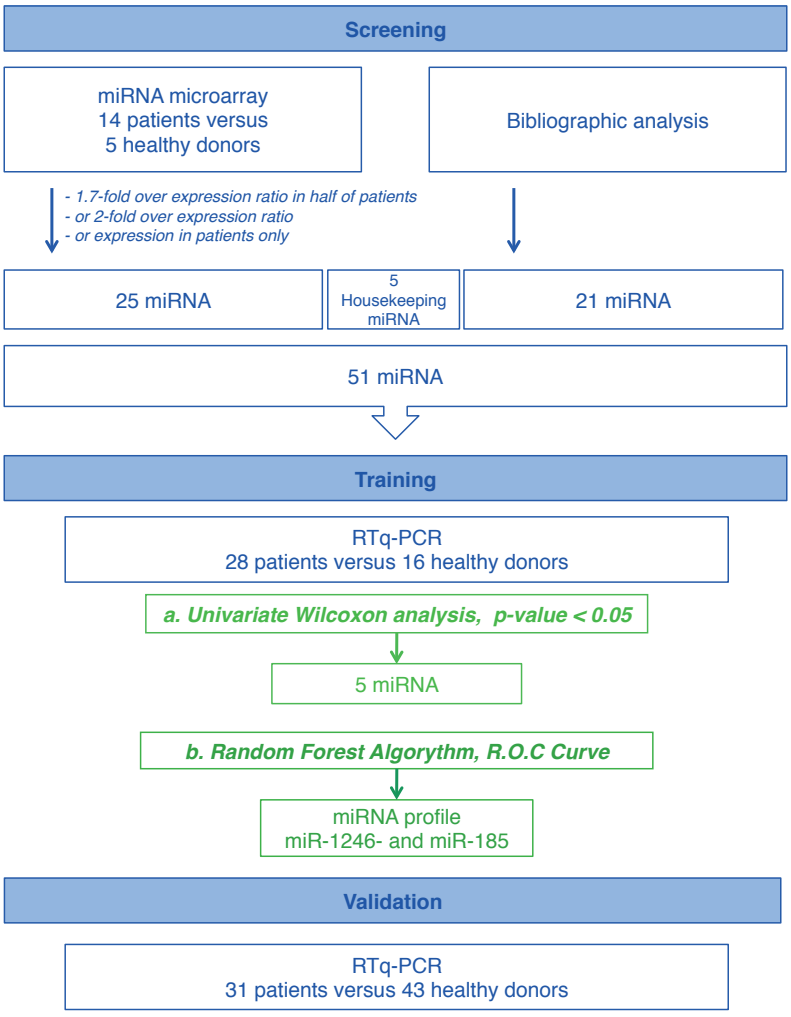

Fig. S1. Study design: a 3-step single-centre case-control study was conducted.

