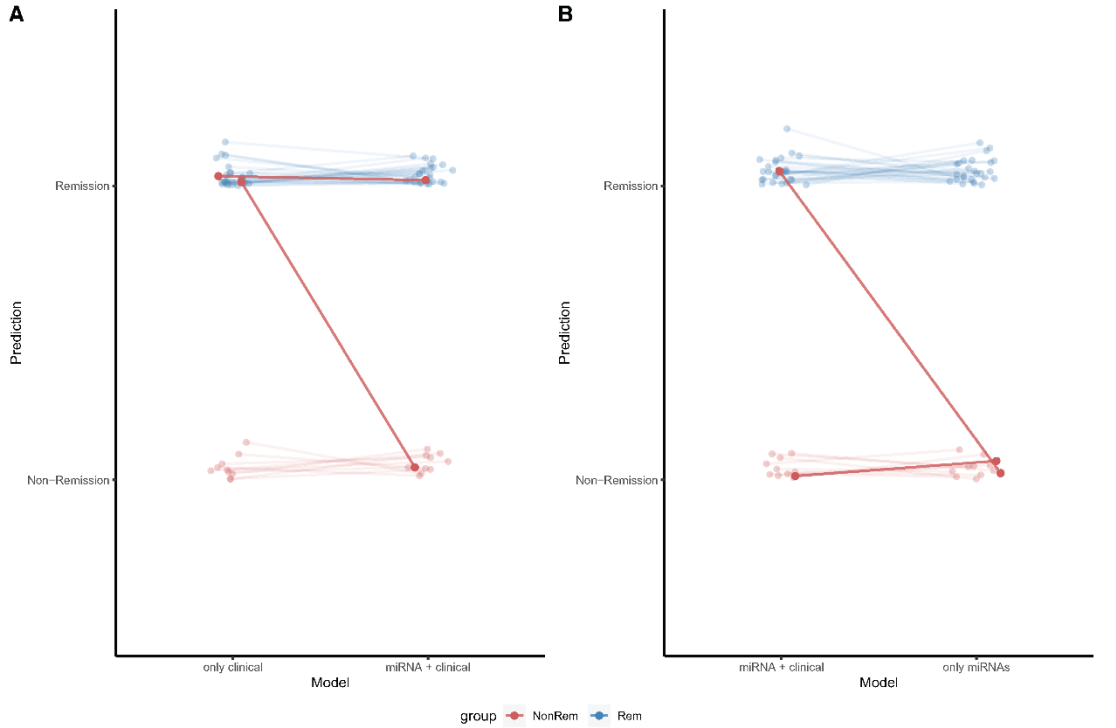

FIGURE 2 Adding miRNA information increases model accuracy. (A) Two nonremission patients (highlighted as dark red) were misclassified in a model with four clinical variables (accuracy = 0.947). One patient was correctly classified when four miRNAs were added (accuracy = 0.974). (B) The second patient was correctly classified in an miRNA-only model (using 10 miRNAs, accuracy = 1). Other patients remained correctly classified. miRNA, microRNA; NonRem, nonremission; Rem, remission

and hsa-miR-21-5p were added into the clinical model, patient 1 was correctly predicted to have nonremission. Patient 2 was still predicted as remission. When 10 or 15 miRNAs were used instead of clinical variables, both patients were classified as nonremission. Considering that patient 2 seemed to be borderline remission, the model with both clinical variables and miRNAs appears to be most accurate.

Data from the six patients with unclear remission status also agree that clinical variables are essential in the prediction model. Models with clinical predictors matched the most with postsurgery information. Using only miRNAs increased the disagreement between prediction and postsurgery data. Although more samples are needed to confirm, this suggests that our miRNA-only models are likely to be an overfit, and clinical variables should be kept in prediction models.

When available, miRNA information can help improve prediction for difficult patients and provide additional information to potentially imprecise clinical measures. Two out of four variables used in our clinical model can be inaccurate: fasting plasma glucose and T2D medication information. We requested for our patients to fast before the OGTT but we could not guarantee that they genuinely fasted. T2D medication was obtained through the patient questionnaire, which is subject to recall bias.

