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Pre-storage centrifugation conditions have significant impact on measured microRNA levels in biobanked EDTA plasma samples

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ABSTRACT

Background: In the past few years, an increasing number of studies have reported the potential use of microRNAs (miRNA) as circulating biomarkers for diagnosis or prognosis of a wide variety of diseases. There is, however, a lack of reproducibility between studies. Due to the high miRNA content in platelets this may partly be explained by residual platelets in the plasma samples used. When collecting fresh plasma samples, it is possible to produce cell-free/platelet-poor plasma by centrifugation. In this study, we systematically investigated whether biobanked EDTA plasma samples could be processed to be suitable for miRNA analysis.

Materials and methods: Blood samples were collected from ten healthy volunteers and centrifuged to produce platelet-poor-plasma (PPP) and standard biobank plasma. After one week at −80 °C the biobanked EDTA plasma was re-centrifuged by different steps to remove residual platelets. Using RT-qPCR the levels of 14 miRNAs in the different plasma preparations were compared to that of PPP.

Results: We were able to remove residual platelets from biobanked EDTA plasma by re-centrifugation of the thawed samples. Nevertheless, for most of the investigated miRNAs, the miRNA level was significantly higher in the re-centrifuged biobanked plasma compared to PPP, even when the platelet count was reduced to 0–1 × 10^9/L.

Conclusion: We found, that pre-storage centrifugation conditions have a significant impact on the measured EDTA plasma level of miRNAs known to be present in platelets. Even for the miRNAs found to be less effected, we showed that a 1.5–3 fold change in plasma levels may possible be caused by or easily overseen due to sample preparation and/or storage.

1. Introduction

MicroRNAs (miRNAs) are short, single-stranded non-coding RNAs acting as posttranscriptional regulators of gene expression [1]. In the circulation cell-free miRNAs are protected against degradation as they are included in microvesicles or exosomes or they are bound to high-density lipoproteins or to the argonaute 2 protein complex [2]. MiRNAs were first identified in Caenorhabditis elegans in 1993 [3], but it took almost a decade before they were discovered in mammals [4,5], and the first reports of circulating miRNAs came in 2008 [6,7]. In the past few years, an increasing number of studies have reported the potential use of miRNAs as circulating biomarkers for diagnosis or prognosis of a wide variety of diseases such as cancers [8,9], cardiovascular diseases [10], diabetes [11] and mental illness [12]. However, in only a minority of studies where miRNAs were reported to be differently expressed in healthy and diseased individuals, the results have been confirmed by others [13]. Pre-analytical conditions are a major source of variation in and between miRNA studies, and have been addressed by several studies in the past few years [2,14–19]. An important factor to consider is cellular remnants in the samples, for example in hemolysis where disruption of erythrocytes causes a significant increase in the plasma/serum levels of many miRNAs [15]. Furthermore, due to residual platelets, the levels of some miRNAs are higher in routine plasma samples compared to routine serum samples [14], and certain other miRNAs are found in higher levels in serum compared to plasma, probably due to their release from leucocytes and/or platelets during the coagulation process [20]. When collecting fresh samples, it is possible by additional centrifugation steps to produce cell-free/platelet-poor plasma, but many studies are performed using biobanked routine plasma samples with variable number of residual platelets. In this study we will systematically investigate

Abbreviations: PPP, platelet-poor-plasma; RT-qPCR, reverse transcription polymerase chain reaction; °C, degrees Celcius

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whether biobanked EDTA plasma samples can be processed to be suitable for miRNA analysis, when investigating the plasma levels of miRNAs known to be present in platelets.

2. Materials and methods

From each of 10 healthy volunteers 3×10 mL of K_2-EDTA anticoagulated venous blood were collected using a 21 gauge needle after a minimum of venous stasis, and discard of the first 5 mL of blood drawn (tubes and needles were obtained from Becton-Dickinson, Franklin Lakes, NJ, USA).

A schematic overview of the sample preparation can be seen in Fig. 1. All the centrifugation steps were carried out at 20 °C.

In order to obtain platelet-poor-plasma (PPP) 10 mL of EDTA anticoagulated whole blood was centrifuged at 3000 g for 15 min. After centrifugation the plasma phase was carefully transferred to another tube, leaving approximately one mL of plasma on top of the buffy coat. The centrifugation step was repeated on the remaining plasma, and again approximately one mL was left in the bottom of the tube when the platelet-poor-plasma was transferred into cryo-tubes (labeled PPP) for storage at −80 °C.

Ten mL of EDTA anticoagulated whole blood was centrifuged at 2000 g for 10 min, which is standard procedure in our laboratory when collecting plasma for biobanking. After centrifugation the total amount of plasma from the 10 mL collection tube was transferred to another tube (labeled biobank), and stored at −80 °C. After one week the biobank-plasma was thawed and gently mixed. A volume of 1.5 mL was transferred to a cryotube (labeled biobank A). The remaining material was centrifuged at 3000 g for 15 min, and the plasma was transferred to a cryotube (labeled biobank B) leaving approximately one mL in the bottom of the centrifuge tube.

