A methodological procedure for evaluating the impact of hemolysis on circulating microRNAs

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Abstract. Circulating microRNAs (miRNAs) are promising non-invasive biomarkers whose expression may be affected by confounding factors, including hemolysis, that should be considered in studies of miRNA discovery. The present study proposes a methodology for evaluating the impact of hemolysis on the expression of miRNAs. An experiment of *in vitro* controlled hemolysis was designed for assessing if changes in the expression of eight miRNAs observed to be circulating in plasma may be associated with hemolysis, and also to estimate the level of red blood cell (RBC) contamination in plasma samples where the expression of these miRNAs will be measured. It was confirmed that four miRNAs, miR-16, miR-92a, miR-451 and miR-486, known to be present in blood cells, were influenced by contamination of RBCs. Furthermore, it was demonstrated that miR-378 and miR-30c are hemolysis-independent and that the expression of miR-320 and miR-324-3p was associated with the level of RBC contamination. This procedure is proposed as a tool for the evaluation of the influence of hemolysis on candidate circulating miRNA biomarkers prior to their analysis in plasma samples.

Introduction

Recent studies have revealed that microRNAs (miRNAs) released by cancer cells and circulating in the plasma are promising candidates for non-invasive cancer detection (1-3). However, the evidence that blood also contains miRNAs resulting from the lysis of hematopoietic cells (hemolysis), and whose levels correlate with blood cell counts (4,5), makes their identification complex. Certain cellular miRNAs have been proposed to be tumour-associated circulating biomarkers (6); however, differences in their levels may reflect blood cell-based phenomena rather than the presence of cancer.

Hemolysis is therefore an important factor that should be considered in studies of miRNA. Various hemolysis indices based on absorbance measurements have previously been suggested (3,5,7,8) to identify hemolysed samples that can be discarded from studies. However, their removal limits the search for circulating biomarkers and prohibits the development of a tool for the detection of circulating miRNAs that may be used in clinical practice. An alternative approach to sample removal would be to test the potential miRNA biomarkers for their sensitivity to hemolysis and to proceed only with miRNAs that are not influenced by hemolysis. The present study proposes a procedure that integrates the quantification of miRNAs within the levels of an ad-hoc designed hemolysis calibration curve and directly evaluates the influence of hemolysis on their expression. In addition, the present study used the same calibration curve to estimate the percentage of red blood cells (RBCs) in plasma samples and therefore evaluate how the expression in RBCs of the potential miRNA biomarkers may be critical in the series of samples in analysis. The present study describes the procedure by applying it to data obtained from plasma samples collected for studies conducted at the Scientific Institutes for Research and Treatment Foundation 'National Cancer Institute' (Milan, Italy).

Materials and methods

Patient cohort. The series of plasma samples used to estimate the unknown percentage of RBCs was collected from 110 individuals (57% female; 43% male) who tested positive on a fecal immunochemical test and underwent colonoscopy at the Scientific Institutes for Research and Treatment Foundation

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'National Cancer Institute' (Milan, Italy). All patients provided written informed consent to donate their blood for research purposes. Plasma samples were collected between February 2013 and April 2014. Patient age at blood collection was between 50 and 70 years old (median age, 61 years old). The study design was approved by the institutional review board of the Scientific Institutes for Research and Treatment Foundation 'National Cancer Institute'.

In-vitro controlled hemolysis experiment. Plasma and RBC samples, separated from a blood sample collected from a healthy donor, were kindly provided by Dr Appierto from the Scientific Institutes for Research and Treatment Foundation 'National Cancer Institute' (7). Hemolysis was artificially introduced in the plasma by adding RBCs starting from a 2% concentration and by performing ten 1:2 serial dilutions (range, 0.002-1% v/v) for a total of twelve samples (S01-12), including the uncontaminated plasma (0%, S12). Fig. 1 displays the dilution scheme of the experiment and the RBC colorimetric scale of the twelve samples.