Our prediction models can help decision-making for newly diagnosed T2D patients who qualify for SG. Some of our patients were unaware of their T2D status and were diagnosed during their presurgery visit, which might explain the relatively low percentage of patients taking T2D medication. We found that most patients who did not report taking T2D medication achieved remission after SG, but not everyone. SG is a simpler surgery procedure but it has a lower T2D remission rate than Roux-en-Y gastric bypass (RYGB) (4,11). Therefore, deciding on bariatric surgery for new T2D patients is not straightforward. Our prediction models might help predict whether SG would result in rapid T2D remission or not for these patients.

Previous prediction models, which used similar clinical variables, predicted remission in SG patients with sensitivity and specificity up to 0.92 and 0.83, respectively (12). Our clinical model with four variables achieved sensitivity and specificity of 0.83 and 1, respectively, and adding four miRNAs increased the sensitivity to 0.917. Confirmation in external cohorts is vital to confirm the usefulness of our models.

TABLE 3 Pre- and postsurgery characteristics of the two misclassified patients and predictions shown from LASSO models: with only clinical variables, with clinical and miRNA variables, and with 10 miRNAs

		Pre surgery				12 months post surgery				Remission prediction		
Patient	Sex	Age	T2D medication	Fasting plasma glucose	HbA1c	T2D medication	Fasting plasma glucose	HbA1c	Remission	Only clinical variables	Clinical and miRNAs	Only miRNAs
1	M	63	No	193	8.1	Yes	117	6.3	No	Yes	No	No
2	M	66	No	135	6	No	128	5.9	No	Yes	Yes	No

Abbreviations: HbA1c, hemoglobin A1c; LASSO, Least Absolute Shrinkage and Selection Operator; M, male; miRNA, microRNA; T2D, type 2 diabetes.

TABLE 4 Pre- and postsurgery characteristics of six unclear patients and predictions shown from post-surgery data and LASSO models: with only clinical variables, with clinical and miRNA variables, with 10 miRNAs, and with 15 miRNAs

		Pre surgery				12 months post surgery			Remission prediction				
Patient	Sex	Age	T2D medication	Fasting plasma glucose	HbA1c	T2D medication	Fasting plasma glucose	HbA1c	Based on postsurgery data	Only clinical variables	Clinical and miRNAs	Only 10 miRNAs	Only 15 miRNAs
A	F	63	Yes	137	NA	No	NA	6.1	Yes	No	No	No	No
B	F	41	Yes	118	6.4	No	NA	5.7	Yes	Yes	Yes	Yes	No
C	M	43	Yes	127	6	NA	102	5.4	Yes	Yes	No	No	No
D	F	49	Yes	NA	7.6	No	NA	NA	Yes	No	Yes	Yes	Yes
E	F	54	Yes	135	7.5	Yes	NA	5.7	No	No	No	No	No
F	M	37	No	110	6.6	NA	95	4.9	Yes	Yes	Yes	No	No

Abbreviations: F, female; HbA1c, hemoglobin A1c; LASSO, Least Absolute Shrinkage and Selection Operator; M, male; miRNA, microRNA; NA, not available; T2D, type 2 diabetes.

