Serum miR-365b-5p/miR-222-5p as a potential diagnostic biomarker for long-term weight loss in patients with morbid obesity after bariatric surgery

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ABSTRACT

Background

The successful weight loss following bariatric surgery is not achieved in all patients with morbid obesity (MO). This study aims to determine whether a serum miRNA profile can predict this outcome.

Design

Thirty-three patients with MO were classified in “good responders" (GR, percentage of excess weight loss (%EWL)≥50%) or "non-responders" (NR, %EWL<50%) according to the %EWL 5-8 year following bariatric surgery. Baseline serum miRNA sequencing was performed to find predictor biomarkers and human adipocyte culture were performed to determine their effect.

Results

Fifty-six differentially expressed miRNAs were found between GR and NR. Multiple logistic regression models showed two miRNAs, hsa-miR-365b-5p (upregulated in GR) and hsa-miR-222-5p (upregulated in NR) associated to %EWL. Receiver operating characteristic curves showed that the combination of these miRNAs was the best serum miRNAs profile that distinguished between GR and NR. The experimentally validated target genes of these miRNAs were involved in processes related to the response to stress, cell cycle, transduction, and development and proliferation processes. The *in vitro* expression of six genes involved in adipogenesis and adipocyte differentiation (STAT3, ILR7, PARP1, SOD2, FGF2 and TMEM18) was downregulated in lipogenic and upregulated in lipolitic conditions in human adipocytes incubated with the combination of a hsa-miR-365b-5p mimic and a hsa-miR-222-5p inhibitor.

Conclusions

Baseline serum hsa-miR-365b-5p and hsa-miR-222-5p were able to predict %EWL 5-8 years following bariatric surgery. The combination of these potential predictive biomarkers was involved in regulating the expression levels of genes associated with obesity. However, these effects could be modified depending of other stimuli.

Keywords: Morbid obesity; bariatric surgery; percentage of excess weight loss; miRNA; miR-365b-5p; miR-222-5p.

INTRODUCTION

There is a strong association between obesity and adverse health outcomes [1]. A number of conservative therapies have demonstrated limited efficacy with regard to weight loss and the management of comorbidities. Bariatric surgery has been regarded as the most efficacious therapeutic approach for severe obesity, as it induces weight loss and modifies metabolism to control morbid obesity and systemic comorbidities [2]. However, there are some discrepancies about it in the literature since 5-20% of the patients do not achieve a successful long-term weight loss [3,4]. Therefore, the identification of biomarkers associated with successful weight loss following bariatric surgery would facilitate the development of personalized medicine, as well as enable the integration of additional interventions in cases where adequate weight loss is not anticipated. However, the specific biomarkers that can predict surgical outcomes remain unclear, representing a crucial step in the advancement of personalized medicine.

In recent years, significant research has been conducted with the aim of identifying genes associated with obesity. This research has sought to enhance our understanding of the underlying mechanisms, identify new targets for clinical therapy, and facilitate the early prediction of metabolic complications and treatment response. Indeed, there is an accumulating body of evidence that epigenetic regulation of gene expression represents a significant contributor to the variation in predisposition to obesity and associated comorbidities [5]. In this context, miRNAs have been demonstrated to regulate a multitude of cellular processes at the post-transcriptional level, including cell growth, proliferation, differentiation, DNA repair, and apoptosis [6]. Deregulation of miRNA profiles has been associated with a number of pathological conditions [7-9]. Furthermore, miRNAs have been identified as a key regulator of various biological processes associated with obesity, including inflammation, adipogenesis, adipocyte differentiation, metabolic integration, fat metabolism, and insulin sensitivity [10-11]. Although miRNAs act at the subcellular level, they can also be found in the circulatory system. The biological implications and physiological role of circulating miRNAs remain unclear, despite their potential involvement in intercellular communication. Consequently, these miRNAs have gained clinical and therapeutic relevance as novel biomarkers, with changes in their profile indicating the presence of physio-pathological conditions [12].

A number of studies have indicated that specific stimuli can influence the miRNA profile of patients, thereby promoting health improvement. The intake of macronutrients, the composition of energy-restricted diets with varying carbohydrate and fat ratios, and the physical condition of the individual all influence the pattern of circulating miRNAs [13-14]. In the context of bariatric surgery, some studies have demonstrated alterations in postoperative circulating miRNA expression, which have been associated with an improvement in the metabolic state of patients [15]. However, given the genetic background of obesity, it is reasonable to hypothesize that predictors may be identified within the genetic context. Therefore, the present study aims to determine whether the circulating miRNA profile can be used as a predictive biomarker for the success of a bariatric surgery in patients with morbid obesity.

MATERIAL AND METHODS

Study design and subjects

A total of 90 patients with morbid obesity (BMI>40kg/m^2), who were scheduled for bariatric surgery at the Virgen de la Victoria University Hospital and Regional University

Hospital in Malaga between 2010 to 2013, were invited to participate in the present study. The patients underwent one of the following bariatric surgery procedures: laparoscopic Roux-en-Y gastric bypass (RYGB) or sleeve gastrectomy (SG) [16,17]. Of the 90 patients who underwent bariatric surgery, only 53 consented to participate in this prospective study. Patients were classified into two groups based on their bariatric surgery outcome: "good-responders" (GR) and "non-responders" (NR), as defined below. Of these, only 33 patients had baseline serum samples available for predictive biomarker analyses. No significant differences were observed in the variables analyzed between patients with and without baseline serum samples (Supplementary Table 1). The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided written informed consent and the study was reviewed and approved by the Malaga Provincial Research Ethics Committee (Malaga, Spain) (protocol code PI-0194-2017 (1 February 2018)).

Clinical and biochemical Data

The data were prospectively collected prior to bariatric surgery and over a period of between 5-8 years in the postoperative period (long term). The percentage of change of variables 5-8 years after bariatric surgery was calculated as 100 x (variable at the time of evaluation – preoperative variable)/preoperative variable.

Assessment of Weight Change

The percentage of excess weight loss (%EWL) was based on the excess weight compared to the weight corresponding to a BMI of 25 kg/m^2 for each patient: 100 × (preoperative weight - weight at the time of evaluation)/(preoperative weight - weight corresponding to BMI=25 kg/m^2) [18]. Weight loss was considered insufficient when %EWL<50% in analogy with the Reinhold criteria [19], and modified by Christou et al. [20]. This %EWL was calculated at 5-8 years after bariatric surgery. Those patients with a %EWL≥50% were considered as GR. On the other hand, patients with %EWL<50% were considered as NR.

miRNA extraction and small RNA sequencing

Total serum RNA, with an enhanced miRNA enrichment, was extracted and used to perform an ultrasequencing of miRNA libraries to analyze the miRNA expression pattern by Next-Generation Sequencing.

Statistical analysis for algorithm designed as predictor to distinguish between GR and NR patients

Diagnostic efficacy analysis of these differentially expressed-miRNAs (DE-miRNAs) were carried out with the subjects of the prospective study (GR *vs*. NR) through step-by-step logistic regression analysis, with which the best predictor model was selected after combining different variables. The predicted probabilities of the model were dichotomized based on the best cut-off calculated by the receiver operating characteristic (ROC) curve. Next, the predictive values were calculated based on this cut-off.

Enrichment analysis of miRNAs selected as predictor biomarkers

An analysis of the targets and pathways regulated by the miRNAs selected as predictor biomarkers was performed by miRTarBase (last access June 2023 https://miRTarBase.cuhk.edu.cn/) [21].

Incubation of human adipocytes with miRNAs selected as predictor biomarkers

Human mesenchymal stem cells from subcutaneous adipose tissue (subASCs; PromoCell, C-12977, Heidelberg, Germany) were differentiated to adipocytes as previously described [22], and cultured for an additional 48 hours in either lipogenic or lipolytic medium. At the start of the culture in lipogenic or lipolytic medium, adipocytes were stimulated with either a mirVana™ miRNA mimic for hsa-miR-365b-5p (Assay ID: MC15283), a mirVana™ miRNA inhibitor for hsa-miR-222-5p (Assay ID: MH12656), or a combination of both, along with their respective non-targeting controls (Thermo Fisher Scientific Inc, Waltham, MA). After these 48 h, the mRNA was isolated as previously described [23], and analyzed the mRNA expression.

A complete description of the Material and Methods is provided in the Supplementary Material and Methods.

RESULTS

Characteristics of patients with morbid obesity

Clinical and biochemical variables in the GR and NR groups prior to bariatric surgery is presented in Table 1. No differences were identified in the variables analysed.

Differentially expressed miRNAs in good responders *vs.* no responders prior to surgery

The serum miRNA expression profile was determined prior to bariatric surgery. Using a p-value of ≤0.05, we identified 56 DE-miRNAs between the GR and NR groups (Figure 1A). Of these, 38 miRNAs were significantly upregulated in the GR group, while 18 miRNAs were significantly downregulated in the GR group, or conversely, upregulated in the NR group. The log2FC and p-values of these DE-miRNAs are presented in Supplementary Table 2.