Absorbance measurement. A total of 2 μl of each sample were used to measure absorbance on a NanoDrop™ 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). As demonstrated in Table I, the level of hemolysis for each plasma sample was computed according to the hemolysis indices previously used in other miRNA-related studies: The absorbance peak at 414 nm (5), the hemolysis ratio (3), the H-score (7) and the Harboe method, which measures the concentration of hemoglobin (8).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA (including small non-coding RNA molecules of between 50 and 250 nucleotides in length) was extracted from 600 μl of each of the vials as previously described (3). A total of two independent RNA extractions were performed.

TaqMan MicroRNA assays were used for miRNA quantification. A total of 2 μl of RNA was reverse transcribed to complementary DNA using the TaqMan MicroRNA Reverse Transcription kit and the commercially available primers specific to the 8 miRNAs analysed (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, in a total reaction volume of 15 μl. The names of the miRNAs analysed, the assay catalogue number and the mature miRNA sequence amplified, are listed in Table II. No-template-controls were used as a negative control of each assay. qPCR was performed using the TaqMan FAST Universal PCR Master Mix, no AmpErase® UNG according to the manufacturer's protocol in a PRISM 7900HT Real-Time PCR system (both Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 95˚C for 20 sec; 40 cycles of 95˚C for 60 sec and 60˚C for 20 sec. Technical duplicates of Cq (threshold cycle) measurements were performed. Normalization was performed using the $2^{-\Delta\Delta Cq}$ method, according to the criteria specified in the NqA algorithm as described previously (9,10).

Statistical analysis. The ΔCq values (11) were used as pivotal variable to evaluate the influence of hemolysis on each miRNA of interest. Specifically, for each sample (12 samples, s), for each replicate (two replicates, j) and for each extraction (two extractions, i) the ΔCq was obtained as follows: $\Delta Cq_{ijs} = Cq_{ijs} - Cq_{ijs}REF$, where $Cq_{ijs}REF$ is the Cq average of reference miRNAs identified on the bases of the NqA algorithm (9,10). Subsequently, for each miRNA, the relative quantity (RQ) was computed by subtracting the ΔCq value of each sample from the 0% sample (S12) as follows: $RQ_{ijs} = 2^{(-\Delta\Delta Cqijs)}$, where $\Delta\Delta Cq_{ijs} = \Delta Cq_{ijs} - \Delta Cq_{ijS12}$.

The significance of the changes in expression among the 12 samples was evaluated by resorting to the non-parametric Brown-Mood test, based on the comparison of the median scores (12). To consider the simultaneous determination of the various miRNAs on the same sample, the P-value of the test was adjusted according to the Bonferroni method by considering the significance level of 0.05. In addition, to evaluate the relevance of the change of expression compared with the uncontaminated plasma sample (S12) and to consider the simultaneous determination of the miRNAs, the 95% simultaneous confidence interval (SCI) of the $\log_2(RQ_{ijs})$ was computed for each miRNA, according to the bootstrap percentile method (13).

Following the conventional two-fold threshold rule, the low levels and high levels of a specific miRNA were considered statistically relevant due to RBC contamination, if the upper limit of the 95% SCI of $\log_2(RQ)$ was ≤-1 and the lower limit of the 95% SCI of $\log_2(RQ)$ was ≥1. In addition, by appropriately fitting the values of the considered hemolysis index computed starting from the absorbance values as a function of the known induced percentage of RBCs of the 10 serial dilutions (samples S02-11), a calibration hemolysis curve was generated and, by applying the inverse regression method (14), the unknown percentage of RBCs in the series of 110 plasma samples was estimated. Statistical analysis was performed by developing ad hoc programs with the SAS software version 9.2 (SAS Institute, Inc., Cary, NC, USA).