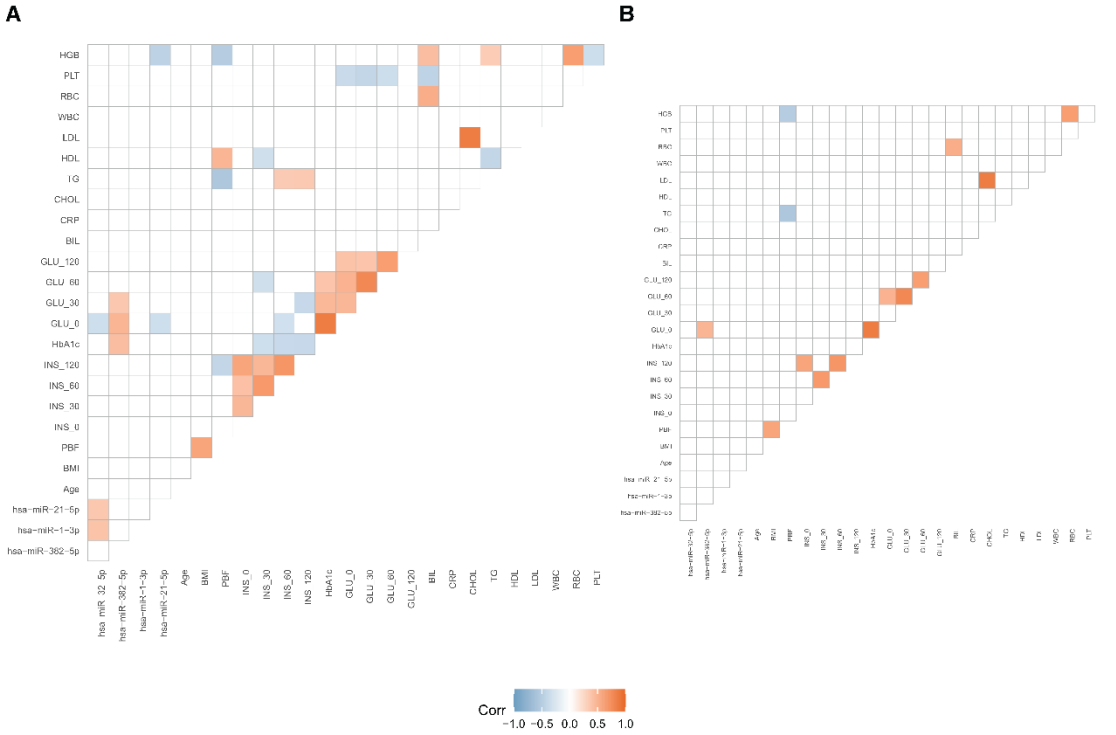

FIGURE 3 Significant Pearson correlations between selected miRNA and clinical variables. The analysis was done using R packages Hmisc and ggcorrplot. Nonsignificant correlations based on (A) $p < 0.05$ and (B) adjusted $p < 0.05$ are set to blank. Red boxes indicate positive correlations, whereas blue boxes represent negative correlations. BIL, bilirubin levels; CHOL, cholesterol levels; CRP, C-reactive protein levels; GLU_0, fasting blood glucose levels; GLU_30, plasma glucose levels measured at 30 minutes during OGTT; GLU_60, plasma glucose levels measured at 60 minutes during OGTT; GLU_120, Plasma glucose levels measured at 120 minutes during OGTT; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein levels; HGB, hemoglobin cell count; INS_0, fasting blood insulin levels; INS_30, plasma insulin levels measured at 30 minutes during OGTT; INS_60, plasma insulin levels measured at 60 minutes during OGTT; INS_120, plasma insulin levels measured at 120 minutes during OGTT; LDL, low-density lipoprotein levels; miRNA, microRNA; OGTT, oral glucose tolerance test; PBF, percentage of body fat; PLT, platelet blood count; RBC, red blood cell count; TG, triglyceride levels; WBC, white blood cell count

To our knowledge, these four serum miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) have not been studied as predictive biomarkers for T2D remission after surgery. However, studies have reported associations between these miRNAs with obesity and T2D. The miRNA hsa-miR-382-5p is involved in cholesterol homeostasis (23). Plasma and serum levels of hsa-miR-21-5p are associated with T2D (24-26), as well as with obesity (27,28). The miRNA hsa-miR-32-5p is also associated with T2D (29) and obesity (29,30). Our pathway analysis identified 19 obesity- and T2D-related pathways regulated by these miRNAs, including mechanistic target of rapamycin (serine/threonine kinase) (mTOR), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase-protein kinase b (PI3K-Akt), fatty acid elongation, and degradation pathways. The miRNA hsa-miR-1-3p has regulatory roles in cardiac muscle tissues and tumor suppressors in various cancers (31). It is also dysregulated in pancreatic cancer patients (32).

These miRNAs have been studied in bariatric surgery patients to measure differential expression before and after surgery (16). An RYGB study reported that plasma hsa-miR-32-5p and hsa-miR-21-5p were significantly reduced 9 and 12 months after surgery (33). However, another RYGB study reported an increase of plasma hsa-miR-21-5p 12 months after surgery (28). The miRNAs hsa-miR-1-3p and hsa-miR-382-5p were reported to be not significantly differentially expressed after RYGB (33). It appears that predictive miRNAs do not need to be differentially expressed after surgery. However, these studies were primarily done in RYGB patients, and more studies with SG patients are needed.