To identify groups of samples with specific patterns of miRNA expression, a hierarchical cluster analysis was conducted, which permitted an investigation of similarities or differences between the samples from both groups on an individual basis. A heat map was generated, and the results demonstrated a distinctive expression pattern of the 56 DE-miRNAs, as illustrated in Figure 1B. Notably, a principal division was observed between the clusters based on the postoperative response, with 38 DE-miRNAs exhibiting upregulation in the GR group and only 18 DE-miRNAs demonstrating upregulation in the NR group.

Target prediction, enrichment and functional analyses

Following the identification of these DE-miRNAs, they were subjected to further *in silico* enrichment and functional analyses. The analysis of the DE-miRNAs that were upregulated in the GR group revealed 9073 potential target genes, while the analysis of the DE-miRNAs that were upregulated in the NR group predicted 3626 potential target genes. It was found that there were 2360 target genes that were shared between the two groups.

The predicted target genes were subjected to further analysis for KEGG pathway enrichment, with a cutoff p-value<0.05. A total of 91 KEGG pathways were identified as being associated with the GR group, while 104 KEGG pathways were associated with the NR group. Between these KEGG pathways, a total of 78 KEGG pathways were identified as shared between both groups, while 13 KEGG pathways were exclusive to the GR group and 26 to the NR group. In order to identify potential differences according to the %EWL

after bariatric surgery (GR and NR), we focused on the exclusive KEGG pathways for each group. It is noteworthy that numerous pathways associated with metabolism, the endocrine system, and immunology were identified. Of the 13 GR-specific KEGG pathways, several merit further investigation for their potential association with obesity-related biological effects. The Fc gamma R-mediated phagocytosis pathway was found to be statistically significant (p=0.001), as were the Fc epsilon RI signaling pathway (p=0.001), aldosterone synthesis and secretion (p=0.015), and the oxytocin signaling pathway (p=0.016). Furthermore, the pathways of purine metabolism (p=0.022), ovarian steroidogenesis (p=0.024), arrhythmogenic right ventricular cardiomyopathy (p=0.025) and biosynthesis of unsaturated fatty acids (p=0.038) were identified (Figure 2A).

Furthermore, the bioinformatic data analysis revealed 26 NR-KEGG exclusive pathways, some of which are potentially associated with biological pathways involved in obesity. These include leukocyte transendothelial migration (p<0.001), T cell receptor signaling pathway (p=0.001), and C-type lectin signaling pathway. The insulin signaling pathway (p=0.004), Th17 cell differentiation (p=0.01), prolactin signaling pathway (p=0.024) and diabetic cardiomyopathy (p=0.05) were also identified (Figure 2B).

Furthermore, a functional enrichment analysis was conducted using a p-value threshold of 0.05 to examine the GO terms involved in biological processes (GO:BP). A total of 1,588 GO:BP terms were predicted in the GR group and 1,308 GO:BP terms in the NR group, with 905 GO:BP terms being common to both groups. A total of 683 GO:BP terms were exclusive to the GR group, while 403 were exclusive to the NR group. To gain insight into the biological processes that may be affected by the different miRNA expression patterns, the exclusive GO:BP for each group were subjected to further analysis. The top 20 differentially detected GO:BP terms, categorized by the number of related mRNA targets, were selected, as they may represent the most affected biological processes at the gene target level. In the GR group, the exclusive GO:BP terms identified were: establishment of organelle localization (GO:0051656; p<0.001), organelle fission (GO:0048285; p=0.013), nuclear division (GO:0000280; p=0.031), ribonucleotide metabolic process (GO:0009259; p<0.001), ribose phosphate metabolic process (GO:0019693; p<0.001), purine nucleotide metabolic process (GO:0006163; p=0.001), nucleotide metabolic process (GO:0009117; p=0.001), purine-containing compound metabolic process (GO:0072521; p=0.001), nucleoside phosphate metabolic process (GO:0006753; p=0.001), T cell activation (GO:0042110; p=0.001), mononuclear cell differentiation (GO:1903131; p=0.002), phospholipid metabolic process (GO:0006644; p<0.001), fatty acid metabolic process (GO:0006631; p=0.003), glycoprotein metabolic process (GO:0009100; p=0.005), lipid localization (GO:0010876; p=0.013), signal release (GO:0023061; p=0.001), modulation of chemical synaptic transmission (GO:0050804; p=0.031), regulation of trans-synaptic signaling (GO:0099177; p=0.034), positive regulation of response to external stimulus (GO:0032103; p=0.035) and cytokine-mediated signaling pathway (GO:0019221; p=0.043) (Figure 3A).

Similarly, the GO:BP terms identified in the NR group were as follows: regulation of translation (GO:0006417; p<0.001), negative regulation of translation (GO:0017148; p<0.001), negative regulation of cellular amide metabolic process (GO:0034249; p=0.001), ribonucleoprotein complex biogenesis (GO:0022613; p=0.003), RNA splicing (GO:0008380; p=0.003), pattern specification process (GO:0007389; p=0.004), calcium ion transport (GO:0006816; p=0.006), methylation (GO:0032259; p=0.007), negative regulation of hydrolase activity (GO:0051346; p=0.007), regulation of metal ion transport

(GO:0010959; p=0.011), muscle system process (GO:0003012; p=0.015), negative regulation of locomotion (GO:0040013; p=0.019), regulation of endopeptidase activity (GO:0052548; p=0.026), negative regulation of proteolysis (GO:0045861; p=0.027), cognition (GO:0050890; p=0.027), regulation of peptidase activity (GO:0052547; p=0.031), regulation of ERK1 and ERK2 cascade (GO:0070372; p=0.032), cellular divalent inorganic cation homeostasis (GO:0072503; p=0.047), negative regulation of cellular component movement (GO:0051271; p=0.047) and calcium ion homeostasis (GO:0055074; p=0.047) (Figure 3B).

Correlations between serum DE-miRNAs with the %EWL and clinical variables

Spearman correlation analyses were conducted to identify the baseline serum DE-miRNAs that were most closely associated with %EWL at 5-8 years after bariatric surgery. Of the 56 DE-miRNAs, only 32 demonstrated a significant correlation with %EWL (Figure 4A). At baseline, Spearman correlation analyses were conducted to ascertain any potential associations between the DE-miRNAs and the anthropometric and clinical characteristics of the patients. Figure 4A presents only those significant correlations. The majority of these were found with DBP (negatively), glucose and HOMA-IR (positively).

Additionally, Spearman correlations were conducted between the baseline serum DE-miRNAs and the percentage of change in anthropometric and biochemical variables 5-8 years following bariatric surgery (Figure 4B). The results demonstrated that hsa-miR-1295a, hsa-miR-1295b-5p, hsa-miR-3652, hsa-mir-365b-5p, hsa-miR-3690, hsa-miR-4666a-5p and hsa-miR-7977 were those DE-miRNAs more closely associated (correlated with four or more variables). The majority of the associations identified were negative. A more negative value of the percentage of change of the anthropometric/clinical variable indicates a greater percentage of decrease in that variable following bariatric surgery. Consequently, a negative correlation (red colour) implies that as the expression of the DE-miRNA increases, the percentage of decrease in the anthropometric or clinical variable also increases.

Hsa-miR-365b-5p and hsa-miR-222-5p as predictor biomarkers to distinguish between GR and NR patients

In order to develop a prognostic biomarkers panel, multiple logistic regression models (GR=1 and NR=2) were used to evaluate associations between these 32 DE-miRNAs and the %EWL, after adjusting for age, gender and BMI. This selection procedure only identified hsa-miR-365b-5p (B=-1.688, p=0.032, OR=0.68) (upregulated in GR group) and hsa-miR-222-5p (B=2.71, p=0.049, OR=1.72) (upregulated in NR group) for inclusion in the combination panel. Then, we generated ROC curves for the best single biomarker and the combination model to distinguish between GR *vs*. NR patients (Figure 5). The area under the curve (AUC) for hsa-miR-222-5p was 0.670 (p=0.049, 95%CI: 0.48-0.86), for hsa-miR-365b-5p was 0.248 (p=0.014, 95%CI: 0.08-0.41) and for the combination of these two DE-miRNAs was 0.874 (p<0.001, 95%CI: 0.76-0.99).

ROC curves were performed on the combined data of the number of hsa-miR-222-5p and hsa-miR-365b-5p readings, resulting in a cut-off point of 0.174, which exhibited the highest sensitivity and specificity. To obtain the value for each patient, a calculation was made using the following equation:

$$P = 1 / (1 + e^{-(-0.477 - (2{,}71 \times \text{number of readings hsa-miR-mir222-5p}) + (1{,}69 \times \text{number of readings hsa-miR-365b-5p}))})$$

where P represent the predicted probability of achieving adequate long-term (5-8 years) weight loss following bariatric surgery (%EWL≥50%) in patients with morbid obesity, and number of readings hsa-miR-222-5p and hsa-miR-365b-5p is the number of hsa-miR-222-5p and hsa-miR-365b-5p readings obtained in accordance with the specified methodology. The cut-off point determined for this algorithm is 0.174. A value of $P<0.174$ would indicate that patients with morbid obesity have achieved long-term (5-8 years) weight loss (%EWL≥50%) following bariatric surgery, with a positive predictive value and a specificity of 100%. The algorithm yielded a correctly diagnosed patient population of 78.8% (95%CI: 60.6%-90.4%), with a sensitivity of 61.1% (95%CI: 36.1%-81.7%), a specificity of 100% (95%CI: 74.6%-99.4%), a positive predictive value of 100% (95%CI: 67.8%-99.2%) and a negative predictive value of 68.2% (95%CI: 45.1%-85.3%).

