Article | Received 11 November 2024; Accepted 5 March 2025; Published 12 March 2025
https://doi.org/10.55092/exrna20250005

Influence of pre-analytical conditions on cell-free microRNA stability in blood plasma samples

Brayann Martínez Pabón†, Ivan Zaporozhchenko†, Maria Konoshenko, Ekaterina Murina, Olga Bryzgunova* and Pavel Laktionov

Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Novosibirsk 630090, Russia

* Correspondence author; E-mail: olga.bryzgunova@niboch.nsc.ru.

† These two authors contributed equally.

Highlights:

- miRNA degrade at storage.
- miRNA integrity depend on storage conditions.
- Individual miRNA differ in stability.

Abstract: Numerous preanalytical variables (sample collection, pretreatment and storage conditions, miRNA extraction, *etc.*) can influence miRNA detection. Understanding the various properties of miRNA, especially its stability in biofluids, is important in various types of miRNA studies, both fundamental and applied. This study aimed to evaluate the influence of plasma storage conditions and certain RNA extraction parameters on stability of endogenous miRNAs in human blood plasma. We report stability kinetics of four endogenous miRNAs (-16, -19b, -23a, -451a) and cel-miR-39 as exogenous miRNA under short and long-term incubation at different temperatures as well as the effect of long-term storage on extracellular vesicles miRNAs stability. The most stable of the endogenous ones was miRNA-23a. When studying archival samples (1–2 and 9–10 years of storage) of blood plasma from healthy donors, it was shown, that the concentrations of all endogenous miRNAs are steadily decreasing. These findings further show that endogenous miRNA levels do not remain stable during prolonged storage at −20°C. Although packaging of miRNA in extracellular vesicles stabilizes miRNA to some extent, it nevertheless the level decreases over a period of time from 6 months to 6 years. We have also evaluated the effect of the reagents used in the extraction process on miRNA recovery. The addition of guanidine isothiocyanate containing denaturation buffer alone prevented degradation of the synthetic cel-miR-39 miRNA spiked-in to blood plasma. In the presence of denaturation buffer with or without 2-mercaptoethanol the yields were higher than with just 2-mercaptoethanol or in the absence of any agents. Addition of the commercial RNA-stabilizing agent RNAlater did not result in significant retention of miRNA in plasma, but significantly worsened the efficiency of miRNA isolation. Thus, the

degradation rate of miRNAs can be affected by their structure and packaging. Addition of various stabilization solutions to biofluids can affect the efficiency of miRNA extraction.

Keywords: miRNA; liquid biopsy; blood plasma; extracellular vesicles; stability

1. Introduction

MicroRNAs (miRNAs) are a class of short (~22 nt) endogenous, non-coding RNAs found to be highly conserved in animals, plants, invertebrates, protozoa and certain viruses [1]. They function by posttranscriptionally regulating gene expression through mRNA degradation or inhibition of translation and thus take part in key of cellular processes such as of differentiation, development, proliferation, and metabolism [2]. Release miRNAs can occur actively or passively [3] and extracellular or cell-free miRNAs found in biological fluids are believed to be relatively stable, resisting degradation at room temperature for several days and under such conditions as high or low pH, boiling, and multiple freeze/thaw cycles [4]. Despite this, several reports have shown that at least certain miRNAs can exhibit variable stability under these conditions and during storage at various temperatures [5–7]. Stability of extracellular miRNAs is attributed to shielding by other biopolymers (primarily Argonaute proteins AGO1-4 [8–10]), packaging into extracellular membrane-bearing vesicles such as exosomes [11], microvesicles (MVs) [12], and apoptotic bodies, or association with high- and low-density lipoproteins (HDL and LDL, respectively) [13]. Besides, more recent researches have suggested possible role of miRNA sequence, indicating a correlation of miRNA stability with the AU/GC ratio [14,15].

Critical role in regulation of cell function and relative abundance in clinical samples such as plasma/serum, lymph, urine, and other body fluids, has attracted huge interest to cell-free miRNAs as source of biomarkers for diagnostic and prognostic purposes, however, none of these biomarkers have made it through clinical trials yet [16–18]. Currently no universally accepted systematic guidelines exist for storage and processing of clinical samples for subsequent detection of circulating miRNAs biomarkers. We know that numerous pre-analytical variables (sample collection, pre-processing and storage conditions, miRNA extraction, *etc.*) can influence miRNA detection [19] but very little is known about factors that determine the stability of individual miRNAs [19,20]. For example, cell-derived miRNA contamination is one of the major sources of variation in plasma and serum. Therefore, fast and adequate processing of blood into plasma/serum is crucial to avoid blood cell lysis leading to release of intracellular miRNAs [21]. Pre-analytical conditions yielding reproducible results should be explored in detail and properly validated in order to establish a gold standard protocol for sampling and processing of biological fluids for analysis of miRNA expression [22].

This study aimed to evaluate the influence of plasma storage conditions and certain RNA extraction parameters on stability of endogenous miRNAs in human blood plasma. We report stability kinetics of four miRNAs under short and long-term incubation at different temperatures as well as the effect of long-term storage on EVs (extracellular vesicles) miRNAs stability. Additionally, we have compared miRNA yields from fresh and archived blood plasma samples and effects of variations in miRNA extraction protocol and RNA protective agent on isolation efficiency. Important disclaimer is that in this study we use single-phase miRNA isolation method, which we have successfully used in several studies aimed at searching miRNA biomarkers of lung cancer in blood [23]. Due to inherent differences of miRNA extraction techniques results obtained here should not be generalized without additional validation.

