The Journal of Clinical Endocrinology & Metabolism

Altered expression of miR-181a-5p and miR-23a-3p is associated with obesity and TNFα-induced insulin resistance

--Manuscript Draft--

Manuscript Number:	jc.2017-01909R2
Article Type:	Clinical Research Article
Full Title:	Altered expression of miR-181a-5p and miR-23a-3p is associated with obesity and TNFα-induced insulin resistance
Short Title:	miR181a and miR23a improves insulin signalling
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Section/Category:	Obesity and Adipocyte Biology
Manuscript Classifications:	Diabetes; Insulin resistance; Type 2 Diabetes; Metabolism / Obesity; Adipose Tissue; Inflammation
Keywords:	miRNA, insulin resistance, TNFα, obesity, diabetes, adipose tissue
Abstract:	Context: The proinflammatory cytokine TNFα is a key player in insulin resistance (IR). While several miRNAs are believed to be involved in the development of adipose tissue (AT) IR, the role of miRNAs in the association between inflammation and IR is poorly understood. Objective: To investigate the expression profile of miR-181a-5p and miR-23a-3p in obesity and to study their role in TNFα-induced IR in adipocytes. Design: Two separate cohorts were employed. Cohort 1 was used for AT expression studies and included 28 subjects with BMI<30 and 30 subjects with BMI≥30. Cohort 2 was used for circulating serum miRNA studies and included 101 subjects with 4-years follow-up (48 cases and 53 controls). miR-181a-5p and miR-23a-3p expression was assessed in subcutaneous (SAT) and visceral (VAT) AT. Functional analysis was performed in adipocytes utilizing miRNA mimics and inhibitors. Key molecules of the insulin pathway, AKT, PTEN, AS160 and S6K, were analyzed. Results: Expression of miR-181a-5p and miR-23a-3p was reduced in AT from obese and diabetic subjects and was inversely correlated to adiposity and HOMA-IR. Overexpression of miR-181a-5p and miR-23a-3p in adipocytes upregulated insulin-stimulated AKT activation and reduced TNFα-induced IR, regulating PTEN and S6K expression. Serum levels of miR-181a-5p were reduced in cases vs controls at baseline, pointing towards its prognostic value. Variable importance in projection scores revealed miR-181a-5p had more impact in the model than insulin or glucose at 120 minutes. Conclusions: miR-181a-5p and miR-23a-3p may prevent TNFα-induced IR in adipocytes through modulation of PTEN and S6K expression.
Funding Information:	

	Fondo de Investigación Sanitaria (PI14/00465, PI17/00877)	Senior Fellowship Matilde R Chacón
Author Comments:	Disclosure statements: The authors have nothing to disclose	
Additional Information:		
Question	**Response**	
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PRECIS: The precis is a brief description of your paper that will appear on the Table of Contents underneath your article title, should your paper be accepted (see the current issue of JCEM for examples). The description should be **no longer than 200 characters, including spaces,** and should briefly explain what was done in your study and what was concluded. Please ensure that the precis does not simply repeat the article title.	We have identified miR-181a and miR-23a as miRNAs downregulated by TNFα in human adipocytes and in human obese visceral adipose tissue and we tested their functional mechanism in TNF-IR adipocytes	

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R. Paul Robertson, MD

Editor-in-Chief

JCEM / jc.2017-01909R1

Tarragona 21st December 2017

Dear Editor:

Thank you very much for giving us the opportunity to revise again our manuscript JCEM / jc.2017-01909R1 entitled *"Altered expression of miR-181a-5p and miR-23a-3p is associated with obesity and TNFα-induced insulin resistance*". Our responses to the specific comments of the reviewers and to the editor's suggestions to can be found below. We hope this new version will now be suitable for publication in JCEM.

We look forward to hearing from you at your earliest convenience.

Sincerely

Matilde Rodríguez Chacón, Ph.D

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Editor's Comments:

I think the authors would need to do further normalizing only if they were making a point about relative amounts of Akt in total, for each treatment condition. They should a) include methodology for the quantitation of the Westerns; and b) put in some information about the variability of the control condition. They might, for example, include this quantitation within the figure legend or put numbers into the actual narrative of the results.

We are very grateful to the editor for making this clarification. We have now explained in the methods section the procedure followed for western blotting quantification and data analysis.

The corresponding relative quantification values for each control condition is included in each Figure legend and highlighted in yellow in the revised version.

Reviewers' Comments:

Reviewer #1: The authors have responded to some of my comments and suggestions and have made improvements. However, major concerns remain.

There is no indication of how the Western blots were converted into quantitative data. No methods in the manuscript or supplemental material.

We apologize for this oversight. We have now included in supplemental methods the procedure followed for western blot quantification and its corresponding data analysis.

Figures 2 and 3 and the corresponding data MUST take into account the loading control! The authors state that, "We would like to stress out that βactin was never used as a normalizing control; it is just shown to demonstrate equal loading." In other words, we used it as a loading control, but we never took the loading control into account in our calculations. All calculations must be re-done after normalizing to the loading control.

We apologize for this misunderstanding. In Figure 2, since the phosphorilation/unphosphorilation ratio for each condition is calculated relative to the same βactin value, βactin disappear in the same calculation. However in Fig 3 b and Fig 3c, as stated now in methods section, βactin was used as the normalizing value.

Control variability must be added directly to the figures. Although I stressed Figure 2 in my initial review, this suggestion applies to all figures. While controls can certainly be used to calculate a relative value for other conditions, the same can be done within the control condition, i.e., by comparing each individual control value with the average control value.

As suggested by the reviewer, we have added further clarification about the variability of each NTC, which we placed in **all** figure legends plus the number of biological replicates performed. We agree with the reviewer's suggestion about "**comparing each individual control value with the average control value as a way to introduce control variability in each figure"** which can be another way of evaluate the control variability in some experiments procedures, however due to the inherent variability that exists between independent experiments analyzed by western blotting technique and, to be able to notify significant changes between controls and miRNAS treatments, the relative effect of each miRNA was normalized to their respective control within each experiment that is given a value of 100% and, thereafter the S.E. is equal to 0.

Altered expression of miR-181a-5p and miR-23a-3p is associated with obesity and TNFα-induced insulin resistance

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Abbreviated title: miR181a and miR23a improves insulin signalling by targeting PTEN and S6K

Word count: 3598 **Figures:4 Tables:2**

Supplementary information: supplementary Table 1, supplementary Figure 1- 4, supplementary methods

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Disclosure statements:

The authors have nothing to disclose

ABSTRACT

Context: The proinflammatory cytokine TNFα is a key player in insulin resistance (IR). While several miRNAs are believed to be involved in the development of adipose tissue (AT) IR, the role of miRNAs in the association between inflammation and IR is poorly understood.

Objective: To investigate the expression profile of miR-181a-5p and miR-23a-3p in obesity and to study their role in TNFα-induced IR in adipocytes.

Design: Two separate cohorts were employed. Cohort 1 was used for AT expression studies and included 28 subjects with BMI<30 and 30 subjects with BMI≥30. Cohort 2 was used for circulating serum miRNA studies and included 101 subjects with 4-years follow-up (48 cases and 53 controls). miR-181a-5p and miR-23a-3p expression was assessed in subcutaneous (SAT) and visceral (VAT) AT. Functional analysis was performed in adipocytes utilizing miRNA mimics and inhibitors. Key molecules of the insulin pathway, AKT, PTEN, AS160 and S6K, were analyzed.

Results: Expression of miR-181a-5p and miR-23a-3p was reduced in AT from obese and diabetic subjects and was inversely correlated to adiposity and HOMA-IR. Overexpression of miR-181a-5p and miR-23a-3p in adipocytes upregulated insulin-stimulated AKT activation and reduced TNFα-induced IR, regulating PTEN and S6K expression. Serum levels of miR-181a-5p were reduced in cases *vs* controls at baseline, pointing towards its prognostic value. Variable importance in projection scores revealed miR-181a-5p had more impact in the model than insulin or glucose at 120 minutes.

Conclusions: miR-181a-5p and miR-23a-3p may prevent TNFα-induced IR in adipocytes through modulation of PTEN and S6K expression.

Key words: miRNA, insulin resistance, TNFα, obesity, diabetes, adipose tissue

INTRODUCTION

Obesity is associated with chronic low-grade inflammation of white adipose tissue (AT), which can subsequently lead to insulin resistance (IR), impaired glucose tolerance, and ultimately diabetes (1). TNFα is a proinflammatory cytokine whose expression in AT is elevated in obesity, where it can contribute to the modulation of lipid metabolism by altering insulin signaling (2)(3).

microRNAs (miRNAs) are small (17–24 nucleotides in length) noncoding RNAs that bind to 3'-untranslated regions (3' UTRs) of target mRNAs to regulate gene expression by translational repression or degradation. miRNAs have been shown to regulate metabolic processes that are associated with type 2 diabetes mellitus (DM), including insulin signaling and glucose homeostasis (4), highlighting their potential as therapeutic targets for obesity and metabolic syndrome. Moreover, the finding of circulating miRNAs in biological fluids supports the potential utility of harnessing miRNAs as biomarkers in many diseases ranging from cancer to DM (5). Along this line, differential expression of miRNAs in tissues has been reported in obese *vs* non-obese human individuals and in humans and animals with diabetes (6). However, the function of most miRNAs that are deregulated in obesity and IR is unknown.

miRNAs may provide a link between inflammation in obesity and IR. TNFα is a major initiator of inflammation and has been shown to differentially regulate miRNA expression in several contexts (7)(8). While various miRNAs modulated by TNFα have been described in AT, very little is known about those directly involved in the regulation of the insulin pathway in mature adipocytes (6)(9)(10). Consequently, the underlying mechanisms linking miRNAs and TNFα-induced IR in adipocytes remain to be deciphered.

A recent microarray analysis identified miR-181a and miR-23a as being deregulated in blood from obese and non-obese subjects with and without DM (11). Furthermore, miR-181a expression has been found to be inversely related to adiponectin levels in AT (12), and its expression is known to alter hepatic insulin sensitivity (13). In relation to miR-23, diminished

expression levels have been detected in the plasma of patients with cardiovascular disease (14) and a very recent study has implicated miR-23 in glucose metabolism in the muscle of dogs (15).

Given the scarcity of data on miR-181a-5p and miR23a-3p within the context of obesity and TNFα-induced IR, here we aimed to examine their expression in AT from obese and non-obese subjects, to investigate their role in TNFα-induced IR, and to test their possible functional mechanism in adipocytes. We also explored their potential utility as predictive serum markers of prediabetes in a prospective case-control study.

