# NOVEL CLINICAL APPLICATIONS OF EXTRACELLULAR VESICLES

EDITED BY: Matías Sáenz-Cuesta, David Otaegui and María Mittelbrunn
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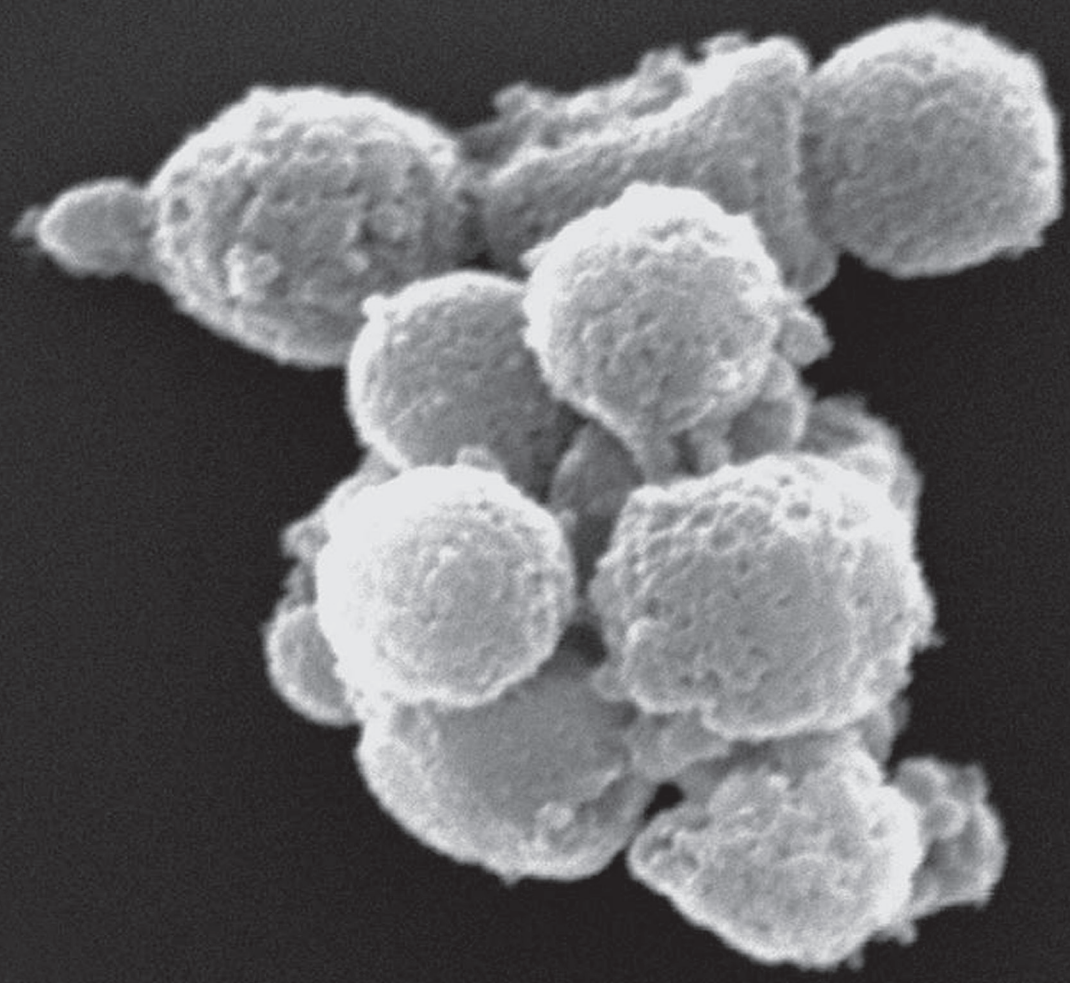

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# NOVEL CLINICAL APPLICATIONS OF EXTRACELLULAR VESICLES

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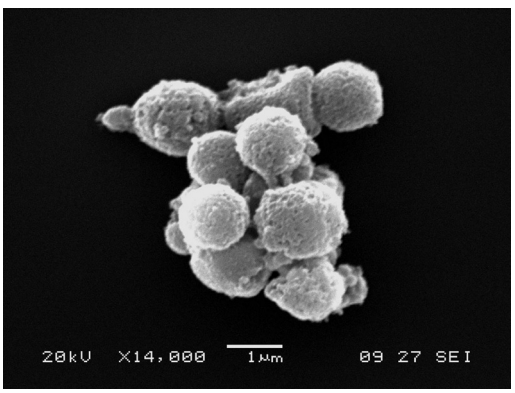


An electron microscopy image of an EV cluster obtained from peripheral blood.

Image by Matías Sáenz-Cuesta.

During the last years, the research on extracellular vesicles (EVs) has raised giving new insights into pathophysiology of several diseases. EVs are membrane-bound particles secreted by almost all cell types. Depending on their biogenesis and size they include exosomes, microparticles / microvesicles and apoptotic bodies. Characteristically, EVs carry markers from the source cell membrane and contain genetic material, lipids and proteins inside. They are known to play a role in cell-to-cell communication and to produce genotypic and phenotypic modifications in the target cell including: antigen presentation, apoptosis induction, cellular activation, inhibition or differentiation. In particular, increasing concentrations of EVs have been found in many diseases such as cancer, autoimmune and cardiovascular diseases, among others. Most of the studies in EVs are focused on the characterization of EVs compounds, identifying mechanism of action, their potential use as biomarkers, and few of them investigate a therapeutic usage. However, there are some issues to be achieved on the path to their clinical application. This research topic offers a common place to discuss current and novel clinical applications of EVs pointing on future directions. We encouraged the submission of original articles, reviews, hypothesis, controversies, future perspectives and personal viewpoints on the following topics of interest, but not limited to:

- Contribution of EVs to better understand the pathology of immunological diseases.
- Standardization of isolation and quantification protocols in the daily clinical practice.

- Possible applications of EVs as clinical biomarkers (diagnostic, prognostic and evolution marker).
- Therapeutic role of EVs being vehicles of specific cargo: current clinical trials?
- Novel immunological functions of EVs.

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EDITORIAL
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# Editorial: Novel clinical applications of extracellular vesicles

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**Keywords: extracellular vesicles, protocol standardization, clinical application, exosomes, biomarkers, immunotherapy, microvescicles, omics-technologies**

Cells employ several modes of communication, from direct contact to paracrine signaling. Understanding what they are whispering one to another has long been a goal for immunologists. In this scenario, the potential of a newly discovered way of sending specific messages between cell types, extracellular vesicles (EVs), has started a revolution in the field.

During recent years, research on EVs has provided new insights into the pathophysiology of several diseases. EVs are membrane-bound particles secreted by almost all cell types. Depending on their biogenesis and size, they include exosomes, microparticles/microvesicles, and apoptotic bodies (1). Characteristically, EVs carry markers from the source cell membrane and contain genetic material, lipids, and proteins. They are known to play a role in cell-to-cell communication and to produce genotypic and phenotypic modifications in the target cell including antigen presentation, apoptosis induction, cellular activation, and differentiation (2). In particular, elevated concentrations of EVs have been found in many diseases including cancer (3), and autoimmune (4) and cardiovascular diseases (5). Most research on EVs is focused on their characterization, identifying mechanisms of action, and their potential use as biomarkers, with some studies investigating therapeutic applications. In relation to the promising results obtained in the EV field; however, there are some technical issues concerning standardization to be resolved and these are being addressed by researchers. Further, even though EVs have a growing future as biomarkers, drug delivery systems, or therapeutic targets, there remain milestones to be achieved on the path to their clinical application. This research topic aims to provide a forum for the discussion of current and novel clinical applications of EVs, opening new avenues in this field.

We focus the topic on two closely related fields where EVs have produced the most impact, namely, cancer and immunology. First, de Toro et al. have summarized the current roles of EVs in physiological and pathological and physiological states, including neurodegenerative, cardiovascular, and immune diseases (6). They conclude with an interesting section on the potential applications of EVs in diagnosis and therapy.

In relation to cancer, Benito-Martín et al. have reviewed the functions of innate immune-derived EVs in relation to modification of microenvironment and the control of tumor progression (7). The authors also provide a detailed description of the role of EVs derived from innate immune cells in specific cancers (colorectal cancer, osteosarcoma, neuroblastoma, and neurofibromatosis-1-related tumors). They point to the lack of information on these EVs under physiological conditions.

The proposal suggested by Carvalho and Oliveira is the use of EVs as a "liquid biopsy" overcoming the limited information provided by a single fragment of a tumor (8). They also assert that EVs may be circulating biomarkers with a potential role in the detection of the early stages of cancer. However, they note that the identification of EVs and characterization of their cargo should be carefully analyzed depending on whether they have been isolated in a human cancer sample or in a cancer cell-line culture. Finally, they propose a longitudinal approach involving the sampling of EVs over the course of a disease.

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Moving specifically to the biomarker area, Gámez-Valero et al. have conducted a comprehensive review of urine-derived EV candidates for monitoring kidney diseases (9). These authors affirm that urinary EVs reflect the state of urinary system; however, depending on the isolation protocol used, published data are not always comparable. In relation to this, they detail current and some novel methods for isolating EVs from urine and provide a clear review of urinary EV biomarkers by type of kidney disease. They also stress the urgent need for a consensus on methods and for these to then be applied to larger cohorts.

In an attempt to respond to the aforementioned suggestion, Sáenz-Cuesta et al. have compared five different EV isolation protocols (including the standard ultracentrifugation approach), starting with blood and urine samples as these are the most readily available, and several types of analysis (flow cytometry, nanoparticle tracking analysis, and electronic microscopy, among others) (10). The authors propose an interesting workflow for the study of EVs in a hospital setting, taking into account the facilities of a non-specialized core laboratory and based on an easy and quick medium-speed centrifugation protocol.