2. Materials and methods

2.1. Sample collection

According to ISEV recommendations, collection of the EV-containing fluid must be gentle, limiting cell disruption [24]. We acted within the framework of these recommendations. Whole blood samples of 10 healthy male donors were obtained from E. Meshalkin National medical research center of the Ministry of Health of the Russian Federation (Novosibirsk, Russia). All procedures were carried out in accordance with relevant guidelines and the ethical standards of the institutional research committees. The study was approved by ethical committees of ICBFM SB RAS (N 10 from 22.12.2008). Written informed consent was obtained from all subjects before the blood extraction.

Venous blood was collected in ethylenediaminetetraacetic acid (EDTA) spray-coated vacutainers, stored at 4 °C and processed into plasma and blood cells within 4 h of blood sampling. To obtain blood plasma, samples were centrifuged at 400 g for 20 min at 4 °C, followed by centrifugation at 800 g for 20 min at 4 °C to remove cell debris and remaining platelets. Resulting blood plasma was aliquoted into 1.5 mL tubes and stored at −20 °C for no more than 3 months. Archived samples were processed according to the same protocol and stored at −20 °C for a period of 9–11 years before RNA isolation.

2.2. Isolation and characterization of blood EVs

Total extracellular vesicles were obtained from 2 mL plasma by ultracentrifugation as described in [25]. The pellets were resuspended in PBS (1 mL), frozen in liquid nitrogen, and kept at −80 °C for later miRNA isolation or EVs analysis. According to the recommendations of the ISEV [24], Transmission Electron Microscopy was performed using the technique described in [25].

2.3. Storage experiments

To determine miRNA stability blood plasma aliquots were incubated at 37 °C, 25 °C, or 4 °C to induce artificial aging for up to 6 months. Synthetic exogenous microRNA cel-miR-39 spiked-in at the isolation stage was used as a control. At certain time intervals, aliquots of plasma were taken to room temperature and miRNAs were isolated. For each time point 10 independent blood plasma aliquots were analyzed.

To determine miRNA stability a half of plasma EVs samples (n = 10) was incubated at −70 °C during 6 month, another group (n = 10) was incubated at −70 °C during 6 years.

2.4. Assessment of a stabilizing agent

Plasma samples from healthy donors were thawed and incubated with RNAlater (Ambion, USA) for 5 minutes before RNA isolation. Ratio of RNAlater relative to plasma volume (150 µL) were as follows: 10%–15 µL, 25%–37.5 µL, 50%–75 µL or 250%–375 µL.

2.5. miRNA isolation from blood plasma and plasma EVs

RNA was isolated from 150 µL of blood plasma and 250 µL of plasma EVs (resuspended in PBS) using a single-phase extraction protocol described previously [26]. Briefly, frozen samples were thawed and an aliquot is mixed with 0.03 V of 2-mercaptoethanol and 2 V of denaturing buffer (DB, 6 M guanidine

isothiocyanate and 15 mM Tris–acetate, pH 6.5). Subsequently, if applicable, synthetic miRNA spike-in control (cel-miR-39) was added to monitor the RNA extraction and immediately 9 V of 2:1 ethanol/chloroform solution was added and mixed thoroughly, followed by RNA purification on silica-based spin column (BioSilica Ltd, Novosibirsk, Russia) and isopropanol precipitation into a final volume of 15 µL of RNAse-free water.

To assess the influence of reagents, plasma samples were incubated at room temperature for 2 hours in the presence of each component separately or a complete denaturation mix - 2-mercaptoethanol, guanidine-containing denaturing buffer or without any additives. After incubation the remaining components were added and miRNA purification was performed as usual.

2.6. Reverse transcription and quantitative TaqMan PCR

Quantification of miRNA (cel-miR-39) added to every sample at the isolation step (see above in the text) as a spike-in control was used as normalizator within and between experiments.

Reverse transcription (RT) on miRNA templates was performed as described by Chen *et al.* [27]. Reactions were performed in a total volume of 10 µL and contained 2 µL of RNA, 50 nM each of miRNA-specific primers, 100 units of MMLV-RH reverse transcriptase (Biolabmix, Novosibirsk, Russia), 2 µL of 5× MMLV reaction buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), and 250 µM of each dNTP. The reaction conditions were as follows: 16 °C for 30 min, 42 °C for 30 min, and 72 °C for 10 min. Samples without RNA template were used as negative controls. Resulting cDNA was diluted with 3 volumes of DNAse-free water.

Real-time PCR was carried out on the CFX 96^{TM} Real-time System (Bio-Rad, USA). All reactions were carried out in duplicate in a total volume of 30 µL. Each reaction contained 5 µL of diluted cDNA, 1 unit of Taq DNA polymerase (Biolabmix, Russia), 15 µL of 2X PCR buffer [750 mM TrisHCl (pH 8.8 at 25 °C), 200 mM $(NH_4)_2SO_4$, 0.1% (V/V) Tween 20], 3.2 mM $MgCl_2$, 200 mM of each dNTP, 600 nM miRNA-specific forward primer, 800 nM universal reverse primer, and 300 nM specific TaqMan probe. After an initial denaturation at 95 °C for 3 min, the reactions were run for 50 cycles of 95 °C–15 s and 60 °C–45 s. The detection limit was calculated as the measured minimum concentration of a specific oligonucleotide for each miRNA without interference from their respective primers.

Primers and probes for reverse transcription and TaqMan qPCR (Table 1) were synthesized in the Laboratory of Medicinal Chemistry (ICBFM SB RAS, Novosibirsk).

Table 1. Sequence of primers and probes for quantitative PCR.