METHODS

Experimental subjects

Cohort 1: adipose tissue expression

We recruited 28 subjects with BMI <30 kg/m^2 and 30 subjects with BMI ≥30 kg/m^2, age- and gender-matched, at Hospital Joan XXIII of Tarragona (Spain) (**Table 1**). All patients were of Caucasian origin and reported that their body weight had been stable for at least 3 months before the study. They had no systemic disease other than obesity, and all had been free of any infections in the month before the study. Liver and renal diseases were specifically excluded by biochemical work-up. Samples of visceral (VAT) and subcutaneous (SAT) adipose tissue were obtained from the same patient during abdominal elective surgical procedures. Samples and data from patients included in this study were provided by the BioBanc IISPV (B.0000853+B.0000854) integrated into the Spanish National Biobanks Network, and were processed following standard operating procedures with appropriate approval of the Ethics and Scientific Committees.

Cohort 2: miRNA circulating serum analysis

We selected 101 subjects from the *Pizarra* study with 4 years follow-up (48 incident diabetes and 53 controls). The *Pizarra* study is a population-based prospective study undertaken in a

119 population from southern Spain. The characteristics of the study have been reported elsewhere
120 (16).

121 **Analytical methods**

122 Venous blood samples were drawn between 9:00 a.m. and 10:00 a.m. or two hours after an oral
123 glucose tolerance test. Samples were centrifuged at 4°C and serum and plasma from each
124 subject were stored at -80°C for later analysis. Plasma glucose, cholesterol, and triglyceride
125 levels, high-density lipoprotein cholesterol and insulin were measured as described (17). Insulin
126 resistance was determined by HOMA-IR. Cytokines (TNFα and its receptors R1 and R2, and
127 IL-6) were measured by enzyme immunoassay as described (18). Leptin, adiponectin, FABP4,
128 REDOX, SHBG (sex hormone-binding globulin) were measured by ELISA as described (18).

129 **Cell culture and treatments**

130 Human preadipocytes were purchased from the European Collection of Cell Cultures
131 (Salisbury, UK). The Simpson Golabi Behmel Syndrome (SGBS) cell line was kindly provided
132 by Dr. M. Wabitsch (University of Ulm, Germany). Cells were differentiated to adipocytes as
133 described (19) and then either incubated with or without 50 ng/mL TNFα for 8 h. Cell lysates
134 were collected for RNA extraction.

135 *miRNA mimics and inhibitors:* mimic-miRNAs (miScript mimic-miR-181a-5p**,** miScript mimic-
136 miR-23a-3p), non-target control siRNA and miRNA inhibitors (miScript anti-miR-181a-5p
137 inhibitor and miScript anti-miR-23a-3p inhibitor) and a negative control inhibitor were all
138 purchased from Qiagen (Madrid, Spain). SGBS adipocytes were transfected at day 9 of
139 differentiation with 50 nM of mimic or 50 nM of inhibitor in 0.66 μL/cm^2 Lipofectamine 2000
140 (ThermoFisher Scientific, Madrid, Spain)(for dose-response assays, see supplemental methods).
141 Twenty-four hours after transfection, adipocytes were left either unstimulated or were
142 stimulated with 100 nM insulin (Actrapid Novo Nordisk, Demmark) for 10 min. In some
143 experiments, post-transfected adipocytes were stimulated with 50 ng/mL TNFα for 8 h followed
144 by a 10 min stimulus with 100 nM insulin. Cells were then collected for protein analysis.

Luciferase reporter assays

Potential 3'UTR specific binding sites for miRNAs were predicted by Microrna.org and microT-CDS, revealing potential sites for miR-181a-5p and miR-23a-3p in PTEN and S6K genes. LightSwitch™ 3´UTR reporter GoClone™ RenSP luciferase reporter constructs with the full-length 3'UTR sequence of PTEN or RPS6KB1 were co-transfected into HEK293 for 24 h as detailed in supplemental methods.

RNA isolation, cDNA synthesis, and real-time PCR

Frozen AT (400–500mg) was homogenized with an Ultra-Turrax 8 (Ika, Staufen, Germany). Tissue total RNA was extracted with the RNeasy Lipid Tissue Midi Kit (Qiagen Science, Hilden, Germany). Adipocyte total RNA was extracted with the miRCURY™ RNA Isolation Kit–Cell & Plant (Exiqon, Vedbaek, Denmark). RNA quality control was assessed spectrophotometrically by Xpose® (Gentbrugge, Belgium).

The miRCURY™ RNA Isolation Kit–Biofluids (Exiqon) was used to extract total miRNA from serum. The Universal cDNA Synthesis Kit II (Exiqon) was used for total RNA retrotranscription. qRT-PCR gene expression was performed using the ExiLENT SYBR® Green master mix (Exiqon). miRNA expression levels were quantified on the 7900HT Fast Real-Time PCR platform (Applied Biosystems). Data were analyzed by RQ manager software (for more details see supplemental methods).

Western blotting

Cellular proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes and western blots were performed using standard protocols. The following primary antibodies were used: pAKT, AKT, pAS160, AS160, S6K and PTEN, all from Cell Signaling Technologies (Werfen, Spain). An antibody to βactin was purchased from Sigma Aldrich (Madrid, Spain) (detailed information can be found in supplemental methods).

Statistical analysis

For clinical and anthropometrical variables, data are expressed as mean (SD) or median (25^{th}–75^{th}) quartiles when appropriate. Differences in clinical/laboratory parameters or expression variables between groups were compared using the Kruskall-Wallis one-way analysis and Mann-Whitney U test for non-normally distributed data or one-way ANOVA with *post hoc* Bonferroni correction and Student's *t* test for normally distributed data. The chi-square test was used for categorical data to assess differences among groups. For paired plasma samples, Wilcoxon signed-rank test was performed. Spearman's Rho test was used to assess the strength of association correlations between variables. Receiver Operating Characteristic (ROC) curve analysis was performed to evaluate the best predictive model. Variable importance in projection (VIP) analysis and Partial Least Squares Discriminant Analysis (PLS-DA) model were developed using R programming platform. For *in vitro* studies, mean comparison was performed by one-way ANOVA and Student's *t* test. Statistical analysis was performed using version 19 of the Statistical Package for the Social Sciences (SPSS, Chicago, IL). Significance was considered at $P < 0.05$.

RESULTS

miR-181a-5p and miR-23a-3p expression is reduced by TNFα in human adipocytes *in vitro*

TNFα is known to alter the expression of miR-181a-5p and miR-23a-3p in several cell types (20)(21). To determine whether this also occurred in human mature adipocytes, we treated differentiated human primary preadipocytes with 50 ng/mL TNFα for 8 h and measured miRNA expression using qRT-PCR. We found that the expression of both miR-181a-5p and miR-23a-3p was significantly lower in treated than in untreated adipocytes [**Fig. 1(a)**], whereas the expression of miR-155-5p, a validated TNFα-regulated miRNA (22), was elevated under the same conditions. Identical results were obtained in differentiated SGBS adipocytes [**Fig. 1(a)**], leading us to hypothesize that these microRNAs may be deregulated in inflammatory-activated obese AT.

miR-181a-5p and miR-23a-3p expression in adipose tissue from obese and diabetic subjects

To test the aforementioned idea, we compared miR-181a-5p and miR-23a-3p expression in paired VAT and SAT samples from subjects with BMI <30 or ≥30 (Cohort 1). As shown in **Table 1**, subjects with BMI ≥30 presented a worse metabolic profile than those with BMI <30 concomitant with elevated mRNA levels of TNFα in VAT (**supplementary Fig. 1**), as previously described (2). Analysis showed that miR-181a-5p and miR-23a-3p expression was significantly lower in VAT of patients with BMI ≥30 than in those with BMI<30 **[Fig. 1(b)]**, whereas in SAT this significant deregulation was observed only for miR-23a-3p. When subjects were reclassified according to glucose tolerance status, we found that miR-181a-5p expression in VAT was significantly lower in subjects with DM than in normal glucose tolerant (NGT) subjects, irrespective of adiposity [**Fig. 1(c)**]. A similar result was seen in SAT only from subjects with BMI <30. Analysis of miR-23a-3p expression showed that it was significantly lower in SAT and VAT of patients with DM and with BMI <30 than in subjects with NGT [**Fig. 1(d)**]. We also observed that only miR-23a-3p expression levels were significantly lower in NGT patients with BMI ≥30 than in those with BMI <30 [**Fig. 1(d)**]. Finally, expression of both miRNAs was lower in VAT from DM patients with BMI ≥30 than in DM patients with BMI <30, pointing to a combined effect of the co-morbidity.

Correlation analysis between miRNA expression and clinical and anthropometrical characteristics revealed that miR-181a-5p and miR-23a-3p expression inversely correlated with adiposity (measured by BMI and waist circumference) in VAT, whereas this correlation was found only for miR-23a-3p in SAT. The same correlation was observed with both miRNAs and HOMA-IR (**Table 2**). Moreover, the expression of both miRNAs was inversely related to TNFα expression in VAT, where the proinflammatory cytokine plays a prominent role in the development of IR (2). miR-155-5p expression levels were below detection levels in the majority of VAT samples; however, in those samples with detectable expression levels (N=30), no significant correlation was observed with TNFα ($r = 0.302$ $P = 0.316$).

miR-181a-5p and miR-23a-3p modulate insulin-stimulated AKT activation and reduce TNFα-induced insulin resistance in human adipocytes

Our findings suggest a possible link between miR-23a-3p and miR-181a-5p expression and TNFα-induced IR in obesity. To evaluate the contribution of both miRNAs to insulin signaling, we transiently overexpressed them using mimic-miRNAs in mature adipocytes, which we then stimulated with insulin or insulin plus TNFα. We then measured phosphorylated (p)AKT and AKT substrate of 160 kDa (pAS160) levels as a surrogate measure of insulin signaling. We observed that individual transfection of miR-181a-5p and miR-23a-3p significantly elevated the insulin-stimulated increase in pAKT levels by up to 28% and 32%, respectively, and pAS160 up to 36% (by miR-181a-5p), with respect to non-target control siRNA (NTC) [**Fig. 2(a)**]. Furthermore, overexpression of miR-181a-5p prevented, in part, TNFα-induced IR measured as a suppression of insulin-induced phosphorylation, by significantly increasing pAKT levels up to 32%, and pAS160 levels showed a clear tendency for upregulation [**Fig. 2(b)**]. By contrast, no preventative effect was detected when miR-23a-3p was overexpressed [**Fig. 2(b)**]. We next wondered whether combined overexpression of both miRNAs would have additive effect for improving insulin signaling. Co-transfection of both miRNAs in adipocytes significantly upregulated pAKT levels up to 20% after insulin stimulation and we observed a clear tendency for upregulation of pAS160 signaling; however, no improvement over single miRNA transfection was observed [**Fig. 2(a)**]. Nevertheless, miRNA co-transfection minimized the TNF-induced IR effect with greater efficiency than did individual miRNAs by upregulating the level of AKT phosphorylation up to 135.8%, and up to 62% for AS160 phosphorylation although the latter effect did not reach statistical significance [**Fig. 2(b)**]. These results suggest that both miRNAs may share targets in the insulin pathway regulated by the action of TNFα.