The application of the study of EVs in a daily clinical setting requires suitable technologies and quality controls that could be managed by the hospital itself or delegated to specific facilities. Regarding the facilities provided by culture platforms, Aiastui posit that they should offer a basic level of quality control for the production of large quantities of EVs in good manufacturing practice conditions (11). For that, close collaboration between clinicians from hospitals, the biotech industry, and basic researchers is necessary to turn what is currently an idea into a product. As an example, Aiastui suggest that according to current requirements, culture platforms should become EV sample quality control services before EV products are applied as a therapy.

Research into EV cargo is continuously growing thanks to the spread of these new technologies. One of the most recently applied omic approaches, metabolomics, is focused on metabolites, cytosolic small molecules up to 1 kDa. Palomo et al. analyze the benefits of adding this to the spectrum of omic approaches applied to EVs, discussing which platform is the most suitable and also warning about the importance of additional background controls (e.g., exosome-depleted media analysis) (12).

Finally, Romagnoli et al. hypothesize about the possibility of using dendritic cell (DC)-derived EVs as cancer immunotherapy enhancing tumor antigenicity (13). They demonstrate the boost effect of DC-derived EVs in primed T-cell activation against an adenocarcinoma cell line and conclude that both DCs and tumor cells became more immunogenic after the incorporation of specific EVs.

It is the desire of the editors that this topic should help to connect all interested authors and readers, and join our efforts to understand what and how the cells are whispering.

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REVIEW
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# Emerging roles of exosomes in normal and pathological conditions: new insights for diagnosis and therapeutic applications

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From the time when they were first described in the 1970s by the group of Johnstone and Stahl, exosomes are a target of constant research. Exosomes belong to the family of nanovesicles which are of great interest for their many functions and potential for diagnosis and therapy in multiples diseases. Exosomes originate from the intraluminal vesicles of late endosomal compartments named multivesicular bodies and the fusion of these late endosomes with the cell membrane result in the release of the vesicles into the extracellular compartment. Moreover, their generation can be induced by many factors including extracellular stimuli, such as microbial attack and other stress conditions. The primary role attributed to exosomes was the removal of unnecessary proteins from the cells. Now, several studies have demonstrated that exosomes are involved in cell–cell communication, even though their biological function is not completely clear. The participation of exosomes in cancer is the field of microvesicle research that has expanded more over the last years. Evidence proving that exosomes derived from tumor-pulsed dendritic cells, neoplastic cells, and malignant effusions are able to present antigens to T-cells, has led to numerous studies using them as cell-free cancer vaccines. Because exosomes derive from all cell types, they contain proteins, lipids, and micro RNA capable of regulating a variety of target genes. Much research is being conducted, which focuses on the employment of these vesicles as biomarkers in the diagnosis of cancer in addition to innovative biomarkers for diagnosis, prognosis, and management of cardiovascular diseases. Interesting findings indicating the role of exosomes in the pathogenesis of several diseases have encouraged researchers to consider their therapeutic potential not only in oncology but also in the treatment of autoimmune syndromes and neurodegenerative disorders such as Alzheimer's and Parkinson's disease, in addition to infectious diseases such as tuberculosis, diphtheria, and toxoplasmosis as well as infections caused by prions or viruses such as HIV. The aim of this review is to disclose the emerging roles of exosomes in normal and pathological conditions and to discuss their potential therapeutic applications.

**Keywords: exosomes, therapeutics, cancer, neurodegenerative disorders, infectious disease therapy, biomarkers, pharmacological**

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In recent years, the study of extracellular vesicles (EVs) has mainly focused in a type of vesicles secreted into the extracellular compartment that were termed exosomes by Johnstone, who isolated them from sheep reticulocytes and described their endocytic origin (1). Later, other authors have described this type of vesicles and their secretory pathway. These findings have been extensively described in many comprehensive reviews (2–7).

The interest in EV and, in particular, in exosomes is reflected in many reports published earlier and the constitution of the International Society for Extracellular Vesicles in 2011 to discuss important findings in the field and a database named Exocarta (http://www.exocarta.org), created in 2009 as a free web-based resource to compile proteins and RNA identified in exosomes (8).

Extracellular vesicles are constitutively released from many cell types including neurons, tumor and immune cells, among others. They can also be found in different body fluids such as serum, breast milk, saliva (9), cerebrospinal fluid (10), semen (11), and urine (12).

Eukaryotic cells release vesicles into the extracellular environment either by direct membrane budding (ectosomes or microparticles: apoptotic vesicles, membrane particles and exosome-like vesicles) or by fusion of internal multivesicular compartments (exosomes) (7).

Exosomes are small particles of 30–100 nm with a membrane that is rich in lipids such as sphingolipids, ceramide, and cholesterol with a density between 1.15 and 1.19 g/ml (3).

Exosomes have an endocytic origin and their development begins when early endosomes, loaded with ubiquitinilated proteins, are recognized by the endosomal sorting complex required for transport (ESCRT). This recognition allows the formation of intraluminal vesicles which, in turn, give rise to the multivesicular bodies (MVBs) containing proteins. This process continues with the fusion of the MVBs with the plasma membrane releasing their content to the extracellular space; the microvesicles released are exosomes (3, 6, 13, 14). However, an independent ESCRT pathway for exosomal biogenesis and release has recently been described (6).

When exosomes are secreted outside the cells, they can follow one of these three pathways: (1) they can be captured by neighboring cells (or by the same cells that have given rise to them); (2) they can be internalized by cells that are within a certain distance; or alternatively, (3) they can enter the systemic circulation and then be taken up by different tissues (15).

Canonical exosomes can be described by the presence of molecules which are specifically associated to them, regardless of the cell type they derive from (16). For example, exosomes express some typical cytosolic proteins such as tubulin and actin, molecules involved in MVB biogenesis as TSG101 and Alix, proteins that participate in signal transduction such as protein kinase, metabolic enzymes, heat shock proteins (HSP 70, HSP 90), Annexin and Rab family proteins, tetraspanins (CD9, CD63, CD81), various transmembrane proteins, and major histocompatibility complex class I (MHC I) molecules. Particular expression of some proteins may be present, such as major histocompatibility complex class II (MHC II) on exosomes derived from antigen-presenting cells such as B-cells or dendritic cells (DCs) or some tumor antigens such as MelanA/Mart-1 (16, 17). Because of their endosomal origin, nuclear, mithochondrial, or endoplastic proteins have not been found in exosomes (13).

In addition to this protein content, exosomes bear molecules such as lipids, mRNA, and non-coding RNAs, including micro RNAs (miRNAs) that are delivered and properly translated in target cells (6, 17). The latter is an important feature because the presence of mRNA in exosomes results in the transfer of genetic information allowing protein expression to take place at a distance.

Overall, all these features have allowed characterization and isolation of exosomes from other microvesicles secreted by cells into the extracellular compartment. The classic method to isolate exosomes, is the differential centrifugation and ultracentrifugation. However, over the last few years, new methods have been developed either to obtain large quantities of exosomes to be used in clinical trials which include, for example, the ultrafiltration (18, 19) or in an attempt to obtain pure exosomes preparations, as the use of monoclonal antibodies directed to marker proteins on the surface of exosomes bound to magnetic beads (20). In addition, microvesicles have been differentiated and classified according to various characteristics such as morphology, density, expression of marker proteins, size and a very important feature: their intracellular origin.

Until recently, it has generally been accepted that EV differ in their physiochemical characteristics such as size, density as determined by their sedimentation equilibration in sucrose gradient, and the expression of main protein markers. Most of the works that have been done in the field relay on these properties to develop methods as those mentioned earlier to isolate and characterize the different EV. However, recent works have reported overlapping characteristics and functions for the EV. In fact, a single cell can produce different EV, including exosomes. In this sense, there is a general concern among scientists working in the field regarding the representativeness of the current literature data. The International Society for Extracellular Vesicles has published a paper proposing standardized methods to isolate EV. Thus, new methods and more accurate markers are under evaluation (21). Detailed evidences of the heterogeneity of EV preparations have recently been reviewed by Colombo et al. (7).

Although the primary role attributed to exosomes has been the removal of unnecessary proteins from the cells, their principal function described is their role in cell–cell communication (17). In addition, they participate in several different functions and in a large variety of pathways as they are biologically active vesicles secreted to the extracellular environment (13).

## The Role of Exosomes in Immune Function

The role of exosomes in the stimulation of the immune system has been extensively studied and a recent study in deep review has been published discussing how exosomes regulates immune response (6). In this review we will discuss some of the most relevant findings.

The original work carried out by Raposo et al. demonstrating the importance of exosomes derived from B-cells in antigen presentation and T-cell stimulation has changed the idea that these microvesicles serve merely as a clearance system for obsolete

proteins (22). Since the publication of this work, numerous studies have confirmed that exosomes derived from professional antigen presenting cells, such as DCs, express class I, class II MHC, adhesion, and co-stimulatory molecules. These characteristics enable these exosomes to activate directly CD8+ and CD4+ T-cells inducing a strong immunogenic response (2, 4, 23). DCs pulsed with tumor peptides release immunogenic exosomes and elicit a strong CD8+ T-cell-dependent anti-tumor immune response (2). In fact, it has been demonstrated that exosomes can activate T-cells either by a direct antigen presentation or by an indirect presentation through transfer of antigenic peptides to APCs (24–26).