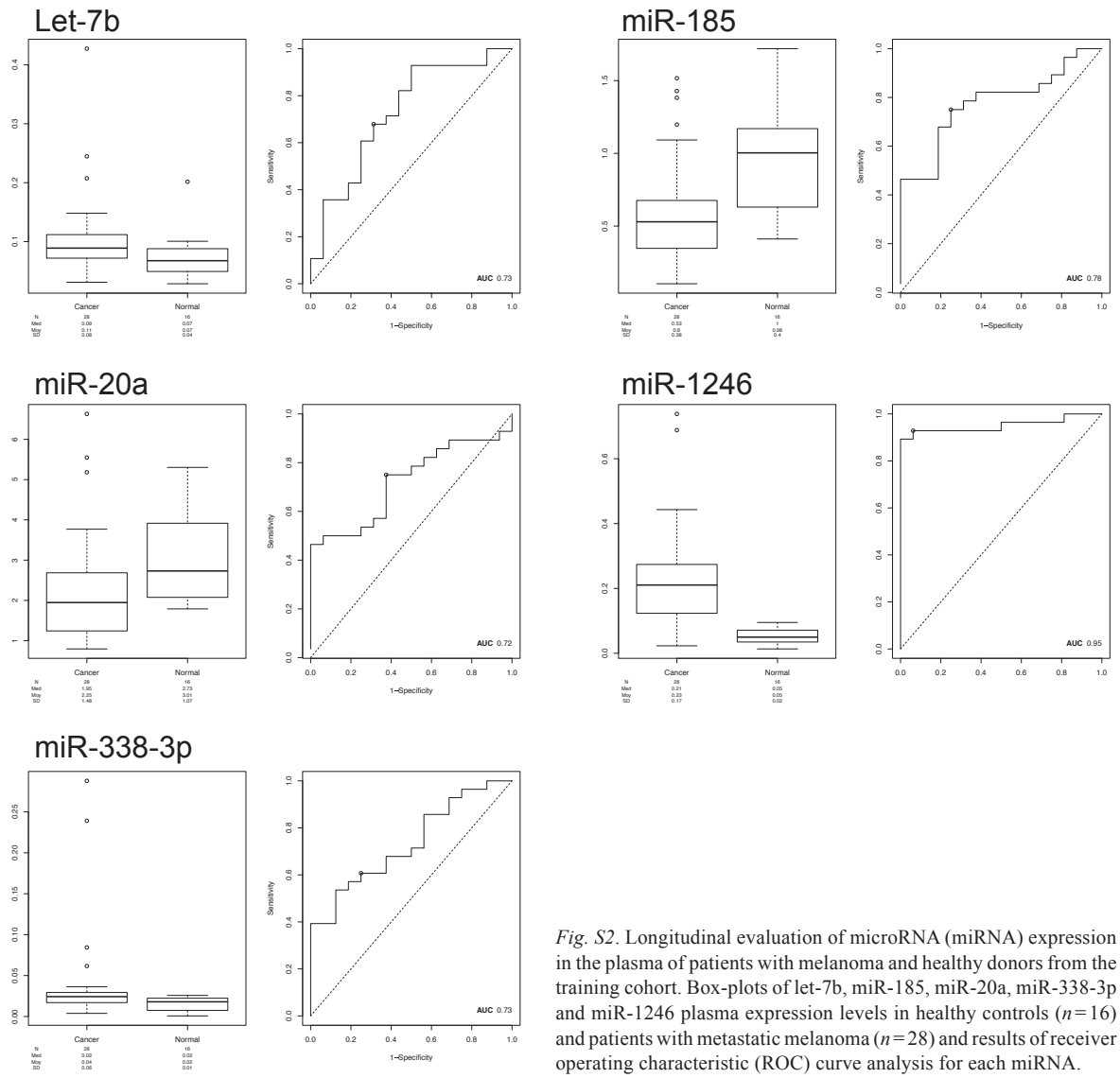

Fig. S2. Longitudinal evaluation of microRNA (miRNA) expression in the plasma of patients with melanoma and healthy donors from the training cohort. Box-plots of let-7b, miR-185, miR-20a, miR-338-3p and miR-1246 plasma expression levels in healthy controls ($n = 16$) and patients with metastatic melanoma ($n = 28$) and results of receiver operating characteristic (ROC) curve analysis for each miRNA.

