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Research paper

The impact of various preanalytical treatments on the phenotype of small extracellular vesicles in blood analyzed by protein microarray



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ABSTRACT

The research field of extracellular vesicles (EVs) is increasing immensely and the potential uses of EVs seem endless. They are found in large numbers in various body fluids, and blood samples may well serve as liquid biopsies. However, these small membrane-derived entities of cellular origin are not straightforward to work with in regard to isolation and characterization.

A broad range of relevant preanalytical issues was tested, with a focus on the phenotypic impact of smaller EVs. The influences of the i) blood collection tube used, ii) incubation time before the initial centrifugation, iii) transportation/physical stress, iv) storage temperature and time (short term and long term), v) choice of centrifugation protocol, vi) freeze-thaw cycles, and vii) exosome isolation procedure (ExoQuick™) were examined. To identify the impact of the preanalytical treatments, the relative amounts (detected signal intensities of CD9-, CD63- and/or CD81-positive) and phenotypes of small EVs were analyzed using the multiplexed antibody-based microarray technology, termed the EV Array. The analysis encompassed 15 surface- or surface-related markers, including CD9, CD63, CD81, CD142, and Annexin V.

This study revealed that samples collected in different blood collection tubes suffered to varying degrees from the preanalytical treatments tested here. There is no unequivocal answer to the questions asked. However, in general, the period of time and prospective transportation before the initial centrifugation, choice of centrifugation protocol, and storage temperature were observed to have major impacts on the samples. On the contrary, long-term storage and freeze-thawing seemed to not have a critical influence. Hence, there are pros and cons of any choice regarding sample collection and preparation and may very well be analysis dependent. However, to compare samples and results, it is important to ensure that all samples are of the same type and have been handled similarly.

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1. Introduction

In recent years, the interest in extracellular vesicles (EVs) has increased immensely (Løtvall et al., 2014) and several studies have shown the potential of utilizing them in a clinical setting, as diagnostic, prognostic and as therapeutic agents, as reviewed by Revenfeld et al. (Revenfeld et al., 2014) and György et al. (György et al., 2015). Furthermore, it has proven relevant to investigate immune cell-derived EVs, as they appear to be important in several immunological relations (Robbins and Morelli, 2014; Pugholm et al., 2016). EVs are small membrane-derived entities produced from a diverse range of cell types throughout the human body and, therefore, they are accessible in various body fluids (Caby et al., 2005; Admyre et al., 2007; Ogawa et al., 2008; Gonzales et al., 2009). These vesicles can be divided into several subgroups according to specific characteristics such as cellular origin,

size, protein/RNA composition, and biogenesis. When sorting using the latter characteristic, the major subgroups are exosomes (30–100 nm in diameter), microvesicles (MVs, 100–1000 nm) and apoptotic bodies (500–4000 nm) (Pugholm et al., 2015), although MVs are also often denoted microparticles (MPs) (Colombo et al., 2014). Each of these subgroups possess numerous biological functions, and it is of interest to determine them thoroughly to fully understand and utilize the vesicular biology (Mulcahy et al., 2014).

A major challenge when working with EVs is the pronounced impact that the preanalytical treatment has on the analysis outcome. Many investigators have highlighted the importance of a consistent protocol for sample collection and preparation of EVs (Lacroix et al., 2010; György et al., 2011; Yuana et al., 2011; Løtvall et al., 2014); however, it is also relevant to consider which protocols are the best suited for the research question addressed (Witwer et al., 2013). The choice of anticoagulant in the blood collection tube has a considerable influence on the MV/MP count (Jayachandran et al., 2012; György et al., 2014), as has the incubation time between blood collection and centrifugation, with an up to 80% increase in the MP count after 4 h (Lacroix et al., 2012). One very

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well-described preanalytical factor is the centrifugation procedure. A standardized protocol for the preparation of platelet-free plasma (PFP) has been suggested and it has shown to be of great importance when analyzing MVs/MPs (Lacroix et al., 2012). However, it is hard to obtain strictly platelet-free plasma and consequently it is urged that the same protocol is applied to all samples that are to be compared (Witwer et al., 2013).

Factors such as freezing, storage temperature and time, freeze-thaw cycles, and transportation have been examined as well (György et al., 2014; Jayachandran et al., 2012; Dey-Hazra et al., 2010; Lacroix et al., 2012). Nevertheless, these features are somewhat influenced by the method of isolation and subsequent choice of analysis. However, all of these factors have primarily been investigated regarding MVs/MPs, whereas there is little knowledge of the impact of the smaller vesicle types (exosomes and exosome-like vesicles), especially in regard to protein load.

Analysis of biobank samples is typically an ongoing process years after collection; hence, a challenge is that the material is often quite old. Other issues are that the samples may have been exposed to several freeze-thaw cycles, and/or that the blood has been collected in tubes that were most optimal for the first analysis in mind. Therefore, it is critical to determine the preanalytical impact as thoroughly as possible and optimally for all types of analyses and for all types of EVs.

To determine the phenotypes of small EVs (sEVs), we have established a protein microarray-based analysis, which is termed the EV Array. This analysis platform, described by Jørgensen et al. (Jørgensen et al., 2013), is optimized to catch and detect the smaller types of EVs, such as exosomes and exosome-like vesicles, with diameters up to ~150 nm. The detection is performed by utilizing a cocktail of antibodies against the tetraspanins CD9, CD63, and CD81, which are found on exosomes (Vlassov et al., 2012). The detection antibodies are easily exchangeable, as are the capture antibodies, which can be combined as desired and target up to 60 different markers simultaneously in the same well (Jørgensen et al., 2015). The analysis is performed in a 96-well setup and consumes only 10 µL of plasma, which makes the platform very cost-efficient, multiplexed and high-throughput. It has already demonstrated great diagnostic potential in non-small cell lung cancer (NSCLC), where cancer patients were distinguished from non-

cancer lung-diseased patients with up to 75.3% accuracy (Jakobsen et al., 2015; Sandfeld-Paulsen et al., 2016).

As Choi et al. (Choi et al., 2013) previously noted, a number of limitations and challenges prevent EVs from being used diagnostically at present. The goal is a rapidly performed and low-cost analysis platform, which meets the limited decision time of the clinician. However, aspects such as preanalytical standardization and development of a reliable technique must be addressed initially. Here, we aim to complement the current knowledge of preanalytical factors with a focus on phenotyping sEVs using an antibody-based platform designed to include 15 relevant markers. Using four different types of blood collection tubes, blood from five healthy volunteers was analyzed and a broad range of preanalytical factors was tested, as illustrated in Fig. 1.

2. Materials and methods

2.1. Blood sampling

All research involving samples from human subjects was approved by the local ethics legislation. Each person signed a written consent form allowing for the use of their blood for research purposes. Venous blood samples were obtained from five healthy volunteers and collected into four different types of Vacuette® blood collection tubes (Greiner Bio-One GmbH, Germany): CPDA (citrate phosphate dextrose adenine), EDTA (K3EDTA, ethylenediaminetetraacetic acid), heparin (Lithium Heparin) and serum (Z Serum Clot activator) tubes. The same number of tubes was collected for each Vacuette® type from the same donor at once within the same laboratory where the samples were to be handled afterwards. Subsequently, the preanalytical treatments and tests were performed simultaneously on all four Vacuette® types as specified in the following.