Tumor-derived exosomes express tumor antigens that can activate DCs, thereby priming the immune system to recognize and promote a specific cytotoxic response with a higher immunogenicity than that accomplished by tumor cell lysates or soluble antigens when used as vaccines (24). Moreover, a single intraperitoneal injection of tumor peptide-loaded DCs-derived exosomes can induce a very strong immune response leading to a delay in tumor growth or to a complete tumor rejection. This phenomenon is probably because of the high tumor antigen density and also of the presence of HSP with an adjuvant capacity in the microvesicles, as observed for exosomes derived from melanoma cells (24, 27–30). In this regard, Lancaster and Febbraio have determined that exosomes expressing HSP70 can activate *natural killer cells* (NKs) and macrophages (31).This rational approach has been probed in several pre-clinical studies and in Phase I (32, 33) and Phase II clinical trials that are already under investigation in patients with inoperable non-small cell lung cancer.

Other immune cells release microvesicles with immune functions, for example, NKs-derived exosomes enclose perforin and granzyme B and mediate anti-tumor activities either *in vitro* or *in vivo* (34). Furthermore, peptides expressed in exosomes released by mast cells are presented by DCs and induce specific immune responses *in vivo* (35). It has also been reported that macrophages release IL-1β on inflammasome activation, suggesting a role of these microvesicles in the pro-inflammatory activity and the innate immune response (36).

Exosomes have also been shown to induce immune suppression. Over the last years many authors have reported the immunosuppressive properties of tumor exosomes which may explain the low immunogenicity observed in some studies. For example, tumor exosomes can suppress NK cells by modulating the expression of the NKG2D receptor (37). These exosomes can promote the generation of regulatory T-cells (38); induce T-cells apoptosis through the activation of Fas (39) or tumor necrosis factor ligands (40) or by the expression of galectin 9 (41) and affect the maturation of DCs (42). The immunosuppressive properties of exosomes derived from IL-10 treated DCs or DCs genetically modified to express IL-4 have also been demonstrated in a collagen-induced arthritis model (43, 44).

Moreover, it has been demonstrated that exosomes derived from infected cells also display pathogenic antigens that can induce a specific anti-microbial immune response. For example exosomes released by endothelial cells infected with Cytomegalovirus (45)or *Mycobacterium bovis*-infected macrophages (46) can induce a specific immune response. Besides, DCs pulsed with *Toxoplasma gondii* promote anti-parasite immunity in mice (47).

## Exosomes in Cancer Disease

It is known that a direct interaction between tumor cells and their environment is essentially required for cancer progression. To achieve this cell–cell communication, an efficient information exchange must exist, being exosomes of paramount importance to induce a pro-tumoral microenvironment for carcinogenesis and regulating the immune response to promote tumor progression and survival. To accomplish these tasks, exosomes are involved in multiple mechanisms: exosomes released into the extracellular milieu participate in the remodeling of the extracellular matrix and promote angiogenesis, thrombosis, and tumor cell proliferation (48, 49).

Owing to their stability, the specific tissue uptake and their ability to transfer micro or mRNA and proteins to recipient cells, exosomes may travel to distant sites and promote a pro-tumor environment to harbor metastatic niches (50). As mentioned earlier, exosomes may additionally exert an immune suppression profile, thus favoring a tumor escape mechanism to evade the immune attack.

It has been demonstrated that exosomes can modulate nearby or distant target cells by direct contact of their surface molecules to activate intracellular pathways. Alternatively, on internalization by membrane fusion or endocytosis, exosomes deliver their protein or RNA content. Many proteins such as mutant KRAS and MET oncoprotein have been found to be transported by exosomes and that their uptake was favored by the hypoxic tumor microenvironment. Recently, studies have demonstrated that tumor cells contain different levels of miRNA and also onco-miRNA which can post-transcriptionally modulate the acceptor cell function (51). Exosomes obtained from normal prostate epithelial cells with normal levels of miRNA-16, 143, and 205 could inhibit the *in vitro* proliferation of a prostate cell line with lower concentrations of these miRNA (52).

Cytokines and soluble mediators in combination with tumor-derived exosomes can recruit bone marrow-derived cells to tumor pre-metastatic tissue where they contribute to modulate the permissive microenvironment for tumor establishment. Recently, Peinado et al. have demonstrated that melanoma exosomes containing MET oncoprotein modulate bone marrow-derived cells to generate a pro-vascular phenotype in the lung (53).

As the result of the many cellular functions in tumor development and dissemination where exosomes take place, these microvesicles represent novel biomarkers for a non-invasive and more accurate diagnosis and prognosis of the disease progression.

Exosomes have several properties that make them preferable over EV for the purpose of therapeutics, including that exosomes are stable *in vivo* and *in vitro*, bioavailable, well distributed in the organism, cross the blood–brain barrier, are well tolerate and may regulate gene expression by transferring miRNA and siRNA to target cells. Overall, these characteristics highlight the importance not only as ideal vaccines for cancer treatment, as initially considered, but also more important as natural liposomes for the delivery

of biologics, allowing multiple opportunities for developing new alternatives for cancer treatment.

## Exosomes Associated to the Generation and Progression of Neurodegenerative Diseases

Exosomes have been proposed to be novel actors during normal development and physiology of the nervous system, acting as cell–cell communicators and playing functional roles not only during the development but also during the regeneration of normal neurons. Lachenal et al. have elucidated the role of exosomes in the normal physiology of the central nervous system by demonstrating the secretion of exosomes in culture by completely differentiated cortical and hippocampal neurons, being this secretion regulated by the calcium influx and by the glutamatergic synaptic activity (54). Furthermore, Frühbeis et al. have reported a reciprocal cell–cell communication between neurons and oligodendrocytes mediated by exosomes (55). Oligodendrocytes release exosomes in response to neuronal stress signals and are internalized by neurons via an endocytic pathway to deliver protective proteins, glycolytic enzymes, mRNA, and miRNA to axons, exerting neuroprotection (56).

Apart from the function of exosomes in the normal development and physiology of the nervous system, the generation and progression of many neurodegenerative diseases have been associated with exosome-mediated transport of misfolded proteins. Moreover, exosomes have been termed The *Trojan horses of Neurodegeneration* because of their capability of shipping toxic agents from unhealthy neurons to their own neighboring cells (57). This nickname has then, and not entirely correctly, associated exosomes to pathological transport mechanisms involved in several kinds of pathways that finally end in neurodegeneration.

In Parkinson's disease, for example, α-synuclein mutated proteins form intracellular oligomers (known as Lewy's bodies) can be secreted via exosomes to the extracellular milieu and internalized by nearby cells, thus spreading the disease from cell–cell within the brain. In this regard, Danzer et al. have characterized autophagy as a protective mechanism in cells, considering it the major degradation pathway for α-synuclein oligomers (58). Any deregulation in neuronal autophagy might promote the aggregation of these proteins and their secretion by exosome release, thus spreading the toxic seed and causing neurodegeneration. Mittelbrunn et al. have proposed that promoting autophagy (i.e., preventing cells from exosomal release) may become a novel approach in the treatment of neurodegenerative diseases (59).

Several hallmark protein accumulations have been characterized in other neurodegenerative diseases like Alzheimer's, in which *tau* protein aggregates form filamentous intracellular inclusions that can spread from affected nerve and glial cells to healthy ones thus functioning also as potential *seeds* of the disease. Bolmont et al. and Götz et al. have shown that extracellular α-amyloid aggregates can induce *tau* pathology in transgenic mice and finally promote neurodegeneration (60, 61). Moreover, exosome-associated *tau* and α-amyloid have been described and it has been proposed that the exosomal surface can act as the seed responsible of the β-amyloid aggregation after protein conformational modifications (62).

In this regard, β-site APP-cleaving enzyme 1 (BACE 1) is responsible for the formation of the aggregates as mentioned earlier. Alvarez-Erviti et al. have accomplished the encapsulation of BACE 1 siRNA in exosomes, observing after their administration to a murine model for Alzheimer's, a significant decrease in BACE 1 mRNA and β-amyloid levels (63). They have also demonstrated that exosomes which have the ability to cross the blood–brain barrier are non-toxic and that they can be perfectly well tolerated. These findings imply that the expression of BACE1 levels, controlled by exosome-mediated siRNA delivery, may be an interesting breakthrough in the treatment of Alzheimer's disease.

Exosomes were also implicated in the propagation of PrPSc (the infectious agent associated with prion diseases such as Creutzfeldt–Jakob disease and Gerstmann–Sträussler–Scheinker syndrome) (64–66).

In an interesting study it was demonstrated a differential expression of specific exosomal miRNA in post-mortem brain samples in patients with Scizophrenia and bipolar disorders (miR-497 and miR-29c) compared with those from normal control samples (67).

Owing to the fact that a variety of aggregating proteins involved in neurodegenerative diseases have a direct association with exosomes, these nanovesicles have become an interesting biomarker for diagnostic and prognostic. Moreover, their capability to accomplish targeted neuron-to-neuron transport transforms them in potentially specialized carriers of therapeutic drugs for neurologic and psychiatric disorders. In addition, exosomes might be used for controlling the disease by silencing or restoring the normal content of miRNA.

## The Role of Exosomes in Cardiovascular Diseases

Cardiovascular diseases are one of the leading causes of death worldwide. Although patients can control some of the behavioral risk factors, physiological factors responsible for heart damage are major goals to be attained.

Several authors have found a direct association between cardiovascular diseases and high concentrations of circulating microvesicles. Exosomes have pro-angiogenesis, pro-coagulant and pro- and/or anti-inflammatory effects as well as an opposing impact on the vascular tone and vessel wall. These features of exosomes are probably because of their capability of transporting and cell–cell transferring of proteins, mRNAs, and miRNA, among others (68).