Name	Sequence
cel-miR-39-3p-RT primer	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAGCT-3’
cel-miR-39-3p-Reverse primer	5’-ATTCACCGGGTGTAAATC-3’
cel-miR-39-3p-Probe	5’-(FAM)-CACTGGATACGACCAAGCTGA-(FQ1)-3’
hsa-miR-16-5p-RT primer	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAA-3’
hsa-miR-16-5p -Reverse primer	5’-GCCCGTAGCAGCACGTAAATAT-3’
hsa-miR-16-5p-Probe	5’-(FAM)-GCACTGGATACGACCGCCAA-(FQ)-3’
hsa-miR-19b-3p-RT primer	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT-3’
hsa-miR-19b-3p-Reverse primer	5’-CGCTGTGCAAATCCATGCAA-3’
hsa-miR-19b-3p-Probe	5’-(FAM)-GCACTGGATACGACTCAGTT-(FQ)-3’
hsa-miR-23a-RT primer	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAAAT-3’
hsa-miR-23a-Reverse primer	5’-CTATCACATTGCCAGGGA-3’
hsa-miR-23a-Probe	5’-(FAM)-TCGCACTGGATACGACGGAAAT-(FQ)-3’
hsa-miR-451a-RT primer	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTCA-3’
hsa-miR-451a-Reverse primer	5’-CTGCAAACCGTTACCATTACT-3’
hsa-miR-451a-Probe	5’-(FAM)-TCGCACTGGATACGACAACTCA-(FQ)-3’
Universal Forward primer	5’-GTGCAGGGTCCGAGGT-3’

2.7. Statistics

Statistical comparisons were performed using Microsoft Excel. Group comparisons were made with student test. p-values < 0.05 were considered statistically significant.

3. Results

To normalize data from different experimental synthetic ribooligonucleotide with sequence of cel-miR-39 was added to every sample tested at the isolation step after the sample was mixed with buffer, containing 2-mercaptoethanol and guanidine isothiocyanate inhibiting RNAse activity. Variation of spikes-in control in the range of ± 1 Ct around 27 cycles were the conditions for inclusion of data on the content of microRNAs tested in sample in data analysis. Actually, the amount of spike-in control (cel-miR-39) did not significantly varied in the studied samples throughout the entire study period. The spread of values did not exceed 1 Ct (Figure 1).

Blood plasma storage

First, stability of miRNA during storage at different temperatures was investigated (Figure 1). When incubated at 37 °C, a steady decline of miRNA levels was observed, but the rate of degradation varied between miRNAs. Three miRNAs, namely miR-451a, miR-16 and miR-19b demonstrate similar degradation rate and their concentrations have reached their respective limits of detection in 6 months. Total ΔCt for 24 weeks (relative to the "fresh" sample) for these 3 miRNAs is equal to approx. 10. Decay of miR-23a was less pronounced (ΔCt for 24 weeks relative to the "fresh" sample is about 2), suggesting differences in stability of individual circulating miRNA in blood plasma (Figure 1A).

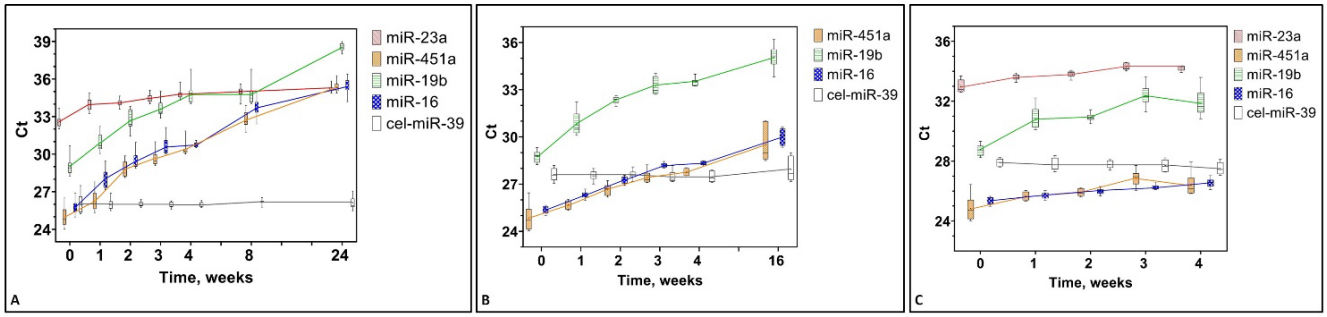

Figure 1. Stability of miR-23a, miR-451a, miR-19b and miR-16-5p in plasma of healthy volunteers at (**A**) 37 °C, (**B**) room temperature and (**C**) 4 °C.

When plasma was incubated at room temperature the degradation occurred slower: ΔCt for 16 weeks relative to the "fresh" sample vary between 5 and 6 for all miRNAs tested (Figure 1B). Interestingly, when comparing the data of miRNA expression in between nearest weeks at room temperature, miR-16 showed a p-value of significance < 0.001 from the first week of incubation, for miR-451 a p-value < 0.05 was observed for the first week and < 0.001 in the following. It is noteworthy that for miR-19b only a p < 0.001 was observed until the fourth week of incubation.

Finally, we showed that during storage at 4 °C for 1 month the degradation curves for all miRNAs were flattened, ΔCt for miR-19 relative to the "fresh" sample do not overexceed and for the resting

miRNAs 4 weeks ΔCt do not overexceed 2, indicating slower decay. Plasma miRNA levels remain relatively stable during this time, and in general, the effect of storage at 4 °C on circulating microRNA levels was less than at higher temperatures. However, levels of less stable miR-19b showed moderate variation compared to miR-451a and miR-16, establishing it as the least stable miRNA in this study (Figure 1C).

