Research Article

Cancer Epidemiology, Biomarkers & Prevention

"Future-Proofing" Blood Processing for Measurement of Circulating miRNAs in Samples from Biobanks and Prospective Clinical Trials

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Abstract

Background: Quantifying circulating nucleic acids is an important new approach to cancer diagnosis/monitoring.

Methods: We compared the suitability of serum versus plasma for measuring miRNAs using qRT-PCR and assessed how preanalytic variables that can affect circulating tumor DNA (ctDNA) quantification in plasma also influence miRNA levels.

Results: Across 62 blood-derived specimens, plasma samples in EDTA, Streck-DNA, and Streck-RNA tubes showed significantly higher C_t values for multiple housekeeping miRNAs, compared with serum samples. For the EDTA-plasma tubes, this difference was only seen when including the high-speed centrifugation protocol used to optimize ctDNA extraction. In plasma samples derived from blood stored at room temperature for up to 14 days (conditions that typically apply to samples processed for biobanking), levels of endogenous housekeeping miRNAs gradually increased, in parallel with the hemolysis marker hsa-miR-451a, consistent with release from blood cells/platelets. It was necessary to normalize levels of the housekeeping miRNAs to those of hsa-miR-451a, to obtain the stable values needed for referencing test miRNA levels.

Conclusions: Our data indicate that plasma samples prepared for ctDNA extraction are suboptimal for miRNA quantification and require the incorporation of multiple data normalization steps. For prospective studies designed to measure both miRNAs and ctDNA, the most suitable approach would be to obtain both serum (for miRNAs) and plasma (for ctDNA). If only plasma can be collected, we recommend an initial low-speed centrifugation step, followed by aliquoting the supernatant into parallel samples, one for direct miRNA quantification, and the other for a further high-speed centrifugation step to optimize ctDNA retrieval.

Impact: These recommendations will help "future-proof" clinical studies in which quantification of circulating miRNAs is a component. *Cancer Epidemiol Biomarkers Prev; 27(2); 208–18. ©2017 AACR.*

Introduction

New approaches to cancer diagnosis and/or monitoring are based on detecting nucleic acids in blood, including circulating tumor DNA (ctDNA; refs. 1–6) and miRNAs (7, 8). Studies using ctDNA have almost exclusively been based on plasma, due to the low yield of ctDNA retrieved from serum (9, 10). Stability of ctDNA in plasma is improved using preservatives; for example, those in Streck tubes (11–13). However, it is clear that multiple preanalytic variables must be considered in prospective ctDNA-based clinical trials, including time to plasma isolation and the types of preservative used (14).

Detection of miRNAs is particularly attractive for many non-epithelial and/or pediatric cancers that have a low mutational prevalence (15, 16). In testicular malignant germ cell tumors (GCT), for example, mutations occur in less than half of all cases (17). We previously identified that miRNAs from the miR-371-373 and miR-302/367 clusters were coordinately elevated in the serum at the time of malignant GCT diagnosis (18, 19) and were also sensitive indicators of disease relapse (20). Subsequent studies, from multiple groups, have all focused on serum samples and have confirmed the clinical importance of this novel approach to malignant GCT diagnosis (20–30). In consequence, collection of biospecimens, including blood for further evaluation of miRNA detection, has been embedded in multiple prospective clinical trials across the spectrum of GCTs.

As future studies of circulating nucleic acids are likely to compare or combine miRNAs and ctDNAs for tumor diagnosis/monitoring (31), we sought to address whether plasma would also be a suitable medium for detecting circulating tumor–derived miRNAs. If so, this would allow combinatorial analysis of miRNAs and ctDNAs from the same sample. We also assessed whether preanalytic factors that can affect ctDNA analysis in plasma samples would also affect miRNA levels in such samples. We

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compared serum obtained using gel separator tubes with plasma derived from EDTA, Streck DNA, and Streck RNA tubes. For the EDTA tubes, we used two-step centrifugation protocols optimized for ctDNA extraction from plasma (32–34). We measured circulating levels of established housekeeping miRNAs and assessed effects on miRNA levels of tube type, time to processing, centrifugation speed, and hemolysis. On the basis of our findings, we recommend protocols for miRNA detection that are optimized for blood samples from biobanks and biological studies linked to clinical trials.

Materials and Methods

Patient samples

In total, we analyzed 62 blood-derived specimens. First, we undertook a comparison study, in which we comprehensively analyzed 27 specimens collected from a cohort of adult male patients ($n = 7$) recruited from a single hospital, via the Cambridge Urology Translational Research and Clinical Trials Biorepository (CUTRACT; cases CUB_001 – CUB_007). This cohort comprised a group with testicular malignant GCTs ($n = 5$) and a control group with non-GCT testicular malignancy ($n = 2$; one relapsed acute myeloid leukemia and one B-cell lymphoma). Second, we performed a time-course study of 26 samples from healthy adult male subjects ($n = 2$), plus nine specimens from a patient with testicular Leydig cell tumor (CUB_008). Details of all specimens, including clinicopathologic information, are provided in Supplementary Table S1. All samples were collected with Local Research Ethics Committee approval (CUB samples reference 03/018; other samples reference 01/128) and informed consent, in accordance with the Declaration of Helsinki.

Sample collection and processing

For the comparison study, blood samples were collected immediately prior to orchiectomy. The tubes used were 4.9 mL Sarstedt S-Monovette Z-Gel [gel separator serum (GSS)], 9 mL Sarstedt S-Monovette K3E [EDTA plasma (EP)], 10 mL Streck Cell-Free DNA BCT [Streck DNA plasma (SDP)], and 10 mL Streck Cell-Free RNA BCT [Streck RNA plasma (SRP)]. Six patients provided samples in all four tubes, while one patient (CUB_003) provided samples in three tubes, as an SDP tube was unavailable at the time of patient recruitment. All specimens were centrifuged and processed at room temperature within 2 hours of venipuncture, according to the recommendations of the manufacturers of each tube. There was no difference in processing times (average or range) for the different tubes in the comparison study. Details of the processing are summarized in Supplementary Table S2. In brief, for GSS tubes, the blood was allowed to clot for 30 minutes and then centrifuged at 3,000 $\times$ g for 10 minutes and the separated serum removed from above the gel layer. For EP tubes, the blood was centrifuged at 1,600 $\times$ g for 10 minutes, and then the separated plasma was aliquoted and subjected to a secondary spin of 14,400 $\times$ g for 10 minutes, as per standard protocol conditions for preparing plasma for optimal ctDNA analysis (32–34). For SDP and SRP tubes, the blood was initially centrifuged at 300 $\times$ g for 20 minutes and the separated plasma aliquoted into fresh tubes, prior to a secondary spin of 5,000 $\times$ g for 10 minutes. For all samples, the final supernatants were aliquoted into fresh tubes and then stored immediately at −80°C until further analysis.