Target prediction and functional analyses of hsa-miR-365b-5p and hsa-miR-222-5p

In order to identify the pathways that may be influenced by the two DE-miRNAs, hsa-miR-365b-5p and hsa-miR-222-5p, the validated target genes obtained from miRTarBase (Figure 6A) were subjected to further analysis using the KEGG pathway enrichment tool. A total of 109 target genes were identified as potentially affected by hsa-miR-365b-5p, while 74 were found to be affected by hsa-miR-222-5p. Of these, only two target genes were found to be shared. Of the remaining 179 (107 from hsa-miR-365b-5p and 72 from hsa-miR-222-5p), there were 30 genes related to enzymes, 23 related to transcription factors, 15 related to membrane trafficking, 12 related to chromosome and associated proteins, 11 related to ubiquitin system, 9 related to exosomes, 8 related to domain-containing proteins not elsewhere classified, 7 related to CD molecules and 6 related to spliceosome, protein phosphatases and associated proteins and transporters. The KEGG pathways with greater relevance (with five or more genes) associated with the 107 target genes from hsa-miR-365b-5p and the 72 target genes from hsa-miR-222-5p are illustrated in Figure 6B. The validated experimentally target genes were also found to be involved in a number of different biological processes (GO:BP), primarily related to the regulation of catalytic activity, response to stress, cell cycle, transduction, and development and proliferation processes (see Figures 6C and 6D for target genes from hsa-miR-222-5p and hsa-miR-365b-5p, respectively).

Experimental validation of some of the target genes of hsa-miR-365b-5p and hsa-miR-222-5p in human adipocytes

Finally, we sought to ascertain whether the level of some of the potential target genes of hsa-miR-365b-5p and hsa-miR-222-5p, which may be associated with obesity, were modified in adipocytes following incubation with these miRNAs. The objective was to simulate the associations identified between %EWL and these miRNAs: hsa-miR-365b-5p upregulated and hsa-miR-222-5p downregulated in the GR group. To achieve this, adipocytes (n=4) were incubated with a hsa-miR-365-5p mimic, a hsa-miR-222-5p inhibitor and a mixture of both.

Under lipogenic conditions (Figure 7A), the incubation of adipocytes with the miR-365-5p mimic resulted in a statistically significant reduction in the expression of STAT3 ($p<0.05$), PARP1 ($p<0.05$) and SOD2 ($p<0.01$). Incubation with the miR-222-5p inhibitor resulted in a significant decrease in the expression of STAT3 ($p<0.01$), IL7R ($p<0.05$), PARP1 ($p<0.01$) and SOD2 ($p<0.01$). The combination of the miR-365-5p mimic and the miR-222-5p inhibitor resulted in a greater enhancement of the inhibitory effects observed with each individual mimic and inhibitor. A significant decrease was observed in the

expression of STAT3 ($p<0.005$), IL7R ($p<0.01$), PARP1 ($p<0.01$) and SOD2 ($p<0.005$). Furthermore, this combination resulted in a notable reduction in the expression of STAT3 ($p<0.05$), PARP1 ($p<0.05$) and IL7R ($p<0.05$) in comparison to the effects observed with the miR-365-5p mimic, and of FGF2 ($p<0.05$) in comparison to the effects observed with the miR-222-5p inhibitor.

Under lipolytic conditions (Figure 7B), only the combination of the miR-365-5p mimic and miR-222-5p inhibitor produced a significant increase in the expression of IL7R ($p<0.05$), FGF2 ($p<0.05$) and TMEM18 ($p<0.05$) in comparison to the control group (without mimic or inhibitor), and of STAT3 ($p<0.05$) and PARP1 ($p<0.05$) in comparison to the effects observed with the miR-365-5p mimic.

DISCUSSION

To our knowledge, we provide the first evidence of a preoperative serum miRNA signature in patients with morbid obesity according to their response to long-term weight loss after bariatric surgery. In particular, we identified 56 miRNAs at baseline that showed differential expression between the two groups of patients (GR vs. NR), which may be associated with the degree of weight loss after this intervention. Among these DE-miRNAs, some were upregulated in the GR group, which were mainly associated with biological processes related to nucleotide and nucleoside metabolism, immune response and lipid metabolism and localization. According to the literature, some of these miRNAs have been well studied, while others appear to be new in the field of obesity research. For example, previous studies have confirmed the importance of hsa-miR-206 in obesity [7,24], with some studies showing its role in the pathogenesis of non-alcoholic fatty liver disease [25,26]. Other miRNAs have been implicated in obesity-related pathologies such as type 2 diabetes mellitus (T2DM). For example, lower levels of hsa-miR-199-5p were found in patients with T2DM compared to prediabetic subjects [27]. Regarding the NR group, only a few of the upregulated DE-miRNAs found in this group have been associated with obesity or related diseases. For example, overexpression of hsa-miR-320a was found in β-pancreatic cells in a mouse model of obesity-induced apoptosis [28]. MiR-31-5p was significantly upregulated in children with obesity/overweight [7]. In addition, miR-31-5p was also significantly higher in visceral adipose tissue of patients with obesity and T2DM than in healthy subjects [29]. But most of these miRNAs were mainly associated with T2DM, such as hsa-miR-320a, hsa-miR-3611, hsa-miR-222-5p, hsa-miR-31-5p, hsa-miR-218-1-3p and hsa-miR-4687-3p [28,30-32].

The present study also demonstrates correlations between certain DE-miRNAs related to weight loss and T2DM-related variables. Some DE-miRNAs were found to be positively associated with glucose levels, including hsa-miR-1228-5p, hsa-miR-3652 and hsa-miR-551a. Moreover, other DE-miRNAs (hsa-miR-1295a, hsa-miR-1295b-5p, hsa-miR-204-3p and hsa-miR-6772-5p) also exhibited a strong and positive correlation with glucose and HOMA-IR. In various murine models of obesity, the downregulation of miR-204 alleviated inflammation and insulin tolerance [33]. Indeed, the genetic deletion of miR-204 enhances glycemic control in the context of obesity in db/db mice [34]. Furthermore, elevated levels of several DE-miRNAs were linked to a greater reduction in glucose, insulin, and HOMA-IR following bariatric surgery, indicating the potential predictive value of these miRNAs in predicting surgical outcomes. Such correlations could perhaps indicate that the upregulation of these DE-miRNAs is intended to compensate for the

elevated baseline glucose level, which could facilitate its reduction following bariatric surgery. However, this hypothesis requires further investigation.

Nevertheless, T2DM has not been subjected to comprehensive investigation in the present study, which is designed to examine the relationship between serum miRNAs and %EWL following bariatric surgery. In this context, the most significant findings pertain to weight loss. Given that the study design divided patients into two groups, with greater or lesser weight loss following bariatric surgery, the majority of DE-miRNAs were associated with the change in BMI, waist and hip circumference following bariatric surgery. As anticipated, the majority of the DE-miRNAs exhibited a correlation with %EWL. Nevertheless, a more detailed examination of the results enabled the selection of hsa-miR-365b-5p (overexpressed in GR) and hsa-miR-222-5p (overexpressed in NR) as predictor biomarkers for distinguishing between GR and NR patients. The combination of these two genes allows the creation of an algorithm that can accurately predict whether patients with morbid obesity will achieve adequate long-term weight loss (%EWL≥50%) following bariatric surgery in 78.8% of cases. Moreover, this algorithm allows us to have a positive predictive value and specificity of 100% for long-term (5-8 years) weight loss (%EWL≥50%) following bariatric surgery. To date, there has been little research linking these two miRNAs to obesity, particularly in the case of miR-365b-5p. Previous studies has demonstrated that miR-365 plays a role in white fat adipogenesis [35]. This miRNA inhibited the preadipocyte differentiation and the accumulation of lipid droplets in preadipocytes in Yanbian yellow cattle [36]. Furthermore, additional evidence substantiates the role of miR-222 in the regulation of obesity. Elevated circulating levels of miR-222 have been observed in patients with morbid obesity [37], accompanied by an increase in adipose tissue [38]. This miRNA has been demonstrated to be reduced during the differentiation of human mesenchymal stem cells [38,39], indicating that it functions as a negative regulator of differentiation [39]. Furthermore, its ectopic expression was found to significantly inhibit adipogenesis [39].

