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ORIGINAL ARTICLE

miR-10b and *miR-223-3p* in serum microvesicles signal progression from prediabetes to type 2 diabetes

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Abstract

Purpose Type 2 diabetes frequently remains undiagnosed for years, whereas early detection of affected individuals would facilitate the implementation of timely and cost-effective therapies, hence decreasing morbidity. With the intention of identifying novel diagnostic biomarkers, we characterized the miRNA profile of microvesicles isolated from retroactive serum samples of normoglycemic individuals and two groups of subjects with prediabetes that in the following 4 years either progressed to overt diabetes or remained stable.

Methods We profiled miRNAs in serum microvesicles of a selected group of control and prediabetic individuals participating in the PREDAPS cohort study. Half of the subjects with prediabetes were diagnosed with diabetes during the 4 years of follow-up, while the glycemic status of the other half remained unchanged.

Results We identified two miRNAs, *miR-10b* and *miR-223-3p*, which target components of the insulin signaling pathway and whose ratio discriminates between these two subgroups of prediabetic individuals at a stage at which other features, including glycemia, are less proficient at separating them. In global, the profile of miRNAs in microvesicles of prediabetic subjects primed to progress to overt diabetes was more similar to that of diabetic patients than the profile of prediabetic subjects who did not progress.

Conclusion We have identified a miRNA signature in serum microvesicles that can be used as a new screening biomarker to identify subjects with prediabetes at high risk of developing diabetes, hence allowing the implementation of earlier, and probably more effective, therapeutic interventions.

Keywords Prediabetes · Progression · Diagnosis · Microvesicle · Biomarker · MicroRNA

Introduction

Diabetes mellitus is the most common chronic metabolic disease in the world, with type 2 diabetes (T2D) being its most frequent clinical manifestation [1]. The last epidemiological study conducted in Spain revealed that 13.8% of citizens suffer from diabetes, almost half of which (6%) do not know it, while a further 15% experience intermediate states of altered glucose metabolism collectively termed prediabetes [2]. These local figures are in accordance with worldwide estimates [1]. Hence, more than 25% of the population displays alterations in glucose metabolism. Chronic complications derived from T2D and prediabetes increase morbidity, so that 10% of the public health budget is dedicated to their treatment [3]. Therefore, early identification of those individuals at higher risk of developing T2D will improve their quality of life.

Prediction of developing T2D relies in phenotypic markers including the body mass index (BMI), percentage of glycated hemoglobin HbA1c, and plasma levels of glucose, lipids and pro-inflammatory cytokines [4]. However, diagnostic based on these markers occurs late during disease development, when considerable damage has been accumulated [5]. Extracellular microRNAs (miRNAs) found in bodily fluids hold promise as novel informative biomarkers for many conditions [6]. MicroRNAs are small RNA molecules

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that modulate gene expression post-transcriptionally [7]. Extracellular miRNAs are found in blood as several populations, either bound to protein complexes and high-density lipoprotein (HDL) particles, or encapsulated inside small vesicles [8]. By analyzing the global extracellular miRNA profile, we previously described two miRNAs up-regulated in serum of prediabetic subjects [9]. However, it is possible that by interrogating specific extracellular miRNA populations more precise information could be gathered. In this regard, the population of miRNAs in microvesicles displays several attractive features.

Extracellular vesicles, particularly exosomes, are small vesicles released by all cells [8]. They are enriched in miRNAs and can transfer their cargo between cells, thus constituting a novel mechanism of intercellular signaling [10]. We and others have recently demonstrated that exosomal miRNAs participate in diabetes development in mice [11, 12]. Therefore, they may act as informative biomarkers for patient screening and stratification. Here, we followed a cohort of normoglycemic and prediabetic individuals for 4 years to record progression to T2D with the aim of identifying new biomarkers for disease progression. We profiled the miRNA content of microvesicles isolated from serum of a subset of normoglycemic subjects and individuals with prediabetes that in the follow-up period had either developed or not T2D. The results were validated in a second subset of samples from the same cohort.