To assess the effect of long-term storage on miRNA stability, archival blood plasma from healthy donors (n = 10) stored at −20 °C for 1–2 years were analyzed alongside collected blood plasma from healthy donors (n = 10) stored at −20 °C for 9–10 years. Levels of endogenous miR-451a, miR-16, and miR-19b were significantly lower in 9–10 years' archival samples (5.2, 6.3, and 4.1 Ct difference, respectively). These findings further show that endogenous miRNA levels do not remain stable during prolonged storage at −20 °C (Figure 2).

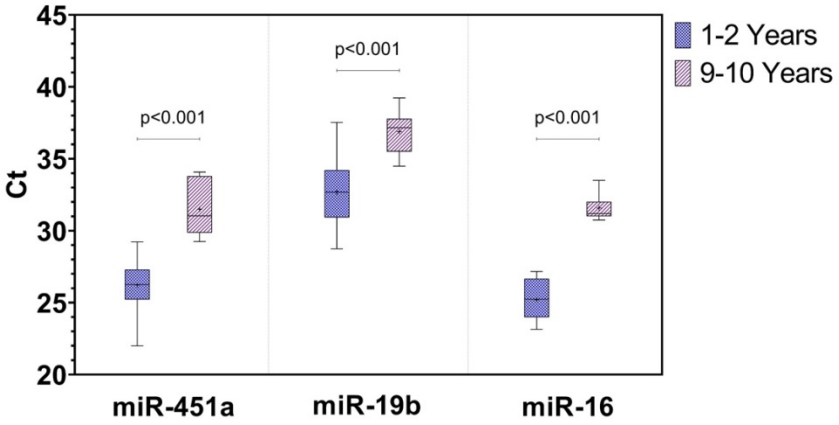

Figure 2. Effect of long term storage on miRNA concentration in plasma of healthy volunteers. Samples were stored at −20 °C for 1–2 years or 9–10 years before thawing and miRNA extraction.

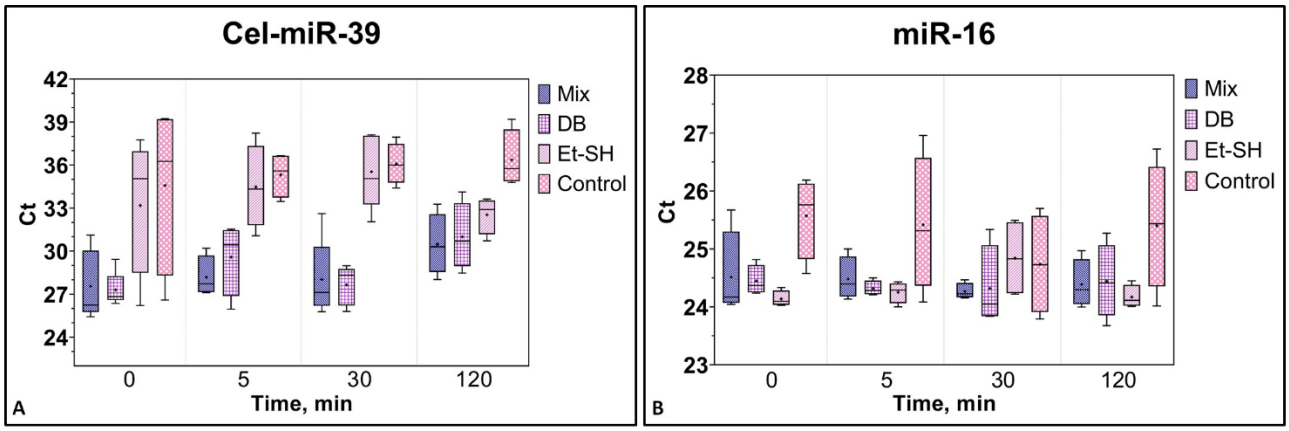

Figure 3. The effect on reagents used in miRNA extraction of miRNA stability. Plasma samples were incubated at room temperature for up to 2 hours in the presence of each component separately or a complete denaturation mix - 2-mercaptoethanol, guanidine-containing denaturing buffer or without any additives (control).

We have also evaluated the effect of the reagents used in the extraction process on miRNA recovery. The addition of guanidine isothiocyanate containing denaturation buffer (DB) alone prevented degradation of the synthetic cel-miR-39 miRNA spiked-in to blood plasma. In the presence of DB with

(Mix) or without 2-mercaptoethanol (Et-SH) the yields were higher than with just 2-mercaptoethanol or in the absence of any agents. Interestingly, in the case of endogenous miR-16, the reagents do not appear to have a significant effect on their levels. The Ct values for miR-16 do not depend on which reagent was added to the reaction. This difference is most likely due to the shielding conferred to endogenous miRNAs by packaging, which is absent from the exogenous control (Figure 3).

In attempt to counteract miRNA decay in blood plasma samples and increase yields, we tested the effect of commercial RNA-stabilizing agent RNAlater. Surprisingly, no apparent positive effects on recovery of endogenous miR-451a and miR-19b and synthetic cel-miR-39 were found when 10%, 25%, 50% or 250% of RNAlater (relative to plasma volume) were added (Figure 4). While addition of RNAlater in a volume of 10% relative to the volume of plasma did not significantly affect the efficiency of miRNA isolation, increasing the volume of the solution to 25% resulted in noticeably decreased yields (cel-miR-39—1.05 Ct, $p < 0.001$; miR-451a—0.98 Ct, $p < 0.001$; miR-19b—1.07 Ct, $p < 0.001$). When 50% of RNAlater was added Ct values increased sharply and reproducibility between repeated experiments was low (data not shown). Differences in miRNA levels were 4–6 Ct compared to control plasma. We tend to believe this decrease in isolation efficiency may be due to the disruption of phase balance in the subsequent miRNA isolation which can lead to lower yields. Surprisingly, at 250% of RNAlater the reproducibility between repetitions of the experiment was again high, but isolation efficiency was even lower (cel-miR-39—3.62 Ct, $p < 0.001$; miR-451a—3.87 Ct, $p < 0.001$; miR-19b—3.90 Ct, $p < 0.001$).