Results

RNA extracted from the twelve plasma samples contaminated with scaling concentrations of red blood cells (S01-12), was used to measure the influence of hemolysis on four miRNAs known to also be present in RBCs [miR-16, miR-92a, miR-451 and miR-486 (4,5)] and on four miRNAs (miR-30c, miR-320, miR-324-3p and miR-378) that were observed to be circulating in plasma. miR-378 was previously identified to be associated with the presence of colorectal cancer (CRC), independently from the hemolysis levels of the samples in analysis (3). Six miRNAs exhibited a significant change in expression in the 12 samples, according to the Brown-Mood test (miR-16, P<0.001; miR-92a, P=0.006; miR-451, P=0.011; miR-486, P<0.001; miR-320, P<0.001; and miR-324-3p, P=0.002). As shown in Fig. 2, significantly elevated levels of miR-16 and miR-486 were observed in contaminated samples (S09-01) compared with the uncontaminated sample (S12) (Fig. 2A and B). Similarly, a significantly increased level of miR-451 was detected starting from the sample with a percentage of RBC contamination of 0.016% (S08) compared with S12 (Fig. 2C). The majority of Cq values of miR-92a were undetected between samples S12 and S09, suggesting a specific association between its expression and the presence of RBCs (Fig. 2D). Accordingly, a significant increase in miR-92a expression was observed starting from

Table I. Descriptive statistics of the considered hemolysis indices.

			Absorbance 414 nm (A414)		Hemolysis ratio (A414/A375)		H-score (A414-A385)+ 0.16xA385		Harboe index, g/l (167.2xA415-83.6x A380-83.6x A450)x1/1,000	
Sample	RBCs, %	n	Median	Range	Median	Range	Median	Range	Median	Range
S11	0.002	3	0.117	0.021	1.000	0.023	0.0204	0.002	0.001	0.0001
S10	0.004	3	0.134	0.014	1.021	0.033	0.0265	0.001	0.002	0.0003
S09	0.008	3	0.151	0.015	1.091	0.042	0.0348	0.005	0.004	0.0004
S08	0.016	3	0.184	0.005	1.170	0.036	0.0540	0.005	0.007	0.0004
S07	0.031	3	0.228	0.022	1.432	0.063	0.0890	0.004	0.014	0.0006
S06	0.063	3	0.329	0.012	1.693	0.087	0.1490	0.003	0.025	0.0006
S05	0.125	3	0.373	0.015	1.673	0.045	0.1690	0.002	0.029	0.0008
S04	0.250	3	0.720	0.062	2.156	0.116	0.3910	0.022	0.071	0.0042
S03	0.500	3	1.506	0.055	2.477	0.043	0.8840	0.023	0.167	0.0078
S02	1.000	3	2.409	0.022	2.145	0.072	1.2380	0.054	0.254	0.0107

RBCs, red blood cells; n, number of replicas; A, absorbance.