In the time-course study, we used 2 mL SDP tubes, in which levels of RNA recovery and endogenous miRNAs were highly comparable with those in 10 mL SDP tubes. Blood samples were collected into 1.2 mL EP and 2 mL SDP tubes and then stored at room temperature for 0, 2, 4, 7, 10, and 14 days before being processed as above. The samples from one subject (patient CUB_008) were also used to study the effects of centrifugation speed on miRNA recovery. Of two 1.2 mL EP tube samples from this patient, one (EP #1) underwent the first low-speed centrifugation (1,600 $\times$ g) only, whereas the other (EP #2) also underwent the second high-speed centrifugation (14,400 $\times$ g).

RNA extraction

RNA was isolated from 200 μL of thawed serum or plasma using the miRNeasy Serum/Plasma kit (Qiagen), incorporating MS2 carrier RNA (Roche) and the exogenous nonhuman miRNA spike-in, cel-miR-39-3p, as described previously (20). RNA was eluted in 100 μL of nuclease-free water. For the comparison study, 750 μL of QIAzol per sample was used. The same protocols were used in the time-course study, except that the volume of QIAzol per sample was increased to 1,000 μL, following an update to the manufacturer's recommendations.

To assess whether the presence of proteins/nucleases within the biospecimens affected RNA retrieval, a subset of samples underwent a further RNA extraction that included a proteinase K digestion step. For this, 15 μL proteinase K (minimum concentration >600 mAU/mL; Qiagen) was added to 200 μL of each sample, then incubated at 60°C for 60 minutes. Subsequently, 750 μL of the QIAzol/MS2/cel-miR-39-3p mix was added, following which the protocol proceeded as described (20).

We also assessed two alternative approaches to RNA extraction from SRP tubes, namely the Plasma/Serum RNA Purification Mini Kit (Norgen BioTek Corp) and the mirVana PARIS Isolation Kit (Ambion). For the Norgen Kit, 200 μL of circulating sample was used, with the addition of cel-miR-39-3p/MS2 as above, to the lysis buffer A. RNA was eluted using 50 μL of nuclease-free water. The standard protocol for the Norgen Plasma/Serum Kit was also adjusted to include a proteinase K digestion step, where 20 μL of the proteinase K solution described above was added to lysis buffer A, mixed with the sample, and incubated for 60 minutes at 60°C. For the mirVana Kit, 400 μL of circulating sample was used, with the addition of 5.6×10^8 copies of cel-miR-39-3p spike-in and MS2 carrier RNA to the denaturing solution. In total, 80 μL eluate was obtained.

qRT-PCR analysis of miRNA levels

In all experiments, levels of circulating miRNAs were detected using our singleplex qRT-PCR method, as described previously (20). In short, 5 μL of eluted RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit (Life Technologies), using the miRNA-specific stem-loop primer from the relevant TaqMan miRNA Assay Kit (Life Technologies). The final volume of 15 μL for each reaction then underwent reverse transcription using a GeneAmp PCR System 9700 (Applied Biosystems) at 16°C for 30 minutes, 42°C for 30 minutes, followed by a final step of 85°C for 5 minutes. A singleplex final PCR was then performed, as per the manufacturer's instructions, on a Mastercycler ep_gradient/S realplex (Eppendorf) at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for one minute, as described previously (20).

The C_t threshold on the PCR machine was set manually to 2,000 fluorescence units across all PCR plates (20). To exclude nonspecific amplification, a no-template control (NTC) was also run

for each assay. None of the test samples had expression levels within 2 C_t values of the relevant NTC samples. Samples with C_t values $\geq$40, or those where the C_t threshold was not reached, were considered nonexpressing and arbitrarily assigned a C_t value of 40. The SDs of C_t values for each set of technical triplicate qRT-PCR reactions were calculated. We compared C_t values and SD values between the different sample types. We defined C_t values for sample sets as being suboptimal if mean miRNA expression values were $\geq$2 C_ts greater than the set with the lowest C_t value (i.e., expression levels were $\geq$4-fold lower). We also defined SD values for sample sets as being suboptimal if they were $\geq$2. For both C_t and SD assessment, samples that were not suboptimal were deemed to be satisfactory.

We quantified levels of six miRNAs, namely the exogenous spike-in miRNA cel-miR-39-3p (added to each sample immediately prior to RNA extraction; ref. 20), the hemolysis-dependent hsa-miR-451a (35), and the endogenous human reference miRNAs hsa-miR-23a-3p, hsa-miR-30b-5p, hsa-miR-30c-5p, and hsa-miR-191-5p, which were previously shown to be readily detectable in both serum and plasma (36, 37). In addition, for the time-course study, we quantified levels of three further endogenous miRNAs, again using singleplex qRT-PCR, namely hsa-miR-130b-3p and hsa-miR-146a-5p, previously shown to be present at stable levels in samples regardless of the degree of hemolysis (38), and hsa-miR-26a-5p, which had also been shown to be stable within the circulation (37).

Hemolysis assessment

In addition to visual inspection of each serum or plasma sample, hemolysis was assessed using two methods (8). First, spectrophotometric analysis of the absorbance of free hemoglobin at 414 nm (A_{414}; refs. 38, 39) was performed using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Products). Absorbance was tested in triplicate and the mean value recorded. Samples were classified as being hemolyzed if A_{414} values were >0.2 arbitrary units, as described previously (38, 39). Second, using the RNA extracted from the sample, hsa-miR-451a C_t (35) and ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) hemolysis values were calculated using singleplex qRT-PCR, as described previously (20). ΔC_t values $\geq$8 were considered to indicate hemolysis (20).

Statistical analysis

Statistics were performed using GraphPad Prism 6 software. Unless otherwise stated, analyses were performed using either a two-tailed paired *t* test (for matched samples) or a two-tailed unpaired *t* test with Welch's correction for unequal variance (for unmatched samples). *P* values <0.05 were considered statistically significant.

