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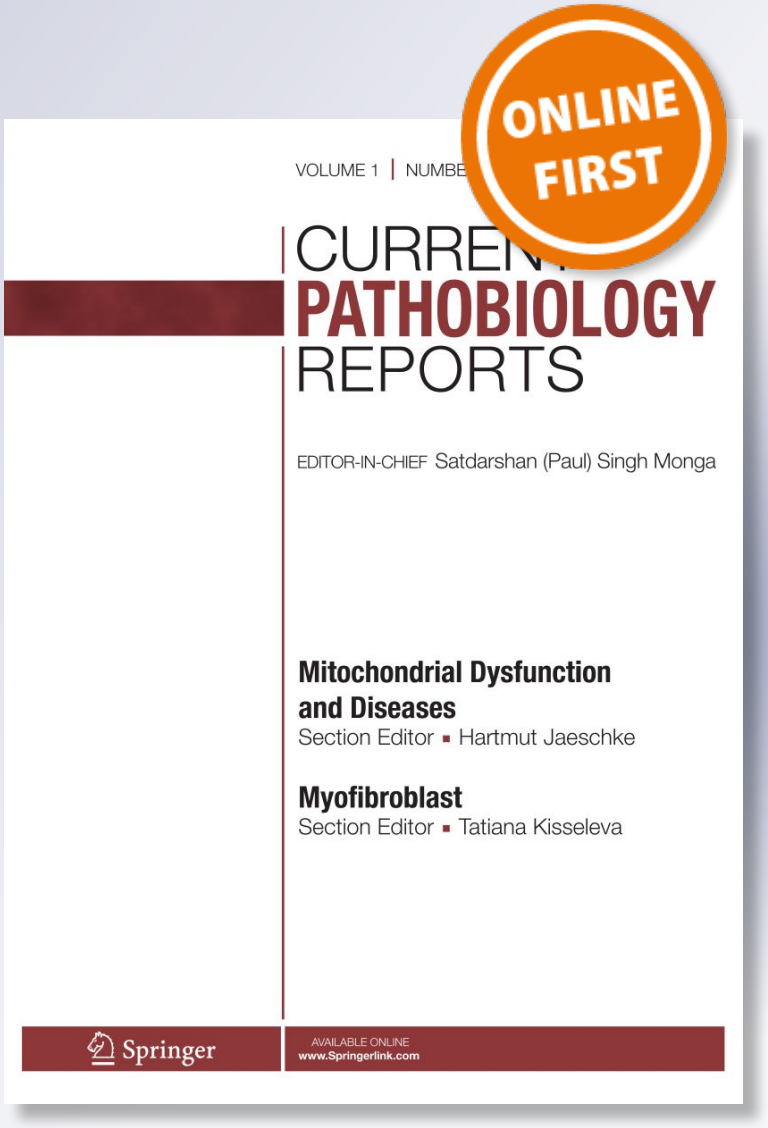

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BIOSPECIMENS SCIENCE AND EVIDENCE-BASED STANDARDS FOR PRECISION MEDICINE (F BETSOU, SECTION EDITOR)

Standardization of Blood Collection and Processing for the Diagnostic Use of Extracellular Vesicles

Marta Venturella[1] · Francesco M. Carpi[1] · Davide Zocco[1]

Abstract

Purpose of Review Extracellular vesicles (EVs) are lipid membrane vesicles released by many types of cells in both health and disease. EVs can be found in most body fluids, carrying a plethora of biomolecules, including proteins, RNA, and DNA, that reflect the biomolecular composition of the tissue of origin. Parenchymal and stromal cells actively release EVs in the extracellular milieu and in circulation, providing valuable information that may be exploited for diagnostic applications. However, isolation of these EV subpopulations in circulation is extremely challenging as they are diluted within more abundant EV subpopulations derived from blood cells (red blood cells, platelets, and white blood cells).

Recent Findings A number of preanalytical variables during blood collection and processing greatly impact the levels of blood-derived EVs, thus affecting sample quality. So far, lack of standard protocols for blood collection and processing as well as quality control metrics has limited the clinical validation and adoption of EV-based diagnostic assays.