PTEN and S6K are potential targets of miR-181a-5p and miR-23a-3p

Key insulin signaling genes PTEN and S6K were identified as the best shared predicted targets for both miRNAs (**supplementary Fig. 2**) (23)(24). To test these predictions, reporter

constructs containing the *Renilla* luciferase gene fused to the PTEN 3′ UTR (luc-PTEN) or to the S6K 3′ UTR (luc-S6K) were transiently transfected into HEK293 cells jointly with miR-181a-5p or miR-23a-3p. As shown in [**Fig. 3(a)**], luc-PTEN 3'UTR luciferase activity was significantly reduced by miR-181a-5p (-36.48%) and miR-23a-3p (-21.94%), and a similar reduction in luciferase activity was observed in luc-S6K 3'UTR with miR-181a-5p (-45.44%) and miR-23a-3p (-46.52%).

Consistent with the results of the *Renilla* reporter assays, a marked reduction of PTEN and S6K protein expression was observed when miR-181a-5p (-32.33%) was over-expressed, but no significant changes where observed for miR-23a-3p [**Fig. 3(b)**]. However, co-transfection of both miRNAs significantly reduced PTEN expression (-21.33%) **Fig. 3(b)**]. Reduced S6K expression (-20.3%) was also observed with co-transfection [**Fig. 3(b)**], moderately affecting its phosphorylation status (**see supplemental Fig. 3**). To bolster these findings, we transfected mature adipocytes with inhibitors for miR-181a-5p and miR-23a-3p, either individually or in combination. Whereas an increase in PTEN expression was observed when miR-181a-5p was inhibited alone (22.3%) or when both miRNAs were inhibited simultaneously (38.3%), this did not reach statistical significance [**Fig. 3(c)**]. By contrast, miR-23a-3p inhibition significantly elevated PTEN protein expression up to 78.7% above control levels. S6K protein expression was significantly increased by single inhibitor miR-181a-5p (13.5%) and miR-23a-3p inhibition (18.4%). An increase in S6K expression was also observed when both miRNAs where inhibited simultaneously, reaching 16.5% when compared with the inhibitor negative control [**Fig. 3(c)**].

miR-181a-5p and miR-23a-3p are found circulating in serum

To assess the potential value of miR-181a-5p and miR-23a-3p as prognostic markers, we measured their levels in serum in a cohort of 101 normoglycemic subjects (Cohort 2) that were followed for 4 years, with 48 diagnosed prediabetic and 53 remaining normoglycemic (**supplementary Table 1**). We observed that miR-181a-5p levels were significantly reduced in the cases *vs* controls at baseline, pointing to its prognostic value [**Fig. 4(a)**]. We also found

274 significant differences for miR-181a-5p and miR-23a-3p between cases and controls after 4
275 years, when prediabetes was already diagnosed [**Fig. 4(a)**]. Interestingly, we observed that
276 levels of miR-181a-5p increased in both cases and controls after 4 years with respect to baseline
277 levels.

278 To evaluate the usefulness of circulating miR-181a-5p as a potential prognosis biomarker of
279 prediabetes, we performed an ROC analysis. The ROC curve of miR-181a-5p at baseline
280 yielded an AUC of 0.633 (95% confidence interval [CI], 0.048–0.632, $P = 0.028$) with 82.7%
281 sensitivity and 33.3% specificity [**Fig. 4(b)**]. We then applied a PLS-DA model to evaluate the
282 potential of miR-181a-5p in the stratification of patients. The model was constructed using
283 biochemical, anthropometrical and clinical variables. Cross-validation analyses showed that a 1-
284 component model had an accuracy of 54.9% (**supplementary Fig. 4**). With regards to the
285 importance of individual variables (VIP), scores showed that HDL-cholesterol, C-reactive
286 protein, and adiponectin had high importance in this model, with miR-181a-5p as the fourth
287 most important variable and having more impact in the model than insulin or glucose at 120 min
288 [**Fig. 4(c)**].

289 Finally, we performed a multivariate logistic regression analysis including miR-181a-3p, HDL-
290 cholesterol, C-reactive protein and adiponectin, variables that had shown significantly different
291 levels at baseline between groups **(supplementary Table 1**). The resulting ROC curve yielded a
292 larger area under the curve (AUC = 0.793; 95% CI, 0.693–0.893, $P < 0.001$) [**Fig. 4(d)**] and
293 significantly higher specificity (72.9%), and the multivariate model correctly classified 72.3%
294 of patients.
295

296 **DISCUSSION**

297 The association between the expression levels of TNFα in AT and obesity and IR has long been
298 recognized (25)(2). In the present study, we have identified miR-181a-5p and miR-23a-3p as
299 miRNAs downregulated by TNFα in human adipocytes *in vitro*. This was corroborated in

human VAT biopsies of obese subjects with BMI ≥30 and was more pronounced in diabetic obese VAT. Although both SAT and VAT have been described to correlate with IR, available data show that the VAT depot more strongly correlates with IR (26). We also show that both miR-181a-5p and miR-23a-3p expression are inversely related to adiposity irrespective of the fat depot and also to IR measured by HOMA-IR. Additionally, both miRNAs are inversely related to TNFα expression in VAT, arguing in favor of their possible role in IR.

TNF-α alters the expression of many proteins that are required for insulin-stimulated glucose uptake in adipocytes, such as the insulin receptor, insulin receptor substrate-1, AKT and AS160, overall affecting the translocation of the glucose transporter GLUT4 to the plasma membrane (27)(28). Other mechanisms implicated in TNFα induced IR include the activation of the JNK signaling pathway and the regulation of the adipogenic master regulator, peroxisome proliferator-activated receptor gamma (29).

We observed that transient overexpression of miR-181a-5p and miR-23a-3p increased insulin-stimulated pAKT and pAS160 expression in adipocytes to levels greater than 25%, but only miR181a-5p blocked TNFα-induced suppression of pAKT and pAS160. Nevertheless, this rescue effect was augmented when both mimics were overexpressed simultaneously in adipocytes, clearly indicating that miR-181a-5p and miR-23a-3p can co-operate to target insulin pathway regulators.

PTEN regulates phosphatidylinositol 3-kinase (PI3K)-dependent insulin signaling pathways in adipocytes (30) and S6K (p70S6K), a serine kinase involved in negative feedback regulation of insulin action (24). The presence of seed regions in PTEN and S6K for miR-181a-5p and miR-23a-3p, which could potentially regulate their expression, was validated experimentally in HEK293 cells, as shown by a down-modulation of the luciferase-reporter genes containing the wild-type PTEN and S6K 3′UTRs. Analysis of the mechanisms through which these miRNAs interfered with insulin signaling revealed a clear down-regulation of PTEN protein expression following combined overexpression of miR-181a-5p and miR-23a-3p and also when miR-181a-

5p alone was overexpressed. The effect of miR-23a-3p on PTEN expression was only evident when inhibition experiments were performed. A possible explanation for this finding is that because miR-23a-3p can target the deubiquitinase A20 (31), which is a negative regulator of NF-κB (32), its suppression may prevent PTEN inhibition by NF-κB (33). S6K expression was reduced when miR-181a-5p and miR-23a-3p were overexpressed simultaneously in adipocytes, but not individually. An upregulation of S6K protein after miRNA inhibition confirmed this target in adipocytes.

Despite their rather mild effect on AKT and AS160 phophorylation targets, our results point to the participation of overexpressed miR-181a-5p and miR-23a-3p in insulin signaling in adipocytes, at least *in vitro*, since these findings were validated by loss of function and by miRNA/mRNA interaction experiments. Nevertheless, one has to be cautious about translating *in vitro* results to an *in vivo* context because changes in individual miRNA levels in tissue or blood do not always induce observable physiologic effects (34).

Many studies have investigated the function of miR-181a in tissues where it is highly expressed, such as thymus (35) and brain (36). miR-181a has been described as an important negative regulator in hepatic insulin sensitivity (13) and belongs to the miR-181 family cluster, which has a crucial role as a positive regulator of PI3K signaling with PTEN as a target in lymphoid development (37). Accordingly, a robust metabolic phenotype might be expected after suppression of the entire miR-181 cluster in adipocytes, which might unveil new functions in the context of obesity and IR. While a role for miR-23a-3p in cancer has been reported, by regulating invasion and migration of osteosarcoma cells *via* its targeting of PTEN (38), to the best of our knowledge the present study is the first to indicate its potential participation in PTEN regulation in adipocyte insulin signaling.

Circulating miRNAs are attractive candidates as biomarkers for disease diagnosis and monitoring (39). The potential involvement of miR-181a-5p and miR-23a-3p in insulin signaling led us to assess their usefulness as serum biomarkers of prediabetes before it is

diagnosed. Interestingly, only miR-181a-5p levels were significantly reduced in prediabetic subjects at baseline when compared to controls, suggesting a possible prognostic role. Changes to miR-23a-3p levels were also observed with prediabetes, which is in concordance with other findings in a smaller cohort study (40). ROC curve analysis of miR-181a-5p showed that the sensitivity and the specificity was too low for a single diagnostic test; even so, miR-181a-5p could be used as a biomarker to support a positive diagnosis of prediabetes rather than the diagnosis being one of exclusion. Similar data were obtained when PLS-DA analysis was applied using all patient clinical and anthropometrical data and also data for miR-181a-5p. In this case, patient stratification had an accuracy of 54.9%. Nevertheless, importance in projection of the first component of the PLS-DA model showed that miR-181a-5p was the fourth most important variable, having more impact than insulin or glucose at 120 min. Thus, although the diagnostic accuracy is moderate for all the indexes we examined, the four serum signatures (HDL-cholesterol, CRP, adiponectin and miR-181a-5p) could represent a potential biomarker panel that allows for early diagnostic of prediabetic patients.

In conclusion, the results presented here outline a potential regulatory role for miR-181a-5p and miR-23a-3p in obesity-related TNFα-associated IR in adipocytes. We also show that miR-181a-5p is deregulated before the onset of prediabetes. Further studies will be needed to establish the molecular mechanisms through which miR-181a-5p and miR-23a-3p modulate adipocyte insulin signaling.

Acknowledgements

We want to particularly acknowledge the patients enrolled in this study for their participation and the BioBanc IISPV (B.0000853 + B.0000854) integrated in the Spanish National Biobanks Platform (PT13/0010/0029 & PT13/0010/0062) for its collaboration. This study was supported by a project from the Fondo de Investigación Sanitaria (FIS): PI14/00465 and PI17/00877 (M.R.CH), co-financed by the European Regional Development Fund (ERDF). Dr Matilde R.

Chacón is supported by the Research Stabilization Program of the Instituto de Salud Carlos III (ISCIII) co-financed by Institut Català de Salut (ICS) in Catalonia.

Author contributions

M.R.CH. and J.L.B. designed the experiments; A.A.C., J. L.B carried out experiments and G. LL., M. P.O, E. R.G analyzed data; R. J., G. R.M., J.V. provided patients and supervised clinical information. M.R.CH drafted the paper. M.R.CH takes the full responsibility for the work as a whole

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Figure 1. **miR-181a-5p and miR-23a-3p expression is reduced in TNFα-treated adipocytes and in obese human adipose tissue**

(a) miR-181a-5p and miR-23a-3p expression is reduced by TNFα in human adipocytes. Mature human and SGBS adipocytes were stimulated at day 9 of differentiation with 50 ng/mL TNFα for 8 h. Relative levels of miRNAs (mean ± SEM) from 4 independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Human adipocytes (Had).