In this regard, exosomes have been proved to reflect stress conditions by modifying their protein or mRNA concentration (69). These molecular components associated with exosomes may play a role in a variety of cellular physiological mechanisms. Waldenström et al. have extracted total RNA from culture-isolated cardiomyocyte exosomes (cardiosomes) and have accomplished the identification of 1520 mRNAs similar to those found in cardiomyocytes. Moreover, almost one-third of these mRNAs had a direct relationship with biological mechanisms, including gene expression changes (70).

Generally, after myocardial infarction there is an alteration in circulating miRNAs which may act in cell–cell communication between cardiac cells and the bone marrow, opening up the possibility of generating a cardioprotective mechanism via the paracrine activation of cardioprotective kinase pathways (71). Chen et al. have demonstrated that exosomes derived from cardiac progenitor cells (CPCs) can protect cardiomyocytes from oxidative stress *in vitro* and *in vivo* by inhibiting ischemia/reperfusion-induced apoptosis, finding enrichment in miR-451 in CPC-derived exosomes when compared with CPC. The transcription of miR-451 responds to GATA4, a transcription factor associated with cardiac morphogenesis, cardiomyocytes survival, and cardiac function maintenance in the adult heart (72).

On the other hand, it has been proved that exosomes derived from cultured cells under hypoxia conditions contain fibronectin, collagen, and lysyl-oxidase-like 2 (LOXL2) in their protein content, suggesting the participation of these proteins in cytoskeletal and extracellular matrix rearrangements of neighboring or distant cells (69).

Moreover, Liao et al. have shown that cardiomyocytes are able to produce TNF-α after induction via hypoxia-inducible factor 1 (HIF-1). This cytokine is present in exosomes during hypoxic conditions and has been demonstrated to contribute actively to inflammation and cardiac remodeling (73).

All these features of exosomes have led Yellon and Davidson to name these nanovesicles "dark matter of the body" – invisible to direct microscopy, but whose existence can be inferred by the effects they have on other cells" (71).

Moreover, their capacity to transport such variety of molecules may allow exosomes to provide a biological "snapshot" of the cell physiological conditions (71) which, in turn, reflects the health status of the individual.

In summary, all these characteristics make exosomes dynamic vesicles endowed with the ability of transporting a great variety of molecules and, taking into account the specificity of their surface proteins, they have been promoted as specific therapeutic transporters for cardiovascular diseases.

## The Role of Exosomes in Pregnancy

To achieve a successful pregnancy, a specific suppression of the maternal immune system and a homeostatic balance to preserve an adequate utero-placental circulation are required. During this process various modulatory signals are released in different forms including EVs.

Pregnancy is an immunological phenomenon where the semi-allogeneic fetus is not rejected because of the immune tolerance induced toward it. In this regard, exosomes with immunosuppressant activities were found to be increased in pregnant women, compared with non-pregnant ones. Moreover, exosomes isolated from sera of women with full-term pregnancies are present at significantly greater concentrations than those from pregnancies delivering pre-term.

The syncytiotrophoblast of the human placenta continuously and constitutively produces and secretes exosomes to the maternal bloodstream. These exosomes exhibit a redundant number of mechanisms that inhibit the function of the maternal immune system during pregnancy and promote the survival of the fetus. These microvesicles express significantly higher levels of the pro-apoptotic molecules Fas ligand (FasL), TRAIL, and PD-L1, inducing T-cell death. Placenta-derived exosomes which express FasL also suppress CD3-ζ chain and the enzyme Janus kinase 3 (JAK3), leading to T-cell anergy. In addition, it has been reported that placental exosomes also carry NKG2D ligand (the ligand molecule for the activating receptor NKG2D express on NK cells) that may downregulate the activity of NK cells by binding to NKG2D receptor and consequently impairing the maternal cytotoxic activity (74–76).

It has been reported that exosomes released in a primary culture of cytotrophoblast cells contain biologically active proteins that can interact with the maternal endothelium and regulate their function (e.g., migration and angiogenesis) (77). Furthermore, exosomes isolated from plasmas of pregnant healthy women in the first trimester promote vascular cell migration from the uterine spiral arteries and may play a role in regulating the endothelium response to maternal adaptation to pregnancy. However, under pathological conditions (e.g., proinflammatory states and pre-eclampsia), the bioactivity of placental exosomes is reduced (78).

Placental miRNAs are abundant in the plasma of pregnant women and are upregulated in pre-eclampsia. It has been proposed that these specific placental miRNA are extracellularly secreted to the maternal circulation from the syncytiotrophoblast through exosomes. The diagnostic and prognostic usefulness of exosomal miRNAs are presently being investigated. However, little is known about the role of placental exosome-associated miRNAs in maternal cells and tissues during pregnancy (79).

During pregnancy, maternal circulation is characterized for the presence of different EV simultaneously secreted by the syncytiotrophoblast, differing in size, morphology, and function (80). Syncytiotrophoblast-derived exosomes as stated earlier are immunosuppressive down-regulating maternal immune system. Syncytiotrophoblast-derived microvesicles/microparticles (STBM) are larger EV and may include apoptotic or necrotic material, the former immunosuppressive and the latter immunostimulatory, anti-angiogenic and procoagulant (80). It is suppose that during normal pregnancy, a delicate balance occurs between STBM and exosomes, favoring STMB according with a mild systemic inflammation distinctive of normal pregnancy. In contrast, in pathological condition such as infertility, recurrent abortions, pre-eclampsia, pre-term labor, and pre-mature birth, this balance is broken. For example, in pre-eclampsia, inflammatory stress could activate the syncytiotrophoblast causing it to release STMB with pro-inflammatory, anti-angiogenic, and procoagulant activities (80, 81).

Although the role of immunosuppressive placental exosomes during normal pregnancy is clear, the contribution of exosomes in pathological pregnancies and related diseases, such as recurrent abortions and infertility, need a more profound evaluation. The knowledge derived in these areas will open up possibilities for novel, exosome-based treatments of pregnancy failure and infertility.

## The Role of Exosomes in Infectious Diseases

Over the last years, it has been well documented that mammalian cells infected by single-cell eukaryotic/prokaryotic pathogens or even prions secrete exosomes with different purposes. Besides, bacteria secrete biological active vesicles named outer membrane vesicles (OMVs); fungi and eukaryotic parasites also produce EVs (82). Moreover, it is known that even parasitic trematodes and nematodes release exosomes as an immunomodulatory mechanism (83). Significantly, these microvesicles were demonstrated to play an important role in infection biology. Apart from the widely studied immunomodulatory effects, pathogen-released exosomes are known to carry specific virulence factors, such as proteins, mRNA, and miRNA, which contribute to spread the infection. As a consequence, microvescicles can either expand or contain the infection being thus beneficial for either the pathogen or the host.

Prions are abnormally folded proteins with the ability to propagate in the central nervous system causing fatal neurodegenerative disorders. Infectious prion proteins (PrPSc) have been identified in exosomes derived from the conditioned media of mammalian neurons (64, 66). These exosomes were internalized by bystander normal cells, transforming naturally occurring cellular prion proteins (PrPc) into misfolded infectious prion proteins (PrPSc), suggesting that exosomes may contribute to intercellular membrane exchange and dissemination of prions throughout the organism. Moreover, the intracerebral inoculation of exosomes obtained from infected cell cultures has proved to cause clinical disease in mice (64, 84). Recently, exosomes inclosing prion proteins were also isolated from plasma of mice bearing transmissible spongiform encephalopathy, suggesting a possible spread of transmissible spongiform encephalopathies via the blood (85).

In viral infections, microvescicles, and particularly exosomes, have been involved in various mechanisms, depending on the type of virus, its life cycle, and the type of infected cell.

HIV-1 virus has developed many exosome-mediated strategies to manipulate the host's cell machinery. For example, exosomes take part in the transfer of proteins, and RNA (miRNA, sRNA) from infected to non-infected cells, transporting these components even to distant cells. Exosome-mediated transport and deliver of functional proteins to recipient cells has been demonstrated in HIV-1 infected macrophages (86). This phenomenon has been demonstrated for crucial proteins for HIV-1 infection such as Gag, p17, and Nef. For example, Nef is incorporated into exosomes released from infected cells and subsequently induces apoptosis of uninfected CD4+ T-cells contributing to viral immune suppression (87). In addition to the proteins, miRNAs transported by exosomes have been demonstrated to be involved in HIV-associated neuronal dysfunction and susceptibility to viral infection (88). In addition, HIV-1 particles captured by immature DCs (iDCs) are exocytosed in association with exosomes and could mediate trans-infection of CD4+ T-cells (89). On the other hand, HIV-1 has also developed a strategy known as *The Trojan horse hypothesis of HIV trans-infection* (90, 91). This model proposes that HIV-1 enters mature DC (mDC). The virus is retained in the MVB compartment and follows the same trafficking pathway used by DC exosomes for antigen dissemination to amplify antigen presentation during pathogen invasion (16, 91). Afterwards, mDC release them to trans-infect CD4+ cells, mainly in the lymph nodes (90–92).

Another retrovirus infection in which exosomes play an important role during pathogenic viral infection is the Human T-lymphotropic virus type 1 (HTLV-1). The extracellular delivery of functional HTLV-1 proteins as the trans-activator protein Tax, as well as viral mRNA transcripts including Tax, HBZ, and Env to uninfected recipient cells via exosomes, protect them from apoptosis under stress conditions and transfers functional HTLV-1 molecules to this uninfected recipient cells contributing to the pathogenesis of HTLV-1 (93).