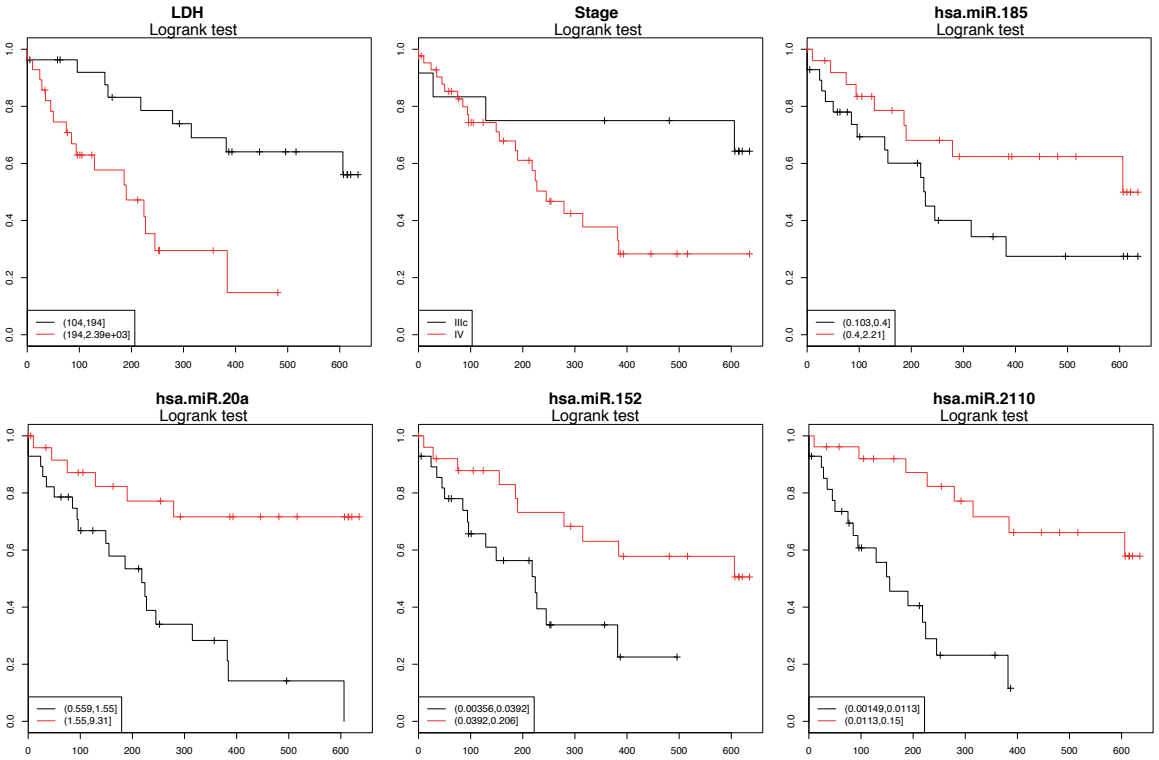

Fig. S3. Prognostic value of lactate dehydrogenase (LDH), age and plasma microRNAs (miRNAs). Kaplan–Meier analysis and log-rank test were carried out.