(b) miR-181a-5p and miR-23a-3p expression from subcutaneous (SAT) and visceral (VAT) adipose tissue samples stratified according to BMI. Relative levels (mean ± SEM) **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

(c) miR-181a-5p expression from subcutaneous (SAT) and visceral (VAT) adipose tissue samples stratified according to BMI and glucose tolerance status. Normal Glucose Tolerance (NGT), Impaired Glucose Tolerance (IGT) and Type 2 Diabetes (DM**)**. Relative levels (mean ± SEM). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

(d) miR-23a-3p expression from subcutaneous (SAT) and visceral (VAT) adipose tissue samples stratified according to BMI and glucose tolerance status. Normal Glucose Tolerance (NGT), Impaired Glucose Tolerance (IGT) and Type 2 Diabetes (DM**)**. Relative levels (mean ± SEM). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 2. miR-181a-5p and miR-23a-3p mediate activation of insulin pathway target genes and block TNFα-induced insulin resistance in human adipocytes

SGBS adipocytes were transfected on day 9 for 24 h with 50 nM mimic miR-181a-5p and/or 50 nM mimic miR-23a-3p (individually or in combination), treated with or without 50 ng/mL

TNFα for 8 h and then further stimulated with or without 100 nM insulin for 10 min. Representative western blots of protein expression levels of phospho (P)-AKT, total AKT, P-AS160, total AS160 and βActin. **(a)** Single and double mimic transfection of SGBS adipocytes under 100nM insulin treatment. **(b)** Single and double mimic transfection of SGBS adipocytes with 50 ng/ml TNFα plus 100 nM insulin. Graphs show relative expression values (mean ± SEM) of 5 independent experiments. P-AKT/AKT and P-AS160/AS160 ratios were normalized to NTC for single transfection or NTC 2× for double transfection. 100% NTC mean ± SEM relative unit values are for n= 5 experiments : NTC+insulin pAKT/AKT: 0.68 ± 0.31; NTC2× +insulin pAKT/AKT: 0.45 ± 0.13; NTC+insulin pAS160/AS160 0.71 ± 0.28; NTC2× +insulin pAS160/AS160 0.53 ± 0.15; NTC+insulin+TNFα pAKT/AKT 0.26 ± 0.08; NTC2× +insulin+TNFα pAKT/AKT 0.25 ± 0.12; NTC+insulin+TNFα pAS160/AS160 0.65 ± 0.31; NTC2× +insulin+TNFα pAS160/AS160 0.45 ± 0.12. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. siRNA non-target control (NTC), double dose (100 nM) of siRNA non-target control (NTC2×), mimic miR-181a-5p (M181), mimic miR-23a-3p (M23); mimic miR-181a-5p + mimic miR-23a-3p (M181+M23).

Figure 3. PTEN and S6K are targets for miR-181a-5p and miR-23a-3p in human adipocytes

(a) HEK293 cells were co-transfected with luciferase (luc)–PTEN 3′UTR or luc-S6K 3′UTR constructs and 50 nM miR-181a-5p mimic, miR-23a-3p mimic or siRNA non-target control (NTC) for 24 h. Relative renSP luciferase activity is expressed as mean ± SEM of 3 independent experiments normalized to NTC + luc-PTEN (or luc-S6K) 3′UTR Relative Light Units (RLU)/µg protein (=100%) are for n= 3 experiments: NTC + luc-PTEN 3′UTR RLU/µg protein 5848 ± 848; NTC + luc-S6K 3′UTR RLU/µg protein 9014 ± 1577. **P* < 0.05; ***P* < 0.01.

(b) Representative western blots of protein expression levels of PTEN and S6K in SGBS adipocytes transfected for 24 h with 50 nM mimics miR-181a-5p (M181) and miR-23a-3p (M23), or in combination (M181+M23).

(c) Representative western blots of protein expression levels of PTEN and S6K in SGBS adipocytes transfected for 24 h with 50 nM inhibitors or their combination: miR-181a-5p inhibitor (iM181) and miR-23a-3p inhibitor (iM23), in combination (iM181+iM23).

Relative values (mean ± SEM) of 3 independent experiments. PTEN/ βActin and S6K/ βActin ratios were normalized to non-target control (NTC), double dose (100nM) of siRNA non-target control (NTC2×), inhibitor negative control (iNC) or double dose (100 nM) of inhibitor negative control (iNC2×) when appropriate. 100% NTC mean ± SEM relative unit values for n= 3 experiments: NTC PTEN/β-Actin 0.37 ± 0.09; NTC2× PTEN/β-Actin 0.51 ± 0.13; NTC S6K/β-Actin 1.35 ± 0.12; NTC 2× S6K/β-Actin 1.31 ± 0.28. 100% iNC mean ± SEM relative unit values: iNC PTEN/β-Actin 0.41 ± 0.04; iNC2× PTEN/β-Actin 0.51 ± 0.06; iNC S6K/β-Actin 1.02 ±0.32; iNC2× S6K/β-Actin 1.44 ± 0.49. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 4. Circulating levels of miR-181a-5p may help to identify the prediabetic phenotype
(a) Concentration of miR-181a-5p and miR-23a-3p in serum of controls and cases at baseline and at 4 years follow-up. **P* < 0.05 between cases and controls at baseline, ¶*P* < 0.05 between cases and control at 4-year follow-up #*P* < 0.005 for 4-year follow-up vs. baseline.
(b) Results of receiver operating characteristic curve (ROC) for miR-181a-5p levels at baseline.

(c) Variable importance in projection representation (VIP) of variables in the discriminating quadratic model. C-reactive protein (CRP), sex hormone-binding globulin (SHBG), fatty acid binding protein 4 (FABP4), waist and hip ratio (WHR).
(d) ROC plot using miR-181a-3p, HDL-cholesterol, C-Reactive protein (CRP) and adiponectin for the analysis.

Table 1. Baseline characteristics of the participants in the study

	BMI<30	BMI≥30	*P*-value
	n=28	n=30	
Age (years)	52.67±14.84	45.8±7.84	0.123
Gender (male/female)	12/16	9/21	0.309
BMI (kg/m^2)	25.74±2.44	43.7±4.91	<0.001
Waist (cm)	87.28±11.77	127.24±11.44	<0.001
Hip (cm)	97.68±11.53	142.16±12.29	<0.001
Waist/Hip	0.89±0.09	0.89±0.09	0.515
SBP (mmHg)	129.57±15	140.03±21.84	0.044
DBP (mmHg)	72.89±8.05	90.5±10.85	<0.001
Glucose Tolerance (Normal /IGT/DM)	22/0/6	12/11/7	0.001
Glucose (mM)	5.7±1.27	5.72±0.99	0.413
Insulin (ρmol/L)	51.32±66.67	116.95±63.96	<0.001
HOMA-IR	1.47±1.15	4.26±2.31	<0.001
Cholesterol (mM)	5.05±1.16	4.89±1.17	0.486
HDL-cholesterol (mM)	1.32±0.36	1.16±0.24	0.101
LDL-cholesterol (mM)	3.7±1.03	3.69±1.11	0.935
Triglycerides (mM)	1.3±0.36	1.7±1.12	0.013
CRP (mg/L)	0.99±0.56	0.89±0.58	0.564

Clinical and anthropometrical characteristics of the study groups classified according to Body Mass Index (BMI) <30 or ≥30: Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Glucose tolerance: Normal glucose tolerance (NGT); Impaired Glucose Tolerance (IGT) and type 2 Diabetes Mellitus (DM), HOMA-IR, homeostasis model assessment of insulin resistance index, High Density Lipoprotein (HDL-) cholesterol, C-Reactive Protein (CRP).

Table 2. Spearman Correlations

	SAT				VAT			
	miR-181a-5p		**miR-23a-3p**		**miR-181a-5p**		**miR-23a-3p**	
	R	***p***	**R**	***p***	**R**	***p***	**R**	***p***
BMI	--	--	-0.467	<0.001	-0.237	0.042	-0.686	<0.001
Waist (cm)	--	--	-0.546	<0.001	-0.269	0.028	-0.651	<0.001
Hip (cm)	--	--	-0.559	<0.001	--	--	-0.584	<0.001
Waist/Hip	--	--	--	--	-0.400	0.003	--	--
SBP (mmHg)	-0.277	0.040	-0.378	0.007	--	--	--	--
DBP (mmHg)	-0.278	0.039	-0.615	<0.001	--	--	-0.506	<0.001
Glucose (mM)	--	--	--	--	-0.391	0.002	-0.315	0.011
Insulin (pmol/L)	--	--	-0.497	<0.001	--	--	-0.545	<0.001
HOMA-IR	--	--	-0.492	<0.001	-0.289	0.033	-0.672	<0.001
Cholesterol (mM)	0.256	0.038	--	--	--	--	--	--
HDL-cholesterol (mM)	0.240	0.048	--	--	0.207	0.071	0.282	0.020
LDL-cholesterol (mM)	--	--	--	--	--	--	--	--
Triglycerides (mM)	-0.254	0.039	-0.314	0.014	--	--	-0.270	0.025
CRP (mg/L)	--	--	--	--	--	--	--	--
gene expression levels								
TNFα mRNA	ND	ND	ND	ND	-0.404	0.031	-0.425	0.024
miRNA expression levels								
miR-181a-5p SAT	1.000		0.748	<0.001	--	--	--	--
miR-23a-3p SAT	0.748	<0.001	1.000		--	--	0.537	<0.001
miR-181a-5p VAT	--	--	--	--	1.000		0.675	<0.001
miR-23a-3p VAT	--	--	0.537	<0.001	0.675	<0.001	1.000	

Significant correlation data between clinical and anthropometrical characteristics and miR-181a-5p and miR-23a-3p expression levels from subcutaneous and visceral adipose tissue depots from the studied patients. SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; R: Spearman Rho; SBP: systolic blood pressure; DBP: diastolic blood pressure; CRP: C-Reactive Protein; HDL; high-density lipoprotein; LDL: low density lipoprotein; ND: Not determined.