Our pilot study suggests that miRNAs could potentially predict T2D remission after the intervention. Our findings agree with a recent study that identified predictive miRNAs for T2D remission after diet intervention (34). The set of miRNAs are different from this study, which might reflect the study population. Our study focused on patients with T2D and obesity, whereas the other study's patients had

TABLE 5 Obesity- and insulin resistance-related pathways regulated by the four predictive miRNAs

No.	KEGG pathway	*p* value	No. of genes	No. of miRNAs
1	Thyroid hormone signaling pathway	9.22E-05	33	3
2	Lysine degradation	2.04E-04	15	2
3	FoxO signaling pathway	2.34E-04	41	3
4	Fatty acid elongation	0.0012	7	3
5	Prolactin signaling pathway	0.0014	21	3
6	Focal adhesion	0.0021	52	3
7	Adherens junction	0.0024	20	2
8	ECM-receptor interaction	0.0025	19	3
9	Valine, leucine, and isoleucine biosynthesis	0.0036	2	2
10	Regulation of actin cytoskeleton	0.0061	50	3
11	MAPK signaling pathway	0.0102	54	3
12	p53 signaling pathway	0.0102	21	3
13	mTOR signaling pathway	0.0133	18	3
14	Protein processing in endoplasmic reticulum	0.0140	39	3
15	Hippo signaling pathway	0.0157	32	3
16	Fatty acid degradation	0.0241	7	2
17	Endocytosis	0.0263	41	3
18	PI3K-Akt signaling pathway	0.0370	68	3
19	HIF-1 signaling pathway	0.0478	26	3

Abbreviations: ECM, extracellular matrix; FoxO, forkhead box protein O; HIF-1, hypoxia-inducible factor 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase; miRNA, microRNA; mTOR, mechanistic target of rapamycin (serine/threonine kinase); PI3K-Akt, phosphatidylinositol 3-kinase-protein kinase b.

BMI around 30 as well as coronary heart disease. Nevertheless, our study has limitations, including the small number of participants and limited external validation. Owing to sample size limitations, we simplified T2D and remission groups as dichotomous traits. Future studies could also investigate T2D subtypes based on β-cell function and insulin resistance measures (35) and include other diabetes-related variables such as C-peptide and T2D duration. Some of the patients were unaware of their T2D status, so we could not obtain an accurate T2D duration for these patients. Patients with differing risk profiles might have different remission rates after surgery. Another limitation is that we focused on SG without comparing other surgery types such as RYGB. RYGB has better long-term T2D remission rates (4,11), but only 8% of our BBSS patients underwent RYGB. Owing to study size limitations, we could not adequately compare miRNA's predictive value between these two surgery types. It would also be interesting to see whether miRNAs can differentiate between the original ASMBS remission groups ("complete remission," "partial remission," "improvement," "unchanged," and "recurrence"). Additionally, we considered only 179 miRNAs that were included in the quantitative PCR profiling platform for serum samples. Using larger profiling platforms such as small RNA sequencing might uncover more or better predictive miRNAs.

In conclusion, we identified four miRNAs (hsa-miR-32-5p, hsa-miR-382-5p, hsa-miR-1-3p, and hsa-miR-21-5p) that might complement clinical models in predicting T2D remission after SG. Further studies in much larger data are needed to confirm the utility of these serum miRNAs as predictive biomarkers. The four serum miRNAs could also be studied further to understand molecular subtypes of T2D that separate remission and nonremission patients.O