Unless otherwise specified, the blood samples were centrifuged once (1800g for 6 min at room temperature, RT) 1 h after collection, before aliquoting and storage at -40°C . Furthermore, the samples were analyzed within a few days of sample collection. The same experienced operator carried out the handling of all samples and analyses.

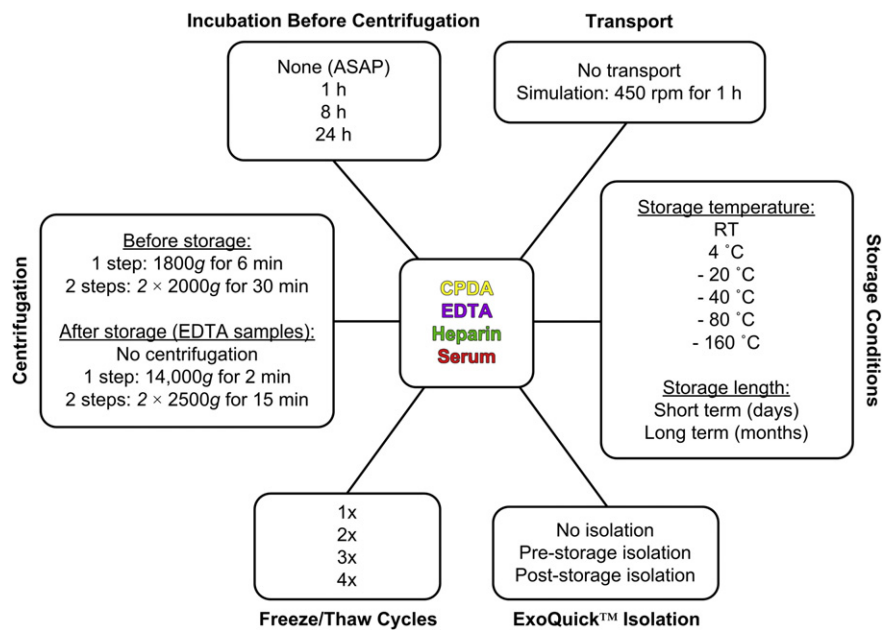


Fig. 1. Schematic overview of the preanalytical factors tested. Five healthy volunteers were included in the study and samples were assembled using the Vacuette® blood collection tubes CPDA, EDTA, heparin, and serum. Unless otherwise specified, blood collection tubes were centrifuged once at 1800g for 6 min 1 h after collection and sample aliquots were stored at -40°C . ASAP, as soon as possible; h, hour; RT, room temperature; rpm, rounds per minute.

2.2. Incubation time before centrifugation

After collection of the blood samples, one tube of each Vacuette® type from all five donors was centrifuged as soon as possible (ASAP) within a few minutes. An equivalent range of Vacuette® tubes was incubated at RT for 8 h and 24 h.

2.3. Transportation

To simulate transportation, one tube of each Vacuette® type from all five donors was placed on an orbital shaker adjusted to 450 rpm during the 1 h incubation period before centrifugation.

2.4. Additional centrifugations

One tube of each Vacuette® type from all five donors was exposed to a two-step centrifugation. The samples were centrifuged at 2000g for 30 min at 15 °C. Then, two-thirds of the plasma/serum was transferred to new 15 mL tubes and the centrifugation was repeated. Finally, the top two-thirds of the supernatant was aliquoted and stored.

For all five donors, one EDTA sample aliquot stored at –40 °C was thawed and two volumes of 50 µL were transferred to new tubes; one aliquot was centrifuged once at 14,000g for 2 min, and one was centrifuged twice at 2500g for 15 min. In those aliquots, 40 µL of sample was removed between the two centrifugations and transferred to a new tube. The obtained aliquots were analyzed immediately after the centrifugations were completed.

2.5. Influence of storage conditions and freeze-thaw cycles

For all donors and Vacuette® types, aliquots from blood samples were stored at different temperatures; RT, 4 °C, –20 °C, –40 °C, –80 °C, and –160 °C. To test the influence of storage temperature and time, blood was collected from the same donors twice with a time gap of approximately 3 months. All samples were analyzed at the same time and were defined as short term and long term sample storage, respectively.

Three aliquots from each donor and Vacuette® type stored at –40 °C were thawed and refrozen one, two, or three times, respectively, to test the influence of up to four freeze-thaw cycles.

2.6. ExoQuick™ isolation of exosomes

Two 50 µL aliquots from each donor and Vacuette® type stored at –40 °C were designated for ExoQuick™ isolation of exosomes. One aliquot was handled before storage while the other was handled after thawing, immediately before analysis. The precipitations were performed according to the manufacturer's instructions. In short, 12 µL ExoQuick™ Precipitation Solution (System Biosciences, CA, USA) was added to each aliquot and the samples were incubated for 30 min at 4 °C. After centrifugation at 1500g for 30 min, the supernatants were discarded. Each pellet was suspended in 50 µL sterile water.

2.7. Production of protein microarrays

Printing of the protein microarray slides to the EV Array analysis was performed on a SpotBot® Extreme Protein Edition Microarray Printer using a 946MP4 pin (ArrayIt Corporation, CA, USA). As a positive control, 100 µg/mL of biotinylated human IgG was printed and PBS with 5% glycerol was applied as the negative control. Antibodies were printed in triplicate on epoxy-coated glass slides (75.6 × 25.0 mm; SCHOTT Nexterion, Germany), which were left to dry at RT overnight prior to further analysis. Two types of EV Arrays were produced; one for phenotyping and one for semi-quantification.

2.7.1. EV Array for phenotyping

The anti-human antibodies used are listed in Table 1. All antibodies were diluted in PBS with 5% glycerol and printed at 200 µg/mL. This EV Array setup was used to analyze all samples and is denoted *Phenotyping Array* in the following.

2.7.2. EV Array for semi-quantification

A mixture/cocktail of anti-human antibodies against CD9, CD63, and CD81 (likewise included in the *Phenotyping Array*), each in the concentration 100 µg/mL, was prepared with PBS containing 5% glycerol and printed with the positive and negative controls. This EV Array was used to analyze all samples and is denoted *Cocktail Array* in the following.

2.8. EV Array analysis

The EV Array analyses were performed as described by Jørgensen et al. (Jørgensen et al., 2013). In short, the printed slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) prior to incubation with 10 µL plasma or serum sample diluted (1:10) in wash-buffer (0.05% Tween®20 in PBS). The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation, CA, USA) at RT for 2 h using an orbital shaker (450 rpm) and subsequently overnight at 4 °C (no shaking). Following a short wash, the slides were incubated for 2 h with a cocktail of biotinylated detection antibodies (anti-human CD9, CD63, and CD81, LifeSpan BioSciences, WA, USA) diluted 1:1500 in wash-buffer. After washing, a subsequent 30 min incubation step with Cy5-labelled streptavidin (Life Technologies, CA, USA) diluted 1:1500 in wash-buffer was carried out for detection. Prior to scanning, the slides were washed first in wash-buffer and then in deionized water, followed by drying using a Microarray High-Speed Centrifuge (ArrayIt Corporation, CA, USA). Scanning at 635 nm and subsequent detection were performed as previously described.