Summary In this review, we describe the preanalytical variables that affect sample quality and suitability for EV-based diagnostic approaches. Furthermore, we suggest biochemical and molecular quality control (QC) metrics to minimize intra- and interstudy variability and improve data robustness and reproducibility.

Keywords Standardization · Extracellular vesicles · Blood · Preanalytical variables · Liquid biopsy · RNA biomarkers

Introduction

Extracellular vesicles (EVs) comprise a heterogeneous population of membrane vesicles with different sizes and origins. EVs can be classified into three distinct classes: large extracellular vesicles, microvesicles, and exosomes. Large extracellular vesicles, such as oncosomes and apoptotic bodies, have a diameter that varies from 800 nm to 5 μm, and they originate from the remodeling of the plasma membrane, usually associated to pathological states (e.g., late-stage cancer). Microvesicles are formed by budding of the cell plasma membrane, their size varies between 150 nm and 1 μm, and they often are referred to as microparticles or shedding vesicles. Exosomes are vesicles of 30–150 nm in diameter, and they are formed within the lumen of multivesicular endosomes (MVEs) as intraluminal vesicles, and secreted in the extracellular milieu upon fusion of MVEs with the cell surface [1]. Recently, a new family of non-membranous nanoparticles has been discovered, called exomeres, which are about 35 nm in diameter and carry unique *N*-glycosylation, protein, lipid, DNA, and RNA profiles [2].

EVs have been found in many body fluids, including blood, saliva, cerebrospinal fluid, and urine. However, most research efforts with diagnostic aims have been performed in plasma and serum as these body fluids are easily accessible, abundant, highly informative, and routinely biobanked [3••].

Diagnostic Potential of Disease-Derived EVs

Extracellular vesicles carry and protect from the extracellular milieu many molecules, including protein, DNA, and RNA (exRNA). In recent years, exRNAs have been the objective of intense research efforts to identify and validate novel diagnostic signatures for the development of a *new class of non-*

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invasive diagnostic assays [4••]. ExRNAs include messenger RNA (mRNA), microRNA (miRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), and other non-coding (nc) RNAs [4••]. Among the ncRNAs, lncRNAs, siRNAs, snRNAs, snoRNAs, piwiRNAs, circRNAs, and Y-RNAs have all been observed within exosomes and detected in body fluids [5, 6]. In cancer and other diseases, altered expression of exRNAs has been reported, suggesting that they may serve as disease biomarkers [4, 7, 8]. This important feature, along with their high abundance and stability, makes them potential non-invasive, blood-based biomarkers that can shed light on early disease onset and development, therapy response, and mechanisms of drug resistance [5, 9••].

Standardization of Blood Collection and Processing for EV-Based Studies

Liquid biopsy approaches are currently under investigation for the development of new clinical algorithms that will guide clinical decision making. Liquid biopsy has the potential to solve some of the issues pertaining to traditional biopsy (Fig. 1). However, it comes with its own set of technical issues. Standardization of body fluid (e.g., blood) collection and processing is considered a key yet unmet prerequisite for the validation of EV-associated biomarkers [10••]. Research efforts to develop EV-based diagnostics or therapeutics are currently limited by the lack of accepted standard protocols that minimize artifacts due to preanalytical steps and improve interlaboratory reproducibility [9]. Indeed, the implementation of different methods in EV research requires validated controls and adequate reporting of experimental parameters [11]. In recent years, many attempts had been taken by researchers to find common guidelines to help the comparison of experimental results. An example is the EV-TRACK knowledge base, developed by an international consortium of researchers, which records experimental parameters of EV-related studies [10••, 11]. Moreover, rigorous documentation and standard guidelines are required also for biobanking activities, in order to obtain high-quality blood specimen for downstream analysis [3••, 12]. Analysis of EV-based biomarkers, including exRNAs, strictly depends on preanalytical parameters [7, 9••]. The preanalytical phase is an important source of artifacts [7, 10••, 13•]. Indeed, blood cells, such as platelets, become easily activated during sample collection and handling, and, as a consequence, they release confounding EVs in the sample that interfere with the isolation of EVs of interest [14]. There are numerous reports of collection, processing, and handling variables that affect the composition of blood and serum; many of these have been demonstrated to have a significant impact on EV analysis [14, 15]. However, the knowledge about preanalytical factors that can alter extracellular vesicles has clear gaps [15]. Figure 1 highlights the preanalytical variables that may affect EV studies and need to be considered for standardization. Another important aspect is the lack of quality control metrics for plasma samples to be used in clinical studies. Only recently, researchers have agreed that the quality of human specimen has to be assessed with standardized metrics before any molecular analysis [14–17] (Fig. 1).