From each of the two plasmas (biobank A/B) 300 µL of plasma was collected for miRNA-isolation, before the remaining plasma was further centrifuged. Biobank A samples were centrifuged at 3000 g for 30 min and biobank B samples at 3000 g for 15 min. The prolonged centrifugation of the biobank A samples was performed, to investigate whether a longer one-step centrifugation could eliminate platelet contamination as efficient as a shorter two-step centrifugation. After centrifugation the plasma was transferred to new tubes (labeled biobank A2 and B2, respectively), leaving approximately 200 µL in the bottom of the centrifuged tubes. From each of the two plasmas (biobank A2/B2) 300 µL of plasma was collected for miRNA-isolation. MiRNA was isolated, as described below, immediately following the plasma preparation procedure without any further freezing/thawing of the samples.

In order to investigate the distribution of platelets in the plasma phase 10 mL of EDTA anticoagulated whole blood was centrifuged at 2000 g for 10 min. After centrifugation the plasma was transferred to new tubes one mL at a time from the top of the collection tubes. In total, 4 mL of plasma (labeled gradient A to D) was collected from each 10 mL whole blood.

After each centrifugation step and from each gradient-sample 200 µL of plasma was collected for platelet count analysis which was performed using the fully automated Sysmex XE 5000 analyzer (Sysmex, Kobe, Japan).

2.1. MiRNA analysis

MiRNA was isolated from 300 µL of each of the plasma preparations PPP, Biobank A, Biobank B, Biobank A2 (only the six samples with a platelet count of 0–1×10^9/L) and Biobank B2 using Nucleospin®miRNA Plasma (Macherey-nagel, Germany) and according to protocol supplied by the manufacturer. As a mean of normalization all samples were spiked with 5 µL Cel-miR-39 (2.75×10^{-12} M) (RiboTask, Odense, Denmark). The spike was added after removal of the plasma proteins in the same step as isopropanol. MiRNA was eluted using 30 µL of RNAse free water, and the samples were frozen at −20 °C. MiRNA-analysis was performed within one month from the time of miRNA-isolation.

cDNA synthesis was performed using TaqMan®MicroRNA Reverse Transcription Kit and Custom TaqMan MIR RT Pool (both from Applied Biosystems, Life Sciences). The RT-primer pool, which was designed for a previous study of platelet derived

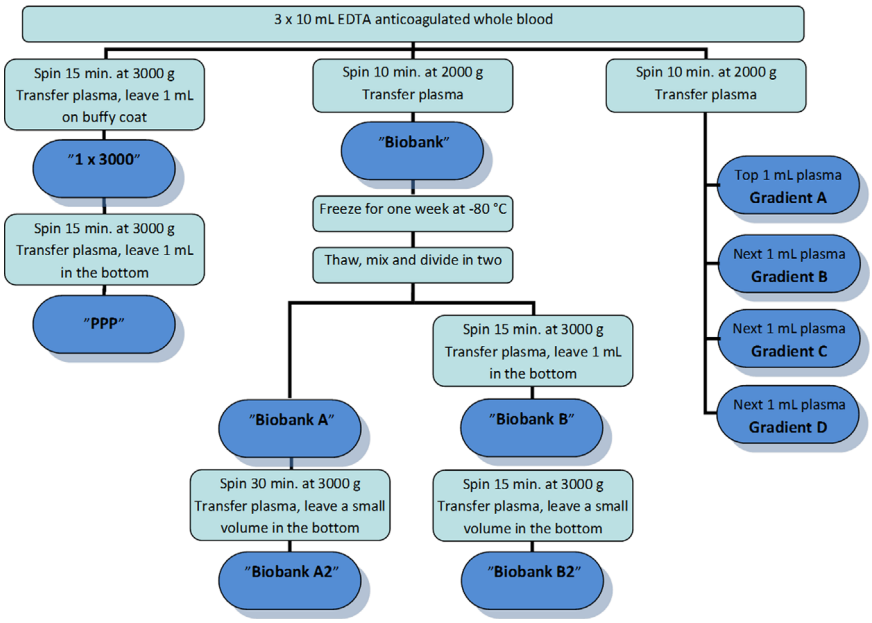

Fig. 1. Schematic overview over the different plasma preparations. A total of 10 mL of whole blood was used for the preparation of platelet poor plasma (PPP). Two tubes of 10 mL whole blood were handled according to our laboratory protocol for preparation of biobank plasma. Plasma from one of the tubes was frozen for one week, thawed and further processed to eliminate platelet contamination (biobank A, A2, B and B2). As illustrated, after freezing and thawing of the biobank plasma we denote it biobank A. Plasma from the other tube was pipetted from the top of the tube mL by mL to investigate the distribution of platelets in the plasma phase (gradient A–D).