Recent evidence indicates that Hepatitis B, C, and E viruses (HBV, HCV, and HEV) also employ the exosomal pathway machinery to mediate alternative active viral transmission and disease persistence. Moreover, it has been demonstrated that exosomes isolated from sera of chronic HCV-infected patients contain HCV RNA, and these exosomes could mediate viral receptor-independent transmission of HCV to uninfected hepatocytes (94). Furthermore, the exosomal export of viral RNA may serve both as a viral strategy to evade the immune system spreading the infection, and as a host's strategy to induce an innate response in bystander cells. In this regard, exosomes derived from HCV-infected cells can also induce the production of type I IFN by the transference of immunostimulatory viral RNA from infected cells to DCs and trigger the production of IFN-α (95).

Members of the Herpes virus family, such as Human Herpes Virus and Herpes simplex, Epstein–Barr virus and human Cytomegalovirus, have also been studied for their interaction with cellular exosomes. As described for retroviruses, the Herpes virus captures the cellular pathway for exosomes biogenesis and release. In addition, enhances virus loaded exosomes production, supporting the persistence and dissemination of viral particles. Furthermore, exosomes may contribute either to the viral escape from the immunological surveillance or to interfere with the transport of antigens to the host's immune system (96).

Exosomes derived from Epstein–Barr virus-infected cells have also been involved in MVB biogenesis, viral egress, and infection to neighboring uninfected cells. In addition, Epstein–Barr virus-infected B-cells can deliver viral BART miRNA to DCs, thus leading to the exosomal-dependent immune suppression in EBV-associated lymphomas (97, 98).

Gram-negative bacteria release OMV to communicate with prokaryotic and eukaryotic cells. They carry and transmit virulence factors, mediate bacterial binding and invasion, cause cytotoxicity, and modulate the host's immune response (99, 100). Furthermore, exosomes secreted by human infected host cells play a relevant role in the bacteria–host interaction. This characteristic is especially significant to identify intracellular pathogens, as bacterial products are not always readily present in fluids for the pathogen detection and diagnosis. In this regard, the works done with *Mycoplasma tuberculosis* and *M. bovis* have yielded relevant results. *M. tuberculosis* or *M. bovis*-infected macrophages release exosomes containing pathogen-derived antigens and these vesicles activate both the innate and acquired immune responses (46, 101, 102) inducing the production of pro-inflammatory cytokines by naïve cells. Exosomes expressing mycobacterial molecules have

been detected in the serum of patients with acute and latent tuberculosis infection. These findings not only enabled the diagnosis but also served as active or latent phase markers of the disease (103).

The expression of specific bacterial proteins either on OM or on exosomes derived from infected as well as from DCs pulsed with microorganism peptides opened up a new alternative for the development of prophylactic or therapeutic bacterial cell-free vaccines.

Finally, exosomes from the fungus *Cryptococcus neoformans* and from parasites such as *Trichomonas vaginalis*, *Trypanosoma cruzi*, *Leishmania* spp., *Plasmodium falciparum, Toxoplasma gondii* (104), and helminthes (83) have also been described. These vesicles express virulence factors and, in addition, stimulate both pro-and anti-inflammatory responses in host cells (104). Interestingly, Regev-Rudzki et al. have reported *P. falciparum* within red blood cells directly communicate to the parasites population using exosome-like vesicles that are capable of delivering genes (105), inducing the promotion and differentiation to sexual form of the parasite.

As stated earlier, the exosomes secreted in response to an infection are implicated in many processes of the infection biology. The exosomes isolated from infected cells may be regulated to eliminate or to attenuate virulence factors to control the spreading of the infection. As they modulate the immune response, exosomes derived from the host's cell or from pathogens could be novel candidates for the design of acellular vaccines. In addition, exosomes can also be employed as infection biomarkers.

## Diagnosis and Potential Clinical Application of Exosomes

The aim of this review was to discuss some significant aspects of exosomes. First, exosomes may be isolated from almost every cell, not only from eukaryotic but also from prokaryotic cells. Second, microvesicles participate in the regulation of central normal biological processes such as the immune response, pregnancy, tissue repair, and blood coagulation, among others. Third, exosomes are involved in pathobiological mechanisms related to the most frequent types of diseases affecting the population such as neurodegenerative disorders, tumorigenesis, and infectious diseases. Fourth, these microvesicles may be captured specifically by cells where they can deliver their content, and finally, they are very stable both *in vivo* (in systemic circulation) and *in vitro* (they can be preserved frozen for a long period of time without losing their biological properties) (15).

Given the importance of exosomes in normal and pathobiological conditions, microvesicles are being studied for their potential therapeutic uses. For example, they are evaluated as biomarkers for the diagnosis and disease follow-up, as immunomodulators to suppress or stimulate the immune system, as vectors for drug delivery, and as therapeutic agents *per se* (**Figure 1**).

One of the hallmark properties of exosomes is the expression of specific proteins or miRNA belonging to the cells from which they derive. In addition, they may be isolated from fluids such as blood, urine, saliva, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, and breast milk. These characteristics were the starting point of many of studies aiming at proving exosomes as potential biomarkers for the diagnosis and prognosis of diseases.

Nowadays, cancer diagnostics rely on biopsies. The potential uses of exosomes have the advantage of being a sensitive and non-invasive method, allowing the detection of tumors at an early stage. Cancer exosomes have been found to be useful for the detection of many types of tumors: prostate, breast, and ovarian cancer; glioblastoma and melanoma, among others (106–110). As mentioned earlier, exosomes transport misfolded proteins associated to neurodegenerative disorders. In Alzheimer's disease, the detection of α-amyloid 42 and tau proteins was possible in cerebrospinal fluid samples at an early stage of the disease, thus opening up the possibility of an early detection (111, 112). A number of works have described the association of exosomes isolated from urine with several kidney pathologies such as renal ischemia/reperfusion, nephrotic syndrome, and acute kidney injury (113–115). The levels of circulating placental exosomes expressing immunosuppressive molecules (FasL) are under investigation to be used as a biomarker to detect complicated pregnancies (75).

Finally, exosomes proved to be a good marker for the diagnosis of infectious diseases. Infectious RNA and proteins are expressed on exosomes, including viral and misfolded proteins allowing the development of sensitive tests, not only to detect the causative agent but also to followup the infection. In this regard, exosomes are especially useful to detect active and latent forms of intracellular infections, such as tuberculosis. In a recent study, 33 unique proteins of *Mycobacterium tuberculosis* were identified from exosomes isolated from human serum that may serve as biomarkers for persistent active and latent tuberculosis (103). Exosomes from human serum are also employed to detect HIV-positive patients and this test is at present commercialized (116).

Another field of intensive study is the use of exosomes to modulate the immune system. Because the pioneer works of Raposo et al. demonstrated that exosomes derived from antigen-presenting cells (B-cells and DCs) contained both MHC I and MHC II molecules and that they could elicit a specific anti-tumoral immune response (22), a myriad of studies employing exosomes as acellular vaccines have been published not only for cancer but also for infectious diseases. As already stated, numerous Phase I protocols have been conducted in cancer patients using an exosomal vaccination. Among them are the vaccination of metastatic melanoma patients (5), advanced non-small cell lung cancer with autologous DC-derived exosomes (117), and patients with colorectal cancer immunized with tumor exosomes derived from ascitic fluid (118).

Following the same strategy, protocols employing DCs pulsed with peptides obtained from infectious agents were developed. For instance, DC pulsed with diphtheria toxin (119), or with *Streptococcus pneumoniae* capsular polysaccharide Cps14 (120), for bacterial diseases and with sonicates from tachyzoites of *Toxoplasma gondii* (47) were investigated.

As immunomodulators, exosomes may be either immunoactivating or immunosuppressive agents. Exosomes were proposed as acellular antigens for the development of vaccines against either

