Review Article

Diagnostic and Prognostic Potential of Extracellular Vesicles in Peripheral Blood

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ABSTRACT

Purpose: Extracellular vesicles (EVs) are small, membrane-enclosed entities released from cells in many different biological systems. These vesicles play an important role in cellular communication by virtue of their protein, RNA, and lipid content, which can be transferred among cells. The complement of biomolecules reflects the parent cell, and their characterization may provide information about the presence of an aberrant process. Peripheral blood is a rich source of circulating EVs, which are easily accessible through a blood sample. An analysis of EVs in peripheral blood could provide access to unparalleled amounts of biomarkers of great diagnostic and prognostic value. The objectives of this review are to briefly present the current knowledge about EVs and to introduce a toolbox of selected techniques, which can be used to rapidly characterize clinically relevant properties of EVs from peripheral blood.

Methods: Several techniques exist to characterize the different features of EVs, including size, enumeration, RNA cargo, and protein phenotype. Each technique has a number of advantages and pitfalls. However, with the techniques presented in this review, a possible platform for EV characterization in a clinical setting is outlined.

Findings: Although EVs have great diagnostic and prognostic potential, a lack of standardization regarding EV analysis hampers the full use of this potential. Nevertheless, the analysis of EVs in peripheral blood has several advantages compared with traditional analyses of many soluble molecules in blood.

Implications: Overall, the use of EV analysis as a diagnostic and prognostic tool has prodigious clinical potential. (*Clin Ther*. 2014;36:830–846) © 2014 The Authors. Published by Elsevier HS Journals, Inc.

Key words: Extracellular vesicles, microvesicles, exosomes, diagnostics, phenotyping, RNA cargo, enumeration.

INTRODUCTION

In recent years, interest in the characterization, biogenesis, and function of extracellular vesicles (EVs) has increased immensely. These membrane-derived vesicles play vital roles in a plethora of processes in several biological systems. In humans, EVs are pivotal to cellular communication for the maintenance of homeostasis and the development and progression of pathologic conditions, such as cancer. Consequently, this communication forms the basis for the use of EV analysis in a clinical setting because EVs seem to be a promising source of biomarkers of diagnostic and prognostic value. EV analysis can likely be used as one component of treatment surveillance. In addition, EVs have the potential of being used as drug therapy entities, delivering a tailored pharmacologic cargo to a specific target.

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Classification of EVs

In general, EVs are a heterogeneous population of membrane-enclosed vesicles released from a variety of cells into the extracellular space *in vivo* and *in vitro*. One general feature for these vesicles is that they are enclosed by a membrane that consists of a phospholipid bilayer. However, the EVs can be divided into a number of subpopulations each with specific characteristics, including their biogenesis, size, cellular origin, protein composition, mRNA and microRNA (miRNA) content, and/or biological function. With biogenesis as a classification tool, the EVs can be divided into 3 major groups: exosomes, microvesicles (MVs), and apoptotic bodies. Many of the properties of EVs, and in particular exosomes, have been reviewed extensively elsewhere[1–9]; therefore, the following section states the overall characteristics of these 3 EV groups.

Exosomes

Exosome is the vesicle type that has been studied most intensely. They are approximately 30 to 100 nm in diameter and originate from inward budding of the limiting membrane of multivesicular bodies, which are late endosomal compartments present in the cytosol of the cell.[4,6,10,11] When the multivesicular bodies fuse with the plasma membrane, the release of the exosomes to the extracellular space is facilitated. The biogenesis of the exosomes causes the orientation of the membrane proteins to be similar to that of the plasma membrane. The exosomal membrane is enriched in cholesterol, ceramide, and sphingomyelin and exposes the phospholipid phosphatidylserine.[6,11] In addition, exosomes contain several proteins that are currently used as markers to identify exosomes. These makers are not ubiquitously expressed on all exosomes but are found in a large proportion of these vesicles. Therefore, they are generally accepted as exosomal markers. These markers include TSG101, Alix, and the tetraspanins CD9, CD63, and CD81.[2,4] Along with these hallmark proteins, the phenotype of exosomes often reflects a molecular signature of the cell from which they originate. This cell-specific signature may provide some indication about the functionality of the exosomes because some of these signature molecules could ensure the delivery of the exosomes to the correct target cell,[12] point to a signal being transduced by a receptor-ligand interaction of exosomes and recipient cell,[13] or simply indicate which cells are the active exosome producers.[14] The composition of the exosome cargo can also be related to the potential biological function of this vesicle type. They have been known to contain proteins from both the plasma membrane and cytosol along with mRNA and the non–protein-coding miRNAs and small interfering RNAs.[2,15–17]

Microvesicles

The size of MVs ranges from 100 to 1000 nm. They are formed from outward budding of the plasma membrane, thus releasing the MVs directly into the extracellular space.[2,4,18] Hence, the membrane proteins of MVs retain the topologic features of those found in the plasma membrane. Generally, most MVs incorporate phosphatidylserine in the outer leaflet of the membrane.[4,6,10,19] This feature has frequently been used to isolate and identify MVs from biological samples along with a combination of cell-specific protein markers to determine their cellular origin.[19,20] However, several studies indicate that phosphatidylserine may only be present in some subpopulations of MVs.[21–24] Currently, there is a less extensive list of markers to identify MVs when compared with exosomes. Nonetheless, CD40 ligand, adenosine diphosphate ribosylation factor 6, and several integrins and selectins have been proposed as MV markers.[2,5,6,18,25] Like exosomes, the phenotype of MVs reflects their parent cell, and the content of the vesicle cargo also includes membrane and cytosolic proteins, mRNA, and miRNAs.[2,5]

Apoptotic Bodies

Apoptotic bodies are the largest vesicle type of the 3 major EV classification groups, with a size ranging from approximately 500 to 4000 nm.[26,27] They are formed from blebbing of the plasma membrane in cells undergoing apoptosis, releasing the apoptotic bodies straight into the extracellular space.[4] Similar to both the membrane of exosomes and MVs, phosphatidylserine can be found in the outer leaflet of the lipid bilayer of apoptotic bodies.[2,6,10,28] In terms of identifying apoptotic bodies, thrombospondin and complement component C3b are in many cases accepted apoptotic body markers.[1] Unlike the 2 other vesicle types, apoptotic bodies are distinguished by containing organelles, DNA fragments, and histones as a part of the vesicular cargo in addition to proteins and other molecules from the cytosol of the parent cell.[2,4,27,28]

EVs and the Immune System

Because some of the first reports of vesicular release were published 3 decades ago,[29,30] an

enormous amount of research has been performed to delineate all aspects of this phenomenon, however, predominantly related to exosomes and MVs. Within the field of immunology, the evidence of the immunomodulatory effects of EVs has steadily increased throughout the years. Several studies document that many of the cells of the adaptive and innate immune system release EVs, including T and B lymphocytes, dendritic cells, and mast cells.[12,15,31–33] Exosomes have in particular been implicated in several contexts of antigen presentation. T cells can be activated by major histocompatibility complex II–bearing exosomes from antigen-presenting cells,[12] and some T cells even acquire these major histocompatibility complex II molecules,[34] enabling them to present antigens to other T cells.[13] On the transcriptional level, miRNA can also be transferred from T cells to antigen-presenting cells by exosomes, and this molecular transfer changes the gene expression of the recipient cell.[13] In relation to pathologic challenges of the immune system, EVs have been associated with the spread of several types of infection. Accordingly, EVs from cells infected with HIV-1, Epstein-Barr virus, hepatitis C virus, and cytomegalovirus mediate the spread of these pathogens by entering the recipient cell.[35,36] In addition, EVs from infected cells can help initiate a proper immune response against the pathogen.[37] Numerous studies have found that tumor-derived EVs can exert immunosuppressive functions, thus promoting tumor progression.[38] This can be facilitated by modulation of T cells, including the inhibition of both function and proliferation of anti–tumor-specific T cells[39] or by promotion of suppressive subsets of T cells.[40,41] Tumor-derived EVs can also play a role in transferring oncogenic activity, as was observed with the vesicular transfer of the oncogenic epidermal growth factor receptor vIII from glioma cells.[42] This receptor could also be detected on tumor EVs in serum from a number of patients with glioblastoma.[17]

Currently, it is apparent that several aspects of the mechanisms and functional consequences of EVs have yet to be determined. Nonetheless, it is also evident from the work already performed that EVs are central to an abundance of cellular processes, including those of a pathologic nature, and consequently have great potential as targets for diagnosis, prognosis, or treatment.