2.9. Data analysis

The statistical analyses and the generation of graphs were performed using Excel 2013 (Microsoft, WA, USA) and GraphPad Prism 6.04 (GraphPad Software Inc., CA, USA). Mean signal intensities of the triplicate antibody spots were used and corrected for nonspecific binding from the detection antibodies by subtracting the mean signal intensities from a blank well containing no sample. For each capturing antibody, the corrected intensities were expressed in relation to the mean signal of negative spots (containing PBS). The antibody signals

Table 1

List of antibodies used to produce the EV Array for phenotyping; the *Phenotyping Array*. Along with the company and catalogue number, the clones are given (when available). CD, Cluster of Differentiation; TNF RI, Tumor Necrosis Factor Receptor 1; TSG101, Tumor Susceptibility Gene 101; ICAM-1, Intercellular Adhesion Molecule 1.

Company	Anti-human antibody	Catalogue no.	Clone
R&D Systems (MN, USA)	AnnexinV	AF399	–
	CD19	MAB4867	4G7-2E3
	CD106	MAB809	HAE-2Z
	CD142	MAB2339	323514
	TNF RI	840237	–
LifeSpan BioSciences (WA, USA)	CD9	LS-C35418	–
	CD42a	LS-C45240	–
	CD81	LS-B7347	–
Biolegend (CA, USA)	Alix	634501	3A9
	CD63	312002	MEM-259
Thermo Scientific (MA, USA)	CD62E	MA1-22165	–
BD Biosciences (CA, USA)	CD3	555337	Hit3a
Abcam (MA, USA)	Flotillin-1	ab41927	–
	TSG101	ab117627	–
eBiosciences (CA, USA)	ICAM-1	BMS1011	R6.5

were log₂ transformed prior to visualization and statistical comparisons, which were performed using non-parametric paired t-tests. The data obtained using the *Cocktail Array* are a total of the signal intensities of all three markers (CD9, CD63 and CD81). Hence, the data are treated as one marker, as described above.

3. Results and discussion

Using the multiplexed, highly sensitive and high-throughput antibody-based platform of the EV Array, we have investigated a broad range of preanalytical factors that could be of importance when analyzing sEVs. Previously, we have examined the technical variability of the EV Array and determined the coefficient of variation as 2.9% (Jørgensen et al., 2015). In this study, we have only focused on sEVs positive for CD9, CD63, and/or CD81, as these molecules are used for detection with the EV Array in the current setup. Since the three markers are detected with a cocktail of antibodies against them, the resulting data are a summation of the obtained signal intensities. The study is based on plasma from five healthy donors. As previously described, the plasma content of sEVs vary greatly among individuals (Jørgensen et al., 2013; Bæk et al., 2016); consequently, the results presented here do not account for this variation. Hence, the data are presented by dot plots to best illustrate the overall influence of the preanalytical treatments on the content of sEVs.

Generally, using only one technique is discouraged, whereas additional analyses could have contributed to a stronger data-set. However, often-used quantitative analyses such as Nano Tracking Analysis and Scanning Ion Occlusion Sensing are observed to be influenced by liposomes/lipoproteins and other aggregates in plasma samples, which

complicates the interpretation of the results. When presenting data obtained with the *Cocktail Array*, the outcome is semi-quantitative and is therefore an indicator for the quantity of the detected sEVs. Hence, the *Cocktail Array* was implemented as an attempt to compensate for the missing additional analysis.

3.1. Does the time between blood sampling and centrifugation have an influence on the sEVs?

To test whether the time range from blood collection to isolation of plasma/serum has an impact on the subsequent analysis, samples from all donors and blood collection tubes were incubated at RT for 1 h, 8 h, and 24 h prior to centrifugation. These samples were compared with samples centrifuged immediately after sample collection, as illustrated in Fig. 2, although it was not possible to obtain useful samples from the serum tubes at this time point.

The mean values for the detected signal intensities of sEVs in samples collected in heparin and serum tubes displayed no significant changes over time (Fig. 2A). However, for samples collected in CPDA and EDTA tubes, the incubation time had an obvious influence on the content of sEVs. The trends were similar for the two glass types; when compared to the samples centrifuged ASAP, the detected signal intensities of sEVs underwent a great decrease after a 1 h incubation. After an 8 h incubation, the detected signal intensities of the sEVs had increased, and after 24 h, the intensities were similar to the values obtained when the centrifugation was performed ASAP. The apparent decrease in the signal intensities of the sEVs happening within the first hour of incubation could be speculated to be caused by the activation of the platelets present in the samples. EDTA almost immediately activates platelets,