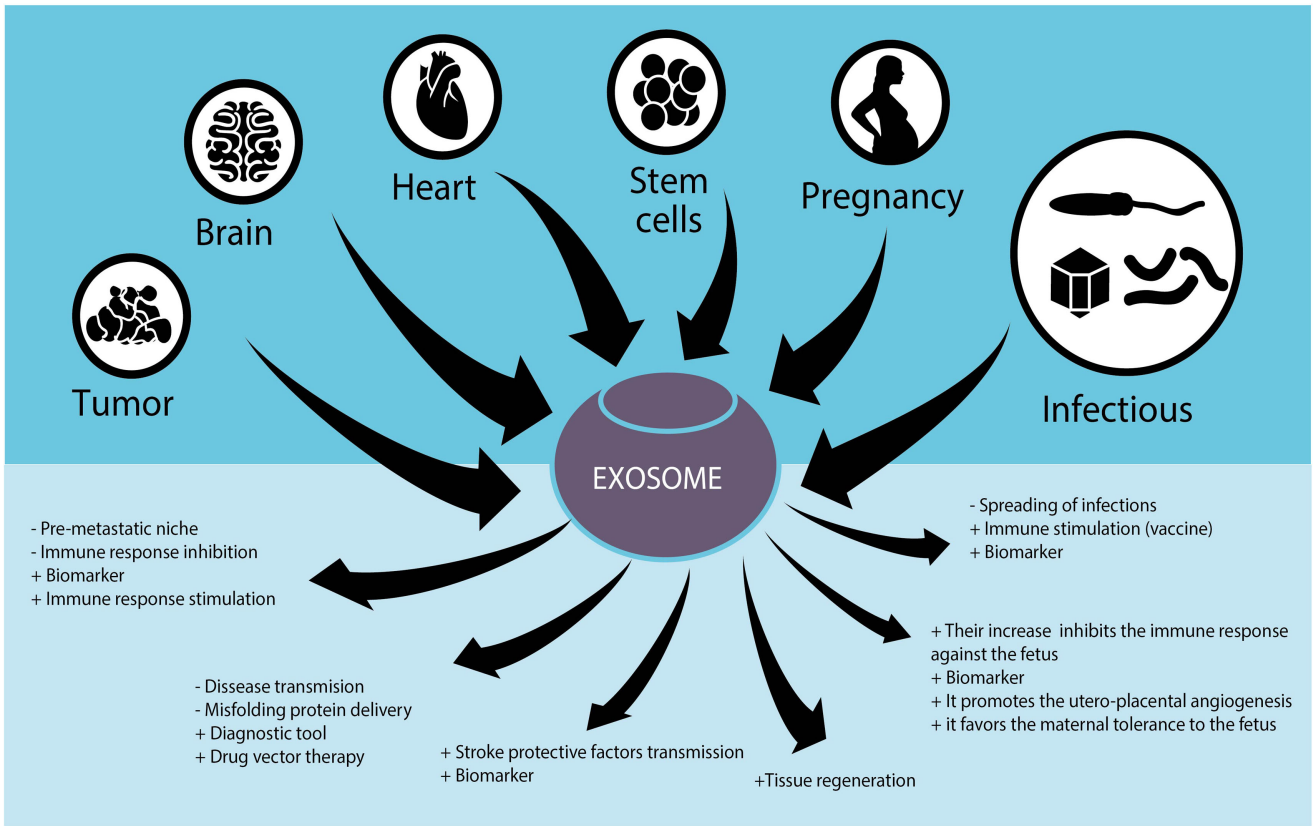


**FIGURE 1 | Role of exosomes in different tissues and their potential use in human diseases**.

infectious diseases or tumors. For example, immunization with exosomes derived from reticulocytes infected with a *Plasmodium yoelli* elicits protective immune responses. This strategy offers a novel platform to develop a vaccine against malaria infection to induce full- and long-lasting protection on this lethal infection (121). Cancer vaccines employing unmodified exosomes (24, 122) or exosomes engineering to express particular molecules derived from tumor cells such as IL-18 or HSP (123–125) induced specific anti-tumor immunity. Using immunosuppressive exosomes from iDCs, it has been possible to induce a donor-specific suppression in a mismatched cardiac allograft model (126, 127). EVs derived from genetically modified DCs expressing FasL, IL-10, or IL-4 successfully suppressed delayed-type hypersensitivity in a model of collagen-induced arthritis (43, 44, 128, 129).

Exosomes are also important therapeutic agents *per se*. EV derived from mesenchymal stem cells have the ability to induce tissue regeneration by delivering growth factors, proteins, miRNA, mRNA non-coding RNA, and lipids. Several reports have demonstrated the feasibility to regenerate cardiac tissue and neovascularization in models of myocardial infarction and kidney injury using exosomes from steam cells (MSC-exo) and endothelial progenitor cells (130). A recent paper by Kordelas et al. (131) has reported a successful therapy for refractory graft-versus-host disease (GvHD) employing MSC-exo in a pediatric patient. In this regard, MSC-exo are under approval for pediatric GvHD treatment in Canada and New Zealand (132). In remarkable studies, it has been demonstrated that systemic administration of MSC-exo effectively improved functional recovery in rats and after stroke (133), and after traumatic brain injury (134) by increasing neurite remodeling, neurogenesis, and angiogenesis.

Owing to their natural origin, exosomes constitute an efficient tissue-specific, non-immunogenic carrier to deliver therapeutic drugs. The fact that exosomes can be engineered to express foreign proteins, miRNA and also siRNA, allows not only the expression of proteins in a tissue-specific manner, but also the silencing of specific genes. For instance, exosomes effectively delivered siRNA can knockout genes inside tumor target cells *in vitro* (135) and *in vivo* into neuronal cells by intravenous injection of the modified vesicles (63). Similarly, mRNA was efficiently incorporated to and expressed in tumor cells (136). These approaches illustrate the possibility to use human exosomes as vectors in RNA-based gene therapy for neurodegenerative disorders, cancer as well as for the treatment of other pathologies.

As the potential therapeutic application of exosomes has gained considerable interest, an important point is starting to be considered. It refers to the kinetics, biodistribution, and clearance of exosomes on systemic administration. Recent studies alert about the rapid clearance of exosomes by the liver and spleen (137–139). In a recent work by Smyth et al., they observed a rapid clearance and minimal tumor accumulation of intravenously injected tumor exosomes, limiting their employment

as drug delivery vehicle. However, a significant greater concentration of the same exosomes were retained in the tumor when delivered intratumorally (137). To overcome these problems Rana et al. demonstrated that exosomes may be selectively taken up by target cell by altering the expression of different tetraspanin proteins (140).

Further studies are needed to overcome these challenges and to establish if exosomes could be utilized effectively for drug delivery.

## Concluding Remarks

The interest in exosomes is mainly because of the important biological and pathobiological functions in which they are involved. In addition, they offer multiple new therapeutic possibilities because of their properties that are used to detect and to ameliorate or cure severe diseases.

Regardless the potential benefits to employ exosomes for therapeutics that were described, some issues such as the purity of the exosomes preparation, the co-expression of different molecules within the microvesicle (proteins and RNAs that may interfere in the biological function required), and the administration route to achieve the targeted delivery and the desired effect remain to be solved. Future research will improve the methods for isolation of pure exosomes necessary to distinguish them from other types of microvesicles and to understand the mechanism in which they are involved. These achievements will help bolster the early diagnosis, control, prevention and therapy.

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# The new deal: a potential role for secreted vesicles in innate immunity and tumor progression

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Tumors must evade the immune system to survive and metastasize, although the mechanisms that lead to tumor immunoediting and their evasion of immune surveillance are far from clear. The first line of defense against metastatic invasion is the innate immune system that provides immediate defense through humoral immunity and cell-mediated components, mast cells, neutrophils, macrophages, and other myeloid-derived cells that protect the organism against foreign invaders. Therefore, tumors must employ different strategies to evade such immune responses or to modulate their environment, and they must do so prior metastasizing. Exosomes and other secreted vesicles can be used for cell–cell communication during tumor progression by promoting the horizontal transfer of information. In this review, we will analyze the role of such extracellular vesicles during tumor progression, summarizing the role of secreted vesicles in the crosstalk between the tumor and the innate immune system.

**Keywords: innate immunity, exosomes, tumor progression, tumor surveillance**

## INTRODUCTION

Cancer is a systemic disease that has mostly been studied by focusing on the primary tumor. Hence, there is little information available as to how the tumor microenvironment influences tumor progression and metastasis. To date, whether a tumor influences its microenvironment or conversely, whether an aberrant microenvironment reinforces tumor progression, is a fundamental question in tumor biology. Metastatic cells conquer new organs after a phenotypic evolution that not only allows them to move from the primary tumor but also to evade innate and adaptive immune responses. Innate immunity protects us against pathogens or potential invaders. However, it is not clear how the innate immune system acts during tumor progression and metastasis. Among the most important questions that remain to be resolved are *whether innate immune cells are present during tumor progression and metastasis and if so, which are their roles*?, *Is the microenvironment involved in the failure of current therapies and if this is the case, can we "educate" our immune system to control or impede metastasis*? In this review, we will focus on how cancer cells interact with the innate immune system during metastasis, highlighting the potential role of secreted vesicles in this process.

## INNATE IMMUNITY IN METASTASIS, THE FIRST LINE OF DEFENSE

There are different factors that can promote tumor progression and eventually metastasis, such as unbalanced growth signaling, alterations to the cell cycle, genomic mutations, and immune evasion. Metastasis is a complex event that accounts for more than 90% of cancer-related deaths, and it reflects the evolution of the primary tumor through its interaction with the microenvironment of local and distant organs (1, 2). Tumor cells migrate from primary tissues by degrading the surrounding tissue and extracellular matrix, spreading through the blood or lymphatic vessels before reaching their newfound land (3). *Lyden and colleagues* defined the novel concept of "*premetastatic niche*" in which stromal cells such as bone marrow-derived cells, under the spell of the primary tumor, are actively involved in the formation of a suitable microenvironment for the metastasis to develop at distant sites (4). Therefore, cancer cells not only need to promote their own immortality and invade propitious territories but they also need to evolve along with the microenvironment and to find strategies that enable them to survive from the constant immune surveillance (5).

Tumors use two major strategies to escape from immune surveillance: immunoediting, in which the least immunogenic tumor cell variants are selected; and active suppression of the immune response along with establishing conditions, within the tumor microenvironment, that facilitate tumor outgrowth (6). Innate and adaptive immunity and their responses must be overcome by tumors in order to evade their effects (7). Escaping from innate immunity, the first barrier of defense against microbial molecules may be one of the earliest events in the progression of the local tumor into a metastatic cancer. However, cancer immunoediting involves the use of the immune system's host-protective events to promote tumor progression and the escape of cancer cells from immune responses, allowing them to develop immune evasive features (8). Indeed, both innate and adaptive immunity appear to contribute to cancer immunoediting (9).

## THE INNATE IMMUNE RESPONSE CONTROLS THE BALANCE BETWEEN THE HOST AND TUMOR MICROENVIRONMENT

Tumor development and metastasis are influenced by the stroma, by angiogenesis, and by the innate and adaptive immune system. The concept of tumor immune surveillance was first contemplated by Paul Ehrlich in 1909, postulating that the immune system can restrict the spontaneous growth of transformed cells by identifying and eliminating them (10). About 50 years later, Burnet and Thomas proposed that tumor-associated antigens can provoke an effective immunological reaction (11). This initial theory of immune surveillance was controversial, especially given the evidence that nude mice that lack an intact immune system are not more susceptible to tumor development (12). In 2001, it was shown that lymphocytes and IFNγ collaborate to prevent tumor immunoediting, thereby preventing the selection of less immunogenic tumor cells (13). Natural killer (NK) cells are the immune effector cells that are active in the elimination of transformed cells but that also promote the maturation and migration of dendritic cells (DCs) with enhanced antigen presentation to T cells (14). In cancer, NK cell activity and the ability to infiltrate tumors may be impaired, and for example, chronic exposure to NK group 2 D (NKG2D) ligand-expressing tumor cells alters NKG2D function in NK cells, promoting NK cell evasion by tumor cells (15). Although the activation of immune cells could result in the eradication of transformed cells, chronic activation of innate immunity, like chronic inflammation, might promote cancer development (16). Cancer has been compared to *a wound that never heals* (17), based on the observation that the tumor is in a state of chronic inflammation. Indeed, immune cells, such as macrophages and mast cells, release soluble agents like cytokines and chemokines promoting the migration and infiltration of leukocytes that can contribute to tumor growth (18). Thus, better understanding how innate tumor surveillance occurs could guide tumor immune therapy that would potentiate the immune system to act against cancer through immunomodulatory approaches.