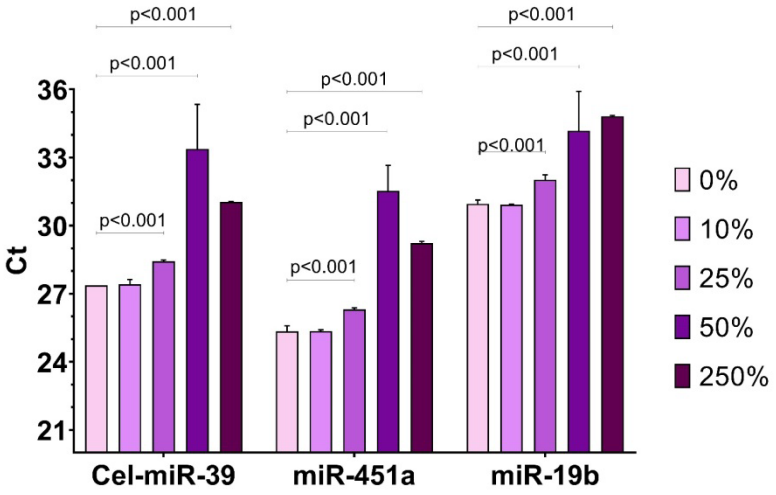

Figure 4. The effect of RNAlater treatment on miRNA recovery. Plasma samples from healthy donors were thawed and incubated with RNAlater for 5 minutes before RNA isolation.

To assess the effect of long-term storage of plasma EVs on miRNA stability (Figure 5), archival blood plasma EVs from healthy donors (n = 10) stored at −80 °C for 6 months were analyzed alongside collected blood plasma EVs from healthy donors (n = 10) stored at −80 °C for 6 years. According to TEM, EVs from the blood plasma samples contained EVs mainly sized from 20 to 220 nm (data not shown). Level of endogenous miR-16 was significantly lower in 6 years' archival samples (2.2 Ct difference), the difference in endogenous miR-19 level was not significant (1.5 Ct difference).

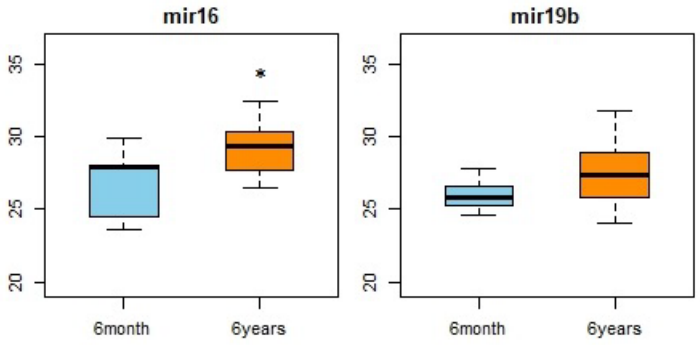

Figure 5. Stability of miR-16 and miR-19b in plasma EVs of healthy volunteers at −80 °C for 6 months and 6 years.

4. Discussion

It was shown that level of cel-miR-39 used like spike-in control have a nice inter experimental reproducibility [28]. The target miRNAs were selected for the current study based on the following considerations: expression level of miR-16 in blood was shown not strongly depend on the state of patient/donor and this miRNA was frequently used to normalize miRNAs expression in early studies of miRNAs [28,29]. MiR-451a is expressed in red blood cells and usually occur in blood plasma at well detectable level [30,31]. This hemolysis indicator can also easily be applied to archival data [30]. According to some data, miR-16 can also serve as an indicator of blood hemolysis, which reduces its value as a normalizer [31]. The levels of miR-16 and miR-451, both present in significant levels in red blood cells, were proportional to the degree of hemolysis [31]. MiR-23a unlike miR-451 is relatively stable in plasma and serum and is not affected by hemolysis [32]. MiR-19b-3p belongs to the miR-17-92 cluster, which possesses oncogenic properties and promoting cell survival [33] and are frequently used in the miRNA panels for different type of cancer detection [34–37]. Nevertheless this miRNA is also presents in normal blood plasma by its level is not so high [35,38]. Totally this set of miRNAs must present the information of the storage of different type miRNAs in blood plasma.

It is know that miRNAs in blood circulated in complexes of proteins, lipids, or may be packed in membrane coated structures like exosomes or microvesicles [39]. Complexes of miRNAs with biopolymers in blood and blood plasma are rather stable, they are not easy to destroy, which is necessary when isolating miRNAs [40,41]. At the same time complexes of miRNAs provide longer circulation time of these molecules. Some of our selections miRNAs like miR-19b and miR-16 were found to be packed in microvesicles and thus are to be more stable in blood [42–44]. Actually the level of RNAses in blood plasma of healthy individuals reach 0.3 μg/mL [45] and RNAs have a limited lifetime in blood; the half-live of endogenous mRNA and miRNA in the blood were reported to reach 16.4 hours [46], whereas more than 99% of the naked added RNA could degrade in plasma after 15 seconds of incubation [47].