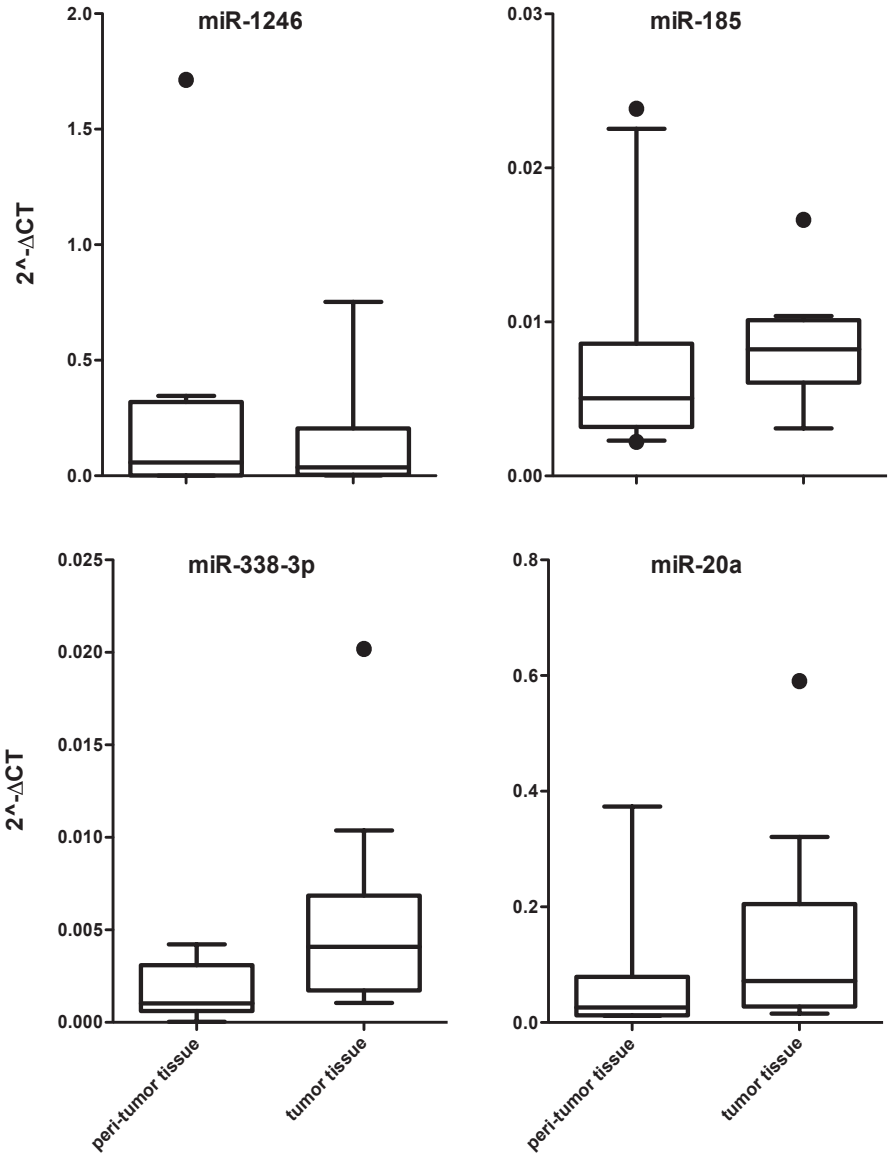

Fig. S4. Tissue expression of miR-1246, miR-338-3p, miR-185 and miR-20a. Manual macrodissection was performed on tumour and peritumoural areas on 10-µm thick sections from formalin-fixed paraffin-embedded (FFPE) biopsies of metastatic melanomas from 10 patients. miRNA levels were analysed by RTqPCR and expressed as $2^{-\Delta Cq}$ after normalization by small RNA RNU6.

	Patients Cohort 2 p-value	Patients Cohort 2 ρ de Spearman	Healthy donors Cohort 2 p-value	Healthy donors Cohort 2 ρ de Spearman	Patients Cohort 1 p-value	Patients Cohort 1 ρ de Spearman
hsa.let.7b	0.896	-0.025	0.742	-0.052	0.17	-0.267
hsa.miR.21	0.199	0.245	0.109	0.248	0.346	0.185
hsa.miR.152	0.591	-0.104	0.246	0.181	0.917	-0.021
hsa.miR.338.3p	0.089	0.322	0.255	0.177	0.743	-0.065
hsa.miR.1246	0.114	0.3	0.087	0.264	0.619	-0.098
hsa.miR.2110	0.553	0.115	0.171	0.213	0.283	0.21
Taux_LDH	0.607	-0.1	0.004	0.427	0.169	0.267