DIAGNOSTIC AND PROGNOSTIC POTENTIAL OF EVs IN PERIPHERAL BLOOD

Numerous studies have established that EVs can be detected in a multitude of biological fluids, such as saliva, urine, blood, ascites, breast milk, and cerebrospinal fluid.[1,8,9] In this context, blood is an immense source of EVs, and serum is estimated to contain approximately 3×10^6 exosomes per microliter.[8] Because EVs are released constitutively into the bloodstream and this release increases on cellular activation, as well as in many pathologic conditions,[43–46] a mere enumeration of EVs may indicate the presence of an aberrant process. In many aspects of diagnostics, the use of blood samples is already implemented in the clinic because it is known that blood harbors a vast amount of biomarkers and other biologically relevant molecules. In addition, most tissues will contribute to this molecular reservoir due to dense vascularization of the body. In line with this, the analysis of EVs in peripheral blood is likely to provide an indicator of the systemic health status, which can be used in clinical settings.

Analysis of EVs in Peripheral Blood

The use of EV analysis encompasses several advantages over the traditional analyses of many soluble molecules in blood, such as hormones and cytokines. One significant advantage is the inherent protection of the EV cargo of proteins and RNA from degradation, thus rendering them intact and functional.[17] Otherwise, they would be rapidly degraded in blood.[8] This has proven to be particularly significant for the use of miRNA as valuable biomarkers because most RNA in blood exists as cargo of EVs. Regarding another aspect of stability, EVs appear to have a relatively long half-life in blood.[8] Therefore, EVs can likely be transported from any location of the body to the bloodstream, thus making them easily accessible for analysis, compared with biopsies. This also has a significant advantage for the patient because the collection of a blood sample is a minimally invasive procedure associated with much less discomfort than a biopsy. Another advantage of EVs links to the great dynamic range of molecules present in the bloodstream. It can often become an issue to detect relevant biomarkers because these diagnostic molecules frequently constitute a small part of the total amount of molecules in a blood sample.[47,48] This is also the case for EV-associated proteins, which for exosomes denote $<0.01\%$ of the plasma proteome.[9] However, on the basis of the presence of a relatively small panel of markers, EVs can be identified, and the phenotype and cargo, holding biomarkers of

otherwise undetectable amounts, can be analyzed and interpreted into clinically relevant information. Furthermore, EV size distribution, phenotype, or cargo content can seemingly change according to the progression of a disease.[43–46,49,50] The access to cell-specific and disease state–specific EV profiles of proteins and RNA from a blood sample renders a sophisticated fingerprint of a disease of diagnostic and prognostic value.

EV Analysis in Risk Stratification

Cases are emerging in which EV analyses of blood samples seem to be good candidates as a part of a diagnostic platform. In addition, some examples exist in which this type of analysis shows a promising prognostic potential. As previously mentioned, the number of detectable EVs in a blood sample seems to increase in several types of cancers compared with healthy individuals.[43–46,49,50] Moreover, in some cases the amount of EVs continuously increase as the disease progresses into later stages.[44,45] In further relation to cancer, protein and RNA biomarkers have been identified in EVs from the peripheral circulation, with the preponderance of cases that involve miRNAs found in exosomes. Accordingly, several cases reveal that either a single or a panel of differentially expressed miRNAs can significantly identify patients with cancer compared with control individuals.[46,50] A selected number of EV miRNAs, which have the potential to become biomarkers of several cancer types, are given in Table I.

Table I. miRNAs differentially expressed pathologic conditions.

miRNA	Cancer Type	Reference
miR-1	NSCLC	51
miR-15b	Melanoma	52
miR-17-3p	Colorectal cancer	53
miR-21	DLBCL	54
	Glioblastoma	17
	Ovarian cancer	55
miR-25	NSCLC	56
miR-30d	NSCLC	51
miR-92	Colorectal cancer	53
	Ovarian cancer	55
miR-93	Ovarian cancer	55
miR-141	Prostate cancer	57
miR-155	DLBCL	54
	Breast cancer	58
miR-182	Melanoma	59
miR-210	DLBCL	54
miR-223	NSCLC	56
miR-486	NSCLC	51
miR-499	NSCLC	51

DLBCL = diffuse large B-cell lymphoma; miRNA = micro-RNA; NSCLC = non–small cell lung cancer.

The use of EV protein markers in risk stratification has also been investigated. One study found that the presence of 2 exosomal proteins in exosomes from plasma distinguished patients with ovarian cancer from controls.[43] In another study, EVs from patients with gastric cancer had significantly increased expression of 2 proteins, including HER2/neu, when compared with controls.[44] From this study, an interesting notion arises, indicating that some EV markers may not exclusively be used in diagnosis but could also indicate which patients are eligible for a specific treatment. A list of disease-related EV proteins from blood is given in Table II.

CHARACTERIZATION OF EVs IN PERIPHERAL BLOOD

The characterization of EVs in peripheral blood can be based on several of their biochemical and biophysical properties. These properties include size, cargo, density, morphologic findings, lipid composition, and protein phenotype. Currently, a wide range of techniques facilitates EV analysis. Some techniques have existed for years and are being further developed to embrace the challenges of this type of analysis. Other techniques have emerged as a consequence of the increasing interest within the field. The focus of the following section is to introduce a toolbox of selected techniques that could be part of a potential platform to rapidly characterize several clinically relevant properties of EVs in peripheral blood. An overview of the steps from blood sample to output of the analysis will be presented for each technique along with advantages and pitfalls. A summary of several key features associated with the presented techniques is outlined in Figure 1.

Isolation of EVs From Peripheral Blood

One aspect that is a prerequisite for almost all techniques used for EV analysis is isolation of EVs

Table II. Selected protein extracellular vesicle markers isolated from blood.

Disease	Markers	Reference
Ovarian cancer	L1CAM, CD24, ADAM10, EMMPRIN	60
	TGFβ1, MAGE3/6	43
	Claudin-4	49
Glioblastoma	EGFRvIII	17
	EGFR, EGFRvIII, PDPN, IDH1	61
Melanoma	CD63 and Caveolin 1	62
Oral cancer	FasL	63
Gastric cancer	HER-2/neu, CCR6	44
Cancer-associated thrombosis	Tissue factor (CD142)	64
Alzheimer disease	Amyloid B	65

from the biological fluid in which they are present. Hence, it is evident that EV isolation is essential, and with no standardized protocols yet, this is a major focus in the field of EV research.[66] This section introduces the key points of EV isolation because many aspects of this procedure have been recently described elsewhere.[8,66–68] Currently, most studies of EVs use differential centrifugation for isolation, sometimes accompanied by a size filtration step.[66,68] The isolation includes numerous sequential centrifugation steps with increasing centrifugal force, thus using size and density properties of the EV subsets to separate these from other components of blood. Initially, low-speed steps (300-500*g*) are applied to remove whole cells.[43,67] This is followed by higher speeds, typically in the range of 10,000 to 20,000*g*, to remove cellular debris and to isolate larger EVs >100 nm.[43,66,68–70] Finally, one or more ultracentrifugation steps are applied to pellet the smallest EVs, using centrifugal forces in the range of 100,000 to 200,000*g*.[43,66–68] It is also possible to combine the ultracentrifugation with a density gradient, such as sucrose or iodixanol,[67] or affinity purification.[71] Nevertheless, the choice of isolation procedure may have a considerable effect on the downstream analyses of EVs from blood. In addition to isolation, the preanalytical procedures also encompass choice of anticoagulant, processing temperature, and storage before analysis. These variables are all likely to affect the dynamic material that blood constitutes, and although standardizations are in the pipeline, several recommendations have been published.[66]