Results

Comparison study of miRNA levels in serum and plasma samples from different blood collection tubes

In the initial comparison study, we assessed levels of six miRNAs in serum samples prepared in GSS tubes, versus plasma samples prepared in EP, SDP, or SRP tubes. Levels of each miRNA were quantified in each sample using technical triplicate qRT-PCR reactions. The SD values for each set of triplicate results were very low for all miRNAs across the GSS, EP, and SDP samples. In contrast, the SD values were much higher for the samples in SRP

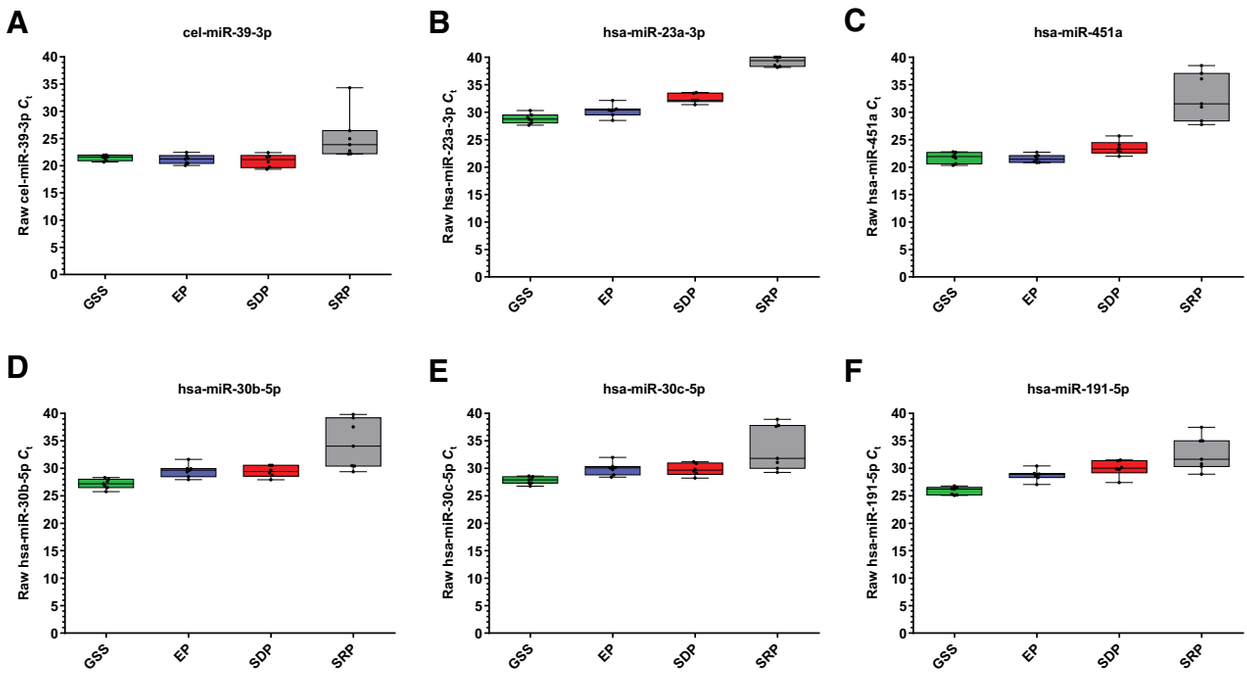

Figure 1.

Levels of individual miRNAs in samples from the four types of blood collection tube in the comparison study. The plots show C_t values for the exogenous nonhuman spike-in miRNA cel-miR-39-3p (**A**), the miRNAs hsa-miR-23a-3p and hsa-miR-451a used for hemolysis assessment (**B** and **C**), and the three established endogenous housekeeping miRNAs hsa-miR-30b-5p, hsa-miR-30c-5p, and hsa-miR-191-5p (**D–F**). GSS, gel separator serum (green boxes); EP, EDTA plasma (blue); SDP, Streck DNA plasma (red); SRP, Streck RNA plasma (grey). Bar, median; box, interquartile range; whiskers, full range of data.

tubes, particularly for hsa-miR-30b-5p and hsa-miR-23a-3p (Supplementary Fig. S1).

Levels of each individual miRNA in samples from each tube type are shown in Fig. 1. In summary:

- For the exogenous normalization miRNA cel-miR-39-3p, C_t values and SDs were satisfactory and similar in the GSS, EP, and SDP groups (Fig. 1; Table 1), indicating acceptable RNA extraction and qRT-PCR efficiency. In contrast, these parameters were suboptimal in the samples in SRP tubes (Table 1), where C_t values were significantly greater than in the EP group (Supplementary Table S3).
- For the endogenous normalization miRNAs hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-191-5p, and hsa-miR-23a-3p, C_t values were satisfactory only for serum samples in GSS tubes (Fig. 1; Table 1). For the EP and SDP plasma samples, C_t values were suboptimal and significantly higher than in the samples in GSS tubes (Supplementary Table S3). For all four of the normalization miRNAs, the SRP samples showed the highest C_t values, which were significantly greater than in the GSS and EP groups (Supplementary Table S3).
- For the hemolysis-associated miRNA hsa-miR-451a, C_t values were satisfactory for the GSS, EP, and SDP groups (Fig. 1; Table 1), but were again suboptimal in the SRP samples, where they were significantly higher than in each of the other sample groups (Supplementary Table S3).

We next compared mean C_t values for different miRNAs in each of the four tube types (Supplementary Fig. S2). Variations in levels of cel-miR-39-3p and those of hsa-miR-30b-5p (Supplementary Fig. S2) were lowest for samples in GSS tubes, which showed very tight clustering of C_t values. There was moderate clustering for the EP and SDP samples, but very poor clustering for the SRP samples (Supplementary Fig. S2). Identical findings were also seen for hsa-miR-30c-5p, hsa-miR-191-5p, and hsa-miR-23a-3p with cel-miR-39-3p. These data indicated that the EP, SDP, and SRP tubes resulted in additional technical variation, when compared with GSS tubes.

We attempted to develop a method that would allow satisfactory RNA extraction and qRT-PCR analysis of miRNA levels in plasma from SRP tubes. Extracting RNA from SRP tubes using the Norgen Plasma/Serum RNA Purification Kit reduced variability in levels of all six miRNAs, compared with RNA extracted from SRP tubes using the Qiagen miRNeasy Serum/Plasma Kit (Supplementary Fig. S3). However, mean C_t values of all six miRNAs in such SRP/Norgen samples remained $\geq$2 higher than those obtained with RNA extracted from GSS tubes using the Qiagen miRNeasy Kit. Indeed, for the exogenous normalization miRNA cel-miR-39-3p, levels in the SRP/Norgen samples were almost 5 C_t values greater than in the GSS/Qiagen samples. As the eluate from the former was 50 µL and the latter 100 µL, with 5 µL from each being used for PCR, this amounted to approximately 58-fold less efficient RNA recovery from the original 200 µL circulating samples. Such differences in miRNA recovery using the Norgen protocol were not improved by modifications such as the incorporation of a proteinase K digestion step. When RNA was extracted from SRP tubes using the mirVana PARIS Kit, expression levels of each of the six miRNAs tested (i.e., those listed in Table 1) were also suboptimal. This large reduction in the efficiency of RNA recovery would be expected to limit the overall sensitivity of a PCR assay for detecting test miRNA biomarkers (particularly those in