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ORCID

Gladys Wojciechowska https://orcid.org/0000-0001-9890-8794

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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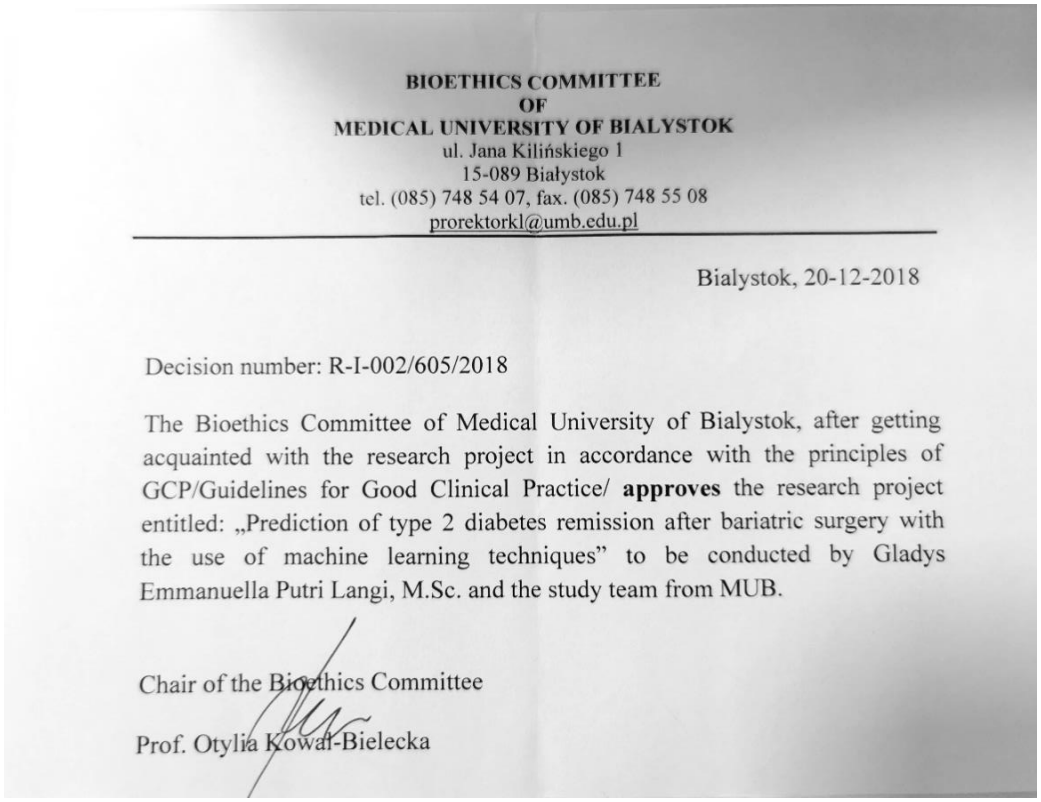
Consent from the Bioethics Committee

Decision number: R-I-002/605/2018



BIOETHICS COMMITTEE
OF
MEDICAL UNIVERSITY OF BIALYSTOK
ul. Jana Kilińskiego 1
15-089 Białystok
tel. (085) 748 54 07, fax. (085) 748 55 08
prorektorkl@umb.edu.pl