Methods

Study population

The PREDAPS study (Primary Health Care on the Evolution of Patients with Prediabetes) is a prospective study of a cohort of 838 subjects with normal glucose metabolism and another cohort of 1184 subjects with prediabetes (fasting glucose between 100 and < 126 mg/dL, or HbA1c between 5.7 and < 6.5%, or both at the same time) recruited from all over Spain starting from 2012 [13]. Participants were monitored annually to determine incidence of new cases of T2D. Detailed inclusion, exclusion and diagnosis criteria for the study have been previously described [13]. We obtained annual serum samples from a sub-cohort of the PREDAPS Study constituted by participants attending primary care health centers in the Barcelona area ($n = 152$), starting from 1 year after the initial observation (2013) and until the end of the study (2017). Blood was collected in the morning after an overnight fast from the median cubital vein with a 21G needle in vacutainer SST tubes. Tubes were maintained at room temperature in the vertical position and transported to the IDIBAPS Biobank (Barcelona) within 3 h after withdrawal for further processing. Samples were then centrifuged for 10 min at 1500 × g and the serum was aliquoted in micronic tubes and stored at − 80 °C until used.

Glucose, glycated hemoglobin, triglycerides (TG), total cholesterol, HDL and low-density lipoproteins (LDL) were determined as described [14, 15]. FLI was calculated as described [16]. Informed consent was obtained from all participants, and the studies were approved by the Research and Ethics Committees of the Hospital Clinic, University of Barcelona and the Parc de Salut Mar Clinical of Barcelona.

Microvesicle isolation and characterization

Microvesicles were isolated from 400 µl serum by reagent-assisted precipitation. Samples from 2013 to 2017 were processed in parallel to minimize variability due to sample handling. Briefly, serum samples were sequentially centrifuged for 30 min at 2000 × g and 45 min at 10,000 × g. Exoquick (System Biosciences) was then added by following the instructions of the manufacturer and the final pellet containing the vesicles was resuspended in 100 µl PBS. Mean diameter size of the vesicles was determined with NanoSight LM10 (Malvern Panalytical) as described [12]. Number of vesicles was estimated by measuring esterase activity with ExoCET Quantification Kit (System Biosciences). Vesicles were stored at − 80 °C until used for RNA extraction.

Extraction and analysis of exosomal miRNAs

For RNA isolation, 25 µl resuspended microvesicles were processed with miRNeasy (Qiagen). An equal volume of each sample was retrotranscribed (Exiqon) and the miRNA profile was analyzed in a subset of samples ($n = 40$) by real-time PCR using 384-well panels with primers specific for the 179 miRNAs most abundant in plasma/serum (Exiqon) in a 7900HT real-time PCR system (Thermo Fisher Scientific). Results were analyzed with GenEx v6.1 (Exiqon) [17], normalizing by the global threshold cycle (Ct) mean of each sample after excluding miRNAs detected in less than 90% of the samples. Fold change was calculated using the mean ΔCt of the control group as calibrator by following the formula: $FC = 2E(\Delta Ct_{CT\_} \Delta Ct_{case})$ [17]. Results were validated by measuring the candidate miRNAs by real-time RT-PCR with specific SYBRGreen primers (Exiqon) in a second subgroup of $n = 30$ subjects from the cohort. Target genes of the miRNAs of interest were obtained in miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and the network was constructed with String (https://string-db.org/).

Statistical analyses

Data are presented as mean ± s.e.m. Differences between groups were determined by one-way ANOVA followed by two-tailed Student's *t* test considering a significance level

of $p < 0.05$. Serum parameters and miRNA abundance were compared by Spearman correlation analysis. Receiver operating characteristic (ROC) curves were plotted using STATA and the AUC (area under the curve) was calculated to determine discrimination accuracy.

Results

Evolution of the study population

A cohort of $n = 52$ control subjects and $n = 100$ individuals with prediabetes were followed from 2013 to 2017. None of the control subjects developed diabetes, and only 11.5% (6/52) developed any of the criteria for prediabetes (Fig. 1). Regarding the group with prediabetes, 54 participants were included based on only elevated glycemia ($n = 13$, 13%) or HbA1c ($n = 41$, 41%), whereas $n = 46$ fulfilled both diagnostic criteria at inclusion (46%). Sixteen participants with prediabetes developed T2D during the study (16%). Interestingly, 14 of those newly diagnosed patients had shown at inclusion both elevated glycemia and HbA1c, whereas just 2 emerged from participants showing only 1 of them (Fig. 1). Similarly, from the group fulfilling both criteria, only 4 participants displayed normalized glycemia and HbA1c values by the end of the study (8.7%), whereas this situation was observed in 20 amongst those presenting only one of the diagnostic criteria at inclusion (37%).

Hence, we selected a subgroup of $n = 70$ subjects from the initial cohort for miRNA analysis (Table 1). Controls that developed any of the criteria for prediabetes were discarded, and only prediabetic individuals fulfilling both inclusion criteria throughout the study were analyzed, as these subjects appeared to be a group at higher risk of developing diabetes, but still not all of them did (Fig. 1).