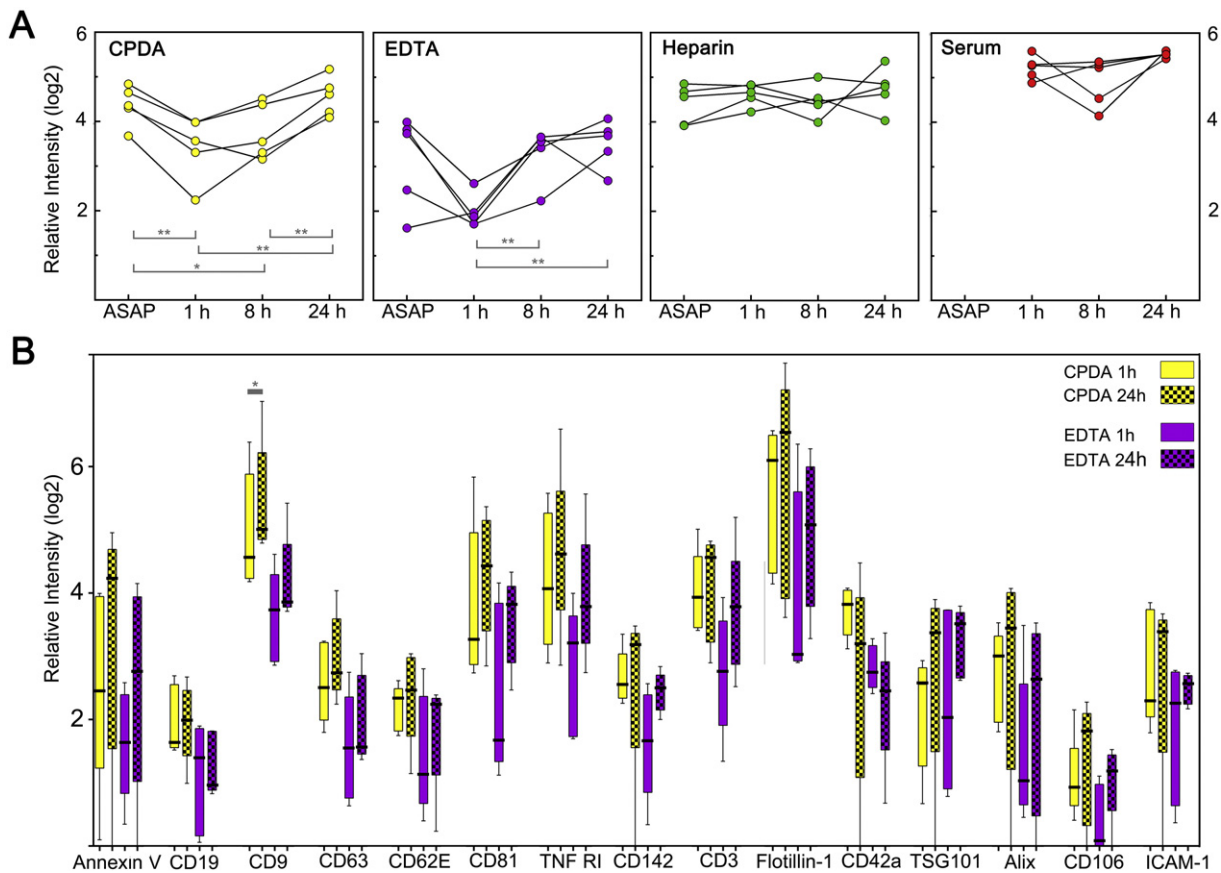


Fig. 2. The influence of incubation time before initial centrifugation. Blood samples were incubated at RT for 1 h, 8 h, and 24 h before centrifugation and compared with samples centrifuged as soon as possible (ASAP) after sampling. The data are presented as log₂ transformed relative intensities of all five donors. A) Dot plots illustrating the semi-quantitative data obtained using the *Cocktail Array* for the Vacuette® blood collection tubes CPDA, EDTA, heparin, and serum. The lines connect the samples from each individual donor. B) Data from samples collected in CPDA and EDTA blood collection tubes are illustrated using box plots indicating mean, min and max values. Data are shown for all markers included in the *Phenotyping Array*. Statistically significant differences are indicated with p-values: *p < 0.05; **p < 0.01.

causing a rapid change in shape (Macey et al., 2002). Sodium citrate causes a less spontaneous activation; hence, when blood is collected into citrate, there is initially little or no change in platelet shape and volume. However, subsequently the platelets slowly adopt a spherical shape and, as in EDTA tubes, swell progressively over a period of 1–2 h (Ahnadi et al., 2003). Using scattering measurements, Macey et al. found that the mean platelet volume decreased significantly during the first 30–60 min when CPDA and EDTA tubes were used. The initial mean platelet volume was regained after 3 h. During the process of activation and change of shape, the platelets rearrange their membrane structures and enhance the amounts of several of their surface molecules (Golanski et al., 1996). Taken together, it could be speculated that the sEVs present in the CPDA and EDTA blood collection tubes are adhering to the platelets during the process of activation and, therefore, are removed with the cells during centrifugation. When the platelet activation and surface rearrangements have been undergone, the nonspecific sEV adhesion is probably relieved by the aggregation of platelets and leukocytes, which could explain the observed increase in detected signal intensities of sEVs after 8 h and 24 h.

During the process of activation, platelets degranulate and produce large amounts of Annexin V positive MPs (size range > 300 nm) (Golanski et al., 1996; Yuana et al., 2011). Lacroix et al. found that the MP count in citrated plasma increased within the first hour compared to the samples centrifuged within minutes. After a 4 h incubation, the MP count had increased approximately 80% (Lacroix et al., 2012). In this study, we measured the sEVs presenting CD9, CD63 and/or CD81 on the surface, whereas Lacroix et al. focused on the larger MPs defined as Annexin V positive vesicles, which explains the disagreements in the results.

In Fig. 2B, the signal intensities obtained with the more specific *Phenotyping Array* of the individual EV markers from CPDA and EDTA samples incubated for 1 h and 24 h are shown. In general, for both sample types, the tendency leans towards an increase in specific sEV signal intensities when increasing the incubation time from 1 h to 24 h, although only CD9 changes significantly ($p < 0.05$). However, CD19, CD42a and ICAM-1 are exceptions to this trend, of which the platelet glycoprotein CD42a diverged the most.

When collecting samples in the clinic, it is difficult to perform the centrifugation within minutes. Of the tested time delays in this study, the 1 h incubation resulted in the lowest detected signal intensities of sEVs, probably because the sEVs are removed with the cells. The standard incubation time in this study was set to 1 h, which matches the recommendations from both the manufacturer of the blood collection tubes and the study of Lacroix et al. (Lacroix et al., 2012). Because of the seemingly induced events in the CPDA and EDTA blood collection tubes, we suggest to either perform the centrifugation immediately after the collection or wait 24 h before centrifugation to avoid the suspected removal of sEVs.

3.2. Does transportation alter the sEV content in blood samples?

To examine the impact of transportation or similar mechanical stress, the blood samples were exposed to agitation for 1 h using an orbital shaker (450 rpm), which should simulate vehicle transportation from one facility to another. The results are shown in Fig. 3.

A general tendency towards a change in the content of sEVs was seen when the blood samples were transported/stressed before isolation of the plasma/serum. The use of CPDA blood collection tubes led to an increase in the detected signal intensities of the sEVs that was not statistically significant (Fig. 3A). Two other studies confirm this tendency observed in the citrate-containing samples, even though other types of citrate-containing tubes were used and the transport simulations were performed differently (Lacroix et al., 2012; György et al., 2014). Both of these studies targeted Annexin V positive MPs and the increases observed were of major orders compared to the increases

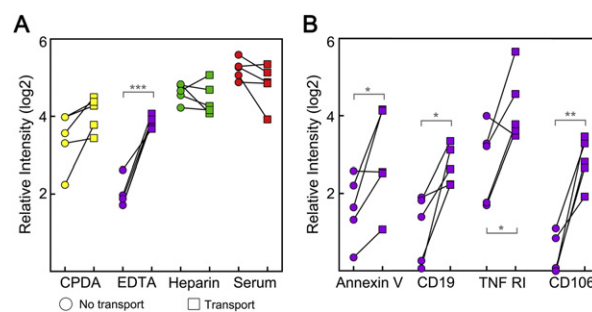


Fig. 3. The influence of transporting the blood samples. Blood samples were placed on an orbital shaker for 1 h at 450 rpm to simulate transport and were compared with samples incubated 1 h without shaking. The data are presented as log₂ transformed relative intensities of all five donors illustrated by dot plots where the lines connect the samples from each individual donor. A) Semi-quantitative data obtained using the *Cocktail Array* for the Vacuette® blood collection tubes CPDA, EDTA, heparin, and serum. B) Data from samples collected in EDTA blood collection tubes. Data are shown for the four markers Annexin V, CD19, TNF RI, and CD106 obtained with the *Phenotyping Array*. Statistically significant differences are indicated with p-values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

presented for the CPDA samples here, which, however, are representative for sEVs. Hence, some differences are to be expected.