The modulation of these two miRNAs may exert varying effects on cells, as predicted by KEGG and GO analysis of their target genes. KEGG pathways show that these two miRNAs are involved in cancer and GO related, including cell proliferation, development, the cell cycle and apoptosis. Our results show that these pathways and GO terms are more closely associated with hsa-miR-222-5p, which is overexpressed in the NR group. Overexpression of hsa-miR-222-5p has been observed in a range of tumour types [40], particularly liver cancer [41]. It plays a pivotal role in promoting cell proliferation, migration and invasion, while reducing cell apoptosis in hepatocellular carcinoma cells. In this context, different studies have shown the relationship between hepatocellular carcinoma (HCC) and obesity [42]. The %EWL has been shown to be positively correlated with fewer hepatocellular adenoma lesions post-bariatric surgery [43]. In addition, this type of intervention may reduce the future incidence of hepatocellular carcinoma (HCC) [44]. Therefore, and according to our data, a higher expression of hsa-miR-222-5p would be associated with a lower weight loss, and perhaps, and based on previous studies, with a higher incidence of HCC. However, given the design of the study, we are not able to establish a relationship between hsa-miR-222-5p and HCC. This is only a hypothesis that needs to be confirmed in further studies. On the other hand, hsa-miR-365b has been reported to be associated with other obesity-related diseases. One study identified a downregulation of hsa-miR-365b-5p in temporal cortex autopsy samples from Alzheimer's disease (AD) [45]. In this context, there is evidence that obesity is associated with AD [46], and that the weight loss following bariatric

surgery is associated with sustained improvement in cognitive function [47]. Therefore, and given the relationship found between obesity and AD, and between this disease and hsa-miR-365b-5p [45], a higher expression of this miRNA would be associated with a greater weight loss, and perhaps, with a lower incidence of AD. However, further long-term studies are needed to investigate the evolution of serum levels of miR-365b-5p after bariatric surgery and its potential effect in the setting of AD.

In order to gain insight into the potential mechanisms through which hsa-miR-365b-5p and hsa-miR-222-5p may influence obesity, we have focused our analysis on a subset of target genes of these miRNAs that have previously been linked to obesity according miRTarBase. Six genes were identified as potential targets of hsa-miR-365b-5p (STAT3, ILR7 and PARP1) and hsa-miR-222-5p (SOD2, FGF2 and TMEM18). To analyse whether modifying the levels of these miRNAs can have an effect on adipocytes, they were incubated with a hsa-miR-365b-5p mimic, a hsa-miR-222-5p inhibitor, and a combination of both, under both lipogenesis and lipolysis conditions. We found that the mRNA expression of these genes was modified by these two miRNAs, especially when adipocytes were incubated with the combination miR-365b-5p mimic / miR-222-5p inhibitor. This combination of miRNAs led to a more pronounced decrease in the expression of the STAT3, PARP1 and FGF2 genes. All these genes are involved in the regulation of adipogenesis (process by which mesenchymal stem cells differentiate into adipocytes), adipocyte differentiation and the regulation of adipose tissue metabolism. The inhibition of most of these genes could reduce adipocyte differentiation, as in the case of STAT3, FGF2 and TMEM18 [48-50], but other genes such as SOD2 seem to increase it [51], and there are even contradictory results for some of them, as in the case of PARP1 [52]. At least through these genes, the levels of these two miRNAs could be involved in the differential weight loss after bariatric surgery. However, we should not forget that other genes could also be involved. In addition, we have observed that the type of stimulus to which the adipocytes are subjected, in our case when they are incubated in a lipogenic or lipolytic medium, can determine the response of these genes to the miRNAs. Under lipogenic conditions, the expression of the six genes studied, with the exception of TMEM18, is decreased. However, under lipolytic conditions, the combination miR-365b-5p mimic / miR-222-5p inhibitor leads to an increase in the expression of these genes, except for SOD2. Therefore, our results suggest that the final effect *in vivo* could be strongly conditioned by the influence of many other stimuli.

The present study has several limitations. The first limitation is the lack of miRNAs expression at the end of the follow-up process, which would improve the association between these miRNAs and the weight loss. In addition, the results of this study need to be confirmed in other patient cohorts in order to shed light on the use of miRNAs as clinical biomarkers. Further animal studies are also needed to confirm the direct relationship between these miRNAs and weight loss.

In conclusion, our study showed that the baseline serum miRNA composition of patients with morbid obesity was able to predict %EWL 5-8 years after bariatric surgery. Our study showed that hsa-miR-365b-5p and hsa-miR-222-5p could be predictive biomarkers related to successful weight loss after bariatric surgery. We also confirmed in an *in vitro* study that the combination of these potential predictive biomarkers was involved in regulating the levels of some genes associated with the development of obesity. However, these effects could be significantly modified depending on, among other things, the subject's metabolic conditions. The results of this study open up new possibilities for

better clinical decision making. These novel findings merit further investigation *in vitro* and in experimental animals, which may lead to a better understanding of the involvement of these two miRNAs in weight regulation. In addition, our study also shows the possible association of different miRNAs with other obesity-related diseases, opening up other areas of research to investigate these possible relationships.

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DATA STATEMENT

The datasets presented in this article are not readily available because they are part of an ongoing study. Requests to access the datasets should be directed to the corresponding author up-on reasonable request.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

V.M., F.M.R., W.O.O., A.S.G., M.T., F.J.M.R., R.S.H., J.L.F.S., R.S.M., F.J.T., E.G.F. and L.G.S.: Investigation. A.S.G., R.S.H. and W.O.O.: Data curation; V.M., A.C.C., A..G.J., E.G.F. and L.G.S.: Formal analysis. V.M., F.M.R., E.G.F. and L.G.S.: Metodology; F.J.T., E.G.F. and L.G.S.: Conceptualization and resources; F.J.T., E.G.F. and L.G.S.: Supervision; V.M., E.G.F. and L.G.S.: Writing-review and editing draft. All authors have read and agreed to the published version of the manuscript.

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Table 1. Biochemical and anthropometric characteristics of the patients with morbid obesity included in the study.

	GR	NR
N (male/female)	18 (2/16)	15 (1/14)
Type of bariatric surgery (RYGB/SG)	6/12	5/10
Age (years)	45.1±10.7	47.3±5.7
Weight (kg)	131.3±20.9	130.8±21.3
BMI (kg/m^2)	47.6±6.3	51.2±10.3
Waist (cm)	132.7±20.5	131.1±14.7
Hip (cm)	145.2±12.5	142.7±22.6
SBP (mmHg)	137.4±17.4	133.3±19.2
DBP (mmHg)	78.4±9.53	78.4±9.8
Glucose (mg/dl)	120.6±59.5	98.8±11.5
Triglycerides (mg/dl)	154.3±101.6	119.6±59.5
Cholesterol (mg/dl)	177.5±39.9	175.2±33.1
HDL (mg/dl)	42.0±12.9	45.0±10.9
LDL (mg/dl)	104.9±39.5	108.6±28.6
Insulin (µUI/ml)	15.5±9.2	15.2±7.9
Hb1Ac (%)	6.3±2.2	5.6±0.4
HOMA-IR	5.4±6.9	3.5±2.1

Data are means ± SD. GR: good responders. NR: non-responders. RYGB: laparoscopic Roux-en-Y bypass. SG: sleeve gastrectomy. BMI: body mass index. SBP: Systolic blood pressure. DBP: diastolic blood pressure. HDL: high-density lipoprotein. LDL: low-density lipoprotein. HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

FIGURES

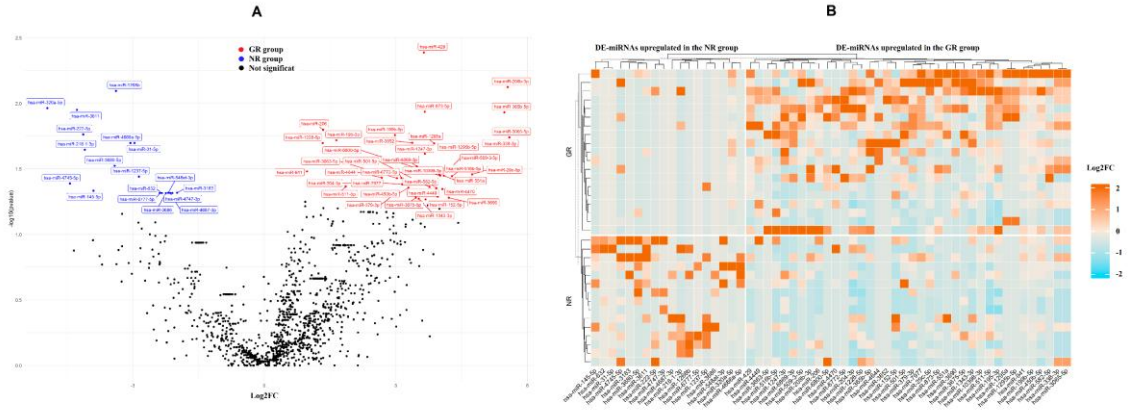


Figure 1. **(A)** Volcano plot illustrating the differentially expressed microRNAs (DE-miRNAs) between the GR (n=18) and NR (n=15) groups. Black dots indicate non-significant DE-miRNAs, whereas colored dots represent DE-miRNAs (adjusted p-value≤0.05). In accordance with the log_2FC values, the red dots represent DE-miRNAs that are upregulated in the GR group, while the blue dots represent DE-miRNAs that are upregulated in the NR group. (**B**) A hierarchical clustering heat map of the DE-miRNAs distinguished by the %EWL after bariatric surgery (GR and NR) is presented. The map depicts the relative expression patterns of the miRNAs, with orange representing relatively high miRNA expression and blue representing relatively low miRNA expression.