In addition, presence or absence of hepatic steatosis was inferred by the fatty liver index (FLI). About 38.5% (15/39) of control subjects displayed FLI ≥ 60, suggestive of hepatic steatosis, whereas 61.5% (24/39) showed FLI < 60. In the group of prediabetic subjects, the situation was inverted with 74% (23/31) displaying FLI ≥ 60. As hepatic steatosis is considered a condition that increases the risk of diabetes [14], we separated the group of normoglycemic individuals into two subgroups according to FLI.

Therefore, the following groups were established for analysis: (1) a control group (CT, $n = 24$) of normoglycemic subjects that throughout the study maintained FLI < 60 values; (2) a group of normoglycemic individuals with FLI ≥ 60 that also remained unaltered during the study (FL, $n = 15$); (3) a group of prediabetic subjects whose clinical status did not change for the duration of the study (PREnoDM, $n = 17$); (4) a group of prediabetic subjects that were diagnosed with

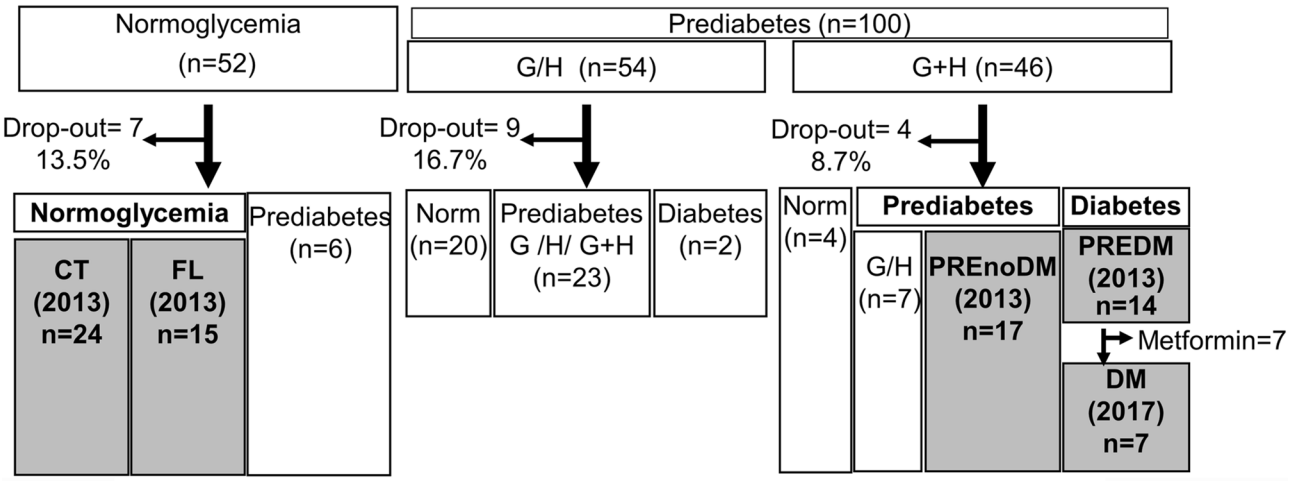

Fig. 1 Flowchart showing patient inclusion and evolution. Fifty-two normoglycemic subjects and 100 individuals with prediabetes were recruited in 2013. By 2017, six normoglycemic subjects had developed one or both criteria for prediabetes and were excluded from the analysis. The remaining normoglycemic subjects were distributed into two subgroups according to the absence or presence of hepatic steatosis as assessed by the fatty liver index (CT and FL groups, respectively). Prediabetic subjects presenting only one of the criteria for prediabetes in 2013 ($n = 54$) were excluded from the analysis. Prediabetic patients presenting both criteria in 2013 that lost one of them ($n = 7$) or reverted to normoglycemia ($n = 4$) in 2017 were also excluded. The remaining prediabetic subjects were distributed into a non-progressor group (PREnoDM group), and a progressor group (PREDM group) including those patients that were diagnosed with T2D after inclusion. Serum samples obtained in 2013 were used for vesicle isolation and miRNA analysis. A fifth group, constituted by the subjects in the PREDM group, but analyzed in 2017, when they had been diagnosed with T2D, was also included (DM group). Seven patients being treated with metformin were excluded from this group. Shaded boxes indicate the five study groups. *G* glycemia ≥ 100 mg/dL, *H* HbA1c ≥ 5.7%, *CT* control group, *FL* fatty liver group, PREnoDM non-progressor prediabetic group, *PREDM* progressor prediabetic group, *DM* diabetic group