Likewise, when collecting blood in EDTA tubes, increased signal intensities were observed in the transported/stressed samples in the data obtained with the *Cocktail Array* (Fig. 3A); however, here the increase was significant ($p < 0.001$). The data obtained with the *Phenotyping Array* revealed that the markers providing the main contribution to the overall increasing signal were Annexin V, CD19, TNF RI, and CD106 (Fig. 3B). Annexin V is often used to identify MVs/MPs; however, it has also been observed in the small EV subsets (Kowal et al., 2016). The fact that Annexin V in this study contributed to the massive increase could indicate that smaller MVs were captured on the EV Array along with exosomes and exosome-like vesicles. The significant increase of the other three markers indicates that other cells than platelets are stressed during transport as well, although platelets are often considered as the main contributor of in vitro vesiculation (Bode et al., 1991). The remaining markers detected with the *Phenotyping Array* also increased; however, these differences were not statistically significant (data not shown). The observed increases could be related to the platelet activation events described in Section 3.1. The lowest detected signal intensities for the CPDA and EDTA plasma samples were seen when samples were centrifuged after 1 h of collection. We speculate that the sEVs adhere to the platelets during the string of activation events and are subsequently released within the following hours. The physical impact of the transport simulation could be causing the sEVs to loosen more rapidly, which would explain the observed increase.

Since transportation does influence EV analyses, the preferable option would be to centrifuge the samples at the site of collection. However, a centrifuge is not always at hand and transportation may be impossible to avoid. Lacroix et al. (Lacroix et al., 2012) did, however, succeed in making the influence insignificant by immobilizing the blood collection tubes in a vertical fashion. This was shown for citrated plasma and whether it is likewise effective for other types of blood collection tubes is uncertain. Nevertheless, it could be an acceptable and easily adaptable solution and should be tested further.

3.3. What impact does storage temperature and period have on sEVs?

To include samples from a pre-collected biobank in a study, a relevant question to answer is what influence different storage temperatures and periods have on the samples in relation to the type of blood collection tube used. Six storage temperatures ranging from -160°C to RT were tested at two time points representing short term (days) and long term (months) storage. The results obtained using the *Cocktail Array* are presented in Fig. 4.

Fig. 4 shows that storage temperature has a high impact on the outcome of the analysis. The detected signal intensities of sEVs change almost randomly when the temperature and type of blood collection tube change, although some trends can be identified (Fig. 4A and B). Initially, the differences in the detected signal intensities are less pronounced when disregarding the results from samples stored at RT and 4 °C. In doing so, the progress of the graphs is somewhat similar within the same Vacuette® types comparing short term with long term storage. However, the results obtained from serum samples diverge, in that the signal intensities of sEVs decrease with lower storage temperature under short term storage (Fig. 4A), whereas the levels appear more constant under long term storage (Fig. 4B). When freezing the samples, it seems that the most stable samples are the ones collected in heparin blood collection tubes and the most unstable are the ones collected in EDTA tubes.

The samples stored at RT and 4 °C displayed a trend towards increasing signal intensities with lower temperatures for long term storage (Fig. 4B) but decreasing signal intensities for short term storage (Fig. 4A). In the latter situation, CPDA plasma samples present somewhat constant signal intensities.

When analyzing the data obtained with the EV Array, signals obtained from corresponding antibody spots in a well only containing wash buffer were utilized to account for nonspecific binding of the detection antibodies. After subtracting the mean signal obtained from the blank well from the corresponding antibody-spot in the sample well, the corrected signal was divided by the mean signal from the blank spots in the same sample well. This accounts for the different background signals individuals may induce; hence, limiting the background noise can be of considerable importance.

In Fig. 4C, the raw scanning of the microarray spots are visualized from the samples stored short term. As the individual sample

background noise is accounted for when calculating the relative intensities, the results are highly influenced by intense backgrounds. As observed in Fig. 4C, the background intensities in the EDTA plasma samples vary greatly with the most severe examples seen for the lowest temperatures. This explains why EDTA plasma samples are more influenced by different temperatures, as observed in Fig. 4A.

The literature is not in agreement as to the effect of temperature and storage period on EVs. One study concluded that freezing of plasma at either –40 °C or –80 °C had no relevant impact on MV count when isolated from PPP (platelet-poor plasma) or PFP (Jayachandran et al., 2012). These samples were stored for more than one year. If the MVs were isolated, however, they could be stored at RT for a few days without being unstable, whereas freezing led to lower counts. In the study of Dey-Hazra et al. (Dey-Hazra et al., 2010), the MP count obtained from citrated plasma stored at –80 °C changed over time, increasing within the first two weeks and then decreasing. Hence, they propose that fresh samples are the most ideal. Lacroix et al. (Lacroix et al., 2012) similarly observed an increase in MP count in citrated plasma samples after one week of storage at the same temperature (–80 °C) followed by a decrease, though the changes were less pronounced. However, after one year of storage, the change in MP count was only minor. Hence, the study of Lacroix et al. agrees with the study of Jayachandran et al. (Jayachandran et al., 2012) that long term storage has only a minor influence on the EV count.

Although we observed contrary results for short and long term storage, and none of the mentioned studies (ours as well as others) fully agree on what should be practice, it should be kept in mind that the compared results were obtained for different EV populations. However, some agreement can be found. It is rarely possible to use fresh samples in large-scale studies. Neither is it always possible to influence the