PHENOTYPING AND PROTEIN VALIDATION

Several methods exist to characterize the protein composition of EVs, related to either a surface marker phenotype or the proteins present in the EV cargo. Although the EV phenotype is particularly important in the determination of cellular and subcellular origin, it can in combination with a protein cargo analysis also provide clues about the functionality of the EVs. The following section focuses on 4 techniques used for characterization of EV proteins because they have great potential as reliable methods used in EV analysis. Common to all 4 methods is the dependence and use of antibodies. Consequently, they all rely on the specificity and sensitivity of the applied antibodies. Currently, no proteins are known to be constitutively sorted into vesicles independently of the subcellular origin of the vesicle and the activation status of the producing cell. This lack of invariant housekeeping markers hampers the quantitative analysis of vesicles. In the 1990s, exosomes derived from B cells were discovered to contain the lysosomal membrane protein CD63,[72] and later other members of the tetraspanin superfamily were found to be enriched in exosomes as well.[73] These early studies also found that exosomal CD63 is present in a much lower amount compared with that of the producing cells. In contrast, CD81 was >10-fold up-regulated in exosomes.[73] The phenotyping of plasma exosomes from 7 healthy donors revealed a similar tendency, indicating that CD63 is less represented and in a heterogeneous manner in relation to CD81 and CD9.[74] This finding could challenge the common

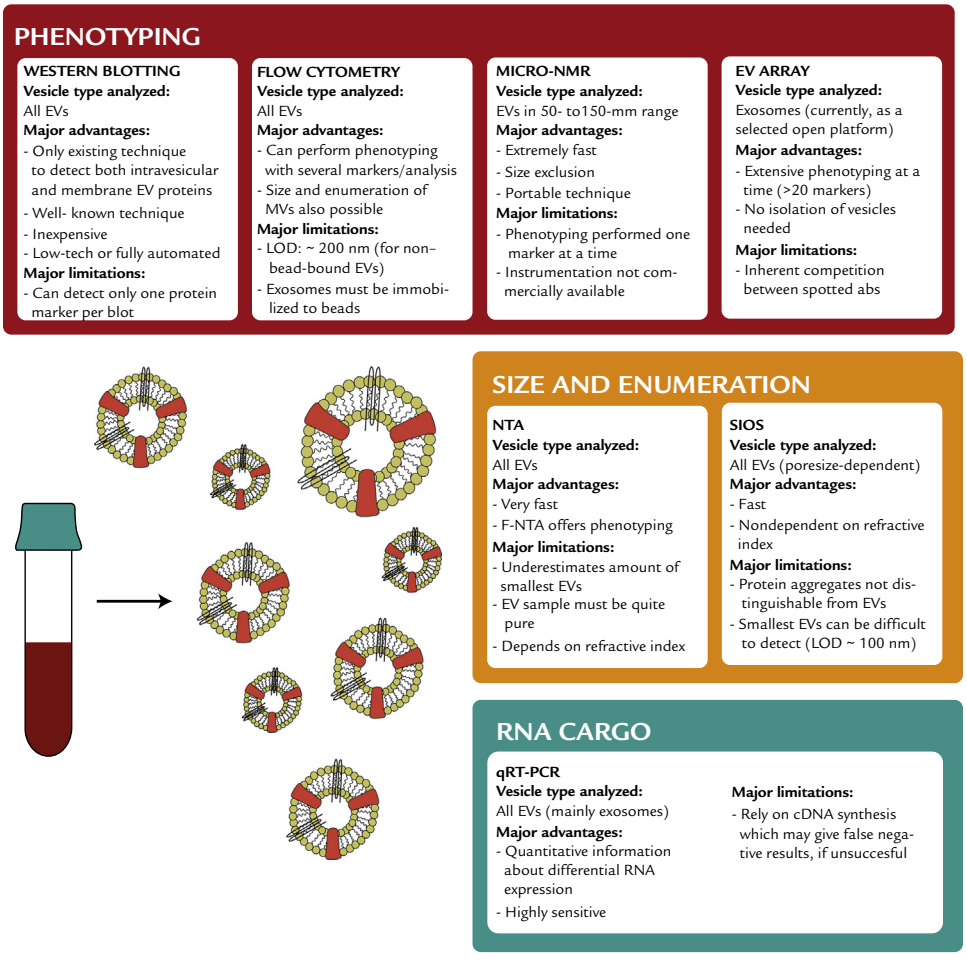

Figure 1. Overview of the techniques used to analyze extracellular vesicles (EVs) present in blood samples from a clinical and diagnostic perspective. The 3 major characterization areas are illustrated (phenotyping, size and enumeration, and RNA cargo) and the major advantages and pitfalls are outlined for each of the techniques presented in this review. abs = antibodies; cDNA = complementary DNA; LOD = limit of detection; F-NTA = fluorescence nanoparticle tracking analysis; micro-NMR = micro–nuclear magnetic resonance; MVs = microvesicles; NTA = nanoparticle tracking analysis; qRT-PCR = quantitative real-time reverse transcription–polymerase chain reaction; SIOS = scanning ion occlusion sensing.

perception of CD63 as an optimal exosomal marker,[72] which may have been attributed solely to its initial discovery and description. Consequently, it is important to choose the markers for EV phenotyping carefully.

Western Blotting

The analytical technique called Western blotting (WB) or immunoblotting is a widely accepted method used to detect specific protein markers in EVs. In general, WB is applied to validate the presence or absence of EV protein markers in purified samples based on the availability of specific antibodies.[75–80]. A major advantage of WB is the possibility of detecting intravesicular and membrane-associated proteins with the same technology (Figure 1). The basic principle of WB[81–83] is outlined in Figure 2A. The results obtained by WB are semiquantitative and can be correlated to the presence of a uniformly expressed protein. Multiple steps of centrifugation of the EV-containing

samples are generally needed before analysis.[71,80,84] In addition, the amount of EV-containing sample used for WB can vary greatly. To illustrate this, it was possible to generate 5 WBs using an EV sample of 200 μL of plasma,[84] whereas another study used WB analysis for the detection of CD63 in EVs and was able to accomplish this with approximately 0.2 μg of EV protein, equivalent to 10^8 EVs.[61] Because WB depends on a high protein concentration, sample preparation with ultracentrifugation is needed, which may be difficult in many clinical laboratories. Nonetheless, if the diagnostic or prognostic marker is of intravesicular origin, WB remains the only usable detection method of those currently applied.

Flow Cytometry

Flow cytometry is a powerful technique for multiparametric analysis of single biological particles and remains the most extensively used technique for enumeration and phenotyping of EVs in clinical samples.[85–90] By suspending particles in a hydrodynamically focused fluid stream passing a laser beam, this technique allows simultaneous analysis of the physical characteristics, including size and granularity, and expression of multiple antigens of up to thousands of particles per second.[91,92] Different approaches are applied when phenotyping either MVs or exosomes by flow cytometry. MVs are typically identified as particles with a forward scatter smaller than an internal standard that consists of approximately 1-μm beads.[93] **Figure 2B** illustrates how MVs can be distinguished using a blend of size-calibrated fluorescent beads. When analyzing a plasma sample, better specificity of MV detection is obtained by identifying those MVs that expose phosphatidylserine and cell-specific markers (**Figure 2B**). In addition, MV count per analyzed volume can easily be calculated by adding a known number of fluorescent latex beads as an internal standard. For the phenotyping of MVs by flow cytometry, use of 50 to 100 μL of platelet-free plasma is recommended.[21] Use of flow cytometry to phenotype exosomes and other EVs <200 nm normally depends on adsorption of these vesicles onto antibody-coated beads (**Figure 2C**). The beads are large enough to facilitate the detection of these small vesicles, which would otherwise fall below the lower limit of detection (**Figure 2C**). A multiparametric analysis of the EVs captured onto the beads can be performed using fluorescent-labeled antibodies against selected EV surface markers.[90] To be able to use flow cytometry for plasma exosome characterization, an isolation procedure is needed. As an example, a 3-step centrifugation isolation on 3 mL of plasma can be used as a preparatory procedure before the phenotyping.[89,90] The previous generation of flow cytometers is challenged by the small size and signals from most EVs, and several laboratories have reported that a size of 0.5 μm is the cutoff value for accurate identification.[94] However, the enhanced sensitivity of modern digital flow cytometers allows the detection of EVs in the range of approximately 200 nm to 1.0 μm[21] (**Figure 1**).