Figure 1

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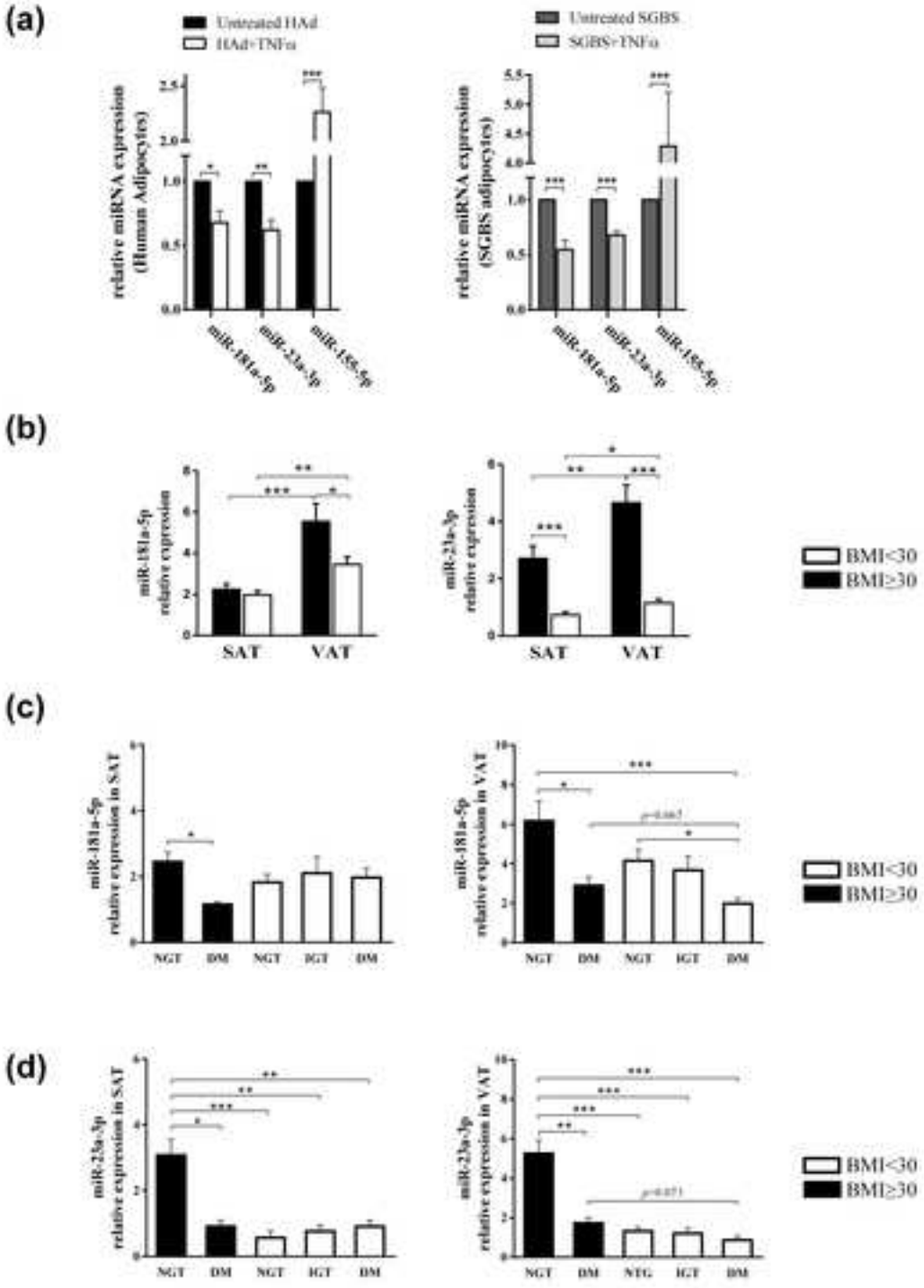

Figure 2

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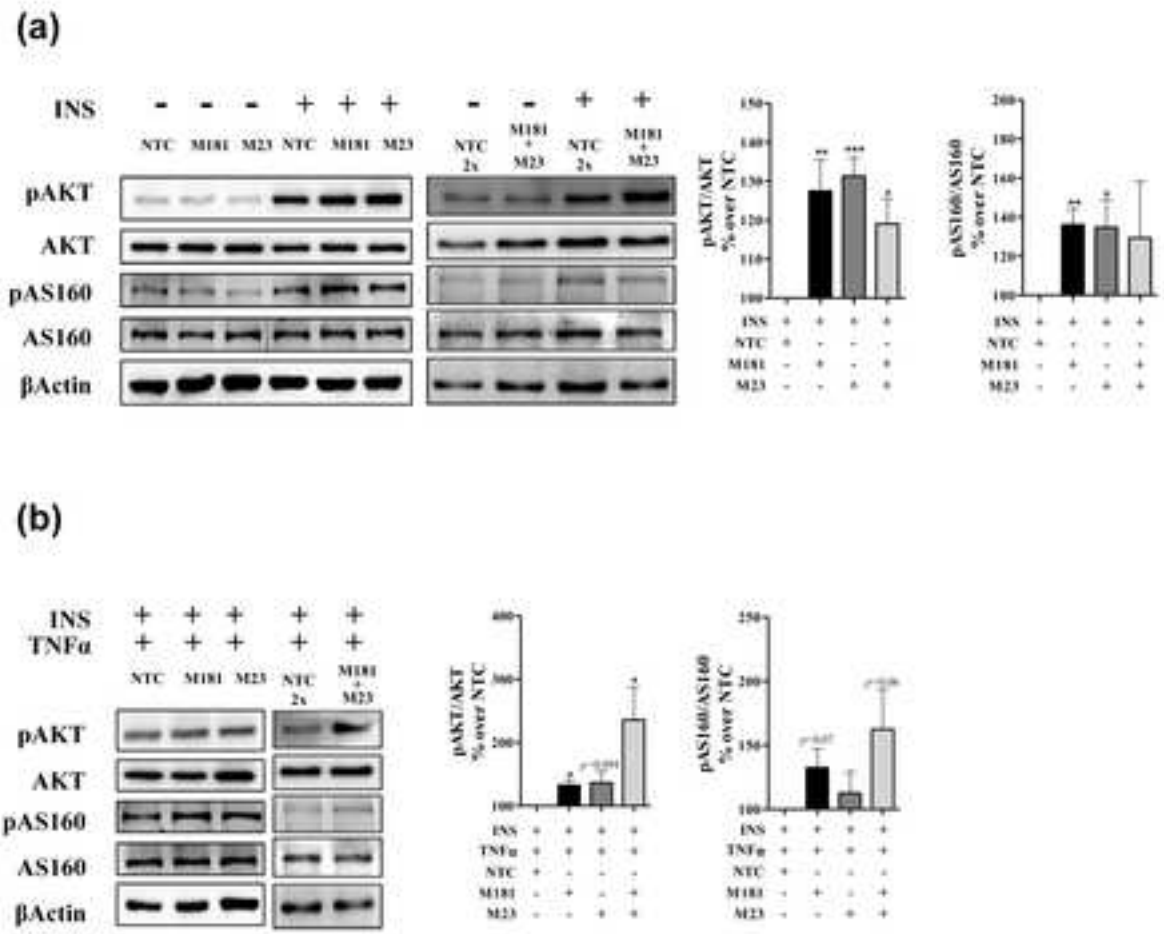

Figure 3

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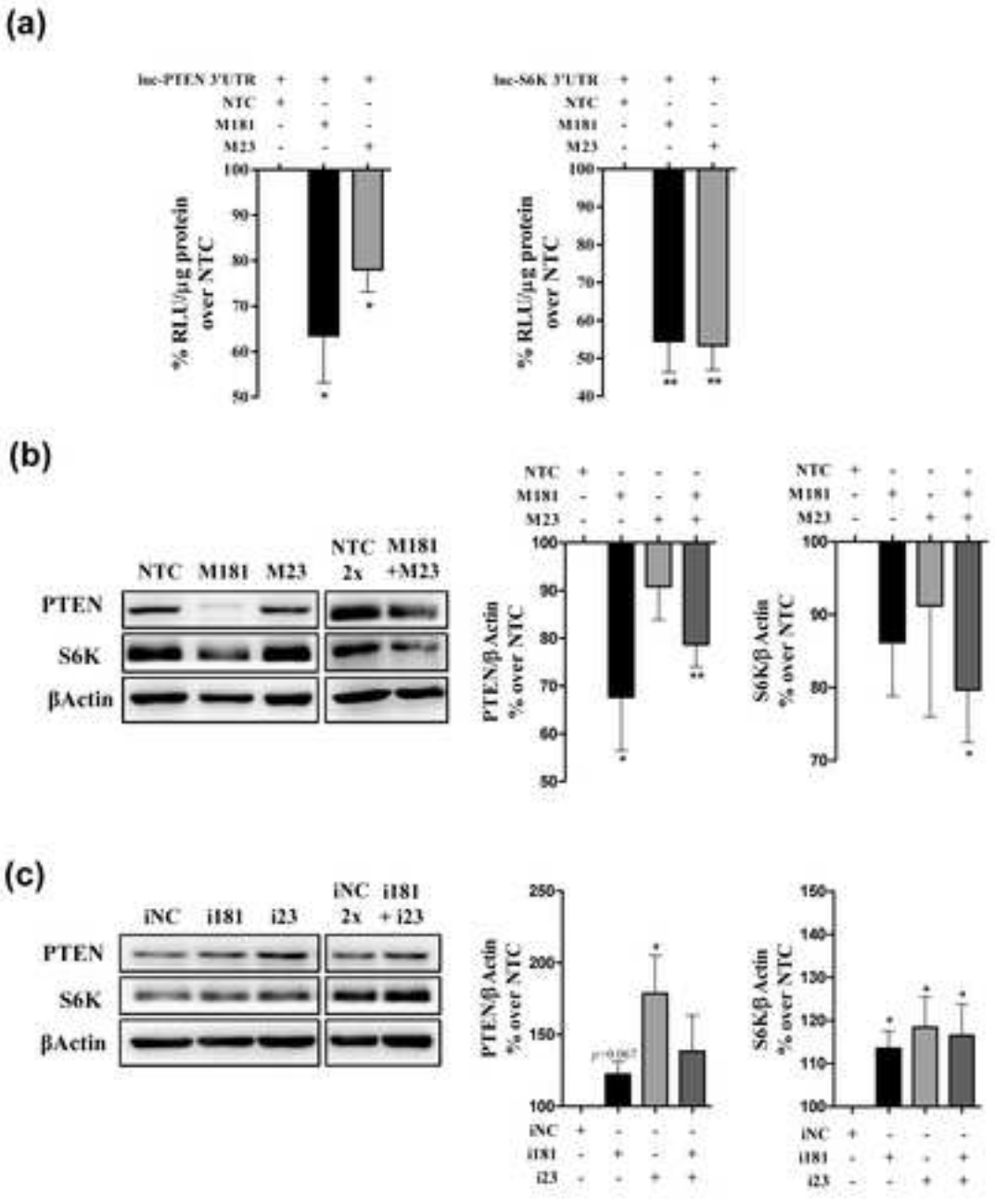
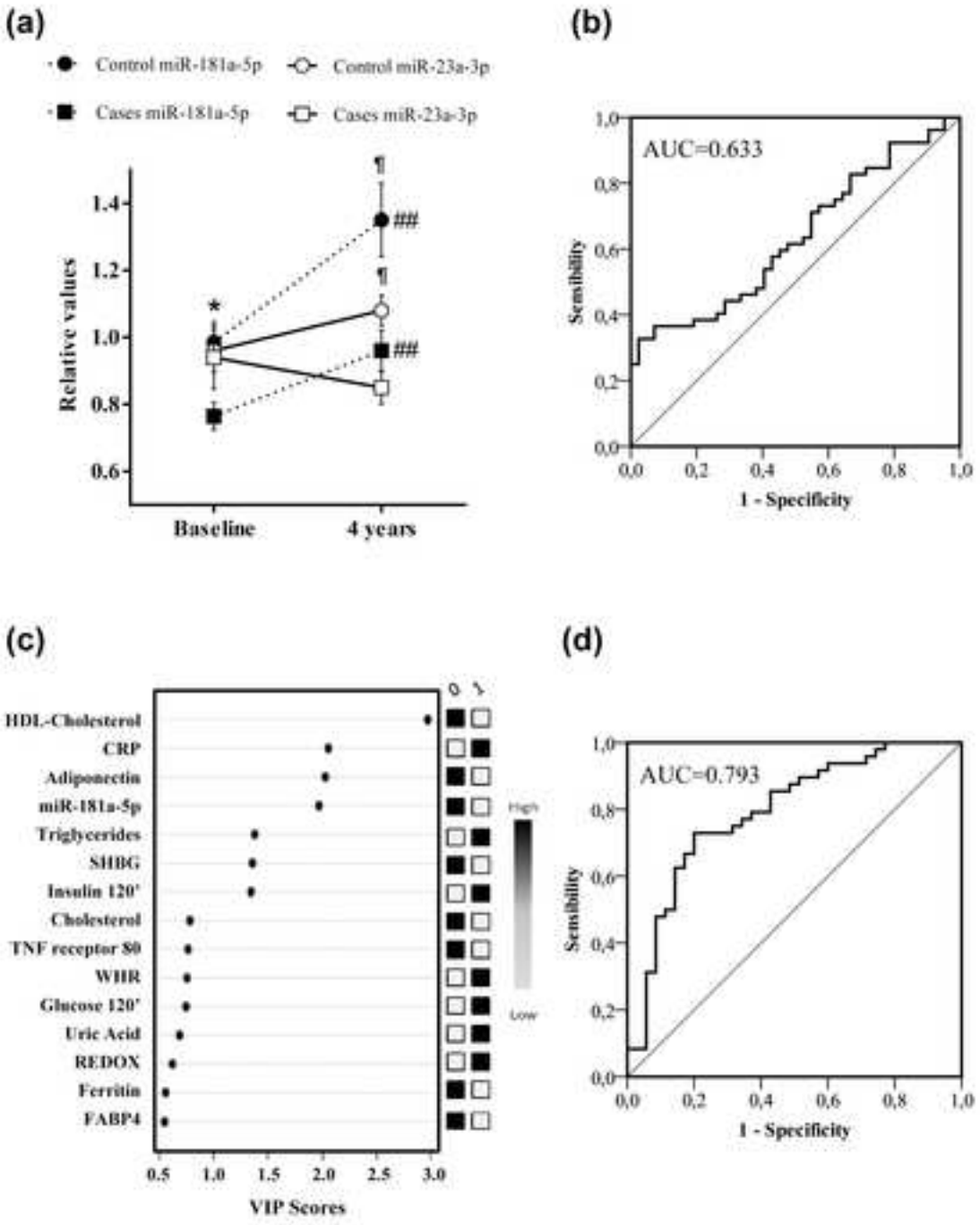