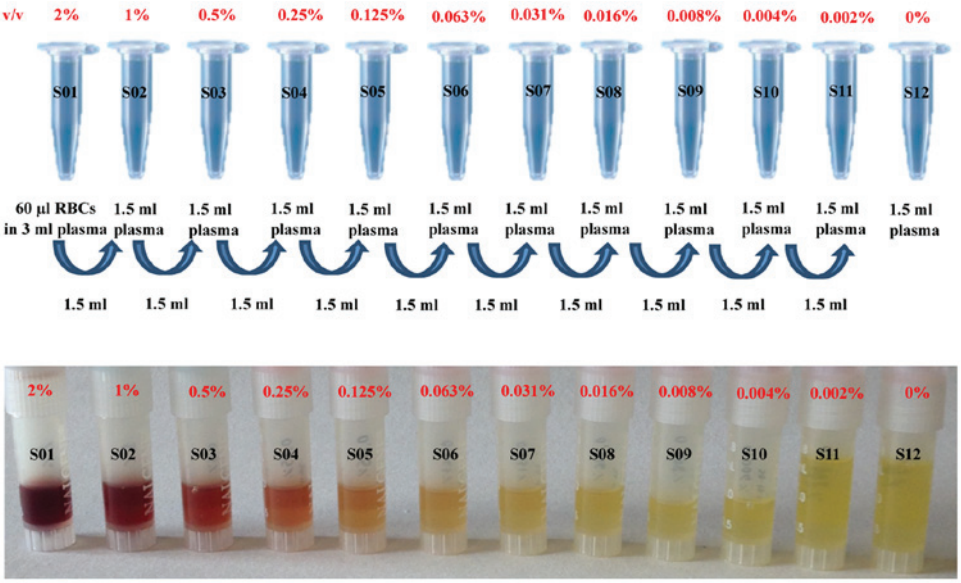

Figure 1. *In vitro* controlled hemolysis experiment. Diagram of dilutions (top panel) and RBCs colorimetric scale (bottom panel) of the twelve samples analysed. RBCs, red blood cells.

S08 (0.016% contamination with RBCs) compared with the unhemolysed sample (Fig. 2D).

miR-320 and miR-324-3p demonstrated a significant increase in expression starting from samples S06 and S05, respectively, compared with the unhemolysed sample (Fig. 3A and B). By contrast, no significant change in expression was observed with respect to S12 (0% contamination with RBCs) for miR-378 (P=0.074) and miR-30c (P=1.000) (Fig. 3C and D).

The present study also used the generated calibration curve to estimate the unknown percentage of RBCs of plasma samples in which miRNA expression investigation was intended. Fig. 4 displays the calibration process performed using, for illustration purposes, the concentration of hemoglobin obtained with the Harboe method as hemolysis index measured on 25/110 plasma samples analysed. According to the calibration curve generated by fitting a linear regression model, the percentage of RBCs of the plasma samples was estimated by considering the regression parameters and the value of the hemolysis index obtained for each sample (represented by grey dots in Fig. 4).

The contemporary measurement of the percentage of RBCs in plasma samples and of the influence of hemolysis on miRNA expression on the same calibration curve provides a real estimation of the number of samples for which the evaluation of the specific miRNA may be considered critical. The vertical dotted lines in Fig. 4B indicate the thresholds identified for miR-320 and miR-324-3p (0.063 and 0.125%, S06

Table II. List of the miRNAs analysed in the *in-vitro* controlled hemolysis experiment.

miRNA ID	Assay type	Mature miRNA sequence
Hsa-miR-16	000391	UAGCAGCACGUAAAUAUUGGCG
Hsa-miR-30c	000419	UGUAAACAUCCUACACUCUCAGC
Hsa-miR-92a	000431	UAUUGCACUUGUCCCGGCCUGU
Hsa-miR-320	002277	AAAAGCUGGGUUGAGAGGGCGA
Hsa-miR-324-3p	002161	ACUGCCCCAGGUGCUGCUGG
Hsa-miR-378	002243	ACUGGACUUGGAGUCAGAAGG
Hsa-miR-451	001141	AAACCGUUACCAUUACUGAGUU
Hsa-miR-486	001278	UCCUGUACUGAGCUGCCCCGAG

miRNA/miR, microRNA.