Micro–Nuclear Magnetic Resonance

Micro–nuclear magnetic resonance (μ-NMR) is a highly sensitive and rapid analytical technique developed for profiling circulating EVs from blood samples.[61] The technique uses size and immunoaffinity to define an EV population and favors EVs in the range of 50 to 150 nm. The EVs are labeled with target-specific magnetic nanoparticles and detected by a hand-sized NMR system[95,96] (**Figure 2D**). A prototype μ-NMR system has previously been used for the detection of whole tumor cells (target size range, >10 μm).[97] The output of the μ-NMR technique is a MV expression of an EV protein marker, which is calculated as the decay rate of the target protein normalized to that of a selected exosomal marker, such as CD63.[61] The output is quantitative in a relative way across samples but cannot be assigned a definitive number of vesicles or number of marker molecules on each vesicle.[61] Before loading the blood sample onto the chip, a 2-step centrifugation purification is needed. The pelleted EVs can be loaded onto the μ-NMR device for analysis, and the relative contents of a single EV marker are determined within seconds. Signals are detectable to approximately 10^4 of EVs, which is equivalent to 0.02 ng of EV protein.[61] Even if it has been established that the expression of CD63 measured by μ-NMR correlates with the number vesicles present,[61] this may not be the case with EVs from all cellular systems. Hence, care should be taken to choose the appropriate exosomal marker for normalization.

EV Array

The EV array is based on the technology of protein microarray and is capable of detecting and phenotyping

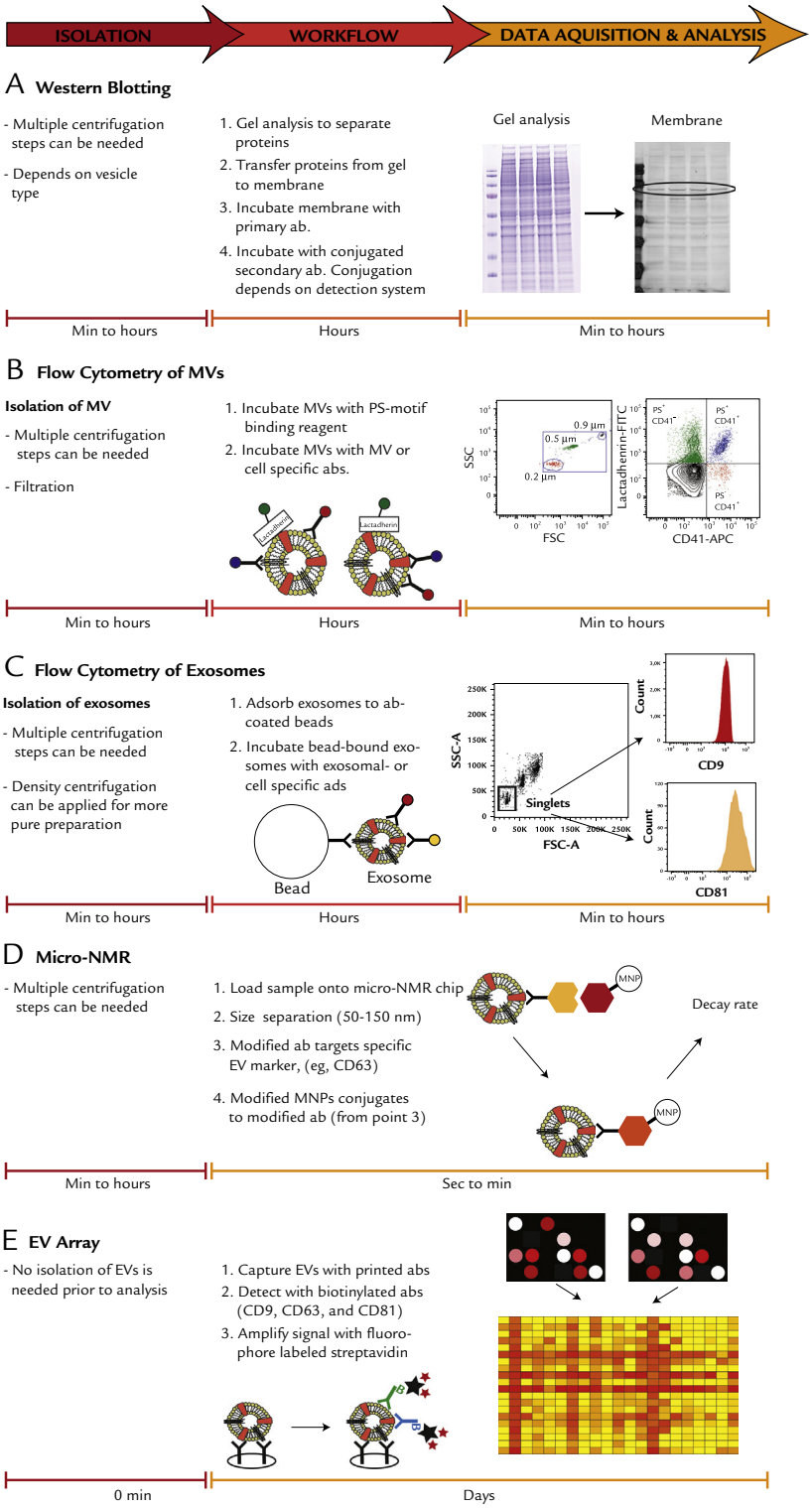

Figure 2. Outline of the workflow and timeframes for the presented techniques used for phenotyping of extracellular vesicles (EVs) from peripheral blood. (A) Western Blotting, (B) flow cytometry of microvesicles (MVs), (C) flow cytometry of exosomes, (D) micro–nuclear magnetic resonance (micro-NMR), and (E) EV Array. abs = antibodies; MNP = magnetic nanoparticles; PS = phosphatidylserine.

EVs from unpurified starting material in a high-throughput manner.[74] The technology is developed to perform multiplexed phenotyping of EVs in an open platform; however, it is optimized to analyze the exosomal EV subset. Protein microarrays are well accepted as powerful tools to search for antigens or antibodies in various sample types.[98,99] Micrometer-sized spots of capturing antibodies against known EV surface antigens are printed in a customized spot setup. The basic principle of the subsequent analysis is outlined in Figure 2E. The captured vesicles are detected using a cocktail of antibodies against the tetraspanins CD9, CD63 and CD81.[72,78] The antibodies are applied to ensure that all exosomes captured are detected, as well as excluding other types of EVs from being detected. The read-out is a fluorescence signal for each individual microarray spot (Figure 2E). The method is semiquantitative and gives the phenotype of EVs for 21 protein markers simultaneously. Plasma samples are analyzed directly without any further purification or isolation steps, and only 10 μL of plasma is needed. It has been established that only 2.5×10^4 exosomes is required for each microarray analysis.[74] The technique uses an overnight incubation to capture the EVs. Because of the incubation time and the remaining development procedure, this method takes approximately 2 days to complete, making it the slowest assay of the ones presented in this review.