Figure 4

Click here to download Figure Fig_4_v3_2.tif

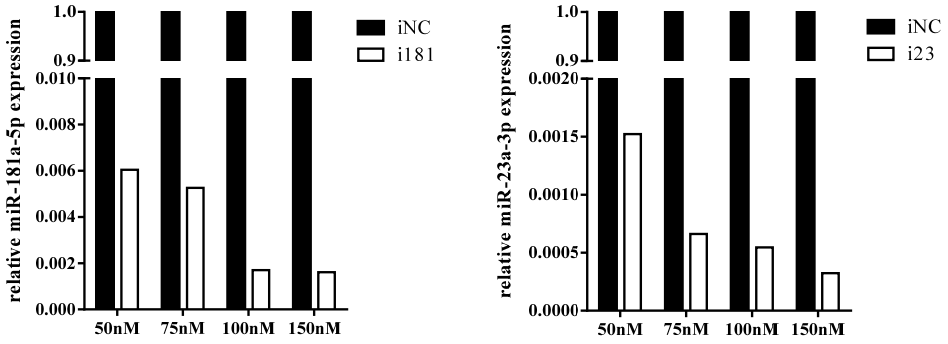


Supplemental materials and methods

Cell culture and treatments

miRNA mimics and inhibitors

Selected doses for mimic transfection on mature adipocytes used have been previously described (1). For inhibitors, a dose-response assay was performed using mature SGBS adipocytes and a dose of 50 nM was established.



Inhibition of miR-181a-5p and miR-23a-3p expression. Graphs show relative expression to negative control inhibitor (iNC) of miR-181a-5p [left] and miR-23a-3p [right] inhibited with their respective inhibitors at different doses: 50, 75, 100 and 150 nM. miR-181a-5p inhibitor (i181). miR-23a-3p inhibitor (i23).

Western blot quantification and Data analysis:

Equal amounts of protein (15 µg) were submitted to SDS-PAGE and then transferred to nitrocellulose membranes, blocked and incubated with various antibodies (as as specified below). Blots were developed with SuperSignal West Femto chemiluminescent substrate (Pierce Biotechnology, Boston, MA, USA) except for β-actin, which was developed with West Pico (Pierce Biotechnology). Proteins were quantified in a VersaDoc Imaging System using Quantity One software (Bio-Rad, Barcelona, Spain) following manufacturer's instructions.

Phosphorilated density values were the results of dividing the phosphorilated density band by its corresponding unphosphorilated band in each case. For other proteins values were normalized to βactin for each condition. Values were then transformed into percentages by normalizing each treatment condition to its respective non-target control (NTC) that was given a value of 100% in each western blot. Statistical analysis were performed on values obtained for n=3-5 western blot data.

Antibodies

Primary Antibodies	**Catalog#**	**Commercialized by**	**Working dilution**
βActin	A2228	Sigma	1:2000
AKT	9272	Cell Signaling	1:1000
pAKT Ser 473	9271	Cell Signaling	1:1000
AS160	2447	Cell Signaling	1:1000
pAS160-Thr 642 (D27E6)	8881S	Cell Signaling	1:1000
PTEN (26H9)	9556S	Cell Signaling	1:1000
S6K	9202	Cell Signaling	1:1000
pS6K- Thr 389	9205	Cell Signaling	1:1000
Secondary antibodies			
Rabbit -IgG	7074	Cell Signaling	1:2000
Mouse-IgG	7076	Cell Signaling	1:2000

Luciferase Reporter Assays

LightSwitch™ 3´UTR GoClone™ RenSP luciferase reporter constructs (Active Motif), including full-length sequence of PTEN 3'UTR or RPS6KB1 3'UTR, were purchased from Condalab (Madrid, Spain). HEK293 cells were plated in 24-well culture plates at 5×10^4 cells/cm^2 in 500 µL DMEM high-glucose medium (Gibco) supplemented with 10% fetal bovine serum (Sigma). Cells were transfected 20 h later with 300 ng of LightSwitch™ 3´UTR GoClone™ RenSP luciferase reporter plasmids and 50 nM of non-target control mimic (NTC), miR-181a-5p mimic or miR-23a-3p mimic (Qiagen) using Lipofectamine 2000 (Invitrogen) at 1:3 ratio [DNA (µg):Lipofectamine 2000 (µl)] in 300 µl of Opti-MEM (Gibco) per well. After 6 h of lipofection, medium was replenished at 500 µl/well with DMEM high-glucose medium

supplemented with 10% fetal bovine serum. Twenty-four hours after lipofection, cells were harvested in 220 µL of PBS. Luminescence was quantified with the LightSwitch Luciferase assay kit (Active Motif), according to the manufacturer's protocol, using a Varioskan LUX System (Thermo Scientific). Relative light units (RLU)/µl were normalized to protein concentration (µg/µl).

Serum miRNA RNA extraction

Serum was thawed on ice and centrifuged at 3000×g for 5 min at 4ºC in a microcentrifuge. Spectrophotometric measurement of oxyhemoglobin absorbance at 414 nm was used to assess hemolysis. miRCURY™ RNA Isolation Kit–Biofluids (Exiqon) was used for the extraction. In brief, an 200 µl aliquot of serum per sample was transferred to a new microcentrifuge tube and 60 µl of Lysis mixture containing 1 µg MS2 RNA bacteriophage (Roche Applied Science) was added, followed by 20 µl of Protein Precipitation Solution (Exiqon). The mixture was centrifuged for 3 min at 11,000×g and the supernatant was transferred to a new tube. Then, 270 µL isopropanol was added and the mixture was placed onto a microRNA Mini Spin colum, incubated for 2 min and centrifuged for 30 sec at 11,000×g. A rinse step (Wash Solution 2 BF) was repeated three times. Total RNA was eluted in 50 µl of RNase-free water and stored at -80ºC.

Synthesis of miRNA cDNA genes and real-time PCR

One microliter of RNA eluate obtained from serum was reverse transcribed in 5 µl reactions using the miRCURY LNA™ Universal RT cDNA Synthesis Kit (Exiqon). The cDNA was diluted 40× in a low-binding 1.5 ml tubes and assayed in 10 µl PCR reactions. Each microRNA was assayed twice by qPCR. A no-template control of water was purified with the samples and profiled as for the samples. Amplification was performed in a LightCycler7900 Real-Time PCR System (Applied Biosystems) in 96-well plates. The amplification curves were analyzed using SDS software, both for determination of Ct (by the second derivative method) and for melting curve analysis.