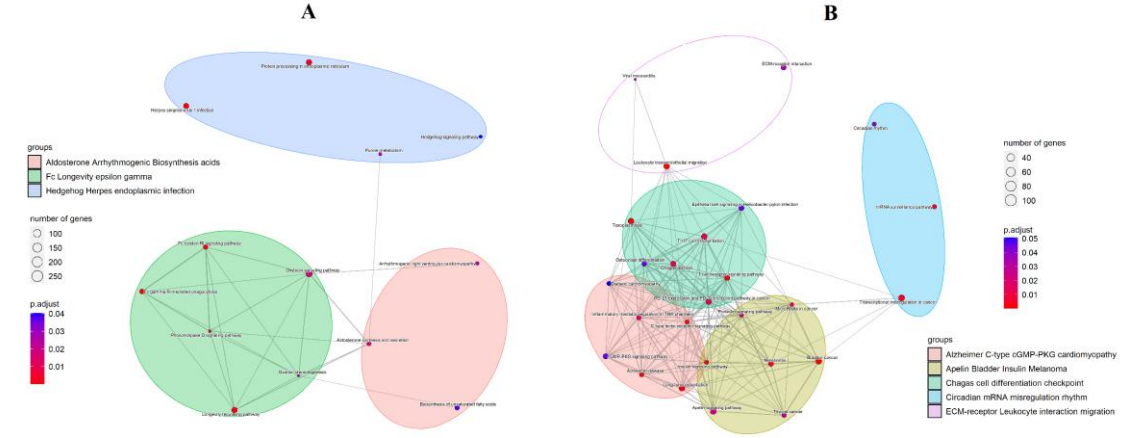


Figure 2. (A) Network of exclusive KEGG pathways associated with the GR group. **(B)** Network of exclusive KEGG pathways associated with the NR group.

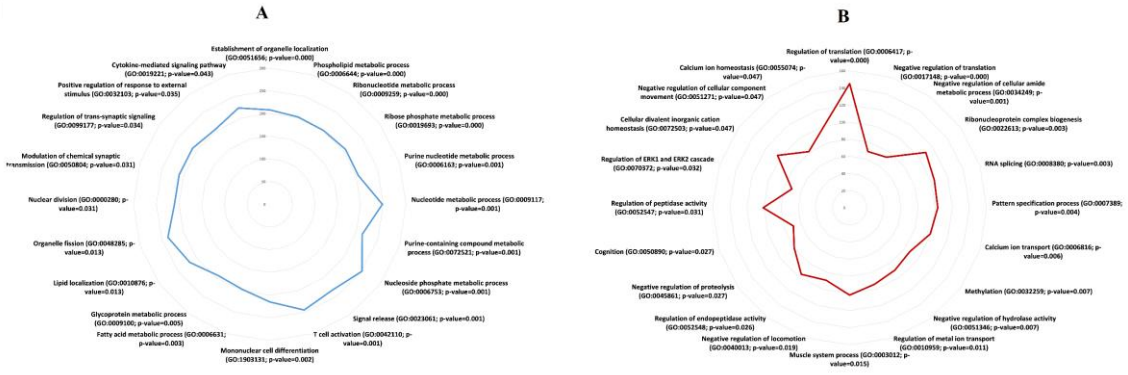


Figure 3. Number of predicted target genes in the top 20 exclusive GO:BP categories that were affected by the DE-miRNAs in the GR **(A)** and in the NR **(B)** groups.

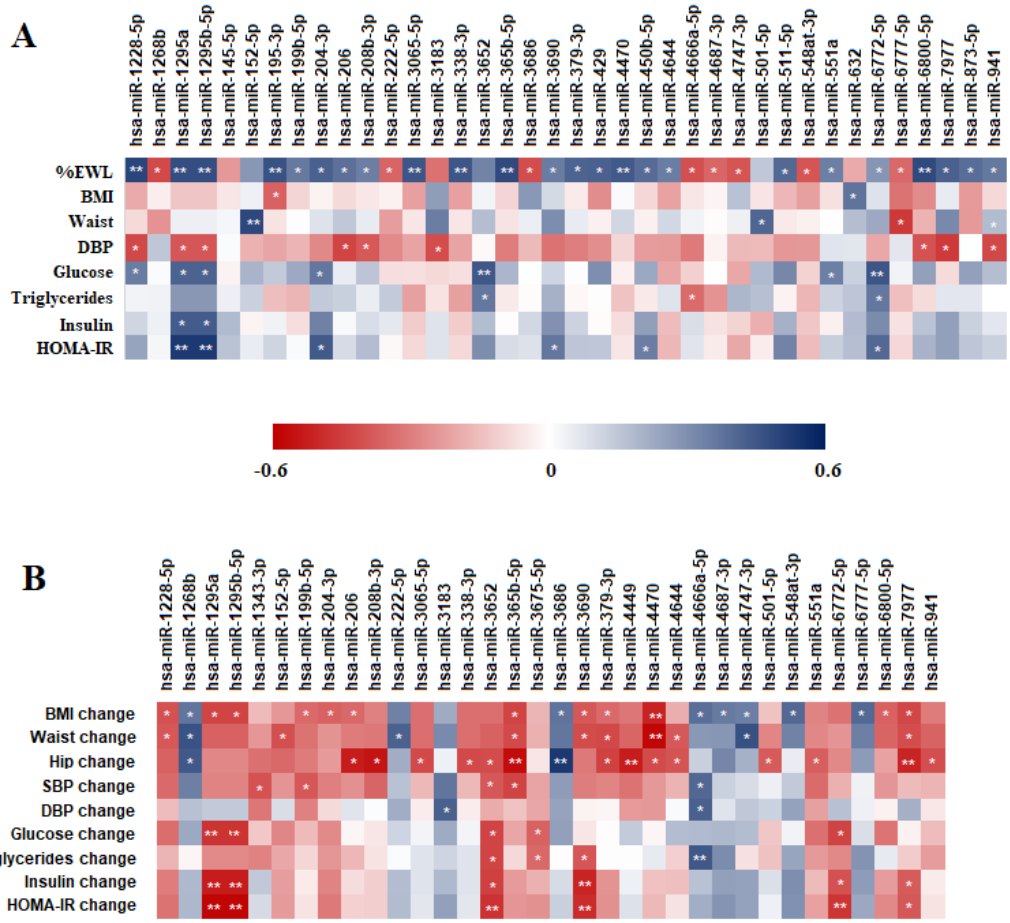


Figure 4. The heat maps illustrate the correlations between DE-miRNAs at the basal level and clinical/anthropometric variables. **(A)** Panel A represents the significant correlations at the basal level. **(B)** Panel B represents the significant correlations between DE-miRNAs at the basal level and the changes in the anthropometric/clinical variables 5-8 years after bariatric surgery. The Spearman correlation coefficient is displayed on a colour scale from red (negative correlation) to blue (positive correlation). Statistical significance is expressed as *p<0.05 and **p<0.01.

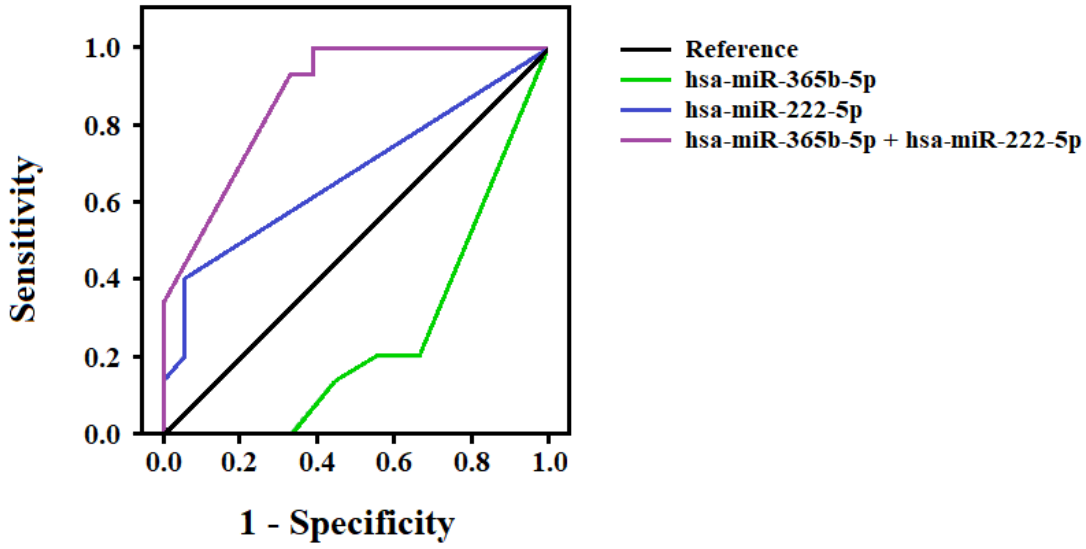
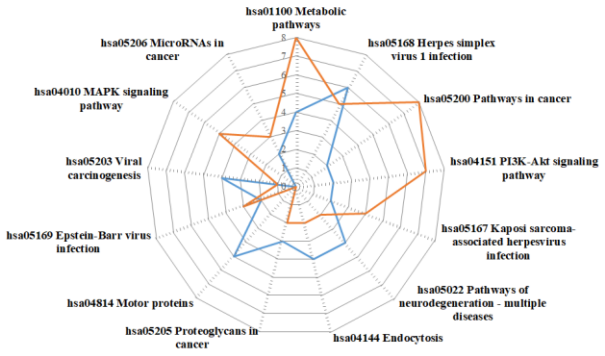


Figure 5. Receiver operating characteristic (ROC) curves for the single biomarkers and the combination model comparing GR and NR patients.