Moreover, RNAs are sensitive to oxidation. It is thought that free radicals that are chemical species containing unpaired electrons in their outer orbit could play a very important role not only in the oxidation of miRNAs [48] but also in the destabilization of its complexes causing their deprotection and subsequent degradation [49]. Some studies indicate that RNA, such as ribosomal RNA and messenger RNA, is more accessible to oxidative damage than other cellular components such as DNA or proteins. Furthermore, free radicals can steal electrons for example from the lipids, resulting in their degradation

and cell damage [50]. Out of a wide diversity of free radicals, the hydroxyl radical ($OH^{\cdot}$) is highly reactive and are directly responsible for most of the oxidative damage in biomolecules, including RNA. The most prevalent oxidized base in RNA is 8-hydroxyguanosine resulting from the loss of an electron and proton of C8-OH product of $OH^{\cdot}$ reaction with guanine. Modification of ribose, base excision, and strand break are in the list of RNA oxidative damages. The list of the oxidative damages of mRNA inhibited reverse transcription [51].

Here we show that natural miRNAs have a different lifetime in blood plasma. The most stable is miR-23a which was shown ΔCt for 24 weeks is about 2 (Figure 1A). MiR-16 and miR-451a have a moderate stability, whereas miR-19a-3p is the less stable (Figure 1). The miRNAs studied showed differentiated degradation rates possibly related to their means of transport or their primary structure. At the same time, the levels of exogenous cel-miR-39 for each extraction did not differ significantly, excluding sample-to-sample variation during extraction (Figure 1).

To stabilize miRNAs in blood many reagents were offered. There are firs targeted to inactivation of RNAses and includes 2-mercaptoethanol to reduce disulfide bounds in RNAses, as well as chaotropic salts and other additives [52]. Currently, there are many known commercially available reagents for stabilizing RNA in blood, for example, PAXgene®, Tempus™, Trizol®LS, RNAlater solution and *etc.* In the current study we investigated effect of RNAlater - the solution according to the specifications avoids RNA degradation during tissue thawing and minimizes the laborious grinding of frozen tissue while protecting the RNA. These qualities that give it an appeal for its use in the protection of miRNA in biofluids as blood plasma and serum. For its implementation, we first evaluated its compatibility with the miRNA isolation procedure (Figure 4) and we have observed that while RNAlater can be successfully used to stabilize and protect cellular RNA in tissues, it is not instrumental in the miRNA isolation process in blood plasma samples, while 5 volumes of the reagent relative to the sample are used with the tissues for RNA protection, only 0.25 volumes relative to the plasma volume were sufficient to significantly affect the amount of miRNA isolated, however, a relative volume of 0.1 did not show to affect the isolated miRNA concentrations, further experiments should be performed in order to find out if this volume is sufficient to effect some protection for these biomolecules.

Others reagents have been used extensively in order to stabilize samples for storage such as guanidinium thiocyanate and phenol (Trizol). Nowadays, there have been described several techniques for isolation of cell-free miRNAs, including; Trizol®LS liquid-liquid extraction and many commercial-kit methods. Nonetheless, Trizol-based method accompanying with the stability of miRNAs in storage conditions of serum, and cDNA derivatives has been questioned. There are many controversial data about this reagent have reported that the efficacy of isolated miRNAs by use of the Trizol-based method was up 35% lower than some commercial-kit methods, such a difference could lead to wrong data analysis in the possible expression of a biomarker so there must be an active search for reagents that do not present this type of inconvenience [19,53,54].

Nevertheless miRNAs degradation under enzymatic degradation looks not the only mechanism led to miRNAs degradation in blood. The results obtained show that endogenous miRNA levels in EVs degrade during long-term storage at −80 °C, however they are much more stable than cell free blood plasma miRNAs during prolonged storage at −20 °C. Earlier the stability of miRNA from plasma EVs isolated by ExoQuick™ Exosome Precipitation Solution stored up to 5 years at −20 °C was studied [55]. It was shown that miRNA EVs remain stable under such conditions. However, in other studies it was

shown that 12 week storage at −80 °C of EVs isolated from cell culture-derived conditioned media by ultracentrifugation or angential flow filtration (TFF) followed by size exclusion chromatography resulted in significant decrease of both particle concentration and RNA amount [56] and 12 days storage of blood EVs at −80 °C also decreased EVs concentration [57,58]. The stability of miRNAs is much higher than long RNAs, however, the integrity violation of EVs will influence their miRNA cargo with high probability. Taken together, all these results indicate that miRNAs are stable during long term storage of plasma EVs which is, in fact, an expected result as soon as EVs membrane should preserve packed miRNAs from degradation and a slight decrease in EVs miRNA levels may be related to the EVs integrity violation.

5. Conclusion

Data suggests that the short term stability of some cell-free miRNAs does not seem to be greatly affected by the fact that miRNA released from cells is usually packaged in some form, including HDL and LDL, exosomes, vesicles, and apoptotic bodies. Due to these different forms of packaging and their primary structures, their rate of degradation may be affected in long term and therefore exhibit greater or lesser stability against different conditions. In contrast, spiked in control miRNAs are rapidly degraded in nondenatured plasma and even after the treatment with some denaturing reagents.

Acknowledgments

This work was supported by the Russian state-funded project for ICBFM SB RAS (grant number 125012900932-4).

Conflicts of interests

The authors have no conflicts of interest to declare.

Ethical statement

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of ICBFM SB RAS (№10, 22.12.2008). Written informed consent was provided by all participants.

Authors' contribution

Conceptualization, I.Z. and P.L.; data curation, P.L., M.K. and O.B.; formal analysis, M.K. and I.Z.; investigation, B.M.P. and E.M.; methodology, B.M.P., E.M., I.Z., M.K. and O.B.; supervision, P.L.; writing—original draft, B.M.P., E.M., I.Z. and M.K.; writing—review & editing, O.B. and P.L. All authors have read and agreed to the published version of the manuscript.

References

[1] Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA Translation and Stability by microRNAs. *Annu. Rev. Biochem.* 2010, 79(1):351–379.