ENUMERATION AND SIZE

Determination of the amount and size distribution of EVs in a blood sample is greatly valuable because of the previously mentioned observation that an increased production of EVs has been detected in several pathologic conditions. In addition, knowing the size of the EVs present can be informative because it can indicate which vesicle type is the most dominant type in an unprocessed sample and provide information about the quality of an isolation procedure.[100] Several methods exist to enumerate EVs and determine their size. The following section focuses on 2 of these because they have great potential as fast methods used in EV analysis.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) allows for a quick size determination and quantification of EVs in a suspension. It was first introduced in 2006 and has in recent years received significant attention within the field of EV analysis.[101] The technique is based on relating the Brownian motions of a particle to its size. Brownian motions are experienced by all particles in a fluid, here constituted by EVs in a suspension. These motions can be tracked when the particles are illuminated by a laser beam and scatter the light[101–103] (Figure 3A). An image processing software subsequently calculates the size distribution and concentration of particles in the solution.[100,101,104] The NTA determines these factors most precisely for EVs in the range of 30 to 1000 nm in a concentration of approximately 10^8 to 10^9 particles/mL.[100,102] Hence, NTA seems to offer a more sensitive enumeration than other conventional methods, such as flow cytometry. For the analysis of plasma, the high content of lipoprotein particles may pose a significant problem.[101] Consequently, it is a prerequisite to isolate the EV type of interest before NTA, when analyzing these vesicles from plasma or serum.[101] As an interesting notion, selected NTA instruments allow for the expansion to fluorescence NTA in which EVs can be labeled with antibodies conjugated to stable fluorophores and subsequently be phenotyped in addition to the enumeration and concentration determination.[103]

Scanning Ion Occlusion Sensing

The scanning ion occlusion sensing (SIOS) method is a relatively new technology that facilitates a fast analysis of EV size, concentration, and biochemical composition. The technology is nonoptical, label free, and based on passing each EV through a nanopore in a membrane by means of single-molecule electrophoresis.[105] The basic principle is shown in Figure 3B. A transmembrane current drives the electrophoresis, and an ionic current through the nanopore channel can be measured.[105] The passing EV shifts this current, and the transient blockade of the pore can be translated into information about size and surface charge.[102] In addition to the size and surface charge, the total number of EVs passing the nanopore is detected, and the read-out can be depicted as a plot of concentration versus size distribution (Figure 3B). The SIOS has a reported usability for particles in the large range of 70 nm to 10 μm and in the concentration of 10^5 to 10^{12} particles/mL.[102] The use of complex samples can pose some difficulties because protein aggregates cannot be separated from MVs, which is explained by their similar size (Figure 1). In contrast to NTA,

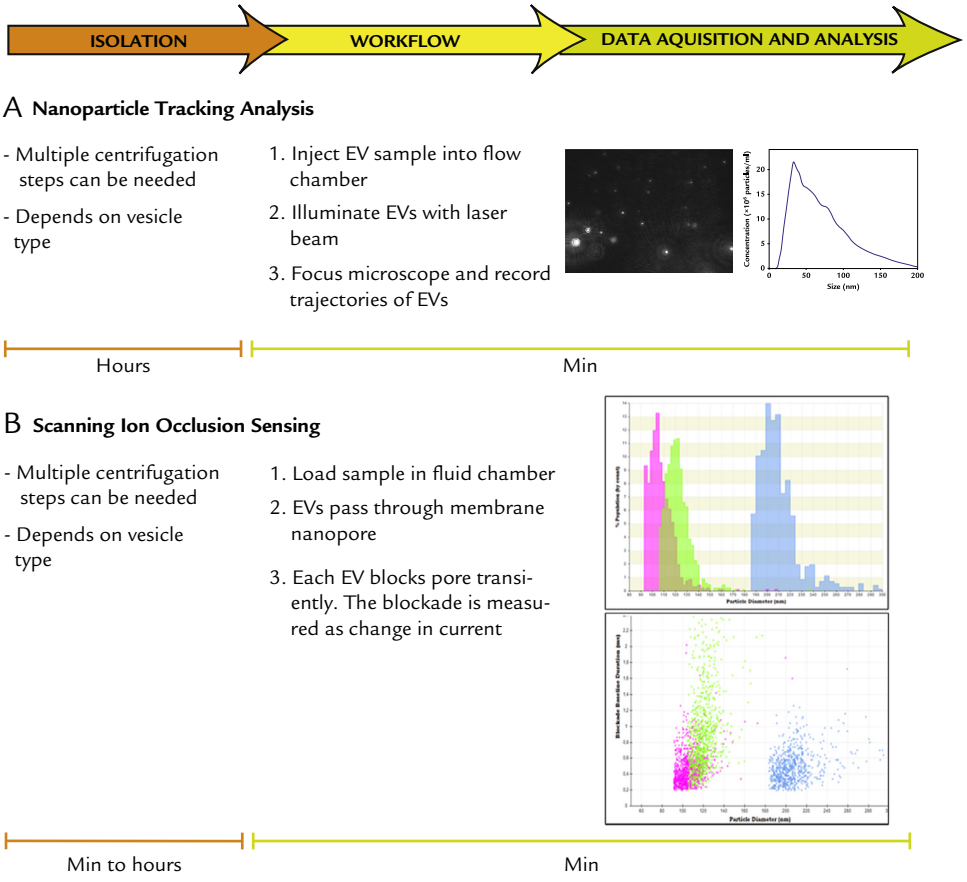

Figure 3. Outline of the workflow and timeframe for the presented techniques used for size determination and enumeration of extracellular vesicles (EVs). (A) Nanoparticle tracking analysis; (B) Scanning Ion Occlusion Sensing.

SIOS does not depend on the refractive index of the particles analyzed because it is a nonoptical technique.

RNA CARGO

Along with the increasing attention on EVs, it has become consistently apparent that their RNA cargo contributes to the potential diagnostic and prognostic value of these vesicles. As mentioned previously, several research groups have reported that the RNA present in serum and plasma is in fact protected from the highly active RNases when being stored inside the different kind of vesicles.[16,57,106,107] In addition, the major content of the EVs has been found to be small RNAs, such as mRNA and miRNA,[17,108] the latter being small non–protein-coding pieces of RNA, consisting of 18–24 nucleotides.[57,109,110] They are known to play important roles in the regulation of mRNA, which they can target for cleavage.[109] The expression of miRNAs seems to depend on the cellular function and in some cases on the cellular stage. In addition, a number of miRNAs are specific to cell type or tissue.[109] As previously mentioned, a rapidly expanding list of miRNAs as potential biomarkers is forming, in which a differential expression can be detected between healthy and pathologic conditions, in particular related to cancer. A selection of such EV miRNA markers is given in Table I. When focusing on the discovery of potential miRNA biomarkers, approaches such as microarray analyses and deep sequencing are useful techniques to perform a wide search. Such a search can be performed with pooled samples of a chosen disease group versus a control group. After single or multiple

candidates are identified, a more targeted manner is chosen to validate and quantify these findings to reveal the true diagnostic potential.[56] This is facilitated by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR).[70] Because the qRT-PCR technique is the most relevant as a possible clinically test to characterize the EV RNA cargo, this is the focus of the following section.

qRT-PCR

The qRT-PCR technique is based on the PCR, which is used to amplify and simultaneously quantify a targeted RNA molecule.[111] For ≥1 specific sequences in a sample, qRT-PCR enables detection and quantification. The quantity can be an absolute number of copies or a relative amount when normalized to RNA input or additional reference genes. Initially, the characterization of the EV RNA cargo requires extraction of the RNA[112] and complementary DNA synthesis[113,114] before qRT-PCR (Figure 4). The output of the quantification is a signal that relates to the amount of RNA present in the sample. Because this has no unit, the results of the relative quantification can be compared across a number of different qRT-PCR analyses. A total of ≥1 reference genes are applied to correct for nonspecific variation, such as the differences in the quantity and quality of RNA used, which can affect the efficiency of reverse transcription and therefore the entire PCR process. In most of the reported work on EV RNA cargo analysis, a comprehensive isolation procedure is applied (Figure 4). The isolation often involves multiple centrifugation steps and filtration.[80,115,116] However, an optimized protocol was recently published, describing the extraction and qRT-PCR analysis of EV RNA directly from plasma and serum isolated with only a single centrifugation step at low speed and time.[70] Furthermore, the protocol describes that a 250-μL sample provides a sufficient amount of RNA to perform a qRT-PCR analysis,[70] which is of high value in a diagnostic relation. Other studies confirm that miRNAs can be extracted from both serum and plasma with similar results.[54,57]