Table 1. miRNA levels in each of the four blood collection tubes from the comparison study

		GSS			EP			SDP			SRP		
miRNA	**Role**	**Mean C_t value**	**SD**	**Conclusion**	**Mean C_t value**	**SD**	**Conclusion**	**Mean C_t value**	**SD**	**Conclusion**	**Mean C_t value**	**SD**	**Conclusion**
cel-miR-39-3p	Exogenous normalization	21.43	0.49	Satisfactory	21.13	0.85	Satisfactory	20.91	1.19	Satisfactory	25.23	4.29	Suboptimal
hsa-miR-30b-5p	Endogenous normalization	27.16	0.90	Satisfactory	29.57	1.18	Suboptimal	29.44	1.04	Suboptimal	34.41	4.45	Suboptimal
hsa-miR-30c-5p	Endogenous normalization	27.77	0.68	Satisfactory	29.93	1.16	Suboptimal	29.73	1.11	Suboptimal	33.78	4.16	Suboptimal
hsa-miR-191-5p	Endogenous normalization	25.91	0.72	Satisfactory	28.76	1.00	Suboptimal	30.02	1.49	Suboptimal	32.74	3.10	Suboptimal
hsa-miR-23a-3p	Endogenous normalization (hemolysis)	28.84	0.89	Satisfactory	30.26	1.10	Satisfactory	32.47	0.86	Suboptimal	39.20	0.84	Suboptimal
hsa-miR-451a	Hemolysis assessment	21.73	0.96	Satisfactory	21.55	0.72	Satisfactory	23.50	1.27	Satisfactory	32.90	4.30	Suboptimal

NOTE: Comparison of C_t values and SD values between the different sample types. C_t values for sample sets were defined as being suboptimal if mean miRNA expression values were $\geq$2 C_ts greater than the set with the lowest C_t value. SD values for sample sets were defined as suboptimal if they were $\geq$2. For both C_t and SD assessment, samples that were not suboptimal were deemed to be satisfactory.
Key to blood collection tubes: GSS, gel separator serum; EP, EDTA plasma; SDP, Streck DNA plasma; SRP, Streck RNA plasma. Red text shows suboptimal values/analyses.

low abundance), when compared with a serum-based approach. As we were unable to resolve the limitations of SRP tubes for quantifying circulating miRNAs, we did not include them in our subsequent analyses.

Time-course study of the effects of blood storage on miRNA levels in plasma

We studied the effects on miRNA quantification of sample storage in SDP tubes at room temperature for up to 14 days, which is the recommended time limit for sample preservation prior to plasma extraction for ctDNA quantification. We compared our findings with plasma samples stored at room temperature for the same time periods in EP tubes. In total, we analyzed 26 separate samples from two healthy subjects, comparing 14 samples in SDP tubes with 12 in EP tubes (Supplementary Table S1).

Levels of hemolysis were lower in the SDP samples compared with the EP samples at matched time points, as indicated by sample inspection (Fig. 2A) and by spectrophotometric measurement of free hemoglobin absorption at 414 nm (A_{414}; Fig. 2B). Using the standard A_{414} threshold of >0.2 arbitrary units (38, 39), the EP samples were hemolyzed within 1 hour of collection (i.e., the day 0 samples). They showed an abrupt increase in hemolysis from day 4, consistently reaching the upper limit of detection threshold by day 14. In contrast, hemolysis was not consistently present in the SDP samples until day 7. Assessment of hemolysis using miRNA ΔC_t values (hsa-miR-23a-3p – hsa-miR-451a) indicated hemolysis at almost all time points in both sample types, albeit with occasional variation in the values observed (Fig. 2C).

Next, we investigated changes in levels of individual miRNAs in plasma obtained from the SDP versus the EP tubes over the 14-day time course (20). As cel-miR-39-3p was spiked-in immediately prior to RNA extraction for each time point, it was not surprising that we saw no difference in levels of this miRNA (Fig. 3A). In contrast, levels of all six endogenous housekeeping miRNAs tested [hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-191-5p (Fig. 3); hsa-miR-26a-5p, hsa-miR-130b-3p, hsa-miR-146a-5p (Supplementary Fig. S4)] all showed similar gradual reductions in C_t values over the time course, indicating gradual increases in expression levels in the blood from which the plasma samples were derived. Similar changes were seen in the red blood cell–derived miRNA hsa-miR-451a, suggesting that the elevations in endogenous housekeeping miRNAs were related to hemolysis. Indeed, normalization of levels of the six endogenous housekeeping miRNAs to those of hsa-miR-451a resulted in stabilization of miRNA measurements over the time course (Fig. 3; Supplementary Fig. S4). These findings were confirmed in a further set of SDP samples obtained from a patient with testicular Leydig cell tumor (CUB_008; Supplementary Table S1; Supplementary Fig. S5).

Comparison of methods of hemolysis assessment in plasma samples

When quantifying test miRNAs, it is important also to measure hemolysis, to exclude any confounding effects of miRNAs released from blood cells (40). We investigated whether previously established parameters for measuring hemolysis in serum using miRNA quantification (8, 20, 41) could also be applied to the plasma samples in our study. The established ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) hemolysis method was applied to the samples from the comparison study in which there was no evidence of hemolysis by spectrophotometry ($n = 20$; Fig. 4A). A ΔC_t value indicating hemolysis ($\Delta C_t > 8$) was seen in only one of seven GSS serum samples but in six of seven EP and five of six SDP plasma samples (Fig. 4B). The false positive results in the plasma samples were attributable to significantly lower detection levels (higher C_t values) of the endogenous housekeeping gene hsa-miR-23a-3p in the EP and SDP samples, compared with the GSS samples ($P = 0.018$ and $P = 0.0002$, respectively; Fig. 1E; Supplementary Table S3).