In this manuscript, we review the impact of preanalytical variables affecting blood-derived EVs and suggest

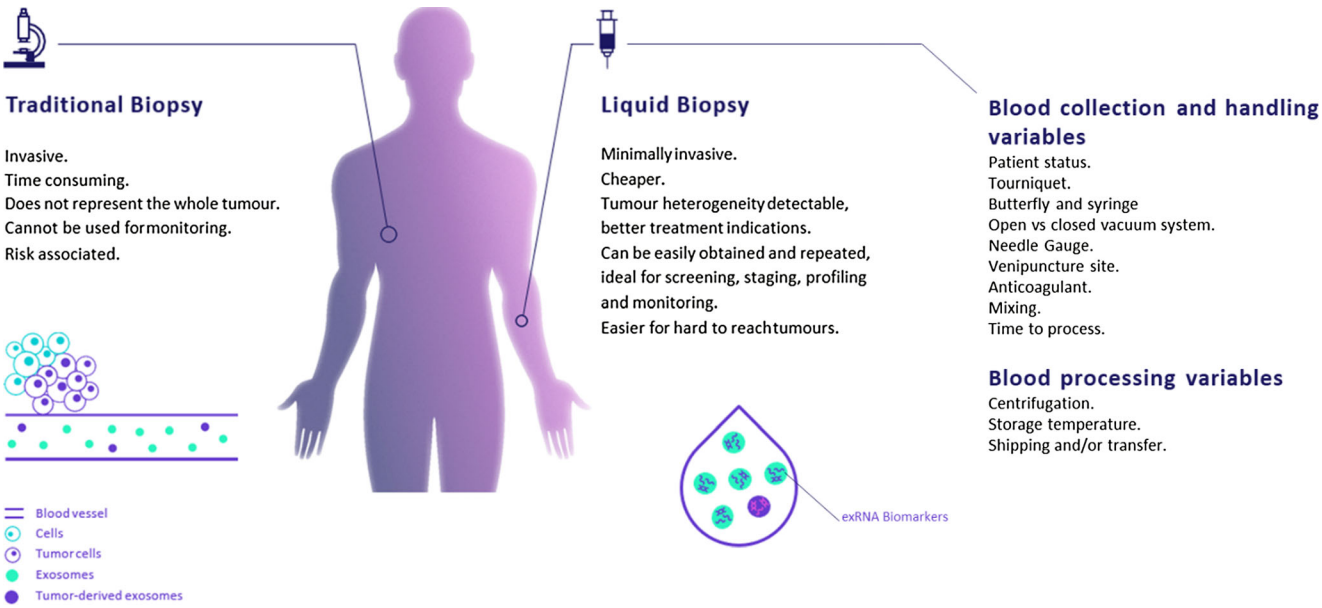

Fig. 1 Preanalytical variables that may affect EV studies

biochemical and molecular metrics to be used to evaluate the quality of blood specimens for translational applications.

Blood Collection, Handling, and Processing for Research and Diagnostic Use of EVs

Preanalytical variables that must be considered when collecting and processing blood for EV research or diagnostic applications are listed below. When known, the impact of each of these variables on the EV population in blood is described.

Patient Status

It is generally accepted that overnight fasting (including smoke, coffee, tea, energy drinks, and alcohol) before blood collection in the morning is beneficial [18••, 19•]. Blood sampling should not be done if patients had meals with a high fat content to reduce the formation of chylomicrons in plasma which might increase the lipemic index [19•]. Patients are also invited to avoid strenuous physical activity the day before sampling as physical activity exerts acute effects on human metabolism and blood composition [18••, 19•]. Moreover, use of anti-inflammatory medicinal products is discouraged within the last 48 h before blood collection. Our suggestion is to provide a checklist to be filled by the donor/patient (Table 1).