## EXTRACELLULAR VESICLES AND INNATE IMMUNITY: A FIRST CONTACT

Most cell types secrete vesicles, and there are many different vesicle types that fulfill a wide range of biological functions. Although the nomenclature is still not fully accepted, one can classify them based on their secretory pathway, and these vesicles can be divided into membrane-derived vesicles and exosomes (19, 20). The term exosome was initially used to name vesicles ranging from 40 to 1000 nm in size that are released by a variety of cultured cells that were defined as "*exfoliated membrane vesicles that may serve a physiological function*" (21). However, this term was later adopted for smaller (30–100 nm) vesicles of endosomal origin that are released as a consequence of the fusion of multivesicular bodies with the plasma membrane, enriched of specific markers such as CD63, CD81, and CD9 (**Figure 1**) (20, 22). Ectosomes, also called shedding vesicles, microparticles, or microvesicles, are variable in size (0.1–1 μm), origin and cargo. Their secretion occurs through budding from the plasma membrane. Ectosomes are secreted by many cell types including platelets, endothelial cells, and leukocytes, and they are released directly into the blood (23). Extracellular vesicles were thought to discard cellular debris from cells; yet during the last decade, they have been shown to play an active role in cell communication and, in particular, cell–cell communication. They carry molecular determinants of their cell or tissue of origin including DNA, RNA, miRNA, proteins, lipids, and other cargo (**Figure 1**) (20). The biological functions of exosomes include antigen presentation, regulation of programmed cell death, angiogenesis, inflammation, and coagulation (24). Most studies of exosomes have been carried out in relation to immune or cancer cells (25), as well as studying the potential effects of tumor-derived exosomes in modulating immune interactions. The mechanisms that guide tumor cells to a specific tissue are largely unknown, although there is evidence that tumor cells themselves modulate immune cells and tissues through the secretion of soluble factors and vesicles (2, 26, 27). Exosomes may be vehicles for many different activities exerted over target cells (20). Indeed, we recently showed that melanoma-secreted exosomes transfer information and reprogram bone marrow progenitor cells toward a pro-vasculogenic phenotype in the premetastatic niche, acting through the receptor tyrosine kinase, MET (28). However, the effects of exosome-enriched proteins on immune cells and, in particular, their effects on tumors and innate immunity are not fully understood (29). Although the innate response involves many cell types, it is particularly dependent on basophils and mast cells (inflammation), and on neutrophils and macrophages (phagocytosis). There are evidence of secreted vesicles in most of the innate immunity cellular components.

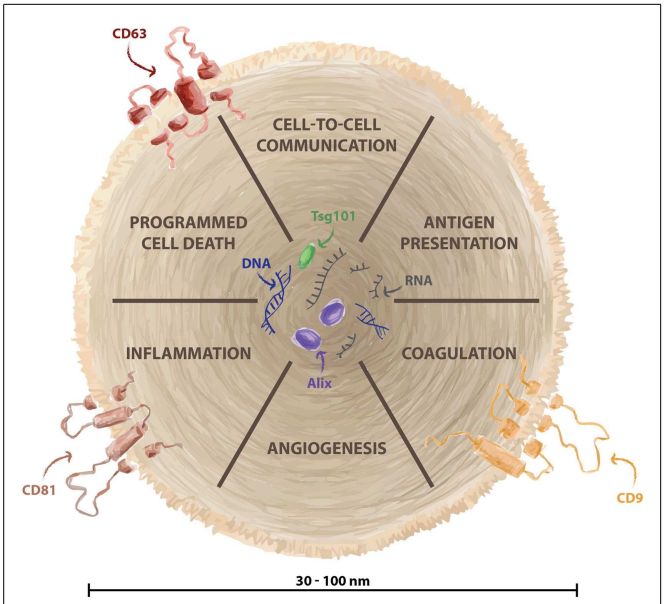


**FIGURE 1 | Biological function of exosomes**. Exosomes are small vesicles (30–100 nm) generated in the endosomal structure and released from several cellular types. They are considered fundamental for cell–cell information exchange, and they contain specific cargo such as DNA, RNA, miRNA, and proteins. Biological functions of exosomes include antigen presentation, regulation of programmed cell death, angiogenesis, inflammation, and coagulation. Exosomes are enriched of specific markers such as CD63, CD81, and CD9.

Accordingly, here we shall address the possible involvement of exosomes in modulating the activity of different elements involved in innate immunity.

## MAST CELLS

As well as fulfilling a key role in allergy, mast cells also have other immunomodulatory functions, both pro-inflammatory and anti-inflammatory (30). The pro-inflammatory role of mast cells has been demonstrated to support the tumor microenvironment in different cancer models with different chemokines and cytokines determining the interplay of mast cells with other members of the immune system (31). Stem cell factor (SCF) is a decisive factor in the differentiation, maintenance, and activation of mast cells (32). Indeed, SCF recruits mast cells, and its expression in experimental models of breast and hepatocarcinoma (33). Mast cells are derived from myeloid progenitor cells in the bone marrow, and then undergo differentiation at peripheral sites, where they become specialized into various subtypes (34). Their location allows them to interact with different insults and external threats, so they could be considered as the first line of the immune defense (35). The levels of some proteases present in mast cells are related to tumor progression. Mast cells release proteins that have been correlated with microvessels density, tumor progression, and angiogenesis such as histamine, which could increase new vessels permeability, promoting leakiness (36, 37). Similarly, MMP-2 and MMP-9 stored in mast cell granules, or the secretion of FGF-2, VEGF, TGF-β, or TNF-α could be involved promoting angiogenesis (38). Mast cells have been reported to release exosomes that contain different proteins, providing an additional mechanism of intercellular communication (39–41). The content of these vesicles has been analyzed in detail and the mast cell exosome-derived cargo identified. Mast cell-derived exosomes contain immunologically determinant factors such as MHC class II proteins (42), co-stimulatory (CD86, CD40, CD40L), and adhesion-related (LFA-1, ICAM-1) molecules (40) (**Figure 2**). They also include CD13, ribosomal protein S6 kinase, annexin V, cdc25, and phospholipases, together with other interacting proteins like aldolase A and heat shock protein 70 (43). The receptor Fcε-RI subunits alpha and beta have been detected in exosomes (44), contributing to the idea that secreted vesicles could amplify immune modulation (**Figure 3**). Bone marrow-derived mast cells can induce resting B cells to proliferate and to produce IgM (45). What was thought to be a non-contact mechanism was described later as exosome-mediated interaction. These mast cell-derived exosomes are responsible of inducing B-cell production of IL-2, IL-12, INF-γ, IgG1, and IgG2, but not IL-4 (40). Functional mRNAs and small RNAs, including microRNAs have also been found in mast cell-derived exosomes (**Figure 2**) (41, 46). The RNA contained in the mast cell-derived exosomes was first described by *Lötvall and Valadi* as exosomal shuttle RNA (esRNA), being functional and transferring information between cells (47). The evidence suggest a role for exosomes in transferring relevant information between stromal and tumor cells (28), exhibiting new potential communication mechanisms (**Figure 3**).

Although the role of mast cells in cancer has been studied widely, this issue is still not yet fully understood. An increase in the mast cell population is associated with poor prognosis in many different cancers, including oral squamous carcinoma, Hodgkin's lymphoma, diffuse large B-cell lymphoma, and prostate cancer (48–52). A positive correlation was described in prostate cancer, colon, and breast cancer (53). The location of mast cells might be an essential factor in their role in tumor rejection or progression. In non-small cell carcinoma, intra-tumoral detection is associated with improved survival (54). In melanoma, mast cell accumulation in the margins of the tumor has been observed (55). c-Kit and Fcε-R1, phenotypical markers for mast cells, are involved in tumor progression and exosomal communication. Interestingly, c-Kit-containing exosomes have been described as a mechanism for progression in lung adenocarcinoma enhancing tumor cell proliferation (56). These data, together with our

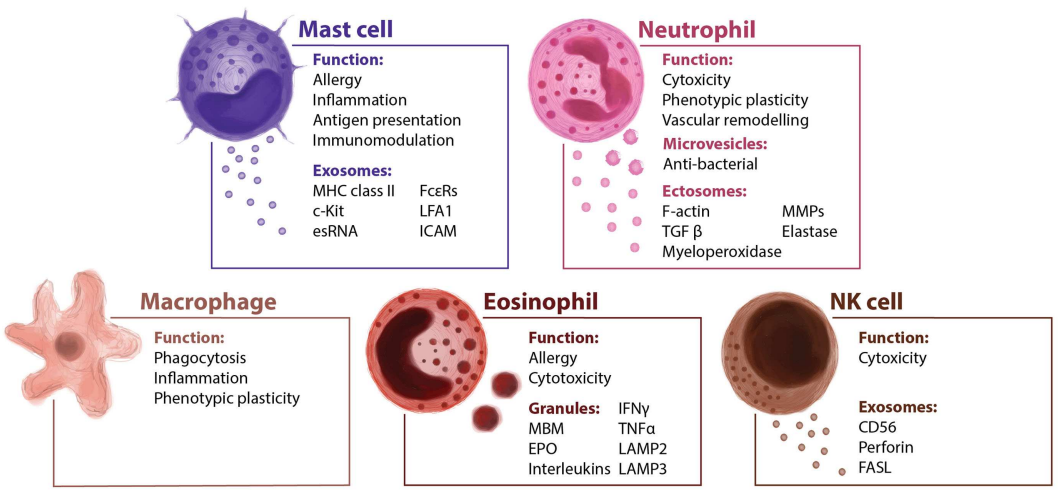


**FIGURE 2 | Innate immune cells and their extracellular vesicles**. The innate immunity is the first barrier of defense against infection and tumoral invasion. It includes humoral factors and many cellular types such as mast cells, neutrophils, macrophages, and NK cells. It is reported that several cellular components of the innate immunity secrete vesicles modulating the direction of the immune response.