RT-PCR data filtering and analysis

All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for each particular assay. Furthermore, any sample assay data point had to be detected with 5 Ct less than the corresponding control assay data point, and with a Ct <35, to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. The data analysis was performed using RQ manager software (Applied Biosystems). $^{\Delta}$CT and $^{\Delta\Delta}$CT were calculated using the following mathematical formula: $^{\Delta}$CT=CT sample - CT endogenous, $^{\Delta\Delta}$CT= $^{\Delta}$CT case - $^{\Delta}$CT control. The geometric mean of 3 selected unchanged miRNAs, hsa-let-7g, hsa-let-7i and hsa-miR-103a-3p, were used as internal controls (normalizing factor). Differential expression was calculated using the $2^{-\Delta\Delta Ct}$ method. For *in vitro* experiments and AT studies, snRNA-U6 was used as normalizing control miRNA.

qPCR primers

Primers were all purchased from Exiqon (Vedbaek, Denmark)

miRNA	Reference
hsa-miR-23a-3p	204772
hsa-miR-181a-5p	206081
hsa-miR-103a-3p	204063
hsa-let-7g-5p	204565
snRNA U6	203907

Supplemental methods References

1. **Enlund E, Fischer S, Handrick R, Otte K, Debatin KM, Wabitsch M, Fischer-**

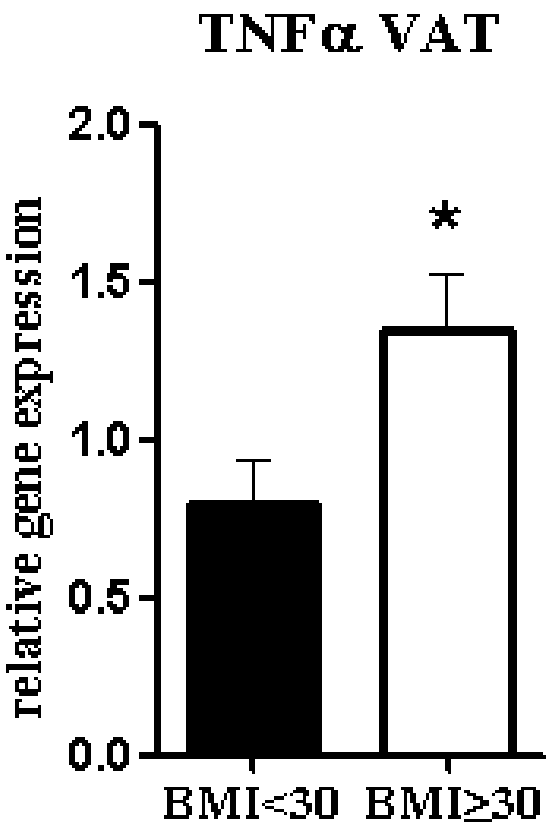
Posovszky P. Establishment of lipofection for studying miRNA function in human adipocytes. *PLoS One* 2014;9(5):1–8.

Supplementary Table 1. Clinical and anthropometrical characteristics of *Pizarra* cohort

		Cases	Controls	*p-value*
n		48	53	
Age (yrs.)		51.1 (12.4)	51.4 (11.7)	0.930
Gender (M/F) (n)		20/28	20/33	0.687
Active smokers (n, %)				
	Baseline	15	14	0.592
	4 years	12*	9*	0.273
Obesity (n, %)				
	Baseline	25	22	0.607
	4 years	28*	22	0.215
Hypertension (n, %)				
	Baseline	26	32	0.528
	4 years	30*	26	0.137
Dyslipidemia (n, %)				
	Baseline	7 (14.6)	15 (28.3)	0.095
	4 years	7 (14.6)	9 (17.0)*	0.742
Anthropometric measurements				
Weight (kg)				
	Baseline	78.6 (14.3)	77.7 (13.2)	0.730
	4 years	80.8 (15.5)*	78.3 (13.6)	0.409
BMI (kg/m^2)				
	Baseline	30.4 (5.0)	30.3 (4.7)	0.944
	4 years	31.4 (5.6)*	30.8 (5.0)*	0.579
Waist (cm)				
	Baseline	104.1 (10.5)	102.7 (11.6)	0.519
	4 years	101.7 (11.6)*	98.8 (12.3)*	0.229
WHR				
	Baseline	0.99 (0.92-1.03)	0.97 (0.92-1.01)	0.846
	4 years	0.92 (0.90-0.96)*	0.90 (0.86-0.97)*	0.129
Blood pressure				
SBP (mmHg)				
	Baseline	135.4 (17.5)	132.9 (19.5)	0.498
	4 years	138.4 (17)	131.9 (19.3)	0.06
DBP (mmHg)				
	Baseline	82.5 (10.7)	80.6 (10.5)	0.350
	4 years	81.5 (11.5)	74.6 (11.5)	0.002
Lipid profile				
Total cholesterol (mmol/L)				
	Baseline	6.4 (1.3)	6.6 (1.2)	0.187
	4 years	5.2 (1)*	5.5 (1)*	0.407
HLD-cholesterol (mmol/L)				
	Baseline	1.5 (0.3)	1.9 (0.5)	0.002
	4 years	1.3 (0.3)*	1.4 (0.3)*	0.065
LDL-cholesterol (mmol/L)				
	Baseline	4.2 (1.2)	4.2 (1.1)	0.879
	4 years	3.2 (0.9)*	3.4 (0.9)*	0.583
Triglycerides (mmol/L)				
	Baseline	1.3 (0.8-1.8)	1 (0.8-1.3)	0.051
	4 years	1.3 (0.9-1.9)	1.1 (0.7-1.6)	0.156
Glucose Profile				
Fasting Glucose (mmol/L)				
	Baseline	5.3 (0.5)	5.3 (0.5)	0.450
	4 years	5.9 (0.7)*	5.1 (0.4)*	<0.001
Glucose 120' (mmol/L)				
	Baseline	5.5 (1.4)	5.1 (1.4)	0.168
	4 years	8.5 (2.3)*	5.1 (1.4)	<0.001
Fasting insulin (pmol/L)				
	Baseline	51.6 (34.7-93.7)	48.6 (31.2-62.5)	0.184
	4 years	ND	ND	
Insulin 120' (pmol/L)				
	Baseline	319.5 (131.9-53.8)	131.9 (90.28-298.6)	0.001
	4 years	ND	ND	
HOMA-IR				
	Baseline	2.35 (1.8)	2 (1.7)	0.262
	4 years	ND	ND	
Uric acid (µM)				
	Baseline	299.5 (111)	279 (76)	0.474

	4 years	297.6 (84)	278 (82.2)	0.211
		Inflammatory parameters		
Leptin (pg/mL)				
	Baseline	14.7 (12.16)	14 (10.5)	0.964
	4 years	ND	ND	
Adiponectin (µg/mL)				
	Baseline	7.6 (5-10.7)	10.7 (7.1-15.4)	0.003
	4 years	ND	ND	
TNF receptor 80 (pg/mL)				
	Baseline	7.1 (4.4-10.4)	7.4 (5-10.3)	0.771
	4 years	ND	ND	
TNF receptor 60 (pg/mL)				
	Baseline	2.3 (1.7-4.8)	2.2 (1.6-2.9)	0.195
	4 years	ND	ND	
TNFα (pg/mL)				
	Baseline	0 (0-6.4)	0 (0-6.2)	0.659
	4 years	ND	ND	
IL-6 (pg/mL)				
	Baseline	2.4 (2.3)	1.8 (1.5)	0.475
	4 years	ND	ND	
CRP (mg/L)				
	Baseline	0.5 (0.2-1.2)	0.24 (0.1-0.5)	0.022
	4 years	ND	ND	
FABP4 (ng/mL)				
	Baseline	27 (16.1-34.3)	28.2 (18.5-36.8)	0.586
	4 years	ND	ND	
SHBG (nmol/L)				
	Baseline	32.4 (21.7-48.5)	42.4 (22.7-63.4)	0.068
	4 years	ND	ND	
REDOX (pg/mL)				
	Baseline	1.3 (1.1)	1.2 (1.1)	0.398
	4 years	ND	ND	
Ferritin (µg/L)				
	Baseline	60.1 (24.4-115.8)	56.4 (28.3-149.2)	0.733
	4 years			
		***miRNA* relative units**		
miRNA-181a-5p				
	Baseline	0.76 (0.26)	0.98 (0.43)	0.028
	4 years	0.96 (0.4)*	1.35 (0.83)*	0.039
miRNA-23a-3p				
	Baseline	0.94 (0.29)	0.96 (0.44)	0.622
	4 years	0.85 (0.56)	1.08 (0.55)	0.047

Data are expressed as mean (SD) or median (25^{th}-75^{th}) percentiles as appropriate. *p<0.005 for 4-year follow-up vs. baseline. Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), High density lipoprotein (HDL-) cholesterol, LDL, Low-density lipoprotein, HOMA-IR, homeostasis model assessment of insulin resistance index, C-reactive protein (CRP), fatty acid binding protein 4 (FABP4), Sex Hormone Binding Globulin (SHBG), ND: not determined



Supplementary Figure 1:
TNFα gene expression is significantly over expressed in visceral adipose tissue (VAT) from obese patients (BMI ≥30). Bars represents the mean ± SEM; *P < 0.05. Mann-Whitney test was used to compare groups.

TNFα-mRNA quantification:
Total RNA (500 ng) from adipose tissue biopsies was retrotranscribed using cDNA High Capacity RNA-to-cDNA kit (Applied Biosystems, #4387406). TaqMan Universal PCR Master Mix (Applied Biosystems, # 4304437) and TaqMan Gene Expression Assays [PPIA #Hs99999904_m1 (Hk) and TNF #Hs99999043_m1, Applied Biosystems] were used for qRT-PCR expression analysis was performed on an ABI 7900HT Fast qPCR system as indicated in the manufacturer's protocol.

(a)

Potential binding sites in PTEN 3'UTR

```
TP  3' ccuuuagggaCCGUUACACUa 5' hsa-miR-23a-3p
                 | :|||||||
1595:5' cuauuguaaaGCUAAUGUGAa 3' PTEN

    3' ugaguggcugucgcaaCUUACAa 5' hsa-miR-181a-5p
                       ||||||
2262:5' uauuauuuuuccuuugGAAUGUg 3' PTEN

    3' ccuuuAGGGACCGUUACACUa 5' hsa-miR-23a-3p
          |||:| | |||||||
2266:5' auuuuUCCUUUGGAAUGUGAa 3' PTEN

    3' ugaguggcugucgcAACUUACAa 5' hsa-miR-181a-5p
                     ||||||||
2295:5' ugaggguuuugauuUUGAAUGUu 3' PTEN
```

Potential binding sites in S6K 3'UTR

```
TP    3' ugaguggcugucGCAACUUACAa 5' hsa-miR-181a-5p
                    :| |||||||
1235:5' uugauggccuuuUGAUGAAUGUc 3' RPS6KB1

    3' ccuUUAGGG-AC-CGUUAC-ACUa 5' hsa-miR-23a-3p
         | |||| || | |||| |||
3152:5' auuAUUCCCAUGAGAAAUGUUGAa 3' RPS6KB1

    3' ugAGUGGCUGUCGCAACUUACAa 5' hsa-miR-181a-5p
          |: | ||: |   |||||||
3582:5' gaUUUCGGAUUG-AAUGAAUGUa 3' RPS6KB1
```

(b)