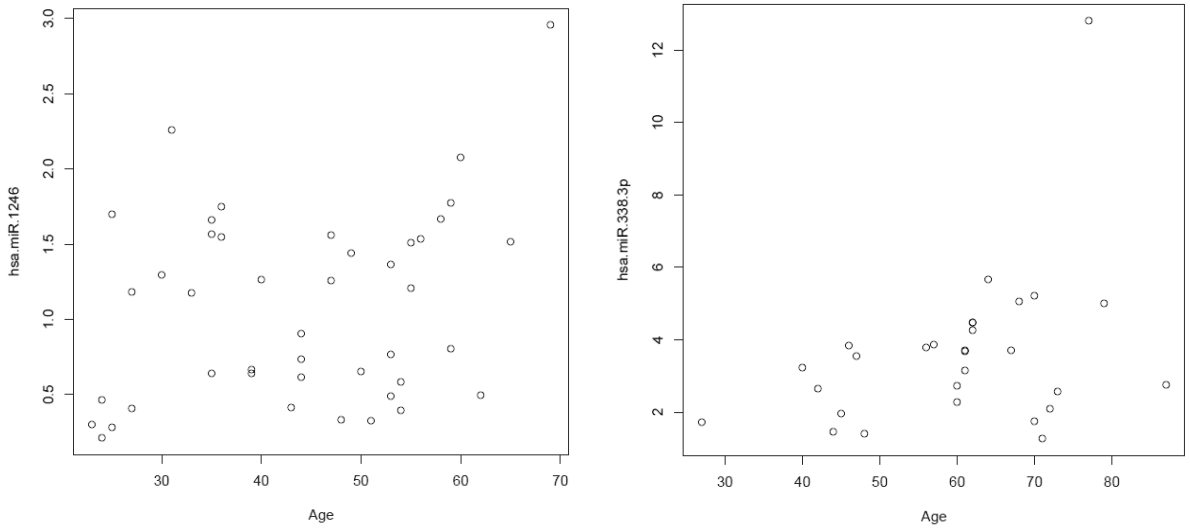

Fig. S5. Analysis of the impact of age on microRNA (miRNA) expression in the different cohorts of healthy donors and patients. miRNA levels (expressed as $2^{-\Delta Cq}$) for several miRNAs from the different cohorts were plotted as a function of the age of donors. Spearman's correlation coefficients for several miRNAs were calculated. Representative examples of plots were added.

Appendix S1

SUPPLEMENTARY MATERIAL AND METHODS

Lactate dehydrogenase assays

LDH activity was measured in the same blood samples as those used for miRNA measurements, using the enzymatic method on a Dimension RxL Max bioanalyser (Siemens, Saint-Denis, France).

Plasma microRNA (miRNA) extraction

Preliminary experiments were performed to compare plasma and serum miRNA extraction rates and stability. As shown previously (13), the rate of miRNA extraction from plasma was superior to that from serum. In addition, miRNA levels remained constant when plasma was subjected to prolonged storage (> 8 h) at room temperature. Plasma was obtained by standard venipuncture and centrifugation in ethylene-diaminetetraacetic acid (EDTA)-coated tubes. Separation of plasma was performed within 2 h after venipuncture, by centrifugation at 2,400 g for 10 min at ambient temperature. Samples were stored at –80°C.

For microarray analysis and reverse transcription quantitative PCR (RTqPCR) analysis, RNA extraction was carried out using 10 ml and 200 µl of plasma, respectively, with a miRNeasy extraction kit (Qiagen) according to the manufacturer's instructions, followed by elution in 12 µl and 30 µl of water, respectively.

As a control for RTqPCR analysis, 2 µl of miSPIKE solution (150 nM) (Integrated DNA Technologies), a synthetic oligonucleotide corresponding to a sequence that does not exist in the human genome, was added to the plasma samples before RNA isolation, and measured for each sample.

microRNA array analysis

Total RNA (3 µg) was ligated to a RNA-linker-Cy3-dye using T4 RNA ligase (Ambion) with overnight incubation at 4°C, followed by ethanol precipitation. Subsequently, overnight hybridization of labelled RNA was performed at 54°C with LNA-modified microarray slides (Exiqon, version 7) containing duplicate copies of 576 miRNAs of human origin. After extensive washing, slides were scanned using an Axon 4000B scanner in which the photomultiplier (PMT) settings were adjusted automatically. Microarray images were analysed using the GenePix Pro 6.0 software (Axon Instruments). GenePix Results (GPR) were processed using BioPlot software (http://biopuce.insa-toulouse.fr/; available upon administrator agreement). All flagged spots, or background-subtracted spot intensities with values below 0, were removed from the analysis. The signal values for each spot were then normalized, using the total mean signal value obtained from all spots of the array as 100%.