All these factors affect the amount, purity, and content of biofluid-derived EVs, and they should be normalized across all study subjects, patients, and controls [4••, 9••].

Blood Collection

Application of Tourniquet

Blood collection should be done preferably without a tourniquet, especially if patients have prominent veins. Instead of a tourniquet, vein illumination devices may be a useful alternative in order to avoid venous stasis and alterations of various blood parameters. In the cases when a tourniquet is necessary, the total tourniquet applying time must not exceed 1 min. If applied for a longer period of time, the tourniquet induces a substantial variation of blood composition, increasing lipoproteins and coagulation factors in the blood (most of these changes are negligible within 1 min of application) [18••, 19•]. No studies have been published yet to evaluate the impact of tourniquet on EV subpopulations of the blood; however, a prolonged use of this tool is likely to increase the levels of platelet or reticulocyte-derived EVs, thus further diluting EV subpopulations coming from the stroma or the parenchyma (unpublished observation).

Table 1 Example of checklist for blood patients or donors

Age
Sex
For females: pregnancies and ovulatory cycle
Race
Old and current pathologies
Current therapies (including oral contraceptives)
Smoker

Use of Butterfly Devices, Syringes, and Intravenous Catheters vs Closed Blood Collection Systems

The European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) discourages the use of open systems, such as syringes with hypodermic needles, that require subsequent transfer of blood into tubes with risks of blood spillage and hemolysis [18••]. EFLM warns on the use of intravenous catheters as they are also associated to an increased risk of hemolysis [18••]. Importantly, there is experimental evidence discouraging the use of open systems like syringes for EV applications [14]. The World Health Organization's (WHO) guidelines recommend the use of a vacuum extraction tube closed system [20••]. This system has an evacuated tube with a needle and tube holder and applies a gentle vacuum pressure that minimizes the risk of hemolysis and platelet activation. In an effort to test vacuum extraction tube closed systems, we compared the syringe-based S-Monovette® system (Sarstedt) to butterfly-needle Vacutainer® closed system (Becton Dickinson) and observed a substantial reduction of artifactual platelet- and red blood cell–derived EVs with the former system (unpublished observation). This observation is consistent with previous reports suggesting that butterfly needles reduce the blood flow and increase the risk of platelet activation [4••, 21].

Choice of Correct Gauge of Needle

The choice of the correct needle gauge plays an important part in minimizing the risk of hemolysis and platelet activation. Needles with a gauge number equal or higher than 23 are considered too small and may cause damage of blood cells during sampling, hemolysis, and release of confounding factors that affect downstream analyses [14, 16, 20••, 21]. The use of these needles may only be justified on patients with difficult veins (geriatrics, oncology, pediatrics) [19•]. For the vast majority of clinical studies, the recommended needle gauge is 21 (or even 19) as it minimizes shear forces responsible for platelet activation and release of platelet-derived and red blood cell–derived EVs [4••, 9••, 21]. High shear forces affect EV content and alter the expression of some plasma miRNAs [9••, 14].

Venipuncture Site

Ideally, the patient should not change his position within 15 min prior to blood sampling [18••]. Blood should be collected from a peripheral vein (the median cubital vein is the most preferable) [18••, 19•, 20••]. The selected venipuncture site must be cleaned with 70% ethyl alcohol because contamination of blood by skin's normal flora may occur [18••, 20••].

Choice Between Plasma and Serum

Serum is obtained from the whole blood sampling without anticoagulant; when clot forms, serum is acquired by centrifugation. Plasma is obtained from the whole blood sampling with different types of anticoagulants by centrifugation. The choice of the blood derivative depends on the analytical requirements (Table 2).

In the EV field, plasma is usually preferred over serum because blood clotting causes the release of additional confounding EVs that may affect biomolecular analyses [4••, 9••, 10••].