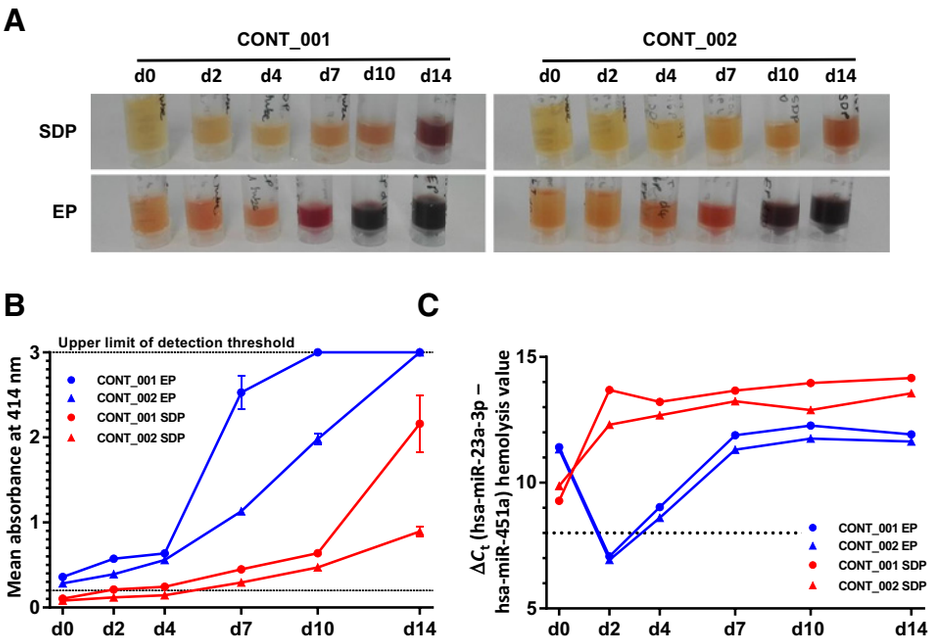

Figure 2.
Levels of hemolysis in plasma samples from the time-course study. **A,** Appearance of the plasma samples derived from the SDP and EP tubes taken from two healthy control subjects (CONT_001 and CONT_002) at the six time points [day (d) 0, d2, d4, d7, d10 and d14]. The darker the sample color, the greater the degree of hemolysis. **B,** Spectrophotometric analysis of the plasma samples in **A**, showing mean absorbance of free hemoglobin at 414 nm (A_{414}). The lower dotted line indicates the threshold (0.2) above which samples were classified as hemolyzed, while the upper dotted line (3.0) indicates the upper limit of detection of the spectrophotometer. **C,** Hemolysis assessment by ΔC_t (hsa-miR-23a-3p – hsa-miR-451a) values. The dotted line indicates the C_t value threshold (8) above which samples were classified as hemolyzed. EP, EDTA plasma (blue lines); SDP, Streck DNA plasma (red lines). Error bars, SD.

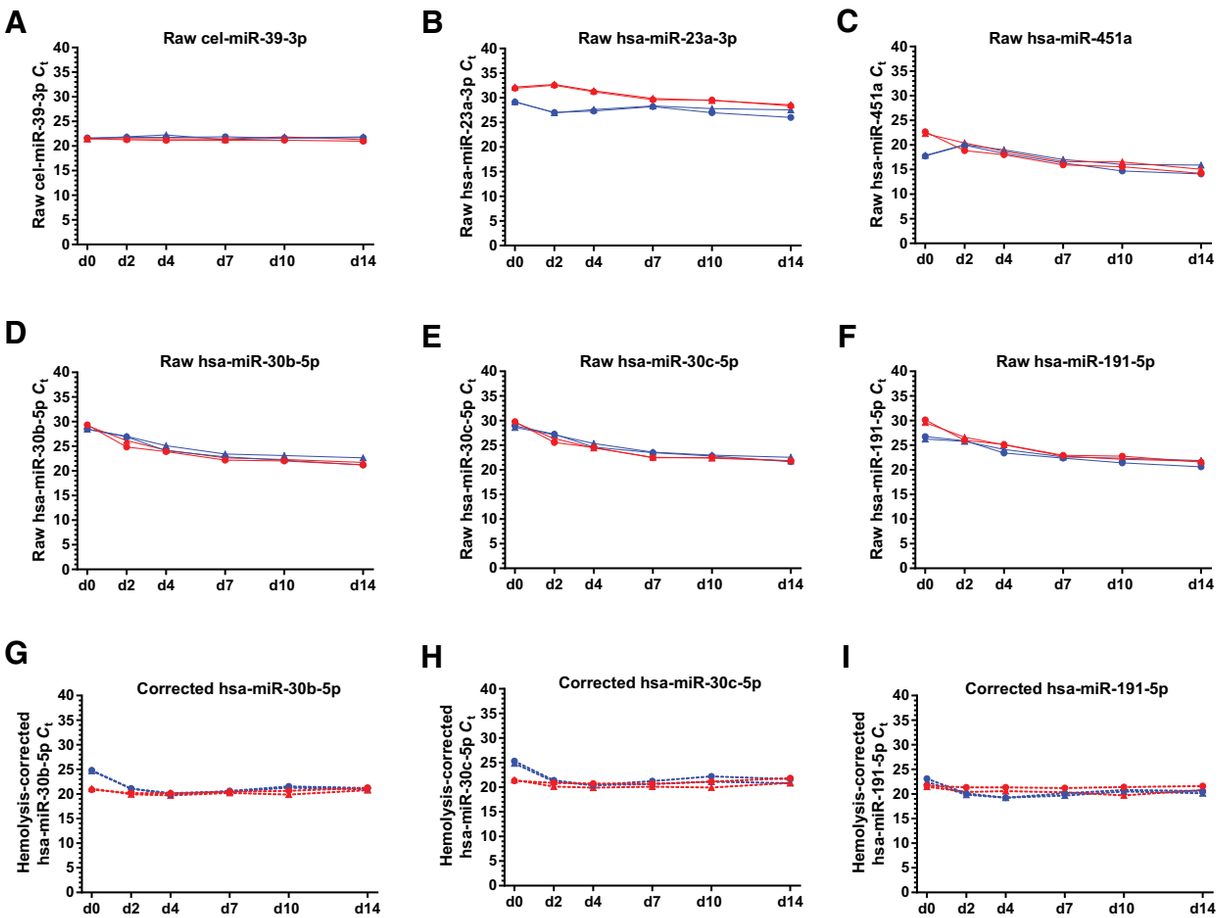

Figure 3.

miRNA levels in plasma samples from the time-course study. Graphs show C_t values for the plasma samples derived from the SDP and EP tubes taken from two healthy control subjects (CONT_001 and CONT_002) at the six time points [day (d) 0, d2, d4, d7, d10, and d14]. **A–F,** Raw C_t values (solid lines) for the exogenous nonhuman spike-in miRNA cel-miR-39-3p (**A**), the miRNAs hsa-miR-23a-3p and hsa-miR-451a used for hemolysis assessment (**B** and **C**), and the three established endogenous housekeeping miRNAs hsa-miR-30b-5p, hsa-miR-30c-5p, and hsa-miR-191-5p (**D–F**). **G–I,** Hemolysis-corrected C_t values (dotted lines) for the three endogenous housekeeping miRNAs shown in **D–F**. EP, EDTA plasma (blue lines); SDP, Streck DNA plasma (red lines). Note that error bars are not visible as they fall within each respective data point.