Potential binding sites in PTEN 3'UTR

```
TP
    3'  UGA____GUGGCUGUCGCAACUUACAA   5'  hsa-miR-181a-5p
         |||    :||:||        ||||||
1261: 5'  UUACUUGAAUACUGAAUACAUAUAAUGUU   3'  PTEN

    3'           UGAGUGGCUGUCGCAACUUACAA  5'  hsa-miR-181a-5p
                 |:||     :||:    ||||||
1869: 5'  CAUCUAAAAUAUUCU____UAGUAAAUAAUGUU  3'  PTEN

    3'  AGUGGCU___GUCGCAA_____CUUACAA    5'  hsa-miR-181a-5p
         |||:|    :|           ||||||
2255: 5'   CACUCUUAUUAUUUUUCCUUUCCAAUGUG    3'  PTEN

    3'  UGAGUGGCUGUC___GC___AACUUACAA    5'  hsa-miR-181a-5p
         || | :|| :|   :|   |||||||||
2289: 5'  UCUGAAUGAGGGUUUUGAUUUUGAAUGUU    3'  PTEN

    3'       UGAGUGGCUGUCGCAACUUACAA     5'  hsa-miR-181a-5p
             :|:||||:||||    ||||||
2780: 5'  AGUGGAGUUUACCGGCAGCAUCAAAUGUU     3'  PTEN

    3'          UGAGUGGCUGUCGCAACUUACAA   5'  hsa-miR-181a-5p
                        :||     ||||||
3923: 5'  AGUGUUAAAAAAAUAAAGUAGAC___AAUGUU   3'  PTEN

    3'       UGAGUG_GCUGUCGCAACUUACAA    5'  hsa-miR-181a-5p
              ||    :|        ||||||
4680: 5'  CUUCCUUCUAAUAUGUAUAUCA_GAAUGUG    3'  PTEN

    3'     UGAGUGGCUGUCGCA__ACUUACAA     5'  hsa-miR-181a-5p
           |:||| :|:|       |||||||
5114: 5'  UCGAAUUCAGUGGCUUAAUCAUGAAUGUC     3'  PTEN

    3'  AGU_GGCUGU__CGCAA_____CUUACAA     5'  hsa-miR-181a-5p
         || ::|:    |:|||     ||||||
5249: 5'  GCAUUUGGAAUUGUGUUAAAUAGAAUGUG     3'  PTEN

    3'  UGAGUGGCUGUCGCAAC_______UUACAA    5'  hsa-miR-181a-5p
         :|:||:| |||:|          ||||||
5881: 5'  GUUUACUG_CAGUGAAAUUCAUCAAAUGUU    3'  PTEN

    3'  _UGAGUGGCU_GUCGCA__AC__UUACAA     5'  hsa-miR-181a-5p
          :||||    || :||  ||  ||||||
6194: 5'  AUUUCACAGUUCAUUGUAAUGAAAAUGUU     3'  PTEN
```

```
TP
    3'     CCUUUAGGGA___C_CGUUACACUA   5'  hsa-miR-23a-3p
           |||:|  ||   | ||  ||||||
364:  5' CAACUGAAGUGGCUAAAGAGCUUUGUGAU   3'  PTEN

    3'        CCUUUAGGGACCG_UUACACUA    5'  hsa-miR-23a-3p
                        || |||||||
1604: 5' UACAACUACUAUUGUAAAGCUAAUGUGA_A   3'  PTEN

    3'           CCUUAGG_GACCGUUACACUA   5'  hsa-miR-23a-3p
                      ||| :||| |||||||
2270: 5' CUGUUAUUAUUUUUCCUUUGG_AAUGUGAA   3'  PTEN

    3'           CCUUUAGGGACCGUUACACUA   5'  hsa-miR-23a-3p
                 ||:   ||     |||||||
3376: 5' UUAAUUAAUGGGCAGCCA____AAUGUGAA   3'  PTEN

    3'           CCUUUAGGGACCGUUACACUA   5'  hsa-miR-23a-3p
                   |||  ::| |:|||||||
4742: 5' UGGUAGCUUUAAAAAGUUU_GUAAUGUGAA   3'  PTEN
```

Potential binding sites in S6K 3'UTR

```
TP
    3'     UGAGUGGCUGUC__GCAACUUACAA   5'  hsa-miR-181a-5p
           |:|:  :|:|     :| |||||||
1232: 5' AAAUAUUUGAUGGCCUUUUGAUGAAUGUC   3'  RPS6KB1

    3' UGAGUGGCUGUCGCA_______ACUUACAA   5'  hsa-miR-181a-5p
       |:|  ::||:  ||        |||||||
3574: 5' AUUAUUUGAUUUCGGAUUGAAUGAAUGUA   3'  RPS6KB1
```

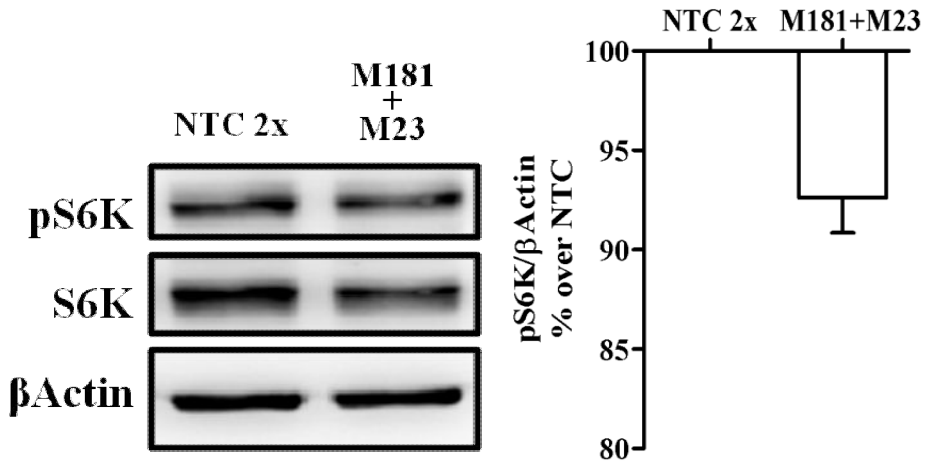
```
TP
    3'  CUUUAGGGACC_______GUU__ACACUA      5'  hsa-miR-23a-3p
         |:|||||:||        |||  ||||||
1998: 5'  CAGAUCCUUGUUCUUUGACAAUUUGUGAU      3'  RPS6KB1

    3'           CUUUAGGGACCGUUACACUA      5'  hsa-miR-23a-3p
                          ||||  ||||||
2089: 5'  AUUAUUUCAGACUGUAAAUGGCU_UGUGAU      3'  RPS6KB1

    3'        CCUU__UAG__GGACCGUUACACUA     5'  hsa-miR-23a-3p
              |||   ||   ||  ||  ||||||
2791: 5'  UAAACUGGGAUUUAUAAACCA_GC__UGUGAU    3'  RPS6KB1

    3'             CUUUAGGGACCGUUACACUA     5'  hsa-miR-23a-3p
                        |:|:||    ||||||
3345: 5'  GAUUAAUACAGUACUUUUUCUUG____UGUGAU   3'  RPS6KB1
```

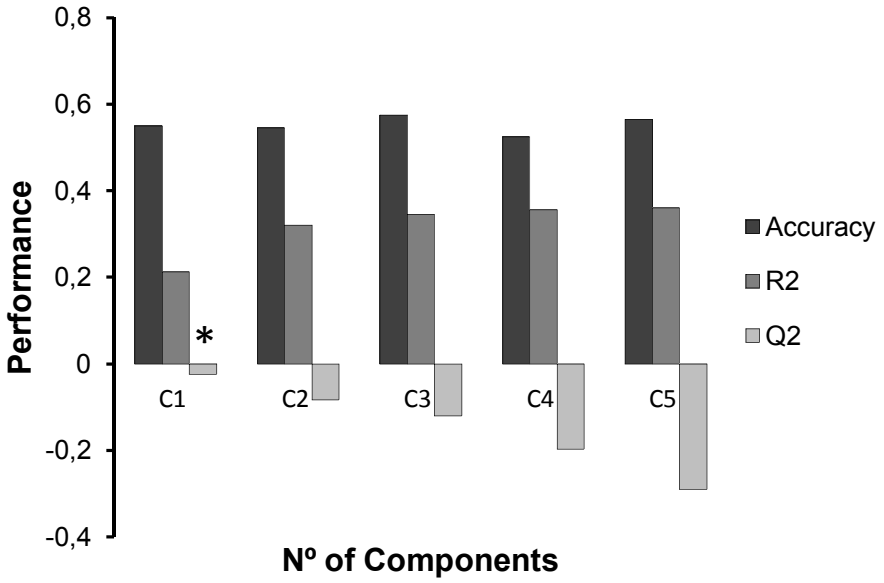
Supplementary Figure 2:
Predicted targets sites algorithms calculated with miTG **(a)** and miRanda **(b)** for miR-181a-5p and miR-23a-3p in both PTEN and S6K (RPS6KB1) 3' untranslated regions (3'UTR). Transcript position in 3'UTR (TP). (|) Watson-Crick base pairing. (**:**) Non-Watson-Crick base pairing.

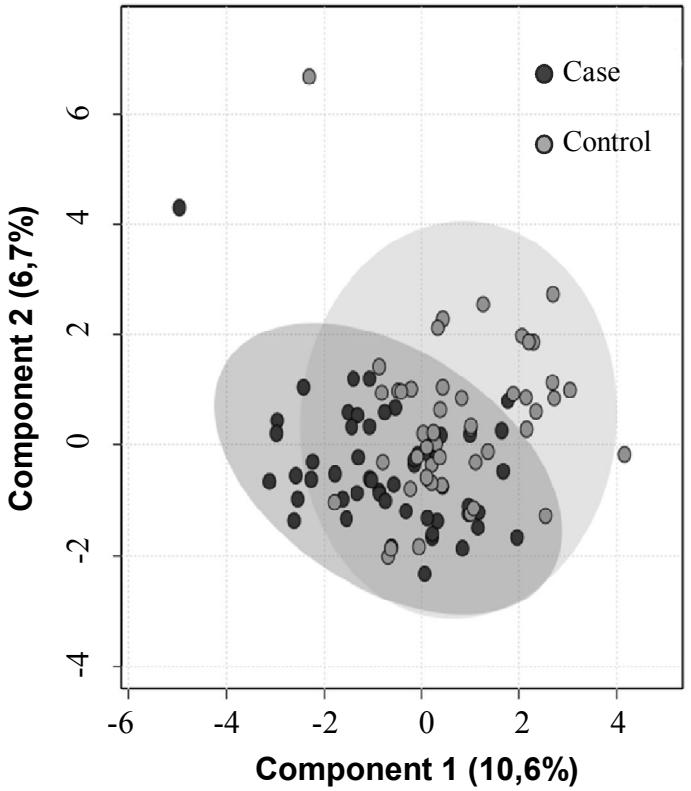


Supplementary Figure 3:
Protein expression levels of pS6K in SGBS adipocytes co-transfected for 24 h with 50 nM miR-181a-5p and 50 nM miR-23a-3p mimics (M181+M23), and treated with 50 ng/ml of TNFα for 8 h and 100 nM of insulin for 10 min. Relative values (mean±SEM) of two independent experiments. pS6K/βActin ratio was normalized to NTC 2×. 100% NTC 2× mean±SEM relative units: 1.14±0.14.

PLS-DA Cross validation details:

Measure0	C1	C2	C3	C4	C5
Accuracy	0.549	0.545	0.575	0.525	0.565
R2	0.212	0.320	0.346	0.356	0.360
Q2	-0.024	-0.084	-0.120	-0.197	-0.290





Supplementary Figure 4:
Partial Least Squares Discriminant Analysis (PLS-DA) model for miR-181a-5p-stratification of cases and control subjects characterized by biochemical, anthropometrical and clinical variables.