FUTURE TECHNICAL PERSPECTIVES

The techniques presented in this review could all potentially be part of a platform to characterize EVs from peripheral blood and transform these data into clinically relevant information. However, to fully harness the diagnostic and prognostic potential of EVs, a number of aspects remain to be delineated. A fundamental aspect, which is imperative to address, is standardization.[66] The standardization relates to several areas of EV analysis and research and includes nomenclature, preanalytical conditions, and isolation procedures. This will give rise to standardized protocols and facilitate unbiased comparisons of results from different studies across laboratories. Furthermore, it will greatly aid in the development of robust clinical assays. The next challenge, before using EVs in a diagnostic manner, is to select the most

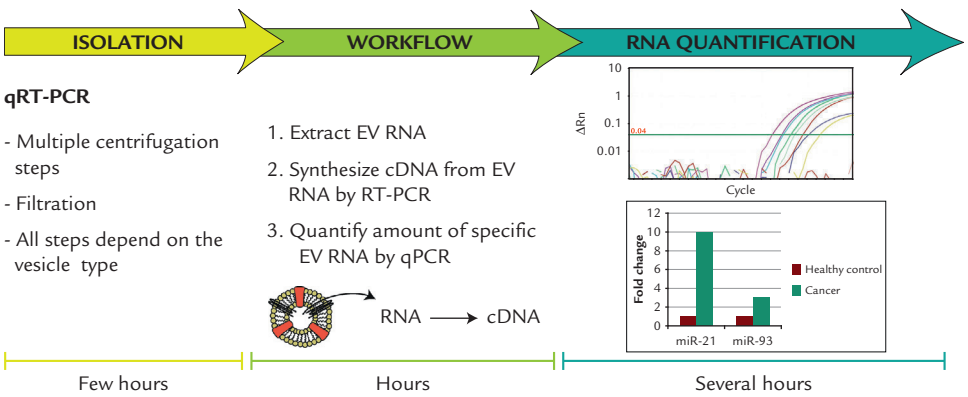

Figure 4. Outline of the workflow and timeframe for the analysis of extracellular vesicle (EV) RNA cargo by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). cDNA = complementary DNA.

optimal methods, concerning sample requirements, apparatus, and analysis accessibility. In addition, selection of the most optimal combination of target markers is essential to several of the methods. A common feature for all the methods outlined is the need for an extensive isolation procedure before EV analysis. In that regard, considerations should be taken concerning the loss of material and thereby valuable information. Therefore, the method of analysis should most optimally work on unpurified plasma or serum samples. A prerequisite for the incorporation of EV analysis into robust clinical assays is the discovery and validation of relevant diagnostic or prognostic EV targets. Currently, thousands of EV-related proteins and RNA components have been identified by proteomic and transcriptomic approaches.[117] In the discovery process, various cell lines have been extensively used.[7,49,71] Even if cell lines are only an approximation of the *in vivo* conditions, they serve as useful model systems and encompass a relevant source of cell surface–expressed biomarkers and intracellular proteins and RNAs, relating to cancer in particular. These *in vitro* model systems are consequently valuable in the matter of identifying which proteins and potential biomarkers are conveyed to the EVs. As an example, this strategy was adopted to identify the protein Claudin as an EV-expressed biomarker for ovarian cancer. Likewise, the model systems of cell lines have a great potential to elucidate the biogenesis of EVs and their subsequent influence on the recipient cells. This finding has been reported in a study in which a mast cell line was exposed to oxidative stress and the subsequent cell-derived EVs revealed a changed RNA profile compared with those derived from cells grown under normal conditions. This study additionally clarified that the stress-derived EVs were able to affect non-stressed cells to become more resistant to the oxidative stress.[32] Nonetheless, the use of cell lines and their autologous production of EVs must be carefully interpreted. Cell lines are kept in a closed and controlled system, mainly as monocultures, and will therefore only receive and respond to the signals specifically applied to them. Because the cells receive no signals or EVs from their original surrounding environment, they will most likely merely autocommunicate. Therefore, cell lines will only partly reflect the authenticity of the biogenesis of the EVs. In the biomarker discovery, the use of mass spectrometry (MS) has for at least 2 decades been essential for the proteomics-driven research of EVs and is the only technique available that allows EV characterization from all common clinical biospecimens, including serum, plasma, urine, synovial fluid, ascites, and feces. Accordingly, the identification of several important EV protein markers has been facilitated by MS.[118] The use of MS has also provided information about aspects of EV biogenesis along with possible pathologic functions of these vesicles.[117] Currently, technical methods for targeted MS platforms are being applied in clinical assays and include multiple reaction monitoring and selected reaction monitoring. These methods enable MS as a high-throughput technique to perform rapid analyses of EV proteins, which could also be used in a diagnostic fashion and not exclusively in the discovery phase for EV biomarkers. Nonetheless, the development of standardizations for EV analysis along with a continued effort to unravel all aspects of EV proteins and RNA will improve the understanding of the molecular mechanisms and biological functions of EVs. Despite being in its infancy, the field of EV research has undoubtedly received an enormous interest. Consequently, numerous initiatives have been implemented in the EV society, including several databases (ExoCarta,[119] EVpedia,[120] Vesiclepedia,[121] and miRandola[122]), conferences (International Society for Extracellular Vesicles conference on microvesiculation and disease), and a dedicated journal (*Journal of Extracellular Vesicles*).[123]

FUTURE CLINICAL PERSPECTIVES

The apparent role of EVs in a vast number of biological processes, along with many of their intriguing features, forms the basis of extending EV analysis beyond basic research and into a clinical and therapeutic context. Despite being a relatively new field, the potential and versatility of EV analysis are supported by an increasing number of publications. The applications of this type of analysis include the areas of diagnostics and prognostics, as well as drug therapy, regenerative medicine, and vaccines. For the purpose of diagnosis and prognosis, the use of EVs seems particularly promising because these vesicles contain a plethora of clinically relevant molecules, such as proteins and RNA from the parent cell. Thereby, the miRNA and protein patterns, which are unique for a specific pathologic condition, can be used. The analysis of EVs could accordingly be incorporated as a screening tool and applied to confirm a diagnosis.

Furthermore, EV analysis has the potential to become an element in treatment surveillance and companion diagnostics, resulting in a patient-optimized treatment. Related to therapeutics, EVs have been proposed as a new type of drug delivery system. Such a system involves engineered EVs loaded with a therapeutic cargo and expressing ligands, which target a particular tissue or cell type. The inherent protection of the cargo and tailored cellular targeting simultaneously enhance the solubility, stability, and specificity of the therapeutic agent. In another approach, the EVs constitute the therapeutic target, and the aim is to inhibit their release or to perform a systemic depletion. In terms of regenerative capabilities, EVs from stem cells have clinical potential.[124] The EVs accordingly constitute a cell-free approach, which mediates many of the regenerative properties from the stem cells. This circumvents several issues related to stem cell transplantations, consequently making handling and storage easier and increasing the stability. This cell-free approach has also been adapted in the field of vaccines and immunotherapy. Particularly in relation to cancer, EVs have been used as potent inducers of antitumor responses.[125,126] In addition, several Phase I and Phase II studies have been initiated, almost exclusively focusing on vaccination with autologous exosomes.[8] The results of these studies indicate a promising future for the use of EV-based treatment. The abovementioned examples of the clinical use of EV analysis clearly indicate its feasibility. In line with the scope of this review, the potential of using EV analysis of a simple blood sample seems encouraging, and this is particularly pronounced in relation to diagnostics. Despite the extensive amount of information, which is possible to obtain from a single blood sample, this complexity is at the same time a challenge that complicates EV analysis. Nonetheless, advances and continued research within the field allow for the development and improvement of techniques and standard protocols, which seek to meet these challenges. The increased understanding of this may lead to a paradigm shift, particularly related to immune regulation and cellular communication in cancer. Taken together, technology and biology will inevitably pave the way for the future use of EV analysis in many clinical applications.

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CONFLICTS OF INTEREST

The authors have indicated that they have no conflicts of interest regarding the content of this article.