Choice of Anticoagulant

The choice of the anticoagulant depends on the analyses to be performed downstream [4••, 10••]. For instance, heparin-based anticoagulants are not recommended for nucleic acid studies because heparin is a potent inhibitor of PCR reactions [4••, 10••]. Furthermore, heparin does not prevent in vitro vesiculation [7]. Citrate is the most commonly used anticoagulant, and it is recommended by the International Society on Thrombosis and Haemostasis [10••]. However, the EDRN (Early Detection Research Network, U.S. National Cancer Institute) recommends EDTA as a universal anticoagulant for standard operating procedures [16]. Consistent to this recommendation, a growing number of reports support the use of EDTA as anticoagulant suitable for EV-RNA analysis [10••] as it prevents the formation of EV-blood cell aggregates and inhibits platelet-derived EV release [9••]. More recently, Buzás E. et al. suggest the use of ACD (acid-citrate-dextrose) tubes which inhibit in vitro vesiculation and do not interfere with RNA analysis [7]. In studies where the same blood must be collected in tubes with different anticoagulants, it is important to follow a precise order of draw as reported by the WHO guidelines [20]. Incorrect order of draw may cause cross-contamination of additives between tubes [18••, 20••]. Furthermore, it is important to discard the first tube to reduce the effects of the vascular damage caused by the venipuncture [4••, 10••, 19•, 21] especially when using a butterfly device [18••]. Finally, we recommend to visually check that the tubes are properly filled during blood collection in order to provide the optimum blood to additive ratio [10••, 16].

Blood Sample Handling

Sample Mixing

Following blood collection, samples must be immediately mixed with the anticoagulant inside the tube. Any delay in this step may affect the quality and integrity of the sample (hemolysis or clot formation) [18••]. Mixing should not be too vigorous in order to avoid hemolysis, platelet activation, or blood clotting [9••, 10••, 18••, 19•, 25]. When sampling is completed, all tubes must be gently inverted at least four times (or following the manufacturers' instructions) and racked in an upright position prior to further processing [4••, 18••].

Time to Process

Blood samples should be kept at room temperature before processing as cold promotes platelet disruption [14] and transported vertically at room temperature in the shortest time possible [10••, 14, 19•, 21]. The time between sampling and blood processing in the lab should be minimized or at least kept constant between samples in order to limit variability in the number and quality of EVs [10••]. These parameters are stable up to 30 min after the blood draw; longer lead times usually increase the number of confounding EVs due to blood cell activation [4••]. Differences in the processing time greatly affect the degree of residual cells and microparticle contamination and, in turn, the results of any biomarker study, because of intra- and interstudy variability [26, 27]. The LacaScore, a

Table 2 Advantages and limitations of plasma vs serum

	Advantages	Limitations	Downstream applications
Plasma	Quick processing; high reproducibility [22]	Plasma retains all clotting factors; anticoagulants can produce matrix effects [23]; hemolysis can occur during blood processing [24]	Biomarker discovery; source of miRNA and cfDNA [3••]
Serum	High sensitivity for biomarker studies [22]	Various clotting times can cause artifacts that influence molecular analyses [16]	Source of miRNA [10••]

new quality control metric based on lactic and ascorbic acid, has recently been proposed to evaluate the effects of precentrifugation temperature/time on the quality of EDTA plasma for biomarker discovery [28].

Blood Centrifugation

Whether centrifugation should be done at room temperature or at 4 °C remains a matter of debate [13, 21]. It has been suggested that refrigeration of whole blood activates platelets leading to an artificial release of microvesicles [4••, 10••]. However, other reports indicate that centrifugation temperature does not influence microparticle count [13•]. It is recommended to use a rotor with swing-out buckets to facilitate the separation of plasma from the cellular components and to minimize remixing of plasma and red cells [19•]. The centrifuge's *k*-factor should be reported, and, when possible, kept constant during sample collection as it dramatically impacts EV content of the sample [4••]. Furthermore, centrifugation with breaks should be avoided to prevent damage of EVs [21].

Centrifugation speed is another matter of debate. Different centrifugation protocols have been applied in different labs in order to obtain a PPP (platelet-poor plasma) or a PFP (platelet-free plasma) [21] (Table 3).