When analyzing 32 available EP and SDP samples from across the comparison and time-course studies, 20 (62%) were classified as hemolyzed by spectrophotometry (Fig. 4C and D), whereas 30 (94%) were classified as hemolyzed by using the ΔC_t value (Fig. 4D). The samples that were classified as hemolyzed by spectrophotometry showed higher ΔC_t values ($P = 0.009$, unpaired t test; Fig. 4D) and lower raw C_t values for hsa-miR-451a ($P < 0.0001$, unpaired t test; Fig. 4C), compared with the samples that were classified as nonhemolyzed by spectrophotometry. The A_{414} absorbance values strongly fitted a nonlinear association with the raw hsa-miR-451a C_t values ($R^2 = 0.818$; Fig. 4E). However, they only weakly fitted a nonlinear association with the ΔC_t (hsa-miR-23a-3p – hsa-miR-451a) values ($R^2 = 0.224$; Fig. 4F), in keeping with the limited accuracy of hsa-miR-23a-3p quantification in the plasma samples. The dynamic range of the A_{414} absorbance values was greater than those of the raw hsa-miR-451a values (Fig. 4E) and ΔC_t (hsa-miR-23a-3p – hsa-miR-451a) values (Fig. 4F), further indicating the limited utility of miRNA quantification for assessing hemolysis in the plasma samples.

Effect of centrifugation speed on miRNA levels in plasma

We sought to determine whether the differences observed between the serum and plasma samples were due to inherent features of the sample types or to the different processing protocols used. We examined the effect of centrifugation speed on miRNA recovery from plasma, using samples extracted from EP tubes, in comparison with serum samples prepared from GSS tubes. We first used samples from patient CUB_008, to compare the effect of a single low-speed centrifugation step (1,600 × *g*; EP #1) with the standard dual centrifugation that is typically used when preparing plasma for ctDNA analysis (1,600 × *g* then 14,400 × *g*; EP #2). The mean expression level of five miRNAs (namely hsa-miR-30b-5p, -30c-5p, -191-5p, -23a-3p, and -451a) was significantly lower (higher C_t values) in the EP #2 sample, compared with the EP #1 ($P = 0.03$) and the GSS sample

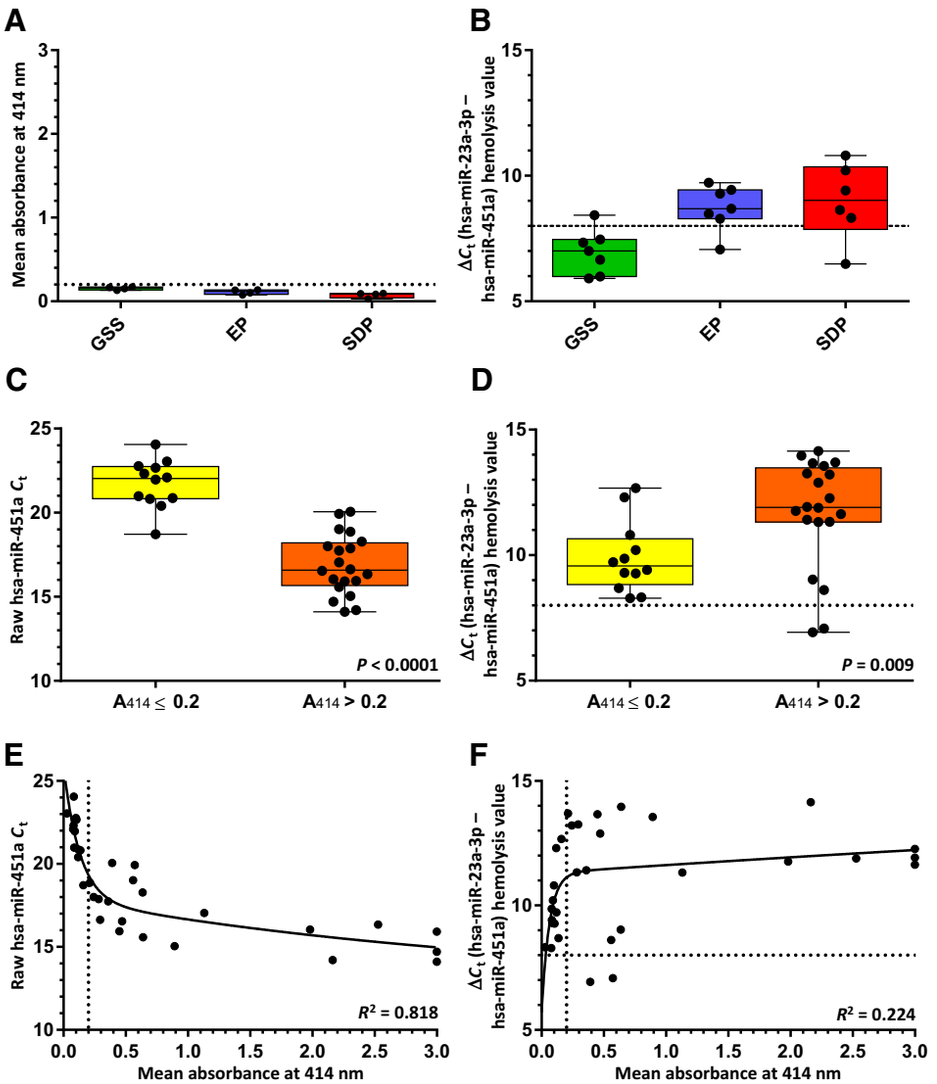

Figure 4.
Comparison of methods of hemolysis quantification. **A** and **B,** Measurement of hemolysis in samples from the comparison study using: mean absorbance of free hemoglobin at 414 nm (A_{414}), as assessed by spectrophotometry (**A**); and ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) values (**B**). **C** and **D,** Raw C_t hsa-miR-451a values (**C**) and ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) values (**D**) in 32 EP and SDP samples from both the comparison and time-course studies, identified by spectrophotometry as nonhemolyzed (yellow box; A_{414} values $\leq$ 0.2) or hemolyzed (orange box; A_{414} values > 0.2). **E** and **F,** Associations of raw C_t hsa-miR-451a values (**E**) and ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) values (**F**) with A_{414} absorbance for the 32 plasma samples analyzed in **C** and **D**, including the lines of best fit. GSS, gel separator serum (green boxes); EP, EDTA plasma (blue); SDP, Streck DNA plasma (red); SRP, Streck RNA plasma (grey). Bar, median; box, interquartile range; whiskers, full range of data. Hemolysis thresholds are indicated by the dotted lines, as defined in the legend to Fig. 2.

($P = 0.01$; Fig. 5A). When analyzing each miRNA individually, levels were also lower in the EP #2 sample compared with the EP #1 and GSS samples (Fig. 5B). There were no differences between the EP #1 and GSS samples in the mean expression levels (Fig. 5A), nor in the levels of each miRNA individually (Fig. 5B).