REFERENCES

1. Akers JC, Gonda D, Kim R, et al. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*. 2013;113:1–11.
2. Andaloussi SEL, Mager I, Breakefield XO, et al. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*. 2013;12:347–357.
3. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013;200:373–383.
4. Gyorgy B, Szabo TG, Pasztoi M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci*. 2011;68:2667–2688.
5. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 2009;19:43–51.
6. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol*. 2009;9:581–593.
7. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics*. 2010;73:1907–1920.
8. Vlassov AV, Magdaleno S, Setterquist R, et al. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta*. 2012;1820:940–948.
9. Pant S, Hilton H, Burczynski ME. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. *Biochem Pharmacol*. 2012;83:1484–1494.
10. Distler JH, Huber LC, Gay S, et al. Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity*. 2006;39:683–690.
11. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Madrid F, et al. Analysis of microRNA and protein transfer by exosomes during an immune synapse. *Methods Mol Biol*. 2013;1024:41–51.

12. Nolte-'t Hoen EN, Buschow SI, Anderton SM, et al. Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood*. 2009;113:1977–1981.
13. Arnold PY, Mannie MD. Vesicles bearing MHC class II molecules mediate transfer of antigen from antigen-presenting cells to CD4+ T cells. *Eur J Immunol*. 1999;29:1363–1373.
14. Choudhuri K, Llodra J, Roth EW, et al. Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. *Nature*. 2014;507:118–123.
15. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun*. 2011;2:282.
16. Valadi H, Ekstrom K, Bossios A, et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9:654–659.
17. Skog J, Wurdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10:1470–1476.
18. Muralidharan-Chari V, Clancy JW, Sedgwick A, et al. Microvesicles: mediators of extracellular communication during cancer progression. *J Cell Sci*. 2010;123(pt 10):1603–1611.
19. Lynch SF, Ludlam CA. Plasma microparticles and vascular disorders. *Br J Haematol*. 2007;137:36–48.
20. Abid Hussein MN, Meesters EW, Osmanovic N, et al. Antigenic characterization of endothelial cell-derived microparticles and their detection ex vivo. *J Thromb Haemost*. 2003;1:2434–2443.
21. Nielsen MH, Beck-Nielsen H, Andersen MN, et al. A flow cytometric method for characterization of circulating cell-derived microparticles in plasma. *J Extracell Vesicles*. 2014 Feb 24. [Epub ahead of print].
22. Connor DE, Exner T, Ma DD, et al. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagulant activity and demonstrate greater expression of glycoprotein Ib. *Thromb Haemost*. 2010;103:1044–1052.
23. Jimenez JJ, Jy W, Mauro LM, et al. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res*. 2003;109:175–180.
24. Perez-Pujol S, Marker PH, Key NS. Platelet microparticles are heterogeneous and highly dependent on the activation mechanism: studies using a new digital flow cytometer. *Cytometry A*. 2007;71:38–45.
25. Muralidharan-Chari V, Clancy J, Plou C, et al. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr Biol*. 2009;19:1875–1885.
26. Berda-Haddad Y, Robert S, Salers P, et al. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. *Proc Natl Acad Sci U S A*. 2011;108:20684–20689.
27. Hristov M, Erl W, Linder S, et al. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells in vitro. *Blood*. 2004;104:2761–2766.
28. Cline AM, Radic MZ. Apoptosis, subcellular particles, and autoimmunity. *Clin Immunol*. 2004;112:175–182.
29. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983;33:967–978.
30. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol*. 1983;97:329–339.
31. Admyre C, Johansson SM, Paulie S, et al. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol*. 2006;36:1772–1781.
32. Eldh M, Ekstrom K, Valadi H, et al. Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS One*. 2010;5:e15353.
33. Saunderson SC, Schuberth PC, Dunn AC, et al. Induction of exosome release in primary B cells stimulated via CD40 and the IL-4 receptor. *J Immunol*. 2008;180:8146–8152.
34. Buschow SI, Nolte-'t Hoen EN, van Niel G, et al. MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic*. 2009;10:1528–1542.
35. Wurdinger T, Gatson NN, Balaj L, et al. Extracellular vesicles and their convergence with viral pathways. *Adv Virol*. 2012;2012:767694.
36. Mack M, Kleinschmidt A, Bruhl H, et al. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med*. 2000;6:769–775.
37. Bhatnagar S, Shinagawa K, Castellino FJ, et al. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. *Blood*. 2007;110:3234–3244.
38. Rak J, Guha A. Extracellular vesicles - vehicles that spread cancer genes. *Bioessays*. 2012;34:489–497.
39. Abusamra AJ, Zhong Z, Zheng X, et al. Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol Dis*. 2005;35:169–173.
40. Wieckowski EU, Visus C, Szajnik M, et al. Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T

lymphocytes. *J Immunol*. 2009;183:3720–3730.
41. Szajnik M, Czystowska M, Szczepanski MJ, et al. Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg). *PLoS One*. 2010;5:e11469.
42. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol*. 2008;10:619–624.
43. Szajnik M, Derbis M, Lach M, et al. Exosomes in plasma of patients with ovarian carcinoma: potential biomarkers of tumor progression and response to therapy. *Gynecol Obstet (Sunnyvale)*. 2013;(Suppl 4):3.
44. Baran J, Baj-Krzyworzeka M, Weglarczyk K, et al. Circulating tumour-derived microvesicles in plasma of gastric cancer patients. *Cancer Immunol Immunother*. 2009;59:841–850.
45. Kim HK, Song KS, Park YS, et al. Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor. *Eur J Cancer*. 2003;39:184–191.
46. Rabinowits G, Gercel-Taylor C, Day JM, et al. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin Lung Cancer*. 2009;10:42–46.
47. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics*. 2002;1(11):845–867.
48. Hortin GL, Sviridov D. The dynamic range problem in the analysis of the plasma proteome. *J Proteomics*. 2010;73:629–636.
49. Li J, Sherman-Baust CA, Tsai-Turton M, et al. Claudin-containing exosomes in the peripheral circulation of women with ovarian cancer. *BMC Cancer*. 2009;9:244.
50. Tanaka Y, Kamohara H, Kinoshita K, et al. Clinical impact of serum exosomal microRNA21 as a clinical biomarker in human esophageal squamous cell carcinoma. *Cancer*. 2013;119:1159–1167.
51. Hu Z, Chen X, Zhao Y, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. *J Clin Oncol*. 2010;28:1721–1726.
52. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. *Int J Cancer*. 2010;126:2553–2562.
53. Ng EK, Chong WW, Jin H, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut*. 2009;58:1375–1381.
54. Lawrie CH, Gal S, Dunlop HM, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008;141:672–675.
55. Resnick KE, Alder H, Hagan JP, et al. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol Oncol*. 2009;112:55–59.
56. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997–1006.
57. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105:10513–10518.
58. Zhu W, Qin W, Atasoy U, et al. Circulating microRNAs in breast cancer and healthy subjects. *BMC Res Notes*. 2009;2:89.
59. Segura MF, Hanniford D, Menendez S, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A*. 2009;106:1814–1819.
60. Keller S, Konig AK, Marme F, et al. Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett*. 2009;278:73–81.
61. Shao H, Chung J, Balaj L, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med*. 2012;18:1835–1840.
62. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*. 2009;4:e5219.
63. Kim JW, Wieckowski E, Taylor DD, et al. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin Cancer Res*. 2005;11:1010–1020.
64. Tesselaar ME, Romijn FP, Van Der Linden IK, et al. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost*. 2007;5:520–527.
65. Matsubara E, Shoji M, Murakami T, et al. Platelet microparticles as carriers of soluble Alzheimer's amyloid beta (sAbeta). *Ann N Y Acad Sci*. 2002;977:340–348.
66. Witwer KW, Buzas EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles*. 27 May 2013. [Epub ahead of print].
67. Momen-Heravi F, Balaj L, Alian S, et al. Current methods for the isolation of extracellular vesicles. *Biol Chem*. 2013;394:1253–1262.
68. van der Pol E, Boing AN, Harrison P, et al. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;64:676–705.
69. Gyorgy B, Modos K, Pallinger E, et al. Detection and isolation of

cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. *Blood*. 2011;117:e39–e48.
70. Moldovan L, Batte K, Wang Y, et al. Analyzing the circulating microRNAs in exosomes/extracellular vesicles from serum or plasma by qRT-PCR. *Methods Mol Biol*. 2013;1024:129–145.
71. Tauro BJ, Greening DW, Mathias RA, et al. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods*. 2012;56:293–304.
72. Peters PJ, Neefjes JJ, Oorschot V, et al. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature*. 1991;349:669–676.
73. Escola JM, Kleijmeer MJ, Stoorvogel W, et al. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem*. 1998;273:20121–20127.
74. Joergensen M, Baek R, Pedersen S, et al. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. *J Extracell Vesicles*. 2013 June 18. [Epub ahead of print].
75. Stoorvogel W, Geuze HJ, Griffith JM, et al. Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein, and mannose 6-phosphate in human hepatoma cells. *J Cell Biol*. 1989;108:2137–2148.
76. Stoorvogel W, Oorschot V, Geuze HJ. A novel class of clathrin-coated vesicles budding from endosomes. *J Cell Biol*. 1996;132:21–33.
77. Blanchard N, Lankar D, Faure F, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol*. 2002;168:3235–3241.
78. Raposo G, Nijman HW, Stoorvogel W, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996;183:1161–1172.
79. Heijnen HF, Schiel AE, Fijnheer R, et al. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood*. 1999;94:3791–3799.
80. Lasser C, Eldh M, Lotvall J. Isolation and characterization of RNA-containing exosomes. *J Vis Exp*. 2012(59):e3037.
81. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
82. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76:4350–4354.
83. Renart J, Reiser J, Stark GR. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci U S A*. 1979;76:3116–3120.
84. Li Y, Jiang T, Zhang J, et al. Elevated serum antibodies against insulin-like growth factor-binding protein-2 allow detecting early-stage cancers: evidences from glioma and colorectal carcinoma studies. *Ann Oncol*. 2012;23:2415–2422.
85. Jy W, Horstman LL, Jimenez JJ, et al. Measuring circulating cell-derived microparticles. *J Thromb Haemost*. 2004;2:1842–1851.
86. Kesimer M, Scull M, Brighton B, et al. Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. *FASEB J*. 2009;23:1858–1868.
87. Mobarrez F, Antovic J, Egberg N, et al. A multicolor flow cytometric assay for measurement of platelet-derived microparticles. *Thromb Res*. 2010;125:e110–e116.
88. Orozco AF, Lewis DE. Flow cytometric analysis of circulating microparticles in plasma. *Cytometry A*. 2010;77:502–514.
89. Caby MP, Lankar D, Vincendeau-Scherrer C, et al. Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005;17:879–887.
90. Silva J, Garcia V, Rodriguez M, et al. Analysis of exosome release and its prognostic value in human colorectal cancer. *Genes Chromosomes Cancer*. 2012;51:409–418.
91. Lacroix R, Robert S, Poncelet P, et al. Overcoming limitations of microparticle measurement by flow cytometry. *Semin Thromb Hemost*. 2010;36:807–818.
92. Gelderman MP, Simak J. Flow cytometric analysis of cell membrane microparticles. *Methods Mol Biol*. 2008;484:79–93.
93. Shet AS, Key NS, Hebbel RP. Measuring circulating cell-derived microparticles. *J Thromb Haemost*. 2004;2:1848–1850.
94. Robert S, Poncelet P, Lacroix R, et al. Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies? *J Thromb Haemost*. 2009;7:190–197.
95. Lee H, Sun E, Ham D, et al. Chip-NMR biosensor for detection and molecular analysis of cells. *Nat Med*. 2008;14:869–874.
96. Issadore D, Min C, Liong M, et al. Miniature magnetic resonance system for point-of-care diagnostics. *Lab Chip*. 2011;11:2282–2287.
97. Haun JB, Castro CM, Wang R, et al. Micro-NMR for rapid molecular analysis of human tumor samples. *Sci Transl Med*. 2011;3. 71ra16.
98. Melton L. Protein arrays: proteomics in multiplex. *Nature*. 2004;429:101–107.

99. Hall DA, Ptacek J, Snyder M. Protein microarray technology. *Mech Ageing Dev*. 2007;128:161–167.
100. Soo CY, Song Y, Zheng Y, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology*. 2012;136:192–197.
101. Gardiner C, Ferreira YJ, Dragovic RA, et al. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles*. 2013 Feb 15. [Epub ahead of print].
102. Momen-Heravi F, Balaj L, Alian S, et al. Alternative methods for characterization of extracellular vesicles. *Front Physiol*. 2012;3:354.
103. Dragovic RA, Gardiner C, Brooks AS, et al. Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine*. 2011;7:780–788.
104. Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res*. 2010;27:796–810.
105. Garza-Licudine E, Deo D, Yu S, et al. Portable nanoparticle quantization using a resizable nanopore instrument - the IZON qNano. *Conf Proc IEEE Eng Med Biol Soc*. 2010; 2010:5736–5739.
106. Kosaka N, Iguchi H, Yoshioka Y, et al. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem*. 2010; 285:17442–17452.
107. Kogure T, Lin WL, Yan IK, et al. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology*. 2011;54:1237–1248.
108. Ekstrom K, Valadi H, Sjostrand M, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *J Extracell Vesicles*. 2012 Apr 16. [Epub ahead of print].
109. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116:281–297.
110. Esquela-Kerscher A, Slack FJ. Oncomirs: microRNAs with a role in cancer. *Nat Rev Cancer*. 2006;6: 259–269.
111. Gertsch J, Guttinger M, Sticher O, et al. Relative quantification of mRNA levels in Jurkat T cells with RT-real time-PCR (RT-rt-PCR): new possibilities for the screening of anti-inflammatory and cytotoxic compounds. *Pharm Res*. 2002;19: 1236–1243.
112. Eldh M, Lotvall J, Malmhall C, et al. Importance of RNA isolation methods for analysis of exosomal RNA: evaluation of different methods. *Mol Immunol*. 2012;50:278–286.
113. de Planell-Saguer M, Rodicio MC. Detection methods for microRNAs in clinic practice. *Clin Biochem*. 2013;46:869–878.
114. Chen C, Ridzon DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*. 2005;33:e179.
115. Crescitelli R, Lasser C, Szabo TG, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles*. 2013 Sep 12. [Epub ahead of print].
116. Lasser C. Identification and analysis of circulating exosomal microRNA in human body fluids. *Methods Mol Biol*. 2013;1024:109–128.
117. Choi DS, Kim DK, Kim YK, et al. Proteomics, transcriptomics and lipidomics of exosomes and ectosomes. *Proteomics*. 2013;13:1554–1571.
118. Choi DS, Kim DK, Kim YK, et al. Proteomics of extracellular vesicles: exosomes and ectosomes. *Mass Spectrom Rev*. 2014 Jan 14. [Epub ahead of print].
119. Simpson RJ, Kalra H, Mathivanan S. ExoCarta as a resource for exosomal research. *J Extracell Vesicles*. 2012 Apr 16. [Epub ahead of print].
120. Kim DK, Kang B, Kim OY, et al. EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *J Extracell Vesicles*. 2013 Mar:19. [Epub ahead of print].
121. Kalra H, Simpson RJ, Ji H, et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol*. 2012;10:e1001450.
122. Russo F, Di Bella S, Nigita G, et al. miRandola: extracellular circulating microRNAs database. *PLoS One*. 2012;7:e47786.
123. Lotvall J, Rajendran L, Gho YS, et al. The launch of Journal of Extracellular Vesicles (JEV), the official journal of the International Society for Extracellular Vesicles - about microvesicles, exosomes, ectosomes and other extracellular vesicles. *J Extracell Vesicles*. 2012 Apr 16. [Epub ahead of print].
124. Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci*. 2014;15:4142–4157.
125. Zitvogel L, Regnault A, Lozier A, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med*. 1998;4:594–600.
126. Andre F, Chaput N, Schartz NE, et al. Exosomes as potent cell-free peptide-based vaccine, I: dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol*. 2004;172:2126–2136.

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