Table 1 Clinical characteristics in 2013 and 2017 of the study population used for exosome analysis

	Normoglycemic ($n=39$)				Prediabetic ($n=31$)			
	CT ($n=24$)		FL ($n=15$)		PREnoDM ($n=17$)		PREDM ($n=14$)	
	2013	2017	2013	2017	2013	2017	2013	2017
Gender (male/female)	8/16	–	11/4	–	10/7	–	9/7	–
Age (years)	57 ± 2.4	–	61 ± 1.3	–	65 ± 1.5	–	61 ± 1.7	–
BMI (kg/m^2)	24.5 ± 0.6	24.6 ± 0.7	30.4 ± 0.9**	31.0 ± 1.0**	30.3 ± 1.5**	31.0 ± 1.4**	31.8 ± 1.1**	32.9 ± 1.1**
Cholesterol (mmol/l)	206 ± 7	212 ± 8	200 ± 7	203 ± 12	215 ± 9	214 ± 10	203 ± 6	206 ± 8
HDL (mmol/l)	62 ± 2.2	61 ± 2.7	52 ± 4.6	51 ± 4.5	55 ± 2.8	54 ± 2.5	59 ± 4.7	52 ± 3.5
LDL (mmol/l)	124 ± 7	128 ± 6	120 ± 8	125 ± 10	119 ± 10	126 ± 8	115 ± 6	126 ± 7†
TG (mmol/l)	99 ± 9	118 ± 11†	142 ± 16*	146 ± 26	207 ± 40**	173 ± 24	147 ± 30	163 ± 35
Glucose (mg/dl)	85 ± 1.4	84 ± 1.5	88 ± 1.5	89 ± 1.7*	109 ± 1.9**	110 ± 1.4**	110 ± 2.8**	136 ± 7.6** ††
HbA1c (%)	5.5 ± 0.05	5.3 ± 0.09††	5.4 ± 0.04	5.4 ± 0.06	6.0 ± 0.09**	6.0 ± 0.08**	6.0 ± 0.09**	6.8 ± 0.20**††
FLI	23 ± 3.7	32 ± 2.0††	75 ± 1.8**	73 ± 4.0**	70 ± 6.4**	78 ± 4.8**	71 ± 6.2**	82 ± 6.1**

CT control group, *FL* fatty liver group, *PREnoDM* non-progressor prediabetic group, *PREDM* progressor prediabetic group, *BMI* body mass index, *FLI* fatty liver index, *HDL* high-density lipoproteins, *LDL* low-density lipoproteins, *TG* triglycerides

*$p<0.05$; **$p<0.01$ with respect to CT on the same year after one-way ANOVA and post hoc pairwise *t* test

$^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$ with respect to the same group in 2013 in a paired *t* test

T2D at any time during the 4 years following inclusion (PREDM, $n=14$); and (5) a group of diabetic patients, constituted by the same patients in the PREDM group, but analyzed in 2017, when they had already been diagnosed with T2D (DM, $n=7$). Seven diabetic patients being treated with metformin in 2017 were not analyzed to avoid interference with the effects of the drug (Fig. 1).

Importantly, at inclusion (2013), both groups of prediabetic subjects were indistinguishable (Table 1). By 2017, the only significant differences were fasting plasma glucose and %HbA1c, as the subjects in the PREDM group had developed overt T2D. At inclusion, the FL group and the two groups of prediabetic subjects differed from the CT group in the FLI, and consequently in BMI and TG levels, both of which are used to calculate it. The PREnoDM and PREDM groups differed from the CT and FL groups in the levels of plasma glucose and HbA1c, as expected. Interestingly, by 2017, the FL group showed significantly increased fasting glucose as compared with the CT group, although still inside normal range, thus suggesting that they may constitute a group at risk of developing prediabetes (Table 1). Accordingly, high FLI values in subjects with prediabetes have been described as predictors of diabetes incidence [14] and diabetes risk increases significantly in individuals with fasting plasma glucose levels at the higher end of normal range [18].

Microvesicle characterization and miRNA profiling

Microvesicles were isolated from serum samples obtained in 2013 and 2017 for the $n=70$ selected individuals. For the initial screening, we selected 40 samples distributed as follows: 8 each for the CT, FL and PREnoDM groups, nine for the PREDM group, and 7 for the DM group (Fig. 1). The isolated vesicles were characterized by nanotracking particle analysis, showing a mean diameter of 120 nm (Fig. 2a). Their number was estimated by measuring the acetylcholinesterase activity known to be associated with the exosomes (Fig. 2b). Significant differences were not observed, although the PREDM and DM group showed higher dispersion and a tendency to be increased ($p=0.07$).