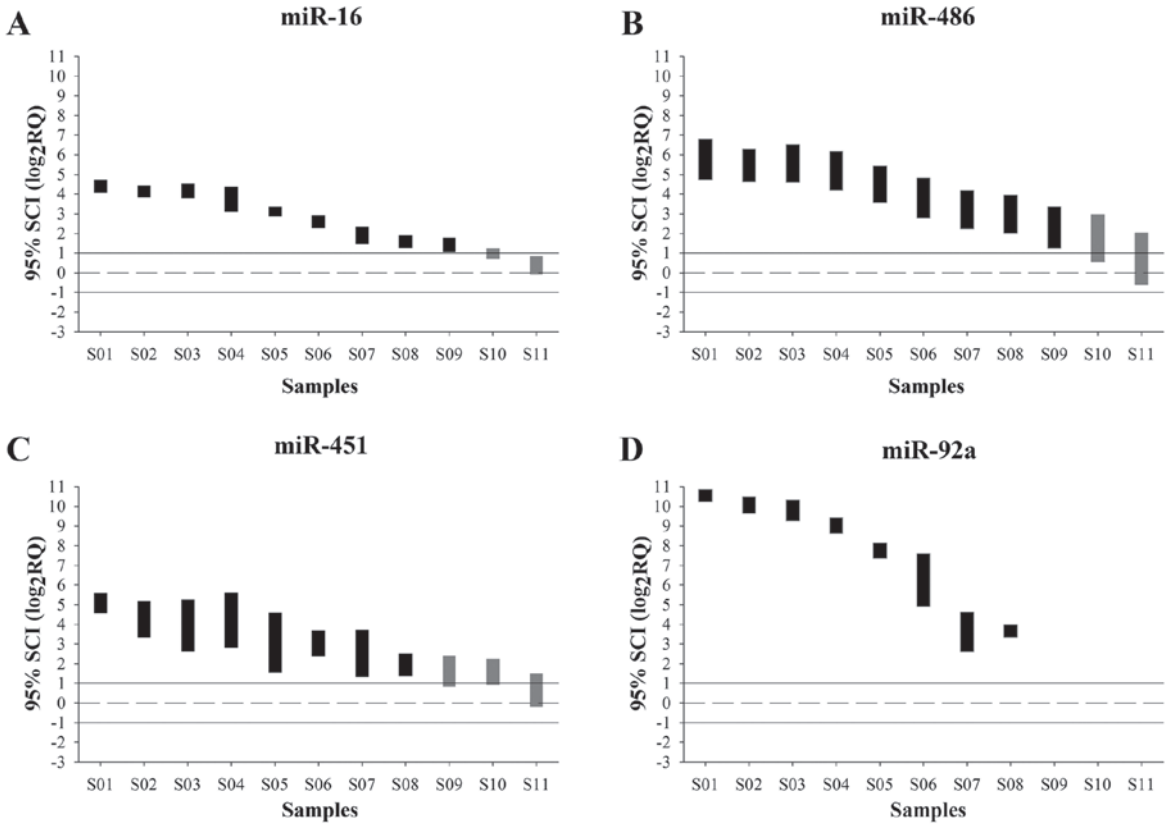

Figure 2. Graphs displaying the 95% SCIs of the log_2RQ for the four miRNAs known to be hemolysis-associated. In panels A-D are reported the 95% SCIs of the log_2RQ for (A) miR-16 (P<0.001), (B) miR-486 (P<0.001), (C) miR-451 (P=0.011) and (D) miR-92a (P=0.006) at each dilution point. Rectangles indicate that differences in expression of each miRNA are linked (black, lower limit of the 95% SCI ≥1) or not (grey) to presence of hemolysis, with respect to the uncontaminated sample. SCI, simultaneous confidence interval; RQ, relative quantity; miR, microRNA.

and S05, respectively) and indicate the percentage of samples (~60 and 15%, respectively) where the expression levels of the miRNA will be affected by the presence of RBCs.

Discussion

In the present study, a comprehensive procedure is presented that allows the evaluation of the influence of hemolysis on the expression of circulating miRNAs, as well as the estimation of the unknown concentration of hemolysis in plasma samples where the miRNAs will be measured. The quantification of miRNAs in samples with known percentages of RBCs permits the determination of the percentage at which RBC changes in miRNA expression may be considered statistically relevant, and indicates whether miRNAs should be discarded or retained for further analyses. By applying the procedure to eight miRNAs observed to be circulating in plasma samples, it was confirmed that four of them (miR-16, miR-92a, miR-451 and miR-486) were hemolysis-associated and that miR-378 was hemolysed independently,