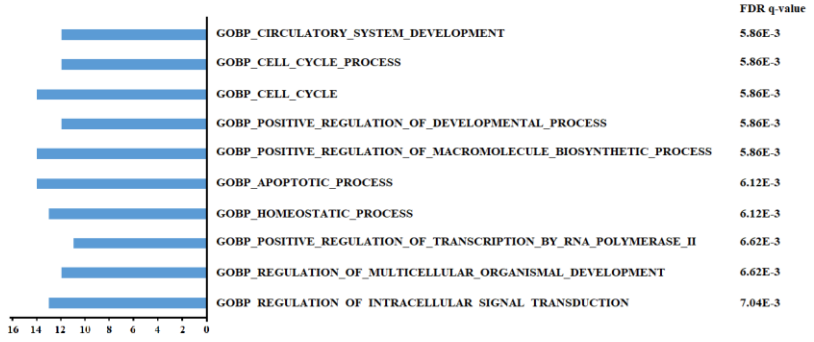
A

Target genes of hsa-miR-365b-5p			Target genes of miR-222-5p	
ADAP2	GRIK3	RRP7A	ADORA2B	LUC7L2
AKR7A2	GTF2H2	S1PR3	ARID2	MKRN3
ALDH5A1	GTF2H2C	SAR1A	ARL15	MTRF1L
ANGEL2	HINFP	SF3B1	ATG10	ND1FB6
ARHGAP26	HMGA2	SGCD	BLOC1S6	NIN
ATP11A	HNRNPC	SLC22A12	C5ORF51	OCIAD2
ATP1B3	ICA1L	SLC25A33	C7orf55-	PAFAH1B2
C11orf98	IGSF9B	SLC25A37	LUC7L2	PCNA
C1RL	IL7R	SLC33A1	CCDC59	PHF8
C21orf59	INTU	SLC35F5	CCDC86	PNRC1
CAPZA2	KCNK3	SPRED3	CCNL2	PRNP
CASP16	KIAA1551	STAT3	CEP55	RFX3
CAV2	KIAA1919	STPG1	CNTLN	RGMB
CCDC117	KLHL21	SYK	COL4A5	RGS17
CCS	KREMEN1	TBC1D13	CREBZF	SAR1A
CD99	LAT2	TIAL1	DDIT4	SEMA3E
CEACAM5	LRRC58	TMEM106B	DUSP5	SGPL1
CIAO1	LRRC75A	TMEM117	E2F2	SLC30A9
COMMD2	MACC1	TNFAIP8L1	EFHD2	SLITRK4
CPE	MASTL	TONSL	ENTPD4	SMNDC1
CSRP1	MCTS1	TPM3	ETNK1	SOD2
DARS2	MRPL17	TRPC4AP	FAM13B	STAU1
DCTN5	MRPS16	TTC38	FGF2	STC2
DNAJC18	MRTO4	TUBD1	FOXK2	TAB2
DNAL1	MTO1	UBE2Q1	FXN	TAF1D
DPY19L4	OIP5	VEZT	GABRA2	TGFA
DUXA	PARP1	WNT2B	GGA3	TMEM18
ELL2	PHAX	ZBTB46	GNG2	UBXN2B
ERMAP	PPAP2B	ZKSCAN1	GOLIM4	UCK2
ESYT2	RAB11FIP4	ZMYM4	GPRC5A	UNC5C
EXOC3-AS1	RAB17	ZNF417	HLA-B	VTA1
FAM118A	RAPGEF1	ZNF454	HSD17B12	WDR26
FBXO48	RDH13	ZNF551	ID2	WNK2
FEM1A	RNF14	ZNF563	IKBIP	ZNF384
FGFR1OP	RNF149	ZNF573	ITGA5	ZNF585B
FPGS	RNPEPL1		KITLG	ZNF99
GMPS	RRP36		LINC00346	ZXDB

B



C



D

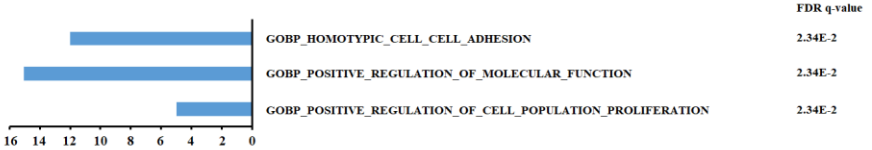


Figure 6. **(A)** Validated experimentally target genes obtained in the miRTarBase of hsa-miR-365b-5p and hsa-miR-222-5p. **(B)** Number of validated experimentally target genes obtained in the miRTarBase in the KEGG pathways with more relevance (with five or more genes). Orange line represents target genes of hsa-miR-222-5p. Blue line represent target genes of hsa-miR-365b-5p. **(C)** Number of validated experimentally target genes in the exclusive GO:BP affected by hsa-miR-222-5p. **(D)** Number of validated experimentally target genes in the exclusive GO:BP affected by hsa-miR-365b-5p.

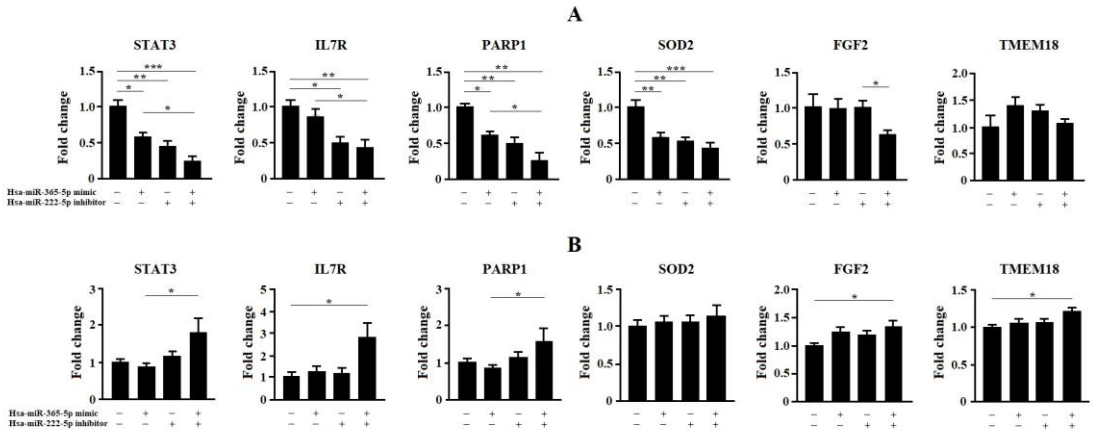


Figure 7. mRNA expression levels of STAT3, IL7R, PARP1, SOD2, FGF2 and TMEM18 in adipocytes (n=4) incubated with a hsa-miR-365-5p mimic, a hsa-miR-222-5p inhibitor and a mixture of both in lipogenic (**A**) and lipolitic (**B**) conditions. *p<0.05; ** p<0.01; ***p<0.005.

SUPPLEMENTARY MATERIAL AND METHODS

Study design and subjects

A total of 90 patients with morbid obesity (BMI>40kg/m^2), who were scheduled for bariatric surgery at the Virgen de la Victoria University Hospital and Regional University Hospital in Malaga between 2010 to 2013, were invited to participate in the present study. For all patients, baseline data were available prior to bariatric surgery, having been collected during their participation in other studies. The patients underwent one of the following bariatric surgery procedures: laparoscopic Roux-en-Y gastric bypass (RYGB) or sleeve gastrectomy (SG) [1,2]. The multidisciplinary team, comprising surgeons and endocrinologists, made the decision regarding the procedure to be performed, taking into account the characteristics of the patient. Of the 90 patients who underwent bariatric surgery, only 53 consented to participate in this prospective study. In order to gain a deeper insight into the long-term outcomes of bariatric surgery, patients were classified into two groups based on their bariatric surgery outcome: "good-responders" (GR) and "non-responders" (NR), as defined below. Of these, only 33 patients had baseline serum samples available for predictive biomarker analyses. No significant differences were observed in the variables analyzed between patients with and without baseline serum samples (Supplementary Table 1). The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided written informed consent and the study was reviewed and approved by the Malaga Provincial Research Ethics Committee of the Biomedical Research Institute of Malaga (IBIMA) (protocol code PI-0194-2017 (1 February 2018)).