We then measured the 179 miRNAs most abundant in human serum/plasma by real-time RT-PCR [19]. The mean number of miRNAs detected per sample was 119 ± 2. Of the 179 miRNAs measured, 82 (46%) were detected in 90% of the samples and constituted the set that was analyzed. One-way ANOVA revealed eight miRNAs showing differences between groups (Table 2). Interestingly, the two groups of individuals with prediabetes were clearly different, with the PREDM group showing more overlap with the DM group than the non-progressor PREnoDM group (Fig. 2c). These data suggest that those individuals with prediabetes that are primed to progress to T2D display a miRNA profile in microvesicles more akin to that of diabetic patients than to other prediabetic subjects.

Identification of candidate miRNA biomarkers

We focused on *miR-10b* and *miR-223-3p*, two miRNAs highly regulated in the PREDM group as compared with the CT group, that were also significantly different between both groups of prediabetic subjects (Table 2). Heatmap representation of *miR-10b* and *miR-223-3p* demonstrates a

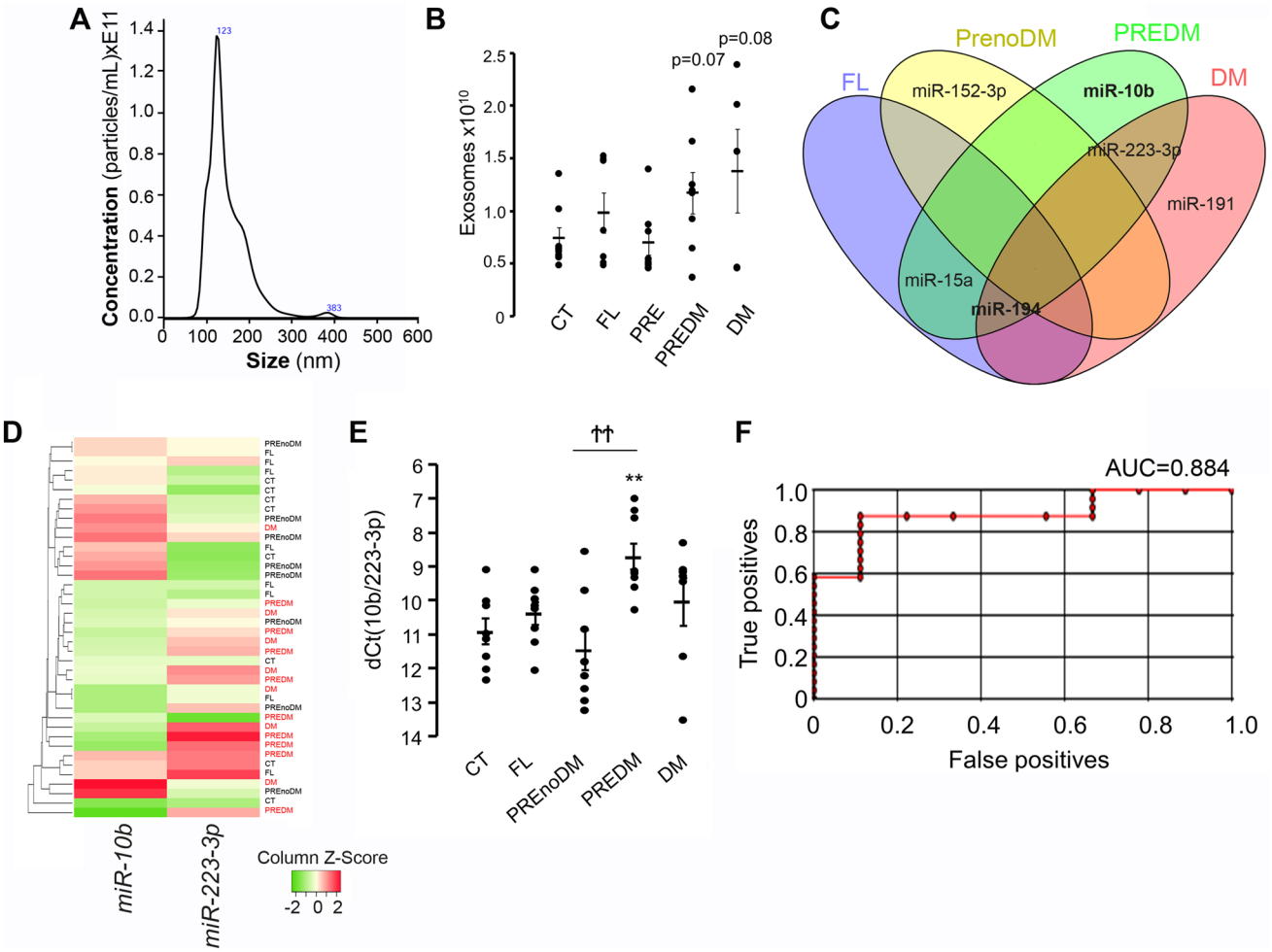

Fig. 2 Exosomal miRNA characterization and identification of candidate biomarkers. **a** Purity of microvesicle preparations was determined by nanotracking particle analysis. A representative plot is shown. **b** Microvesicle number was estimated by ELISA. **c** Venn diagram showing miRNAs down-(regular face) and up-regulated (boldface) as compared to the CT group and common to the FL, PREnoDM, PREDM and DM groups. **d** Heatmap showing the relative values of the candidate miRNAs across the samples and how they allow clustering of the groups. **e** Values of dCt(miR-10b/miR-223-3p). **f** ROC curve showing the specificity and sensitivity of dCt(miR10b/miR223-3p) to identify adherence to group PREDM. $^{**}p<0.005$ with respect to the CT group after one-way ANOVA and post hoc pairwise *t* test. $^{\dagger\dagger}p<0.005$ with respect to the PREnoDM group. $n=8$ (CT, FL and PREnoDM groups), $n=9$ (PREDM), $n=7$ (DM). Where displaying ΔCt values, the axes have been inverted to indicate that lower ΔCts represent increased miRNA abundance