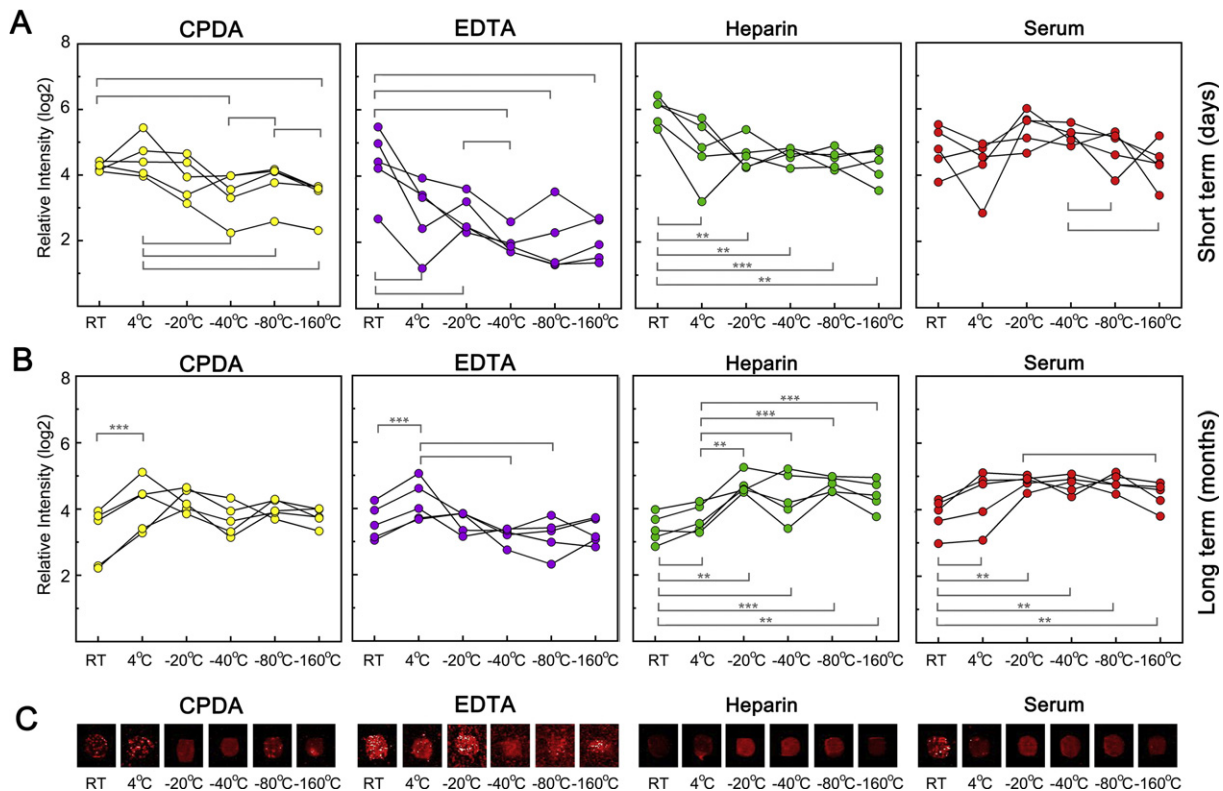


Fig. 4. The impact of storage temperature and period. Blood samples obtained at two different time points were stored at different temperatures (RT, 4 °C, –20 °C, –40 °C, –80 °C, and –160 °C) after isolation of plasma/serum for either A) short term (days) or B) long term (months) periods. The data were obtained using the Cocktail Array for the Vacuette® blood collection tubes CPDA, EDTA, heparin, and serum. Data are presented as log2 transformed relative intensities of all five donors illustrated by dot plots where the lines connect the samples from each individual donor. Statistically significant differences are indicated with p-values, which are * unless otherwise detailed: *p < 0.05; **p < 0.01; ***p < 0.001. C) Unrefined microarray spots from one representative donor obtained from Cocktail Array slides for all Vacuette® blood collection tubes and all storage temperatures after short term storage.

length of the storage time if biobank samples are included in a study. Nevertheless, samples should only be compared if they are of the same type and have been stored similarly.

3.4. Is the analysis of sEVs influenced by the centrifugation protocol of choice?

As mentioned, centrifugation procedures for MPs/MVs are well described, and a protocol for the achievement of PFP has been suggested ($2 \times 2500g$ for 15 min) (Lacroix et al., 2012). The elimination of platelets before storage is of great importance when working with the larger EVs such as MPs/MVs, as residual platelets are able to vesiculate in vitro (Bode et al., 1991), which can alter the subsequent analysis results inconveniently (Artoni et al., 2012; Piccin et al., 2007). However, many different protocols are used to obtain PFP. Trummer et al. (Trummer et al., 2009) used a combined low ($1500g$ for 15 min) and high ($14,000g$ for 2 min) speed approach, whereas Nielsen et al. (Nielsen et al., 2014) combined three low speed centrifugations ($1800g$ for 10 min; $3000g$ for 15 min; $3000g$ for 5 min) to eliminate residual platelets.

To determine if restricting the content of platelets could serve as an advantage to sEV analyses (such as the EV Array), an alternative centrifugation protocol ($2 \times 2000g$ for 30 min) was implemented. This centrifugation approach was adapted from Pereira et al. (Pereira et al., 2006), who aimed for PFP. This alternative protocol was compared to our standard centrifugation protocol ($1800g$ for 6 min). The results are presented in Fig. 5A–C.

In Fig. 5A, a significant difference in the detected signal intensities of sEVs are seen for samples collected in CPDA and EDTA blood collection tubes when changing our standard one-step centrifugation protocol to the alternative two-step protocol. In Fig. 5B, the corresponding background signal intensities are illustrated, which explains the pronounced variation in the CPDA and EDTA plasma samples. Whereas the background intensities for the plasma samples centrifuged by the two-step protocol were insignificant, the plasma samples centrifuged by our standard one-step protocol displayed very high background intensities, in particular for the EDTA plasma samples. The observation that especially EDTA plasma samples can benefit from the two-step centrifugation protocol is confirmed in Fig. 5C, which displays the unrefined microarray spots from the analysis. The background intensity was indeed positively influenced by the alternative protocol.

Having realized the benefit of incorporating the two-step centrifugation protocol when collecting EDTA plasma samples, it was relevant to determine whether it was possible to limit the background noise in already frozen samples. Hence, EDTA plasma sample aliquots were exposed to either a short, high-speed one-step centrifugation ($14,000g$ for 2 min) or a longer, low-speed two-step centrifugation procedure ($2 \times 2500g$ for 15 min) immediately before analysis and compared to sample aliquots not exposed to post-storage centrifugation (Fig. 5D). Again, EDTA plasma samples benefitted from expanded centrifugation procedures. However, based on the appearance of the unrefined microarray spots, the two centrifugation procedures were equally valuable.

Even though the analysis outcome did benefit strikingly at some points from the alternative two-step centrifugation procedure, the results obtained with the *Phenotyping Array* revealed that the residual platelets performing in vitro vesiculation do not alter the analysis outcome of the EV Array analysis of sEVs once the initial centrifugation is performed (data not shown). If this had been the case, all four sample types would have benefitted from the alternative protocol. Furthermore, it was possible to regain clear antibody signals from EDTA plasma samples by eliminating most of the background noise. The ensuing signals were comparable to the ones obtained from the pre-storage double centrifugation (data not shown).

As mentioned, there is a demand for analyses with short performance times. Hence, adapting unnecessary preanalytical steps is not desirable. Since only two of the tested blood collection tubes benefitted