[2] Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *J. Cell. Physiol.* 2019, 234(5):5451–5465.
[3] Zhao C, Sun X, Li L. Biogenesis and function of extracellular miRNAs. *ExRNA* 2019, 1(2):38.
[4] O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* 2018, 9:1–12.
[5] Glinge C, Clauss S, Boddum K, Jabbari R, Jabbari J, *et al.* Stability of Circulating Blood-Based MicroRNAs–Pre-Analytic Methodological Considerations. *PLoS One* 2017, 12(2):e0167969.
[6] Muth DC, Powell BH, Zhao Z, Witwer KW. miRNAs in platelet-poor blood plasma and purified RNA are highly stable: a confirmatory study. *BMC Res. Notes.* 2018, 11:273.
[7] Köberle V, Pleli T, Schmithals C, Augusto Alonso E, Haupenthal J, *et al.* Differential Stability of Cell-Free Circulating microRNAs: Implications for Their Utilization as Biomarkers. *PLoS One* 2013, 8(9):e75184.
[8] Boon RA, Vickers KC. Intercellular Transport of MicroRNAs. *Arterioscler., Thromb., Vasc. Biol.* 2013, 33(2):186–192.
[9] Dueck A, Meister G. Assembly and function of small RNA–Argonaute protein complexes. *Biol. Chem.* 2014, 395(6):611–629.
[10] Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, *et al.* Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci.* 2011, 108(12):5003–5008.
[11] Sanz-Rubio D, Martin-Burriel I, Gil A, Cubero P, Forner M, *et al.* Stability of Circulating Exosomal miRNAs in Healthy Subjects. *Sci. Rep.* 2018, 8(1):10306.
[12] Kosaka N, Yoshioka Y, Fujita Y, Ochiya T. Versatile roles of extracellular vesicles in cancer. *J. Clin. Invest.* 2016, 126(4):1163–1172.
[13] Ishikawa H, Yamada H, Taromaru N, Kondo K, Nagura A, *et al.* Stability of serum high-density lipoprotein-microRNAs for preanalytical conditions. *Ann. Clin. Biochem.* 2017, 54(1):134–142.
[14] López AG, Brogaard L, Heegaard PMH, Cirera S, Skovgaard K. AU Content in the MicroRNA Sequence Influences its Stability after Heat Treatment. *MicroRNA* 2019, 8(3):216–222.
[15] Coenen-Stass AML, Pauwels MJ, Hanson B, Martin Perez C, Conceição M, *et al.* Extracellular microRNAs exhibit sequence-dependent stability and cellular release kinetics. *RNA Biol.* 2019, 16(5):696–706.
[16] Faruq O, Vecchione A. microRNA: Diagnostic Perspective. *Front. Med.* 2015, 2:1–10.
[17] Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*. 2010, 101(10):2087–2092.
[18] Weber JA, Baxter DH, Zhang S, Huang DY, How Huang K, *et al.* The MicroRNA Spectrum in 12 Body Fluids. *Clin. Chem*. 2010, 56(11):1733–1741.
[19] Sourvinou IS, Markou A, Lianidou ES. Quantification of Circulating miRNAs in Plasma. *J. Mol. Diagn.* 2013, 15(6):827–834.
[20] Becker N, Lockwood CM. Pre-analytical variables in miRNA analysis. *Clin. Biochem*. 2013, 46(10–11):861–868.
[21] Borges DP, Cunha-Neto E, Bocchi EA, Rigaud VOC. Impact of Delayed Whole Blood Processing Time on Plasma Levels of miR-1 and miR-423-5p up to 24 Hours. *MicroRNA* 2018, 7(2):115–119.
[22] Makarem N, Mourad A, Nassar FJ. Circulating Micrornas: Potential Biomarkers in Cancer Detection, Diagnosis and Prognosis. *Leban. Med. J.* 2016, 64(2):116–122.
[23] Zaporozhchenko IA, Morozkin ES, Skvortsova TE, Ponomaryova AA, Rykova EY, *et al*. Plasma miR-19b and miR-183 as Potential Biomarkers of Lung Cancer. *PLoS One* 2016, 11(10):1–13.
[24] Lötvall J, Hill AF, Hochberg F, Buzás EI, Di Vizio D, *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J. Extracell. Vesicles* 2014, 3(1):26913.
[25] Konoshenko MY, Lekchnov EA, Bryzgunova OE, Kiseleva E, Pyshnaya IA, *et al*. Isolation of Extracellular Vesicles from Biological Fluids via the Aggregation–Precipitation Approach for Downstream miRNAs Detection. *Diagnostics* 2021, 11(3):384.