good segregation of the PREDM and DM groups from the other experimental groups (Fig. 2d). We then calculated the relationship between both miRNAs in each sample using the formula $dCt(10b/223\text{-}3p) = Ct_{miR10b} - Ct_{miR223\text{-}3p}$. This parameter was significantly different in the PREDM group as compared to the CT and PREnoDM groups (Fig. 2e). ROC analysis evidenced that dCt(10b/223-3p) identified adherence to the PREDM group with AUC = 0.884 (Fig. 2f), thus suggesting that these two miRNAs may act as biomarkers for diabetes progression.

Interestingly, we found that the values of dCt(10b/223-3p) in the samples obtained in 2013 were inversely associated with the glycemia and HbA1c determined in 2016, but no correlation was observed with the same values in 2013, the time at which the miRNAs were analyzed (Table 3). These results suggest a predictive association of dCt(10b/223-3p) in 2013 with the future values of glycemia and HbA1c.

Follow-up analysis

We analyzed the expression of the two candidate miRNAs in the remaining samples of the cohort ($n=30$) and observed that dCt(10b/223-3p) was again able to discriminate between the PREDM and PREnoDM groups with AUC = 0.807 (Fig. 3a, b). Importantly, this value is an improvement above that obtained when using fasting plasma glucose levels in 2013 to predict progression of diabetes in the next 4 years, for which we obtain an AUC = 0.677 (Fig. 3c).

Table 2 Fold change of the miRNAs altered in prediabetes and T2D

MiRNA	CT/FL		CT/PREnoDM		CT/PREDM		CT/DM		FL/PREnoDM		FL/PREDM		FL/DM		PREnoDM/PREDM		PRE/DM	
	FC	*p*	FC	*p*	FC	*p*	FC	*p*	FC	*p*	FC	*p*	FC	*p*	FC	*p*	FC	*p*
miR-10b	−1.2	0.562	1.7	0.198	−2.2	**0.041**	−1.1	0.852	2.1	0.066	−1.8	0.071	1.1	0.796	−3.8	**0.003**	−1.9	0.249
miR-194	−2.3	**0.012**	1.3	0.582	−2.7	**0.001**	−2.3	**0.013**	1.9	**0.013**	−1.2	0.523	1.0	>0.9	−2.2	**0.000**	−1.9	**0.011**
miR-93	−1.1	0.296	−1.2	0.092	1.1	0.357	1.4	0.074	−1.1	0.652	1.3	0.116	1.6	**0.026**	1.3	**0.048**	1.7	**0.012**
miR-132-3p	4.7	0.054	1.3	0.492	1.6	0.166	1.2	0.530	−5.9	**0.034**	−3.0	0.151	−3.9	0.119	2.0	0.076	1.5	0.249
miR-191	−1.2	0.303	−1.2	0.205	1.1	0.598	−1.5	**0.004**	1.0	0.946	1.2	0.156	−1.3	0.138	1.2	0.055	−1.3	**0.045**
miR-152-3p	1.6	0.082	3.5	**0.047**	1.1	0.723	1.2	0.497	2.1	0.203	−1.5	0.147	−1.3	0.262	−3.2	0.050	−2.8	0.104
miR-223-3p	1.2	0.441	1.2	0.405	1.8	**0.028**	1.7	**0.018**	−1.0	0.990	1.7	**0.007**	1.4	0.083	1.8	**0.003**	1.4	0.050
miR-15a	1.8	**0.015**	1.3	0.295	2.0	**0.001**	1.6	0.056	−1.4	0.250	1.1	0.630	−1.1	0.714	1.5	0.081	1.3	0.301