The protocol recommended by the International Society on Thrombosis and Haemostasis is useful to remove most of the platelets from plasma [10••]. However, high-speed protocols (2000–2500*g* or even 13,000*g*) with one or two steps of centrifugation are correlated with decrease of microparticle's number [4••, 13•, 21]. Indeed, a centrifugation step at 10,000*g* is routinely used to pellet microvesicles [8] and, therefore, it is not recommended if large MVs are the objective of a biomarker discovery study [10••]. Moreover, centrifugal force greater than 1500*g* has been reported to induce platelet activation and hemolysis [19•].

In conclusion, centrifugation parameters (speed, deceleration, rotor, *k*-factor, and temperature) must be kept constant during sample processing and carefully reported to further advance biospecimen science for EV-based diagnostics [4••, 10••, 17].

Table 3 Different centrifugation protocols for plasma recovery

PPP (platelet-poor plasma)	PFP (platelet-free plasma)
1500*g* 15 min RT, once 1500*g* 15 min RT, twice	1500*g* 20 min RT; 13,000*g* 2 min RT
	2000–2500*g* 10–15 min RT, twice (protocol of International Society on Thrombosis and Haemostasis)

Sample Stability and Storage

Fresh plasma provides high and pure yields of EVs [29]. Isolated plasma and serum samples are stable at room temperature for at least 4 h and at 4 °C for 24 h. Long-term storage of plasma and serum samples for EV research must be done at − 80 °C [16]. Interestingly, researchers have isolated EVs with intact RNA after storing plasma samples at room temperature for 42 h or at − 80 °C for 12 years [29]. EVs in plasma seem stable after one freeze-thaw cycle [10••]. Repeated freeze-thaw cycles affect the yields of DNA, mRNA, and miRNA [30], partially carried by platelet-derived microparticles increased after thawing PPP [13•, 21]. In any case, samples should be aliquoted into small volumes to minimize the number of freeze-thaw cycles that must be registered and strictly monitored [3•, 10••, 16]. Recently, the use of an antiaggregant additive, trehalose, has been proposed to reduce cryodamage and aggregation of purified EVs after freeze-thaw cycles, thus improving sample quality [31].

In summary, data collection of every critical preanalytical steps (sample collection methods, processing techniques, storage time) is necessary to avoid technical biases and artifacts. Unnoticed differences in operational procedures can lead to different results and poor reproducibility of experiments [3••, 12, 16, 27]. The key rule in any analysis is that patients and control samples must be consistently handled throughout the entire process, from sample collection to data analysis [16].

Quality Control Metrics for Blood Samples

Quality control metrics are essential to evaluate if a blood or blood-derivative sample is suitable for downstream molecular analyses. Canonical biochemical indexes, such as the hemolytic index, have a dramatic impact on exosome number, content, and quality of a plasma or serum sample.

Hemolysis

Hemolysis of the blood is determined by both qualitative and quantitative means. Hemolyzed plasma samples appear red upon visual inspection due to the rupturing of red blood cells during blood processing. Sample hemolysis is usually quantified by measuring the levels of free hemoglobin and established when this protein reaches a concentration higher than 0.3 g/L (18.8 mM). Importantly, hemolyzed plasma samples have been shown to have high levels of reticulocyte-derived EVs or microparticles that may confound molecular analyses. For instance, miR-16 and miR-451 have been found to be more abundant in hemolyzed samples than normal samples, and, for this reason, they are usually ruled out from the list of potential RNA normalizers or disease biomarkers for clinical validation studies [4••]. In general, hemolyzed