Anthropometric Data

The data were prospectively collected prior to bariatric surgery and over a period of between five and eight years in the postoperative period (long term). Anthropometric variables were measured at these two points in all the patients with morbid obesity included in the study. The following measurement were taken: weight, height, waist and hip circumferences, and blood pressure, and body mass index (BMI) calculated. The percentage of change of anthropometric variables 5-8 years after bariatric surgery was calculated as 100 x (variable at the time of evaluation – preoperative variable)/preoperative variable.

Assessment of Weight Change

The percentage of excess weight loss (%EWL) was based on the excess weight compared to the weight corresponding to a BMI of 25 kg/m^2 for each patient. We assessed the %EWL as 100 × (preoperative weight - weight at the time of evaluation)/(preoperative weight - weight corresponding to BMI=25 kg/m^2) [3]. The different patterns of weight loss were defined based on the Reinhold criteria. Weight loss was considered insufficient when %EWL<50% in analogy with the Reinhold criteria [4]. The Reinhold criteria were modified by Christou et al. [5]. This %EWL was calculated at 5-8 years after bariatric surgery. Those patients with a %EWL≥50% were considered as good responders (GR). On the other hand, patients with %EWL<50% were considered as non-responders (NR).

Biochemical Data

Blood samples were collected after a 12-h fast. The serum was aliquoted and immediately stored at −80 °C. Serum glucose, cholesterol, triglycerides and HDL were analyzed using an Advia Chemistry XPT autoanalyzer (Siemens Healthcare Diagnostics, Malvern, PA). The LDL was calculated from the Friedewald equation. Serum insulin levels were measured by immunoassay using an ADVIA Centaur autoanalyzer (Siemens Healthcare

Diagnostics, Malvern, PA). Insulin resistance was calculated by the following formula: HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) = fasting insulin (IU/mL) x fasting glucose (mmol/L)/22.5. The percentage of change of biochemical variables 5-8 years after bariatric surgery was calculated as 100 x (variable at the time of evaluation – preoperative variable)/preoperative variable.

miRNA extraction, library preparation and small RNA sequencing

Total RNA with an enhanced miRNA enrichment was extracted from 200 µl of the serum samples using the Maxwell® 16 miRNA Tissue Kit (Promega, Madison, WI). An aliquot was used to perform an ultrasequencing of miRNA libraries to analyze the miRNA expression pattern by Next-Generation Sequencing (NGS). Total RNA concentration was measured using the Qubit® RNA Assay Kit in the Qubit® 3.0 Flurometer (Thermo Fisher Scientific Inc, Waltham, MA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). For sequencing, the small RNA transcripts were converted into barcoded cDNA libraries using 400-500 ng of RNA per sample as input material for the small RNA library. Sequencing libraries were generated using NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, MA) following manufacturer's instructions. Individual libraries prepared with unique indexes, were pooled and subjected to the Illumina sequencing pipeline, passing through clonal cluster generation on sequencing-by-synthesis 1x 50-75 pb single-end reads on the NextSeq 550 (Illumina Inc., San Diego, CA).

Pre-processing data analysis and miRNA identification.

Quality of samples were analysed with FastQC 0.11.9. In order to obtain miRNA clean reads, raw reads data were processed to remove all the primers using cutadapt 3.4 over the illumine adapters. Then, these files were aligned through bowtie2 (v 2.2.0) with the human reference sequence from the GRCh38 assembly in UCSC (http://genome.ucsc.edu) to find which sequences matched the genome. After, we searched which alignments mapped to coding regions in the human genome, using htseq 0.11.1. Later we counted and selected the transcripts that corresponded to miRNAs, removing the rest of transcripts. In this way, we obtained a list of miRNAs that are differentially expressed (DE-miRNAs) between both groups (GR and NR). These grouped miRNAs were identified using the package biomaRt, in the R software version 4.1.0. To avoid bias in the data, the following corrections were applied: filtering by expression level of at least 10 reads in each gene per group to remove unexpressed genes, correction factor to equalize the number of reads in each sample by trimmed mean of M-values (TMM) and log normalization of counts per million (logCPM). A list of DE-miRNAs with a p-value ≤ 0.05 between both groups (GR and NR) was obtained through Mann-Whitney test. Associations between mirRNAs and patients were shown through several barplots and heatmaps using ComplexHeatmap library from R. Finally, $\log_2$ fold change ($\log_2$FC) were calculated for all the miRNAs and represented versus 1-p-value using a volcano plot with ggplot2.

Enrichment analysis

An analysis of the targets and pathways regulated by miRNAs was performed. First, miRBaseConverter 1.16.0 software was used to convert the data into a mature miRNAs and then, these data was evaluated in order to obtain the specific targets with the package multiMiR 1.14.0. The functional enrichment analysis was performed using the package clusterProfiler 4.0.0 to obtain the related pathways and associated gene ontology (using

the function enrichKEGG (Kyoto Encyclopedia of Genes and Genomes) and enrichGO (gene ontology), respectively). RStudio 1.4.1106 was used to analyse the data using R 4.0.5 language. Statistical significant was assumed when $p<0.05$.

Statistical analysis for algorithm designed as predictor to distinguish between GR and NR patients

Diagnostic efficacy analysis of these DE-miRNAs were carried out with the subjects of the prospective study (GR *vs*. NR) through step-by-step logistic regression analysis, with which the best predictor model was selected after combining different variables. The predicted probabilities of the model were dichotomized based on the best cut-off calculated by the receiver operating characteristic (ROC) curve. Next, the predictive values were calculated based on this cut-off.

Enrichment analysis of miRNAs selected as predictor biomarkers

An analysis of the targets and pathways regulated by the miRNAs selected as predictor biomarkers was performed. First, miRNAs selected as predictor biomarkers were introduced in miRTarBase (last access June 2023 https://miRTarBase.cuhk.edu.cn/) [6]. This database contains the miRNA-target interactions validated experimentally by reporter assay, western blot, microarray and next-generation sequencing experiments. After, the functional enrichment analysis of these target genes was performed to obtain the related pathways (KEGG, https://www.genome.jp/kegg/mapper/color.html) and associated biological process of GO (Gene Set Enrichment Analysis (GSEA; GSEA / MSigDB web site v6.4 version; MSigDB database v7.2, last access June 2023; https://www.gsea-msigdb.org/gsea/index.jsp). We obtained gene sets significantly overrepresented (FDR q-value <0.05).

Incubation of human adipocytes with miRNAs selected as predictor biomarkers

Human mesenchymal stem cells from subcutaneous adipose tissue (subASCs; PromoCell, C-12977, Heidelberg, Germany) were seeded at a density of 10,000 cells/cm² and cultured for seven days in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) supplemented with fetal bovine serum (FBS) (1% v/v), streptomycin (100 µg/mL), penicillin (100 U/mL), and L-glutamine (2.5 mM) to allow cells to proliferate. To induce adipogenesis, this growth medium was replaced with adipocyte induction medium composed of DMEM/F12 supplemented with FBS (1%), streptomycin (100 µg/mL), penicillin (100 U/mL), L-glutamine (2.5 mM), insulin (10 µM), isobutylmethylxanthine (0.5mM), dexamethasone (1.0 µM), pioglitazone (10 µM), rosiglitazone (0.5 µM), biotin (33 µM), panthenonate (17 µM) for 72 hours. After, this medium was replaced for adipocyte maintenance medium, which was similar to the induction media except for deletion of isobutylmethylxanthine, biotin and panthenonate, for an additional 25 days, as previously described [7]. Adipocytes generated after these 28 days of adipogenic differentiation were cultured for an additional 48 hours in either lipogenic or lipolytic medium. Lipogenic medium consisted of DMEM/F12 supplemented with FBS (1%), L-glutamine (2.5 mM), insulin (10 µM), dexamethasone (1 µM), pioglitazone (10 µM), and rosiglitazone (0.5 µM). Lipolytic medium consisted of Minimum Essential Medium Eagle supplemented with dialyzed FBS (0.02% v/v), L-glutamine (2.5 mM), HEPES (25 mM), sodium pyruvate (2 mM), pioglitazone (10 µM), rosiglitazone (0.5 µM), isoproterenol (10 µM), and fatty acid-free BSA (0.02% v/v). At the start of the culture in lipogenic or lipolytic medium, adipocytes were stimulated with either a mirVana™ miRNA mimic for hsa-miR-365b-5p (Assay ID: MC15283, sequence: AGGGACUUUCAGGGGCAGCUGU), a mirVana™ miRNA inhibitor for hsa-miR-

222-5p (Assay ID: MH12656, sequence: CUCAGUAGCCAGUGUAGAUCCU), or a combination of both, along with their respective non-targeting controls (Thermo Fisher Scientific Inc, Waltham, MA). All stimulations were performed at a concentration of 100 nM miRNA using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Waltham, MA) according to the manufacturer's instructions.

mRNA extraction of adipocytes from the in vitro culture with miRNAs

The mRNA of adipocytes incubated with miRNA mimic or inhibitor was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen, GmbH, Germany) as previously described [8]. RNA purity was checked using the NanoVuePlus R spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA). The RNA concentration was measured using the Qubit R RNA Assay Kit in the Qubit R 3.0 Flurometer (Thermo Fisher Scientific Inc, Waltham, MA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). The RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit 200 reactions (Ref 4368814, Thermo Fisher Scientific Inc., Waltham, MA) and RNase Inhibitor Set (Ref N8080119, Thermo Fisher Scientific Inc., Waltham, MA).