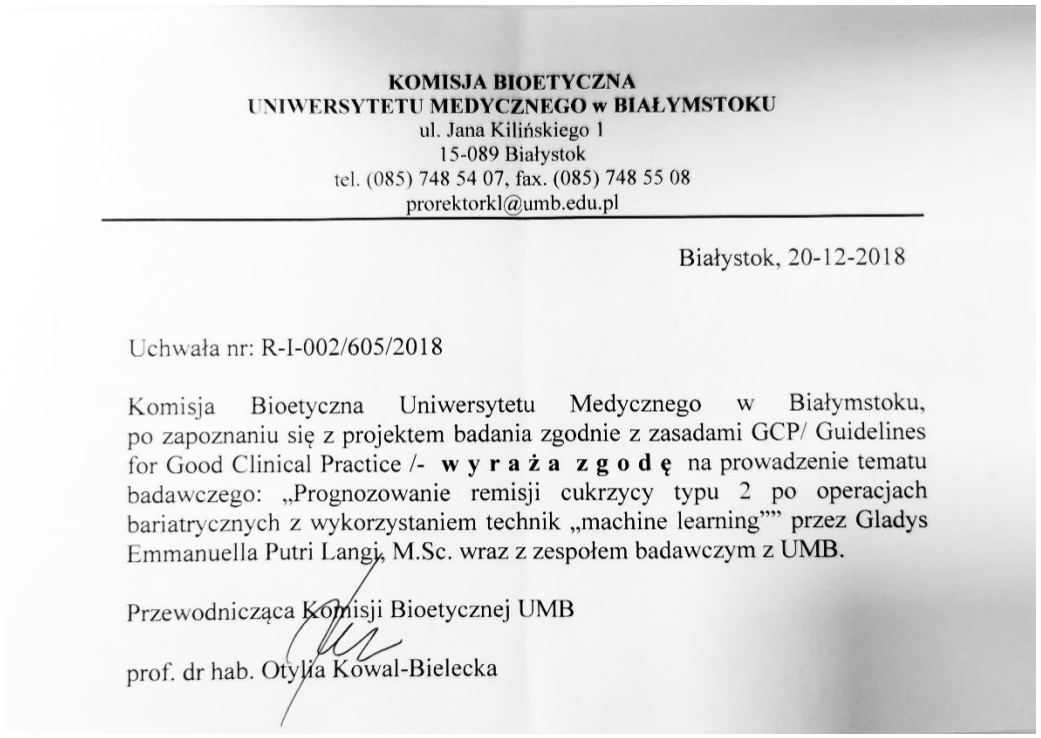
Bialystok, 20-12-2018

Decision number: R-I-002/605/2018

The Bioethics Committee of Medical University of Bialystok, after getting acquainted with the research project in accordance with the principles of GCP/Guidelines for Good Clinical Practice/ **approves** the research project entitled: „Prediction of type 2 diabetes remission after bariatric surgery with the use of machine learning techniques" to be conducted by Gladys Emmanuella Putri Langi, M.Sc. and the study team from MUB.

Chair of the Bioethics Committee

Prof. Otylia Kowal-Bielecka



KOMISJA BIOETYCZNA
UNIWERSYTETU MEDYCZNEGO w BIAŁYMSTOKU
ul. Jana Kilińskiego 1
15-089 Białystok
tel. (085) 748 54 07, fax. (085) 748 55 08
prorektorkl@umb.edu.pl

Białystok, 20-12-2018

Uchwała nr: R-I-002/605/2018

Komisja Bioetyczna Uniwersytetu Medycznego w Białymstoku, po zapoznaniu się z projektem badania zgodnie z zasadami GCP/ Guidelines for Good Clinical Practice /- **w y r a ż a z g o d ę** na prowadzenie tematu badawczego: „Prognozowanie remisji cukrzycy typu 2 po operacjach bariatrycznych z wykorzystaniem technik „machine learning"" przez Gladys Emmanuella Putri Langi, M.Sc. wraz z zespołem badawczym z UMB.

Przewodnicząca Komisji Bioetycznej UMB

prof. dr hab. Otylia Kowal-Bielecka

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KOMISJA BIOETYCZNA
UNIWERSYTETU MEDYCZNEGO w BIAŁYMSTOKU
ul. Jana Kilińskiego 1
15-089 Białystok
tel. (085) 748 54 07, fax. (085) 748 55 08
prorektorkl@umb.edu.pl

Białystok, 25-10-2018

Uchwała nr: R-I-002/386/2018

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Z-ca Przewodniczącej Komisji Bioetycznej UMB
dr n. farm. Krzysztof Chrzanowski

KOMISJA BIOETYCZNA
UNIWERSYTETU MEDYCZNEGO w BIAŁYMSTOKU
ul. Jana Kilińskiego 1
15-089 Białystok
tel. (085) 748 54 07, fax. (085) 748 55 08
prorektorkl@umb.edu.pl

Białystok, 20-12-2018

R-I-002/386/2018

Sz.P.
Prof. dr hab. Adam Krętowski

Komisja Bioetyczna UMB na posiedzeniu w dniu 20.12.2018 r. zapoznała się z wnioskiem do tematu badawczego: „Chirurgia bariatryczna jako metoda leczenia cukrzycy i otyłości – poznanie mechanizmów genetycznych i molekularnych efektu terapeutycznego zabiegów (Bialystok Bariatric Surgery Study – BBSS)" i **wyraża zgodę** na włączenie do zespołu Gladys Emmanuella Putri Langi.

Z poważaniem,

Przewodnicząca Komisji Bioetycznej UMB

prof. dr hab. Otylia Kowal-Bielecka

Statements

CO-AUTHORS
CONTRIBUTION STATEMENT

Title: Meta-Analysis of Differential miRNA Expression after Bariatric Surgery.

Authors: Langi G, Szczerbinski L, Kretowski A.