We extended these findings by interrogating data from a published global miRNA profiling study of EP samples (from breast cancer patients) that had been centrifuged at various speeds (42). For the 106 miRNAs with C_t values <35 (listed in ref. 42), mean levels were significantly lower in EP samples that underwent high-speed centrifugation (10,000 × *g*), compared with EP samples that underwent low-speed centrifugations (either 2,000 × *g* or 1,000 × *g*; $P < 0.001$ for both comparisons; Fig. 5C). There was no significant difference in mean miRNA expression levels between the 1,000 × *g* and 2,000 × *g* centrifugation samples (Fig. 5C). Levels of individual miRNAs were again lower (higher C_t values) in the samples that had received high-speed centrifugation, compared with the low-speed samples (Fig. 5D). The differences in expression levels were particularly large for the three endogenous housekeeping genes hsa-miR-30b-5p, hsa-miR-30c-5p, and hsa-miR-191-5p, but were also seen for hsa-miR-451a (data on hsa-miR-23a-3p levels were not available in the published dataset). Across the 106 miRNAs, high-speed centrifugation had a highly unpredictable effect on miRNA levels compared with low-speed centrifugation (Fig. 5E). C_t values were almost always higher in the high-speed samples, albeit with rare exceptions (Fig. 5E). The difference in expression levels ranged from 0 to >8 C_t values, with a modal difference of 2 to 4 C_t values (Fig. 5F).

Discussion

Biospecimens collected by tissue banks increasingly include blood-derived samples for quantification of circulating nucleic acids. Most effort to date has focused on studying preanalytic variables that affect ctDNA quantification (11–14). Here, we sought to establish how such variables affect miRNA levels and to generate a recommended protocol for collecting and processing blood-derived biospecimens, to maximize the yield of recovered miRNAs. Our comparison study demonstrated that serum samples were better suited for miRNA studies than the plasma samples

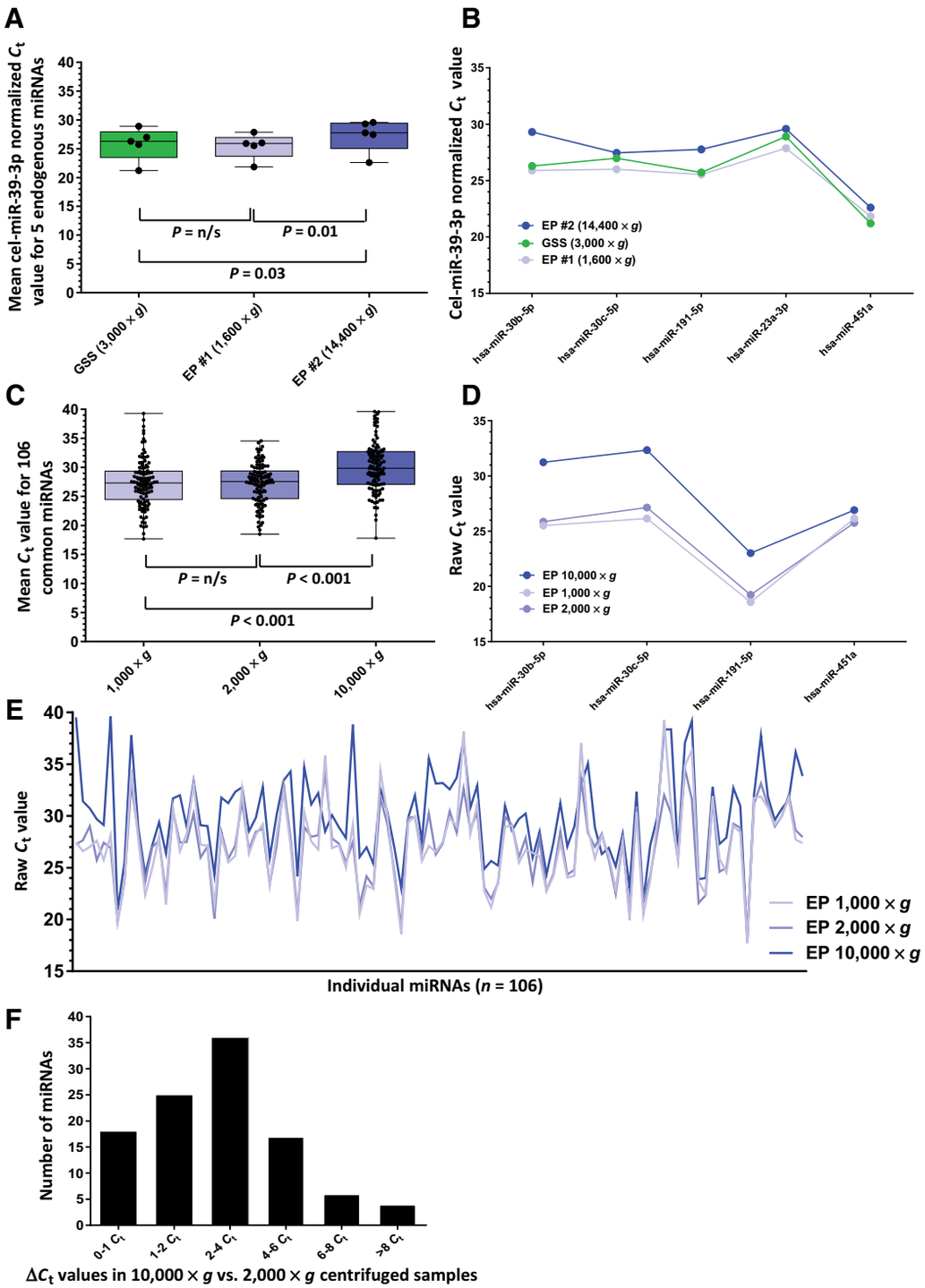

Figure 5.