Table 4 Protein and RNA biomarkers expressed by blood-derived EVs

	Blood cells		Extracellular vesicles	
Peripheral blood cells	Surface marker proteins [33–39]	miRNA profile [38, 40, 41, 42•, 43, 44•, 45, 46]	Surface marker proteins [35, 36, 39]	miRNA profile [38, 42, 45, 47–51]
Platelets	CD9, CD63, CD61, CD31, CD41, CD42a, CD42b, CD62p	miR-126-3p, miR-223-3p, miR-142-3p, miR-26a-5p, miR-328, miR-16, miR-197, miR-574-3p, let-7a, miR-24, miR-21	CD41a, CD42, CD61, CD62p	miR-126-3p, miR-223-3p, miR-197, miR-24, miR-21
T cells	CD3, CD4, CD8, CD28, CD45	miR-150-5p, miR-142-3p, let-7g-5p, miR-26a-5p, miR-16, miR-181	CD3, CD4, CD8, CD45	let-7a-5p, miR-93-5p, miR-23b-3p, miR-142-5p, miR-103a-3p, miR-30b-5p, miR-19a-3p, miR-150-5p, miR-142-3p, miR-25-3p, miR-21-5p, miR-16-5p, miR-92a-3p, miR-29b-3p, miR-24-3p, let-7g-5p, miR-106a-5p, miR-342-3p, miR-146a-5p, miR-155-5p, miR-20a-5p, miR-222-3p, miR-29a-3p, miR-29c-3p, miR-1260a, miR-15a-5p
B cells	CD19, CD20	miR-142-3p, miR-150-5p, let-7g-5p, miR-16-5p, miR-320, miR-191, miR-155	CD3, CD4, CD8, CD19	miR-150-5p, miR-155-5p, miR-92a-3p, miR-21-5p, miR-181a-5p, miR-148a-3p, miR-142-5p, miR-146a-5p, miR-486-5p
Granulocytes	CD66b, CD62P, CD14, CD15	miR-223-3p, miR-142-3p, miR-26a-5p, miR-16-5p, miR-31, miR-107, miR-222	CD66b, CD66e, CD62P, CD11b, CD9, CD63	miR-223, miR-155, miR-146
Reticulocytes	CD235a, CD108a, CD44, CD36	miR-451a, miR-15b-5p, miR-16-5p, let-7a-5p, miR-126-3p, miR-142-3p, miR-486-3p, miR-486-5p, miR-92a-3p	CD235a, CD108a	miR-486, miR-451a, miR-16-5p, miR-92a-3p, let-7a-5p, miR-142-5p

samples should be always discarded to avoid analytical biases and improve data robustness and reproducibility.

Lipemia

Lipemia is defined as a high degree of turbidity after visual inspection of the sample. A lipemic index can be obtained by measuring the absorbance of the sample by spectrophotometric analysis at 660/700 nm. Even if the lipemic index is not a direct measurement of triglyceride concentration, a high lipemic index is usually correlated with more than 300 mg/dL (or 3.4 mM) of these lipids in plasma samples. No systematic studies have been performed to assess the effect of lipemia on EV number, quality, and function. Given the lipidic nature of EVs, it is conceivable that high levels of lipoproteins may affect EV isolation from plasma samples, perhaps copurifying in the same fractions, as observed by some investigators [32].

Jaundice

Identification of jaundiced or icteric samples in the clinic relies solely on visual inspection. Spectrophotometric analysis at 450–575 nm may be performed to determine if hematic bilirubin levels have reached a concentration of 2–3 mg/dL (34–51 μM) typical of jaundiced patients. As for lipemic samples, no systematic studies have been performed to assess the effects of high bilirubin levels on EVs. We recommend to eliminate jaundiced samples from any analysis of EV-based biomarkers.

In addition to these biochemical indexes, we propose to monitor the levels of protein and RNA biomarkers expressed by blood-derived EVs for any given preanalytical protocol. In this way, the confounding effect of these subpopulations can be estimated and minimized to improve the quality of the molecular analyses from parenchymal, mesenchymal, or stromal cell–derived exosomes. Table 4 provides a summary of these biomarkers. In order to promote the adoption of these molecular QC metrics, we recently published a protocol for the detection and analysis of EV-derived miRNA from plasma [52]. The protocol describes the isolation of EV subpopulations using antibodies targeting specific surface markers followed by optimized RNA extraction and analysis of miRNA profiles by real-time quantitative PCR [52].

Conclusion and Final Remarks

Extracellular vesicles are emerging as a promising platform for liquid biopsy approaches in the blood. Standardization of blood collection and processing is required to fully translate this potential into clinically adopted diagnostic solutions. Preanalytical variables affecting sample quality must be taken into account and kept constant to ensure data reproducibility throughout the study. Quality control metrics must be defined

and agreed upon, to enable intra- and interstudy comparison. At present, no unifying protocol has been developed to collect blood and process it into plasma. Industrial efforts are ongoing to identify innovative solutions that would simplify and standardize sample procurement and processing in the preclinical and clinical settings.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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