[26] Zaporozhchenko IA, Morozkin ES, Skvortsova TE, Bryzgunova OE, Bondar AA, *et al.* A phenol-free method for isolation of microRNA from biological fluids. *Anal. Biochem*. 2015, 479:43–47.
[27] Chen C. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*. 2005, 33(20):e179–e179.
[28] Chen X, Lou N, Ruan A, Qiu B, Yan Y, *et al.* miR-224/miR-141 ratio as a novel diagnostic biomarker in renal cell carcinoma. *Oncol. Lett.* 2018, 16(2):1666–1674.
[29] Zhang J, Song Y, Zhang C, Zhi X, Fu H, *et al.* Circulating MiR-16-5p and MiR-19b-3p as Two Novel Potential Biomarkers to Indicate Progression of Gastric Cancer. *Theranostics* 2015, 5(7):733–745.
[30] Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, *et al.* Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods* 2013, 59(1):S1–S6.
[31] Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, *et al.* Haemolysis during Sample Preparation Alters microRNA Content of Plasma. *PLoS One* 2011, 6(9):e24145.
[32] Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, *et al.* Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods* 2013, 59(1):S1–S6.
[33] Olive V, Bennett MJ, Walker JC, Ma C, Jiang I, *et al.* miR-19 is a key oncogenic component of mir-17-92. *Genes Dev*. 2009, 23(24):2839–2849.
[34] Hatiboglu MA, Karacam B, Khan I, Akdur K, Elbasan EB, *et al.* Liquid biopsy for CNS lymphoma: CSF exosomes and CSF exosomal miR-15a, miR-21, miR-155, miR-210, and miR-19b are promising biomarkers for diagnosis. *Mol. Biol. Rep.* 2024, 51(1):1035.
[35] Zhao Q, Shen L, Lü J, Xie H, Li D, *et al.* A circulating miR-19b-based model in diagnosis of human breast cancer. *Front. Mol. Biosci.* 2022, 9:980841.
[36] Duca RB, Massillo C, Dalton GN, Farré PL, Graña KD, *et al.* MiR-19b-3p and miR-101-3p as potential biomarkers for prostate cancer diagnosis and prognosis. *Am. J. Cancer Res.* 2021, 11(6):2802–2820.
[37] Dong L, Ye Y, Huang G, Tao H. The diagnostic value of serum miR-17-92 cluster in ischemic stroke. *Folia Neuropathol.* 2024, 62(2):206–214.
[38] Bulgakova O, Zhabayeva D, Kussainova A, Pulliero A, Izzotti A, *et al*. miR-19 in blood plasma reflects lung cancer occurrence but is not specifically associated with radon exposure. *Oncol. Lett*. 2018, 15(6):8816–8824.
[39] Kumarswamy R, Thum T. Non-coding RNAs in Cardiac Remodeling and Heart Failure. *Circ. Res*. 2013, 113(6):676–689.
[40] Bryzgunova O, Konoshenko M, Zaporozhchenko I, Yakovlev A, Laktionov P. Isolation of Cell-Free miRNA from Biological Fluids: Influencing Factors and Methods. *Diagnostics* 2021, 11(5):865.
[41] Geekiyanage H, Rayatpisheh S, Wohlschlegel JA, Brown R, Ambros V. Extracellular microRNAs in human circulation are associated with miRISC complexes that are accessible to anti-AGO2 antibody and can bind target mimic oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 2020, 117(39):24213–24223.
[42] Wang L, Wu J, Ye N, Li F, Zhan H, *et al.* Plasma-Derived Exosome MiR-19b Acts as a Diagnostic Marker for Pancreatic Cancer. *Front. Oncol.* 2021, 11:739111.
[43] Desmond BJ, Dennett ER, Danielson KM. Circulating Extracellular Vesicle MicroRNA as Diagnostic Biomarkers in Early Colorectal Cancer-A Review. *Cancers* 2019, 12(1):52.
[44] Temilola DO, Wium M, Paccez J, Salukazana AS, Otu HH, *et al.* Potential of miRNAs in Plasma Extracellular Vesicle for the Stratification of Prostate Cancer in a South African Population. *Cancers* 2023, 15(15):3968.
[45] Kamm RC, Smith AG. Ribonuclease activity in human plasma. *Clin. Biochem*. 1972, 5(1–4):198–200.
[46] Wang C, Liu H. Factors influencing degradation kinetics of mRNAs and half-lives of microRNAs, circRNAs, lncRNAs in blood *in vitro* using quantitative PCR. *Sci. Rep.* 2022, 12(1):7259.
[47] Tsui NBY, Ng EKO, Lo YMD. Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin. Chem.* 2002, 48(10):1647–1653.

[48] Wang JX, Gao J, Ding SL, Wang K, Jiao JQ, *et al.* Oxidative Modification of miR-184 Enables It to Target Bcl-xL and Bcl-w. *Mol. Cell* 2015, 59(1):50–61.
[49] Nair U, Bartsch H, Nair J. Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: A review of published adduct types and levels in humans. *Free Radic. Biol. Med.* 2007, 43(8):1109–1120.
[50] Kong Q, Lin CG. Oxidative damage to RNA: mechanisms, consequences, and diseases. *Cell. Mol. Life Sci.* 2010, 67:1817–1829.
[51] Li Z, Wu J, DeLeo C. RNA damage and surveillance under oxidative stress. *IUBMB Life* 2006, 58(10):581–588.
[52] Hermanson GT. *Bioconjugate Techniques*, 3rd ed. St Louis: Elsevier, 2013, pp. 127–229.
[53] McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. Analysis of circulating microRNA: Preanalytical and analytical challenges. *Clin. Chem*. 2011, 57(6):833–840.
[54] Trakunram K, Champoochana N, Chaniad P, Thongsuksai P, Raungrut P. MicroRNA Isolation by Trizol-Based Method and Its Stability in Stored Serum and cDNA Derivatives. *Asian Pac. J. Cancer Prev.* 2019, 20(6):1641–1647.
[55] Ge Q, Zhou Y, Lu J, Bai Y, Xie X, *et al*. miRNA in Plasma Exosome is Stable under Different Storage Conditions. *Molecules* 2014, 19(2):1568–1575.
[56] Görgens A, Corso G, Hagey DW, Jawad Wiklander R, Gustafsson MO, *et al.* Identification of storage conditions stabilizing extracellular vesicles preparations. *J. Extracell. Vesicles* 2022, 11(6):e12238.
[57] Tessier SN, Bookstaver LD, Angpraseuth C, Stannard CJ, Marques B, *et al.* Isolation of intact extracellular vesicles from cryopreserved samples. *PLoS One* 2021, 16(5):e0251290.
[58] Gelibter S, Marostica G, Mandelli A, Siciliani S, Podini P, *et al.* The impact of storage on extracellular vesicles: A systematic study. *J. Extracell. Vesicles* 2022, 11(2):e12162.