Log2 fold change and *t* test *p* value of miRNAs significantly modified when normalizing by the global mean Ct, using as calibrator the CT group
CT control group, *FL* fatty liver group, *PREnoDM* non-progressor prediabetic group, *PREDM* progressor prediabetic group, *FC* log2 fold change

Table 3 Correlational analysis

Variables	Spearman *r*	*n*	*p* value
FLI 2013	−0.24	38	0.141
Glucose 2013	−0.24	40	0.140
HbA1c 2013	−0.20	40	0.209
BMI 2013	−0.15	40	0.361
FLI 2016	−0.16	38	0.338
Glucose 2016	−0.37	40	**0.017**
HbA1c 2016	−0.35	40	**0.025**
BMI 2016	−0.19	40	0.229

Correlations between dCt(miR10b/miR-223-3p) values in 2013 and clinical values in 2016

Finally, we obtained a list of candidate target genes for *miR-10b* and *miR-223-3p* and analyzed them to identify enriched pathways. Interestingly, the targets were involved in insulin-regulated pathways, including phosphatidyl-inositol 3'kinase (PI3 K) and transcription factor FoxO1 signaling (Fig. 3d).

Discussion

Early-stage T2D and its precursor-state prediabetes are mostly silent clinical conditions that may go undiagnosed for years. However, exposure to hyperglycemia may induce

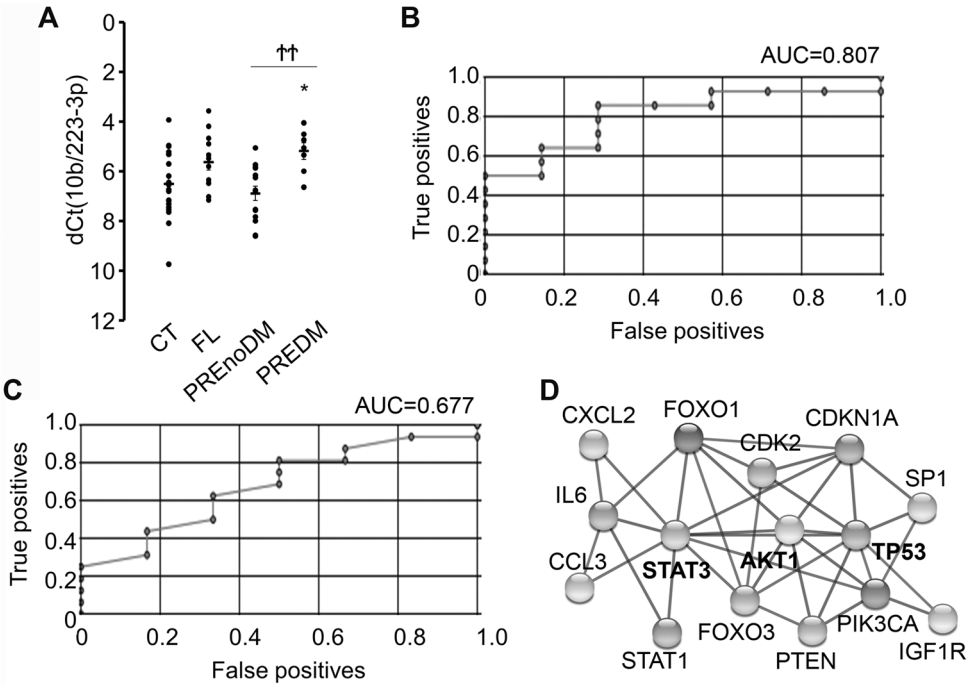

Fig. 3 Follow-up and predictive potential. **a** Values of dCt(10b/223-3p). **b** ROC analysis of dCt(10b/223-3p). **c** ROC analysis of glycaemia as a parameter to discern development of diabetes in the next 4 years. **d** Network analysis of the targets of *miR-10b* and *miR-223-3p*. *$p < 0.05$ with respect to the CT group after one-way ANOVA and post hoc pairwise *t* test. ††$p < 0.005$ with respect to the PREnoDM group. $n = 21$ (CT), $n = 12$ (FL), $n = 15$ (PREnoDM) and $n = 7$ (PREDM). Where displaying ΔCt values, the axes have been inverted to indicate that lower ΔCts represent increased miRNA abundance