Effect of centrifugation speed on miRNA levels in plasma samples. **A,** C_t values (cel-miR-39-3p-normalized) for five endogenous miRNAs (hsa-miR-30b-5p, hsa-miR-30c-5p, hsa-miR-191-5p, hsa-miR-23a-3p, and hsa-miR-451a) in the GSS, EP #1 and EP #2 samples from patient CUB_008. **B,** Individual C_t values (cel-miR-39-3p-normalized) for the five endogenous miRNAs assessed in **A**. Key for **A** and **B**: GSS, gel separator serum (green box); EP #1, EDTA plasma (low-speed centrifugation only, light blue); EP #2, EDTA plasma (including high-speed centrifugation, blue). **C,** Mean C_t values for 106 endogenous miRNAs in EP samples processed using different centrifugation speeds (42). **D** and **E,** Individual raw C_t values for four of the five endogenous miRNAs from **A** and **B** (**D**) and all 106 miRNAs (**E**) in EP samples processed using different centrifugation speeds. **F,** Differences in C_t values for all 106 miRNAs in EP samples centrifuged at 10,000 × *g* versus 2,000 × *g*. Key for **C–F**: EP 1,000 × *g* = EDTA plasma samples with low-speed centrifugation at 1,000 × *g* (light blue); EP 2,000 × *g*, EDTA plasma samples with low-speed centrifugation at 2,000 × *g* (mid-blue); EP 10,000 × *g* = EDTA plasma samples with high-speed centrifugation at 10,000 × *g* (dark blue). In **A** and **C**, bar, median; box, interquartile range; whiskers, full range of data.

tested, as the latter showed greater technical variation, with consistently lower levels of endogenous housekeeping miRNAs. The differences we observed were not attributable to the method used for miRNA extraction, as the Qiagen miRNeasy Serum/Plasma Kit was independently demonstrated to provide the highest yield of miRNAs from plasma samples (42). The difference between serum and plasma from EP tubes (Fig. 1) is likely to relate to the two-spin processing used for preparing the latter, a method that is typically adopted to optimize ctDNA extraction. Samples processed from EP tubes with a low-speed centrifugation step only did not show significant differences in miRNA levels compared with serum samples (Fig. 5A and B), indicating that plasma is not necessarily an inferior substrate for miRNA extraction, if appropriate protocols are adopted. We found Streck RNA (SRP) tubes to be unsuitable for miRNA studies, despite using various RNA extraction methods and protocol modifications, including the incorporation of a proteinase K digestion step.

The uniform clotting process inherent in preparing serum removes a large proportion of protein from a whole blood sample. One practical benefit of this is that the layer of protein obtained when extracting RNA from serum is relatively small. Although endogenous miRNAs are released into the serum during the clotting process, for example, from platelets, the overall pipeline involved in serum processing is straightforward and, most importantly, provides very similar levels of endogenous housekeeping miRNAs across samples. As plasma lacks the clotting step, the protein layer obtained during RNA extraction is large, leading to smaller yields of supernatant. This may necessitate repeated sample processing to obtain adequate volumes of eluate for miRNA quantification, potentially exacerbating the technical variations in measuring miRNA levels in plasma.

A further consideration when evaluating blood-derived biospecimens is the stability of miRNAs during prolonged storage of unprocessed blood. Such conditions typically apply to the blood specimens from which plasma samples are obtained for biobanking, for example, those from a multicenter clinical trial. In plasma samples derived from blood stored over a period of up to 14 days at room temperature, we observed increases in levels of the endogenous housekeeping miRNAs hsa-miR-30b-5p, hsa-miR-30c-5p, and hsa-miR-191-5p. These were associated with parallel changes in the hemolysis marker hsa-miR-451a, suggesting that the miRNAs were likely to have been released into the plasma from red blood cells and/or platelets, either through active shedding or passively as a result of hemolysis. As a result, stabilization of the levels of housekeeping miRNAs in plasma samples over the 14-day time course required correction for levels of hsa-miR-451a.

An additional consequence of the variations in levels of housekeeping miRNAs in the plasma samples studied is that hemolysis could not reliably be indicated by the ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) miRNA quantification method. This is an important contrast with serum samples, where hemolysis can be quantified accurately using this miRNA method (8, 20). In our comparison study, the majority of the plasma samples were falsely classified as showing hemolysis by the ΔC_t method. This was predominantly due to reduced levels of the housekeeping miRNA hsa-miR-23a-3p, rather than alterations in the levels of the direct hemolysis marker hsa-miR-451a. In our time-course study, ΔC_t hemolysis levels in the plasma samples increased then plateaued during a period of ongoing hemolysis (which was indicated by sample inspection and spectrophotometric measurement of hemoglobin absorbance). Normalization of hsa-miR-23a-3p levels to those of hsa-miR-451a, as required for other housekeeping miRNAs in stored plasma samples, would not be appropriate when using the ΔC_t (hsa-miR-23a-3p − hsa-miR-451a) method for hemolysis quantification.

Processing of plasma samples for ctDNA quantification typically involves a double centrifugation process, which includes a high-speed ($\geq$10,000 $\times$ g) second spin, designed to remove background genomic DNA contamination (32–34). Our experimental findings and reanalysis of published data (42) show that such a two-step process significantly alters the profile of circulating miRNAs detected in plasma samples, compared with plasma samples processed by low-speed centrifugation only and also with serum samples. These findings are consistent with a previous global miRNA profiling study showing that the two-step method, including high-speed centrifugation, resulted in lower miRNA levels in plasma when compared with serum (43). Our analyses indicate that the alterations in plasma miRNA levels produced by high-speed centrifugation are highly variable and unpredictable. The miRNAs affected most may predominantly be present in relatively large structures in the plasma, such as platelets or large extracellular vesicles, that are removed by the high-speed centrifugation step (42).

Taken together, our data allow us to make recommendations for the design of biological studies linked to clinical trials, and/or retrospective studies of samples in biorepositories, where quantification of circulating miRNAs is a requirement. Our findings indicate that serum is the optimum biospecimen for quantifying circulating miRNAs, as it is subject to the least preanalytic technical variation. The optimal approach for studies measuring both circulating miRNAs and ctDNA would therefore be to collect both serum (for miRNAs) and plasma (for ctDNA). If only plasma can be collected, then immediate processing of the blood sample to plasma is recommended, to avoid the technical variations that affect miRNA levels after storage and/or transport of whole blood at room temperature. We further recommend that such plasma processing should involve a low-speed centrifugation step only, followed by aliquoting the supernatant into parallel samples prior to storage at −80°C. Such aliquots could then be used directly for miRNA quantification, or subjected to a further high-speed ($\geq$10,000 $\times$ g) centrifugation step to optimize ctDNA retrieval (42).

For retrospective analysis of miRNA levels in plasma samples from biorepositories, particularly those prepared for ctDNA extraction, we recommend normalization of housekeeping miRNA levels using cel-miR-39-3p (correcting for technical differences in RNA recovery) and hsa-miR-451a (correcting for the effects of hemolysis), and also the use of multiple housekeeping miRNAs for referencing the levels of test miRNAs. These steps will improve the likelihood of avoiding biologically inconsistent or even contradictory results, as has recently been highlighted for various urological tumors (7). Despite these steps, our data indicate that the results of retrospective miRNA quantification work in such plasma samples should be interpreted with caution.

In our opinion, these technical considerations are of vital importance to the design of future clinical trials and/or retrospective studies of biobank samples. Our recommendations are practicable and scalable. They will help future-proof clinical studies in which quantification of circulating miRNAs is a component.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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