Analysis of mRNA

We analyzed the mRNA expression of adipocytes from the in vitro culture using quantitative real time reverse transcriptase-PCR (RT-qPCR). Six gene expression levels were analyzed in duplicate (n=4 per group) using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). RT-qPCR reactions were carried out for the following genes using specific TaqManTM Gene Expression Assays (Thermo Fisher Scientific Inc., Waltham, MA): fibroblast growth factor 2 (FGF2) (Hs00266645_m1), transmembrane protein 18 (TMEM18) (Hs00894216_m1), superoxide dismutase 2, mitochondrial (SOD2) (Hs00167309_m1), signal transducer and activator of transcription 3 (STAT3) (Hs00374280_m1), poly (ADP-ribose) polymerase 1 (PARP1) (Hs00242302_m1) and interleukin 7 receptor (IL7R) (Hs00902334_m1). The threshold cycle (Ct) value for each sample was normalized with the expression of ribosomal protein L13a (RPL13A) (Hs04194366_g1). The mRNA level expression was determined by Log_2FC for mRNA expression in adipocytes culture with the miRNA-222-5p inhibitor, miRNA-365b-5p mimic, or a combination of both taking their respective non-targeting controls.

Statistical analysis

The results are expressed as mean values ± standard deviation (SD). The clinical and biochemical data were evaluated using an independent samples t-test between GR vs. NR groups. Following the application of the Shapiro-Wilk test to ascertain the normal distribution of the continuous variables, logarithmic transformation was employed where necessary to ensure the normality of skewed variables. The level of significance was set at $p<0.05$ for the main effects and the interaction. The correlations between clinical/biochemical variables and the serum miRNAs expression were analysed by Spearman's correlation test. All statistical analyses were performed using SPSS statistical software 26.0 (SPSS Inc., Chicago, IL).

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Supplementary Table 1. Anthropometric and biochemical variables of the patients with morbid obesity who agreed to participate in the study.

	GR (n=33)		NR (n=20)	
	Without baseline serum samples	**With baseline serum samples**	**Without baseline serum samples**	**With baseline serum samples**
N (male/female)	15 (2/13)	18 (2/16)	5 (3/2)	15 (1/14)
Type of bariatric surgery (RYGB/SG)	3/12	6/12	1/4	5/10
Age (years)	43.2±10.6	45.1±10.7	44.8±9.7	47.3±5.7
Weight (kg)	137.5±24.7	131.3±20.9	137.6±15.2	130.8±21.3
BMI (kg/m^2)	50.1±7.7	47.6±6.3	49.2±6.9	51.2±10.3
Waist (cm)	136.5±17.7	132.7±20.5	137.8±11.4	131.1±14.7
Hip (cm)	149.6±13.8	145.2±12.5	141.7±11.0	142.7±22.6
SBP (mmHg)	133.8±21.2	137.4±17.4	140.0±19.7	133.3±19.2
DBP (mmHg)	82.7±9.3	78.4±9.53	76.8±6.3	78.4±9.8
Glucose (mg/dl)	110.8±36.1	120.6±59.5	106.±31.2	98.8±11.5
Triglycerides (mg/dl)	140.2±43.2	154.3±101.6	114.0±69.2	119.6±59.5
Cholesterol (mg/dl)	182.6±87.5	177.5±39.9	179.6±29.2	175.2±33.1
HDL (mg/dl)	42.3±12.6	42.0±12.9	44.8±21.6	45.0±10.9
LDL (mg/dl)	109.7±31.9	104.9±39.5	112.0±23.7	108.6±28.6
Insulin (µUI/ml)	21.0±18.1	15.5±9.2	17.3±3.9	15.2±7.9
Hb1Ac (%)	5.8±1.1	6.3±2.2	6.2±1.1	5.6±0.4
HOMA-IR	6.1±5.2	5.4±6.9	4.6±2.6	3.5±2.1

Data are means ± SD. GR: good responders. NR: non-responders. RYGB: laparoscopic Roux-en-Y bypass. SG: sleeve gastrectomy. BMI: body mass index. SBP: Systolic blood pressure. DBP: diastolic blood pressure. HDL: high-density lipoprotein. LDL: low-density lipoprotein. HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.

Supplementary Table 2. List of the miRNAs differentially expressed (DE-miRNAs) between Good Responders (GR) and No Responders (NR) groups with a $p \leq 0.05$.

	miRNA	**Expression in GR (mean)**	**Expression in NR (mean)**	**Log_2FC**	**p-value**
miRNAs upregulated in GR	hsa-miR-1295a	1.68	0.10	4.07	0.02
	hsa-miR-1295b-5p	1.68	0.10	4.07	0.02
	hsa-miR-582-5p	4.84	0.33	3.86	0.04
	hsa-miR-10398-3p	2.21	0.20	3.46	0.03
	hsa-miR-6772-5p	1.05	0.10	3.39	0.04
	hsa-miR-509-3-5p	4.79	0.47	3.35	0.03
	hsa-miR-3663-5p	1.00	0.10	3.32	0.04
	hsa-miR-7977	0.53	0.07	2.98	0.04
	hsa-miR-3652	0.68	0.10	2.77	0.02
	hsa-miR-873-5p	9.37	1.60	2.54	0.01
	hsa-miR-3675-5p	1.53	0.27	2.51	0.05
	hsa-miR-365b-5p	4.37	0.80	2.44	0.01
	hsa-miR-199b-5p	104.37	24.67	2.08	0.02
	hsa-miR-208b-3p	4.16	1.00	2.05	0.01
	hsa-miR-6800-5p	1.58	0.40	1.98	0.04
	hsa-miR-6869-3p	1.00	0.27	1.90	0.03
	hsa-miR-551a	1.74	0.47	1.89	0.04
	hsa-miR-3690	2.68	0.73	1.87	0.05
	hsa-miR-1247-3p	1.68	0.47	1.85	0.02
	hsa-miR-429	24.58	6.93	1.82	0.00
	hsa-miR-4644	0.32	0.10	1.65	0.04
	hsa-miR-4470	2.53	0.80	1.65	0.05
	hsa-miR-29c-5p	11.89	4.27	1.47	0.03
	hsa-miR-516b-5p	5.84	2.20	1.40	0.04
	hsa-miR-379-3p	6.26	2.40	1.38	0.05
	hsa-miR-338-3p	6.37	2.47	1.36	0.02
	hsa-miR-3065-5p	6.37	2.47	1.36	0.02
	hsa-miR-501-5p	7.79	3.27	1.25	0.04
	hsa-miR-1343-3p	7.79	3.27	1.25	0.05
	hsa-miR-941	227.89	97.33	1.22	0.03
	hsa-miR-4449	2.79	1.20	1.21	0.05
	hsa-miR-450b-5p	35.00	15.87	1.14	0.04
	hsa-miR-206	272.74	137.73	0.98	0.02
	hsa-miR-1228-5p	139.79	73.07	0.93	0.02
	hsa-miR-195-3p	52.74	27.60	0.93	0.02
	hsa-miR-204-3p	18.42	9.67	0.93	0.04
	hsa-miR-511-5p	369.68	203.93	0.85	0.04
	hsa-miR-152-5p	2.68	1.60	0.74	0.05
	miRNA	**Expression in GR (mean)**	**Expression in NR (mean)**	**Log_2FC**	**p-value**
miRNAs upregulated in NR	hsa-miR-320a-5p	2.00	2.20	-0.13	0.01
	hsa-miR-145-5p	1.21	2.40	-0.98	0.05
	hsa-miR-4745-5p	1.21	2.73	-0.17	0.04
	hsa-miR-218-1-3p	0.32	1.40	-2.14	0.02
	hsa-miR-1237-5p	0.05	0.40	-2.92	0.04
	hsa-miR-222-5p	0.21	1.87	-3.14	0.02
	hsa-miR-3611	0.16	1.40	-3.14	0.01

	hsa-miR-3680-5p	0.11	1.00	-3.24	0.03
	hsa-miR-1268b	0.00	0.40	--	0.01
	hsa-miR-31-5p	0.00	1.07	--	0.02
	hsa-miR-4666a-5p	0.00	0.80	--	0.02
	hsa-miR-632	0.00	1.07	--	0.05
	hsa-miR-3183	0.00	0.20	--	0.05
	hsa-miR-3686	0.00	0.67	--	0.05
	hsa-miR-4687-3p	0.00	0.47	--	0.05
	hsa-miR-4747-3p	0.00	0.33	--	0.05
	hsa-miR-548at-3p	0.00	0.40	--	0.05
	hsa-miR-6777-5p	0.00	1.00	--	0.05