epigenetic alterations leading to development of complications [5]. Clinical trials have demonstrated that early interventions can delay progression of the disease, in some cases averting its deleterious consequences [20, 21], an effect that has been shown to linger even decades after ending the intervention [20]. But not all subjects with prediabetes display the same risk of progressing to T2D. Our own previously published data indicates an incidence rate of about 4.1/100 person-years in the prediabetic population [15], a result in agreement with the one in the present study, where we find an incidence of 4/100 person-years. Being able to identify in advance this high-risk group of subjects amongst the general prediabetic population would be of great interest.

Here, by analyzing a subset of extracellular miRNAs, those enclosed inside microvesicles, we identify two miRNAs whose combination results in a parameter able to differentiate between progressor and non-progressor prediabetic subjects.

Changes in both *miR-10b* and *miR-223-3p* have been previously shown to be associated with diabetes [22, 23]. The miR-10 family of miRNAs are involved cell differentiation and are frequently dysregulated in cancer [24]. Decreased *miR-10b* expression has been described in insulin-resistant liver [25] and muscle [26] in rodents, whereas its abundance has been shown to be increased in plasma in association with diabetes [22, 23]. On the other hand, decreased plasma levels of *miR-223-3p* have been consistently associated with incidence and progression of diabetes [23], and obesity [27]. *miR-223-3p* has the features of a myeloid gene and is highly expressed during neutrophil and macrophage differentiation [28]. Consequently, *miR-223-3p* participates in the modulation of inflammation and displays promise as a biomarker or therapeutic target for inflammatory diseases [29]. This is an interesting point, as T2D is frequently considered an inflammatory disease. Their known targets include genes involved in insulin function such as transcription factor FoxO1 [29]. Actually, network analysis of the target genes of *miR-10b-5p* and *miR-223-3p* results in nodes centered around kinase AKT1, a crucial regulator of insulin and leptin function [30], the second messenger STAT3 (signal transducer and activator of transcription 3), involved in the control of inflammation [31], and transcription factor TP53, which aside from its well-known role as a tumor suppressor has recently been shown to participate in the regulation of metabolism and, in particular, the progression of diabetes [32].

Our study presents some limitations, the most relevant being the modest sample size. However, the fact that we were able to reproduce our results in a second group gives consistency to our data. It is also important to consider that the analysis of extracellular miRNAs poses numerous technical challenges, chief amongst them the standardization of the methodology for their isolation and quantification. Real-time RT-PCR is a quick and cost-effective method well suited for clinical practice, but has the inconvenient of dealing with relative rather than absolute values [33]. Here, using the ratio between an up-regulated and a down-regulated miRNA, we bypass the problem of identifying invariable controls to effect normalization. The source of material is also of importance, and plasma is considered the preferred choice, as platelet activation during coagulation results in release of microvesicles that contribute to increase the noise [34]. However, the global miRNA profiles of plasma and serum have been shown to be comparable [35].

On the other hand, one of the main strengths of our study is the availability of a cohort of patients that have been monitored during 4 years after inclusion. By attending to their evolution, we have been able to select from a large cohort those groups of patients presenting a more robust phenotype, hence compensating for the relatively small number of samples analyzed.

In summary, we provide evidence for the existence of a microvesicle miRNA signature for prediabetes, which could be used as a new screening tool for patients at risk of developing diabetes, at a stage at which other features, including glycemia, are less adept at separating them.

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Author contributions MP and AN designed the study; XM, XC, SC, LB, CGG, ER, MMC and JFN performed data collection and clinical analysis; CC and MP performed exosome isolation and RNA analysis; SC, MP and CC performed statistical analyses; MP wrote the first draft of the manuscript; all authors provided critical revisions of the manuscript.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest relevant to this article were disclosed.

Ethical approval The study was approved by the Ethical Committees of Parc de Salut Mar Clinical of Barcelona (Register 2011/4274/I) and the Hospital Clinic, University of Barcelona (Register 2011/6945).

Informed consent Written informed consent was obtained from all the participants included in this study, in accordance with the principles of the Declaration of Helsinki.

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