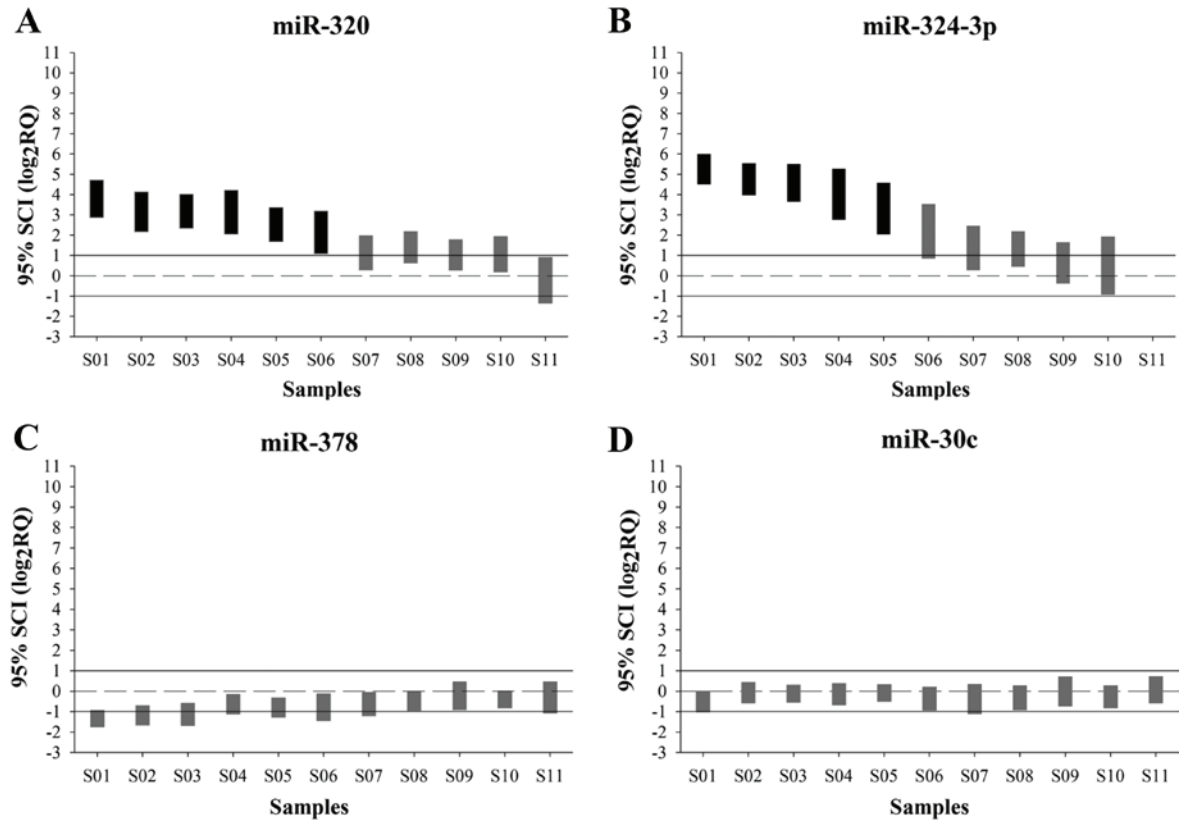

Figure 3. Graphs displaying the 95% SCIs of the log_2RQ of four miRNAs observed to be circulating in plasma. In panels A-D are reported the 95% SCIs of the log_2RQ for (A) miR-320 (P<0.001), (B) miR-324-3p (P=0.002), (C) miR-378 (P=0.074) and (D) miR-30c (P=1.00) at each dilution point. Rectangles indicate that differences in expression of each miRNA are linked (black, lower limit of the 95% SCI ≥1) or not (grey) to presence of hemolysis, with respect to the uncontaminated sample. SCI, simultaneous confidence interval; RQ, relative quantity; miR, microRNA.