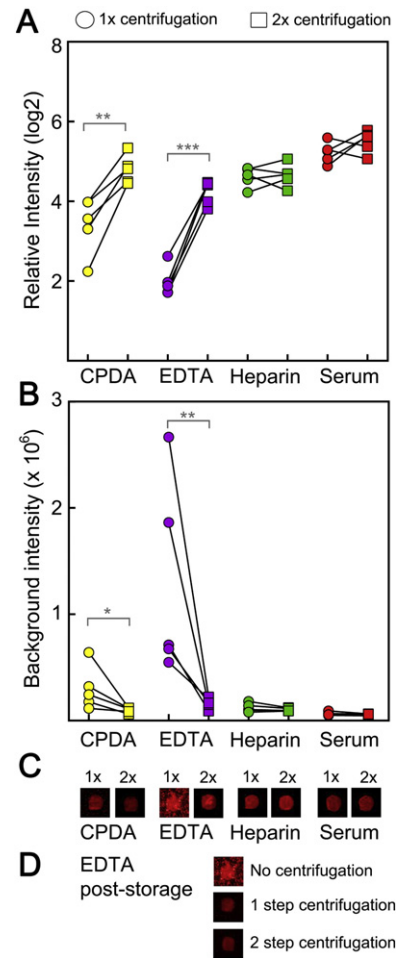


Fig. 5. The outcome of one- versus two-step centrifugations. Blood samples collected in the Vacuette® tubes CPDA, EDTA, heparin, and serum were exposed to two different centrifugation protocols; one-step ($1800g$ for 6 min, standard protocol) or two-step ($2 \times 2000g$ for 30 min) before storage (A, B, and C). The data shown are obtained using the *Cocktail Array*. A) Semi-quantitative data are presented as log₂ transformed relative intensities of all five donors illustrated by dot plots where the lines connect the samples from each individual donor. B) The corresponding background intensities obtained from the same analysis shown by similar dot plots. Statistically significant differences are indicated with p-values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. C) Unrefined microarray spots from one representative donor for all Vacuette® blood collection tubes illustrating the visual influence of spot versus background intensity. D) EDTA sample aliquots stored after one-step centrifugation were exposed to additional centrifugations immediately before analysis. Samples centrifuged using a new one-step ($14,000g$ for 2 min) or new two-step ($2 \times 2500g$ for 15 min) centrifugation procedure were compared to non-centrifuged samples. Shown are unrefined microarray spots from one representative donor.

from the expanded centrifugation procedure it could be relevant to design individual preanalytical protocols for each type of blood collection tube.

3.5. Do several freeze-thaw cycles affect the contents of sEVs?

In continuation of the questions addressed in Section 3.3 relating to storage temperature and period, it is of interest to determine whether samples can be re-frozen and thereby reused. To examine this issue, sample aliquots from all donors and blood collection tubes were exposed to freezing and thawing either one, two, three or four times. The outcome of the subsequent *Cocktail Array* analysis is illustrated in Fig. 6.

Some minor changes in the contents of the sEVs were observed among aliquots of the same sample type, of which few were statistically significant ($p < 0.05$). The changes were mostly minor increases;

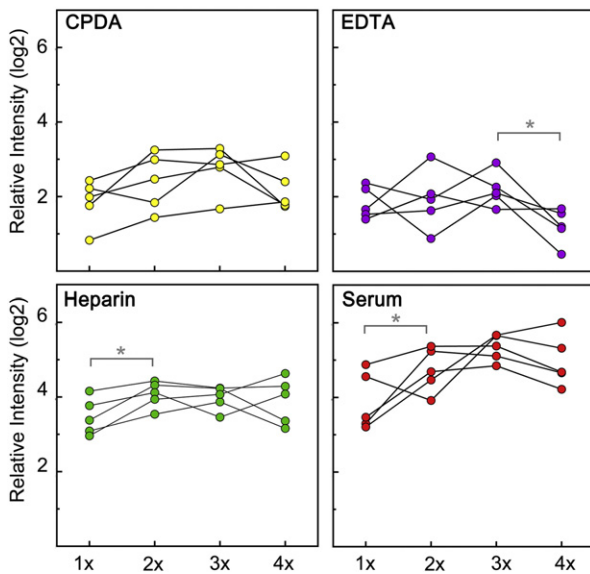


Fig. 6. The influence of freezing and thawing. Sample aliquots from blood collected in the Vacuette® tubes CPDA, EDTA, heparin, and serum for all five donors were thawed and refrozen either 2, 3 or 4 times. The samples are compared to samples thawed only once. Shown are data obtained using the *Cocktail Array* presented as log₂ transformed relative intensities of all five donors illustrated by dot plots. The lines connect the samples from each individual donor. Statistically significant differences are indicated with p-values: *p < 0.05.

however, lower background signals were observed for CPDA, heparin, and serum samples (data not shown), which would contribute to the observed increase. Freeze-thaw cycles therefore had only a small influence on the sEV contents. Hence, it is possible to reuse samples of the four tested blood sample types without compromising the analysis outcome in a critical manner, at least up to four times. This is confirmed by Jayachandran et al. (Jayachandran et al., 2012) who concluded that plasma samples could be freeze-thawed up to three times without altering the MV counts.

3.6. Does the ExoQuick™ isolation procedure influence the EV Array outcome?

Of the increasing interest in the field of EVs, the most attention is focused on exosomes. However, exosomes are hard to purify, as isolates are generally contaminated with other EV subsets (Lötvall et al., 2014). Along with the increasing interest in exosome research, an equally growing demand for isolation techniques has arisen. Various kits and solutions to easily obtain exosome precipitates are now commercially available. One widely tested product is ExoQuick™ Precipitation Solution. Here, we aimed to determine if the EV Array and similar antibody-based analyses can benefit from such a product and hence narrow the research to exosomes exclusively. The results are presented in Fig. 7.

The ExoQuick™ isolation of exosomes was performed either before or after freezing of the sample aliquots, and the results were compared to analyses of crude non-isolated plasma/serum samples (Fig. 7A). For CPDA plasma samples, a minor non-significant increase was observed when performing the isolation before storage compared to non-isolated samples. The detected signal intensities of sEVs were, however, lower in samples exposed to isolation after freezing, although not significantly. In the case of EDTA plasma samples, highly significant ($p < 0.01$) increases were obtained using ExoQuick™ isolation, regardless of whether it was carried out before or after storage. However, the unrefined microarray spots revealed that the increase is mainly caused by a reduction in the background noise. The opposite is the case for CPDA plasma samples, where the background suffered a minor impairment in the precipitates. When performing exosome isolation and thereby

eliminating other CD9-, CD63-, and/or CD81-containing vesicles, a decrease in the detected EV signal intensities would be expected. This was not the case for CPDA and EDTA plasma samples, unlike the heparin plasma samples. The decreases in the signal intensities in the precipitated heparin plasma samples were statistically significant, either $p < 0.01$ or $p < 0.05$ for the isolations performed before or after freezing, respectively. In Fig. 7B, the data obtained with the *Phenotyping Array* of the EV markers contributing significantly to the decrease in heparin plasma precipitates are displayed. Here, the MV/MP marker Annexin V is present along with the platelet-associated CD42a and CD142, which meets the expectations of the exosome isolation procedure. However, for the two platelet-associated markers, the decrease in signal intensity was primarily observed when the isolation was performed before storage. Furthermore, the immune-related markers TNF RI and ICAM-1 and in particular the exosome-associated marker CD9 contribute to the decrease as well. Although CD9 was once regarded a specific exosome marker it has been identified in other EV subsets (Bobbie et al., 2012; Kowal et al., 2016). Hence, this could indicate that CD9-bearing non-exosome sEVs are removed when using the ExoQuick™ Precipitation Solution on heparin plasma samples. In the case of performing the isolation on serum samples, the overall impression is that the sEV content is less influenced by the isolation procedure in this sample type. Only one sample was clearly affected by the procedure, resulting in a major decrease in the detected signal intensities.