Published in: Journal of Clinical Medicine. 2019;8(8):1220. doi:10.3390/jcm8081220

Name	Contribution	Percentage
Gladys Emmanuella Putri Wojciechowska (Langi), MSc.	Conceptualization, data collection, analysis, results interpretation, visualization, writing –original draft preparation, writing –review and editing	90%
dr n. med. Łukasz Szczerbiński	Supervision, conceptualization, writing – review and editing	5%
Prof. dr hab. n. med. Adam Jacek Krętowski	Supervision, conceptualization, writing – review and editing	5%

I hereby declare that all co-authors agreed to the inclusion of this paper in the doctoral degree procedure of Gladys Emmanuella Putri Wojciechowska, MSc.

Signature

dr n. med. Łukasz Szczerbiński
.....
name and last name of the author

Clinical Research Centre,
Medical University of Białystok
.....
affiliation/name of the university

Białystok, 07 March 2022
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signature

Prof. dr hab. n. med. Adam Jacek Krętowski

name and last name of the author

Białystok, 08 March 2022

date, place

Clinical Research Centre,
Medical University of Białystok

affiliation/name of the university

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I confirm that in the article:

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signature

CO-AUTHORS
CONTRIBUTION STATEMENT

Title: Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study.

Authors: Wojciechowska G, Szczerbinski L, Kretowski M, Niemira M, Hady HR, Kretowski A.

Published in: Obesity. 2022;30(2):435-446. doi:10.1002/oby.23342

Name	Contribution	Percentage
Gladys Emmanuella Putri Wojciechowska, MSc.	Conceptualization, data collection, analysis, results interpretation, visualization, writing –original draft preparation, writing –review and editing	80%
dr n. med. Łukasz Szczerbiński	Supervision, conceptualization, data collection, results interpretation, writing –review and editing	5%
Prof. dr hab. inż. Marek Krętowski	Analysis, results interpretation, writing –review and editing	5%
dr Magdalena Niemira	Data collection, results interpretation, writing –review and editing	3%
Prof. dr hab. n. med. Hady Razak Hady	Conceptualization, data collection	2%
Prof. dr hab. n. med. Adam Jacek Krętowski	Supervision, conceptualization, writing –review and editing	5%

I hereby declare that all co-authors agreed to the inclusion of this paper in the doctoral degree procedure of Gladys Emmanuella Putri Wojciechowska, MSc.

Signature

dr n. med. Łukasz Szczerbiński
.....
name and last name of the author

Clinical Research Centre,
Medical University of Białystok
.....
affiliation/name of the university

Białystok, 07 March 2022
.....
date, place

Statement

I confirm that in the article:

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.....
signature

Prof. dr hab. inż. Marek Krętowski

name and last name of the author

Białystok University of Technology

affiliation/name of the university

Białystok, 1 February 2022

date, place

Statement

I confirm that in the article:

Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study

which is a part of doctoral dissertation of Gladys Emmanuella Putri Wojciechowska, my contribution included data analysis and interpretation.

I agree to use this publication by Gladys Emmanuella Putri Wojciechowska, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

M. Krętowski

signature

dr Magdalena Niemira

name and last name of the author

Białystok, 17 February 2022

date, place

Clinical Research Centre,

Medical University of Białystok

affiliation/name of the university

Statement

I confirm that in the article:

Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study

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Magdalena Niemira

signature

Prof. dr hab. n. med. Hady Razak Hady

name and last name of the author

Białystok, 17 February 2022

date, place

1st Clinical Department of General and Endocrine Surgery,

Medical University of Białystok

affiliation/name of the university

Statement

I confirm that in the article:

Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study

which is a part of doctoral dissertation of Gladys Emmanuella Putri Wojciechowska, my contribution included data collection and conceptualization.

I agree to use this publication by Gladys Emmanuella Putri Wojciechowska, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

Hady Razak Hady

signature

Prof. dr hab. n. med. Adam Jacek Krętowski
.....
name and last name of the author

Clinical Research Centre,
Medical University of Białystok
.....
affiliation/name of the university

Białystok, 07 March 2022
.....
date, place

Statement

I confirm that in the article:

Exploring microRNAs as predictive biomarkers for type 2 diabetes mellitus remission after sleeve gastrectomy: A pilot study

which is a part of doctoral dissertation of Gladys Emmanuella Putri Wojciechowska, my contribution included supervision, conceptualization, writing –review and editing.

I agree to use this publication by Gladys Emmanuella Putri Wojciechowska, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

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signature