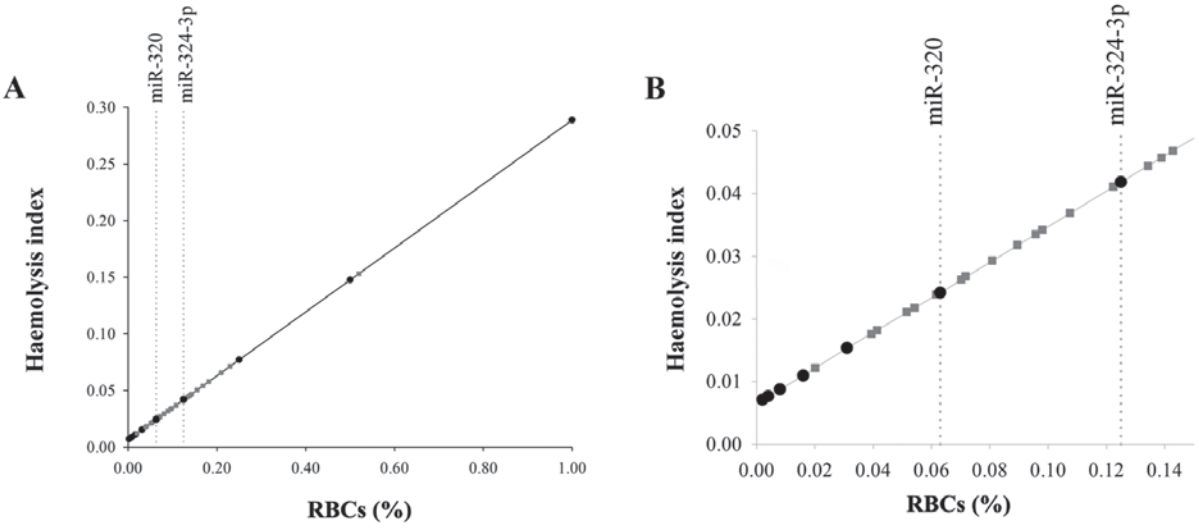

Figure 4. Calibration process. (A) Hemolysis calibration curve calculated by fitting the value of the considered hemolysis index as a function of the known induced percentage of RBCs of the 10 serial dilutions (black dots) and estimation of the unknown percentage of RBCs in 25/110 plasma samples analysed (grey dots), obtained by inverse regression. (B) Magnification of the calibration curve area with %RBCs <0.14. RBCs, red blood cells; miR, microRNA.

supporting the results of a previous study, which identified it to be a hemolysis independent CRC-associated circulating biomarker (3). In addition, the present study highlighted that the expression of miR-30c was not influenced by hemolysis, whereas the expression of miR-320 and miR-324-3p varied according to the levels of hemolysis. These two miRNAs will be discarded from further investigations and caution is advised when using the sets of miRNAs as biomarkers for a particular phenotype (miRNA signatures) that include miR-320 and miR-324-3p.

Furthermore, by exploiting previously proposed hemolysis indices, based on absorbance measurements, a calibration curve was constructed for the classification of plasma samples in terms of percentage of RBCs. As reported by Yamada *et al* (15), a potential method to avoid hemolysis may be to exclude samples with visually recognizable hemolysis from quantification and/or analysis. However, this approach may raise certain issues, including the loss of plasma samples (particularly of diseased patients) and the knowledge that hemolysis affects miRNAs even when its level is not visible (7). A potential solution for considering all the samples and to overcome the hemolysis interference on miRNA expression could be to apply appropriate statistical methods that correct miRNA expression for the level of RBC contamination in the target plasma samples. The present procedure moves in this direction, as it proposes a more accurate quantification of RBC contamination in plasma samples through a standard curve.

In conclusion, the present methodological procedure may be viewed as a tool for evaluation of the influence of hemolysis on candidate circulating miRNA biomarkers. This procedure should be introduced as an important step in the workflow for the identification of new biomarkers.

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