miRNA expression was considered as significant when fold changes in the normalized means of the miRNA expression between the 14 patients and the 5 controls were ≥ 1.5 or ≤ 0.666 and the p-value was ≤ 0.05 according to the Student's t-test (Table SI[1]). In addition, the validity of the results was tested using the Kolmogorov–Smirnov test, and the statistical distance between candidate biomarkers was calculated.

miRNA expression analysis using the 96.96 dynamic array

Reverse transcription (RT) of total RNAs (4 µl) was performed using the miRCURY Universal cDNA synthesis kit (Exiqon) in a 20 µl reaction volume according to the manufacturer's instructions. Specific target preamplification (STA) of cDNA was performed on 1.25 µl of 1:10 RT product using Master Mix TaqMan® PreAmp (Applied Biosystems) and the pool of miRCURY LNA™ primers (Exiqon) with the following protocol: 1 cycle at 95°C for 10 min, 15 cycles at 95°C for 15 s, and then incubation at 60°C for 4 min. qPCR was carried out using the 96.96 Dynamic Array integrated fluidic circuits (Fluidigm Corporation, CA, USA) following the manufacturer's protocol. Briefly, 1.25 µl of 1:10 STA product was loaded for each sample. After loading STA product, runs were carried out in a BioMark® Instrument for PCR using the TaqMan® Gene Expression Master Mix and miRCURY LNA™ primers with the following protocol: 95°C for 10 min, followed by 30 cycles at 95°C for 10 s and incubation at 60°C for 1 min. Each sample was analysed twice. Data were analysed with Real-Time PCR Analysis Software from the BIOMARK instrument (Fluidigm Corp., CA, USA). Only those miRNA assays with an amplification efficiency between 1.7 and 2, a Cq <29 and specific amplification were retained. The $2^{-\Delta Cq}$ method was used to analyse the relative changes in miRNA expression.

Tissue microRNA extraction

RNA was eluted from the spin column membrane with the ultrapure water supplied in the kit. This ultrapure water was used for all subsequent dilutions of RNA samples. RNA yield and purity were evaluated with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific) via absorbance measurements at 260 and 280 nm. The high degree of purity of the isolated RNA was confirmed by ensuring that the A260/A280 ratio was in the range 1.96–2.04 for all samples. Isolated total RNA samples were stored at –80°C before use.

Tissue miRNA expression analysis by RTqPCR using the CFX96 PCR System

RT of 300 ng of total RNA was carried out using the miScript II RT kit (Qiagen) following the manufacturer's instructions with HiSpec buffer. qPCR was carried out using the CFX96 PCR system (Bio-Rad) in a 96-well plate format. Reactions were carried out using 0.5 µl of RT product with the miScript primer assay (Qiagen) and the miScript SYBR Green PCR kit (Qiagen). PCR was performed as follows: 95°C for 15 min, followed by 40 cycles at 94°C for 15 s, 30 s at 55°C and 30 s at 70°C. The data were analysed with Bio-Rad CFX Manager software. The $2^{-\Delta Cq}$ method was used to analyse miRNA expression.

Statistical analysis

Data normalization. Data were analysed with the "R" software and the SlqPCR package (14). As a first step, the miRNAs were ranked according to their stability measure, M. The M values were assessed for all the miRNAs from both cohorts and the miRNA with the highest M value was excluded (selectHKgenes function). M values were then calculated for the remaining miRNAs. This procedure was repeated until 3 reference miRNAs were selected. Data were then normalized by geometric averaging of the 3 reference miRNAs using the normPCR function.