In a study of exosome isolation methods, Lobb et al. (Lobb et al., 2015) concluded that the ExoQuick™ precipitation was inadequate compared to other methods and they observed a high degree of albumin contamination. They proposed ultrafiltration combined with size exclusion chromatography as a better method of isolation. Likewise, Van

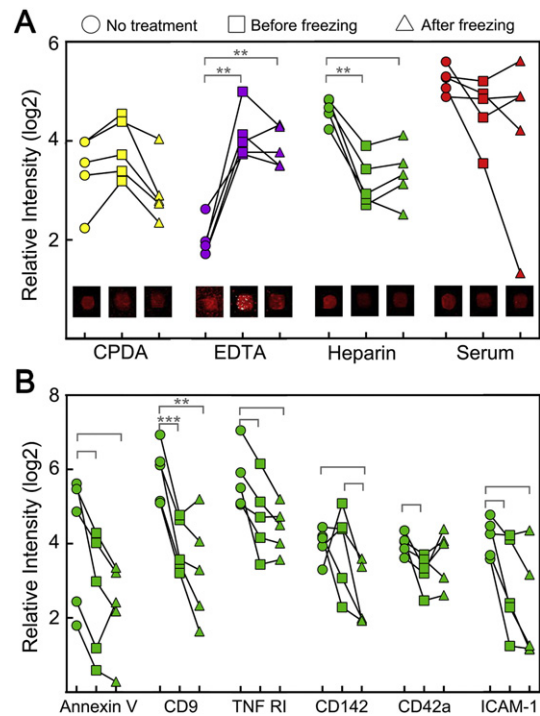


Fig. 7. Exosome isolation using ExoQuick™ Precipitation Solution. Exosome isolation was performed on sample aliquots from blood collected in the Vacuette® tubes CPDA, EDTA, heparin, and serum from all five donors using ExoQuick™ Precipitation Solution. The isolation was performed either before storage or immediately before analysis (after storage). The results are compared to results achieved from crude plasma/serum samples. Data are presented as log₂ transformed relative intensities of all five donors illustrated by dot plots where the lines connect the samples from each individual donor. A) Data obtained using the *Cocktail Array*. Inserted are the unrefined microarray spots from one representative donor. B) Data for the markers Annexin V, CD9, TNF RI, CD142, CD42a, and ICAM-1 obtained with the *Phenotyping Array* from samples collected in heparin blood collection tubes. Statistically significant differences are indicated with p-values: *p < 0.05; **p < 0.01; ***p < 0.001.

Deun et al. (Van Deun et al., 2014) suggested that OptiPrep™ Density Gradient is a better choice because it leads to a higher purity of exosome-specific protein as well as RNA yield compared to the ExoQuick™ Precipitation Solution, among others. Our results agree that the ExoQuick™ product might not be the best suited method for exosome isolation, since only heparin plasma samples benefitted convincingly from the isolation procedure.

3.7. Which blood collection tube is the best suitable for sEV analyses?

From the results presented here, heparin plasma showed good qualities in all the tests. Neither of the other tested blood collection tubes displayed the same degree of stability. However, as reviewed by Witwer et al. (Witwer et al., 2013), the use of heparin-based anticoagulants is discouraged for a number of reasons. Platelet activation is one reason (Gao et al., 2011), which is highly undesirable in EV analyses in general. Another inconvenient property of heparin is that it can block EV uptake by other cells (Maguire et al., 2012), which is a problem when studying the biological functionalities of EVs. The latter issue may not be a problem in the analytical phenotyping aspect described here.

Other studies have tested the use of several types of blood collection tubes as well, but mainly focus on MVs/MPs analyzed by flow cytometry. György et al. (György et al., 2014) tested different types of citrate-containing tubes and concluded that ACD-A plasma seemed the most suitable for MP analyses, which is partly supported by Lacroix et al. (Lacroix et al., 2012), who also recommended citrated plasma. The study of Jayachandran et al. (Jayachandran et al., 2012), on the other hand, showed that EDTA resulted in lower MV (including platelet-derived) counts compared with the other tested anticoagulants and protease inhibitors. In our hands and with the presented analysis approach targeting the smaller EV populations, the EDTA plasma samples were the most troublesome to work with. However, the addition of a few extra steps to the sample preparation made it possible to overcome most issues and to obtain useful results.

The number of EV research projects performed on plasma samples outnumber those involving EV analyses of serum samples. Our results do not exclude serum samples as useless, which, in fact, seemed less influenced by most of the tested factors compared to the often used EDTA and CPDA samples. Actually, on the basis of the results obtained by the EV Array technology presented here, none of the tested blood collection tubes can be ruled out as useless. There are pros and cons of using any of these blood collection tubes, which affect the data outcome differently. Hence, it is very critical to keep the sample type constant when collecting for a study.

4. Concluding remarks

In this study, we have tried to clarify as many aspects of the preanalytical impact of sEVs as possible. Nevertheless, it is important to keep in mind that the statements given here are applicable only for the EV Array analysis and may not be directly transferable to other phenotyping analyses. However, it may be reasonable to assume it applies to other antibody-based methods such as ELISA.

No unequivocal answer exists as to which type of blood collection tube should be used, how it should be stored and for how long it is acceptable to use the samples etc. Any change in the sample collection process may have an impact on the analysis outcome. At first, it is important to stick to the chosen type of blood collection tube, as a change will result in an altered data outcome that cannot be used for comparison. Next, it is critical to keep the period before the initial centrifugation constant, as the content of sEVs seems to be affected by the chemistry that occurs when the blood meets the anticoagulants. This is especially evident in the case of CPDA and EDTA plasma samples, which should be handled either ASAP or the next day (24 h later). This observation, however, contradicts the recommended 1–2 h time frame from the manufacturer of the blood collection tubes and from the study of Lacroix

(Lacroix et al., 2012) involving larger EV populations. If the samples need to be transported before the initial centrifugation, it is important that it applies for all samples. Because of the effect it has on the samples (most pronounced for CPDA and EDTA plasma samples), the most optimal situation would be to centrifuge and store the samples at the site of collection and subsequently transfer all samples together in a frozen state. In this regard, even the storage temperature influences the signal intensities of the sEVs in the samples, which is evident for all the sample types tested. Hence, frozen samples should not be compared with fresh samples and the chosen storage temperature should likewise be kept constant. We observed no major problems with long term storage of frozen samples for any of the tested blood collection tubes. The same applies for the reuse of frozen samples up to 4 times, although freeze-thaw cycles should be kept at a minimum. Furthermore, the choice of centrifugation protocol has an impact on the analysis of sEVs. Both CPDA and EDTA plasma samples benefit from applying a two-step centrifugation procedure (2 × 2000g for 30 min), whereas for heparin plasma and serum samples a one-step centrifugation protocol (1800g for 6 min) is sufficient.

Because the tested preanalytical treatments had different degrees of impact on the analysis outcome, it could be relevant to adjust the sample preparation protocol in relation to the blood collection tube used.

Conflict of interest

The authors declare no conflicts of interest.

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