When the validation cohort was evaluated, 7 individuals from the training cohort (Fig. 1) were studied again; thus 2 sets of measurements were available for these individuals. As we expected to obtain the same results from both sets of measurements, the difference between the 2 sets was seen as an estimate of the drift resulting from uncontrolled parameters. This estimate was used as a correction value and applied to the measurements of the validation cohort. For all miRNAs, the difference between the mean for the validation cohort and that for the training cohort was calculated. This value was then subtracted from that for the miRNAs of the validation cohort.

Thus, the mean of the values obtained for the repeated individuals was the same for the 2 cohorts.

Data analysis. Bivariate Wilcoxon tests were used to test differences between populations. Kaplan–Meier curves, which estimate the survival rates according to time, were used for univariate survival analysis. Kaplan–Meier curves require a categorical classification of patients, so when the data were continuous they were split around the median. The log-rank test was used to determine whether the survival rates in the groups were different. For multivariate survival analysis, a Cox proportional hazard model was used. For all the methods, a test was considered significant if the *p*-value was < 5%. The tests were carried out for description or variable selection purposes, therefore no multiplicity correction was carried out; to eliminate the false discovery risk, a validation phase was performed. The random forest algorithm (R package random Forest, (15)), which operates by constructing several decision trees, was used as the classification method for analysing the data and for generating the rules of assignment. The random forest algorithm was also used to rank the miRNAs with the Gini accuracy index, using variable importance (Vi). The most relevant number of variables to use in the model, namely k, was determined using a nested cross-validation procedure. The k variables with the highest Vi were selected.

Table SI. *MicroRNAs (miRNAs) differentially expressed between patients and healthy donors identified by microarray analysis*

Gene	Patient/control ratio	*p*-value	*n*[a]
hsa-miR-494	0.41	0.01446	
hsa-miR-933	0.41	0.00968	
hsa-miR-1183	0.41	0.00153	
hsa-miR-486-5p	0.47	0.02653	
hsa-let-7e-3p	0.47	0.00040	
hsa-miR-888-3p	0.51	0.00136	
hsa-miR-938	0.53	0.02274	
hsa-miR-153	0.53	0.02089	
hsa-miR-1280	0.54	0.03309	
hsa-miR-29c-5p	0.54	0.02209	
hsa-miR-299-3p	0.61	0.03374	
hsa-miR-766	0.66	0.04199	
hsa-miR-374b-3p	1.53	0.02755	
hsa-miR-219-1-3p	1.54	0.03280	
hsa-miR-423-3p	1.55	0.04134	
hsa-miR-612	1.58	0.04475	
hsa-miR-337-5p	1.61	0.01124	
hsa-miR-130b-5p	1.62	0.03502	
hsa-miR-365	1.62	0.02264	
hsa-miR-99b	1.63	0.02155	
hsa-miR-508-5p	1.66	0.04261	
hsa-miR-433	1.67	0.02118	
hsa-miR-515-5p	1.67	0.01829	
hsa-miR-513b	1.68	0.02351	
hsa-miR-143	1.70	0.00213	8/14
hsa-miR-652	1.73	0.04461	5/14
hsa-miR-139-5p	1.74	0.01178	6/14
hsa-miR-1307	1.74	0.03059	6/14
hsa-miR-148b	1.74	0.00830	6/14
hsa-miR-130a	1.82	0.00344	7/14
hsa-miR-425-3p	1.83	0.04881	10/14
hsa-miR-371-5p	1.85	0.03032	4/14
hsa-miR-338-3p	1.90	0.00124	10/14
hsa-let-7f	1.96	0.03632	5/14
hsa-miR-33a	2.08	0.03246	5/14
hsa-miR-21	2.33	0.04109	10/14
hsa-miR-191	2.51	0.03046	7/14
hsa-miR-1246	3.42	0.00542	11/14
hsa-miR-1290	4.35	0.00362	10/14

[a]Number of patients with a ratio of each miRNA patient value on the median value from the 5 healthy donors ≥ 1.7.

Table SII. *MicroRNAs (miRNAs) detected exclusively in patients by microarray analysis*

Gene	Spot replicate, *n*	Positive patients, *n*
hsa-miR-27b	17	10
hsa-miR-146a	13	9
hsa-miR-302d	13	9
hsa-miR-152	12	7
hsa-miR-92a-1-5p	12	8
hsa-miR-95	12	8
hsa-miR-599	10	7
hsa-miR-520e	9	7
hsa-miR-569	9	7
hsa-miR-597	8	6
hsa-miR-1267	7	5
hsa-miR-1292	7	5
hsa-miR-193a-3p	7	6
hsa-miR-21-3p	7	7
hsa-miR-373	7	6
hsa-miR-517c	7	5
hsa-miR-668	7	5
hsa-miR-876-5p	7	6
hsa-miR-2110	7	4
hsa-miR-19b-2-5p	6	6
hsa-miR-29b-2-5p	6	5
hsa-miR-380-5p	6	5
hsa-miR-485-5p	6	5
hsa-miR-1201	5	4
hsa-miR-1248	5	5
hsa-miR-1537	5	4
hsa-miR-33b-3p	5	5
hsa-miR-491-5p	5	4
hsa-miR-518d-3p	5	4
hsa-miR-539	5	4
hsa-miR-572	5	5
hsa-miR-660	5	5
hsa-miR-664-5p	5	5

Table SIII. *List of MicroRNAs (miRNAs) evaluted in the training and validation cohorts*

	Gene	Selection criteria		Gene	Selection criteria	Reference
☒	miR-1246	Patient > Control		Let-7a	Bibliography	(6, 39)
☒	miR-1290	Patient > Control	☒	Let-7b	Bibliography	(6, 39)
☒	miR-130a	Patient > Control		Let-7e	Bibliography	(6, 39)
☒	miR-143	Patient > Control		miR-125b	Bibliography	(21)
☒	miR-191	Patient > Control		miR-137	Bibliography	(40)
☒	miR-21	Patient > Control	☒	miR-141	Bibliography	(21)
☒	miR-338-3p	Patient > Control	☒	miR-145	Bibliography	(23)
	miR-33a	Patient > Control	☒	miR-155	Bibliography	(22)
	miR-425-3p	Patient > Control	☒	miR-17	Bibliography	(21–24)
	miR-1292	Patient only		miR-182	Bibliography	(41)
☒	miR-146a	Patient only	☒	miR-185	Bibliography	(6, 21, 24)
☒	miR-152	Patient only	☒	miR-18a	Bibliography	(21–22)
	miR-1537	Patient only	☒	miR-200c	Bibliography	(21)
☒	miR-2110	Patient only		miR-204	Bibliography	(21, 24)
	miR-27b	Patient only		miR-205	Bibliography	(21)
	miR-302d	Patient only	☒	miR-20a	Bibliography	(22, 23)
	miR-517c	Patient only	☒	miR-211	Bibliography	(6, 21)
	miR-518d	Patient only		miR-214	Bibliography	(21, 23, 40)
	miR-520e	Patient only	☒	miR-221	Bibliography	(6)
	miR-569	Patient only	☒	miR-222	Bibliography	(6)
	miR-597	Patient only	☒	miR-34a	Bibliography	(24)
	miR-599	Patient only	☒	miR-103	Housekeeping (Exiqon)	
	miR-668	Patient only		miR-16	Housekeeping (Bibliography)	
	miR-92a-1-5p	Patient only	☒	miR-423-3p	Housekeeping (Exiqon)	
☒	miR-95	Patient only		miR-513a-3p	Housekeeping (Microarray)	
☒	miSpike	Extraction control		miR-615-3p	Housekeeping (Microarray)	

☒ miRNA that met the analysis criteria, as defined in the Materials and Methods section, following RTqPCR analysis on the training cohort, and which were retained for the validation cohort analysis.