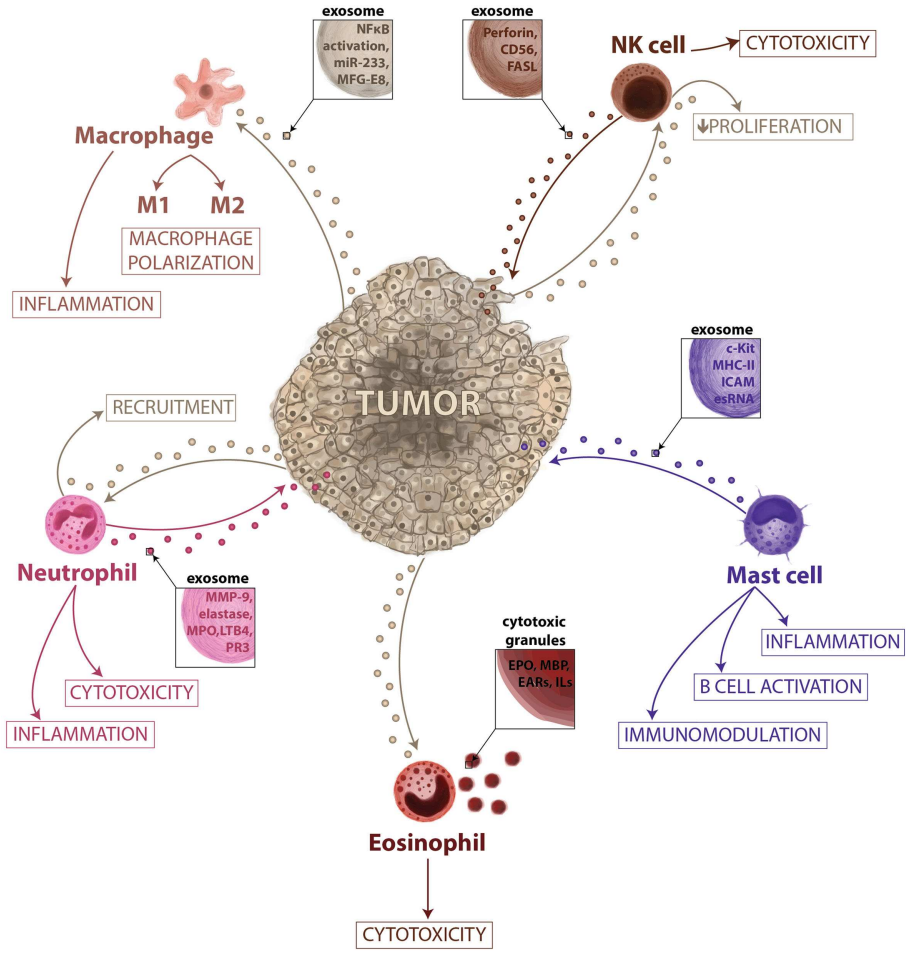


**FIGURE 3 | Vesicle-mediated interactions between tumor and innate immunity**. In this microenvironment–tumor interaction model, tumors secrete exosomes that are responsible for the recruitment of neutrophils and eosinophils, proliferation of NK cells, and activation of macrophages promoting their polarization. When recruited, eosinophils can release their granules having a role in cytotoxic response and immune surveillance. The neutrophils can release vesicles during inflammation activated-process and could have a role in cancer-related inflammation and tissue remodeling. The macrophage-secreted exosomes may regulate the tumor invasiveness and the metastatic behavior; however, specific cargo has not yet been described, while exosomes derived from NK cells may exert a role in immune cytoxicity. Mast cell exosome-derived cargo is described to contain functional mRNAs and small RNAs, immunological proteins and could be involved in immunomodulation, B-cell activation and inflammation.

recently published data about the influence of c-MET secreted on tumor-derived exosomes to bone marrow progenitor cells during melanoma metastatic progression (28), suggest that horizontal transfer of oncoproteins between tumor and bone marrow-derived cells by exosomes play central role in tumor progression. However, the actions of these exosomal proteins in the environment and their role in establishing either a premetastatic niche or promoting tumor growth are yet to be revealed. Secretion of exosomes is a novel mechanism that expands their role far beyond their already known role in allergy. Increasing body of evidence suggest that mast cell-derived exosomes as potential "modulators" of the crosstalk between immune cells and stromal cells. Mast cell-secreted exosomes may have a double-edged function, while they can act through stimulating the immune system during the early events of cancer or when in contact with foreign substances, they could also be involved in the tumorigenic progression by the recruitment of macrophages, fibroblasts, and blood vessels that contribute to tumor growth by secreting a milieu of molecules.

## NEUTROPHILS

Neutrophils are key mediators of the innate immune system, and their activation is essential to protect the host system against infections and to promote healing (57). The short life span of neutrophils and their specific differentiated phenotype has masked their role in cancer-related inflammation, which is why they have been largely ignored. Similar to macrophages, the so-called tumor-associated neutrophils (TAN) can exert pro-tumoral as well as anti-tumoral effects. Evidence from animal models suggests that neutrophils can be polarized toward distinct phenotypes in response to different tumor-derived signals (58). The role of neutrophils in tumor progression is not fully understood. They

gather in metastatic carcinomas modifying the tumor microenvironment by secreting factors like G-SCF or MMP-9 among others (59, 60), and promoting therefore angiogenesis and vascular remodeling. Tumor exosomes act promoting tumor growth through bone marrow progenitor cells education and through neutrophils recruitment (61). Macrophages and DCs are capable of secreting LTB4-producing exosomes (62), which may induce neutrophils recruitment. This calling effect is performed in a gradual fashion that might act like an exosomal gradient (63). Cytokines and secreted factors stored in neutrophils granules may play a role in tumor progression (**Figure 3**). Epithelial lung cancer cells uptake neutrophil elastase (NE) and favor tumor cell proliferation through the hydrolysis of IRS-1 (64). The acquisition of NE by cancer cells confers them a previously unknown mechanism of anti-tumor adaptive immunity (65). Fridlender et al. have demonstrated that TGF-β drives neutrophils to acquire a pro-tumorigenic N2-phenotype, whereas its inhibition enhances the emergence of an anti-tumorigenic N1-phenotype, characterized by cytotoxic activity on cancer cells and an immunostimulatory profile (i.e., high levels of TNF-α, CCL3, and ICAM-1) (58). Human neutrophils release microvesicles with antibacterial properties, and their cargo, in terms of antimicrobial proteins (**Figure 2**), is different in the depending on the stimuli (66). When activated *in vitro* and *in vivo*, in local and systemic inflammation, neutrophil microvesicles are shed through an exocytotic process and they have been denominated ectosomes (**Figure 2**) (67). These vesicles contain cytosolic F-actin indicating their outside-out orientation, but their content and unique characteristics suggest a role in inflammation (68). Ectosomes activate multiple signaling pathways in neutrophils and macrophages, leading to TGFβ1 secretion, and may play a role in macrophage and TAN polarization (69). Neutrophil-derived ectosomes contain TGF-β, MMP-9, myeloperoxidase, proteinase 3, or elastase (**Figures 2** and **3**), postulating them as candidates for determinant roles in inflammation and cancer signaling (**Figure 3**) (70). While the classical role of neutrophils and their secreted vesicles has been described as antibacterial agents, recent data suggest that they could also play a role in inflammation and influence tumor immunosurveillance. Secretion of molecules such as proteases and elastases would impact in the ECM remodeling necessary for tumor progression, recruitment of neutrophils, and release of their exosome cargo to the tumor microenvironment could therefore be involved not only in primary tumor progression but also in the formation of metastatic foci.

## MACROPHAGES

Macrophages are closely involved in the inflammatory responses observed during cancer and metastasis (**Figure 2**). Many observations indicate that tumor-associated macrophages (TAM) have several pro-tumoral functions, including the expression of growth factors and MMPs, the promotion of angiogenesis, and the suppression of adaptive immunity (16). Tumor-secreted exosomes promote the activation of macrophages, as evident by NF-κB activity (71). Cancer cells secrete factors that modulate macrophage activation and polarizations into M2 macrophages (**Figure 3**). However, the mechanisms that mediate such polarizations are not clear. Glycoprotein MFG-E8 is associated with the suppression of pro-inflammatory responses, and it is more concentrated in exosomes from the tissue and serum of prostate cancer patients (72). Indeed, the administration of an antibody against MFG-E8 significantly attenuates M2 polarization (72). Therefore, one of the potential roles of tumor-derived exosomes, in this context, would be the regulation of M1/M2 polarization. The contribution of exosomal miR-233 in autocrine differentiation of macrophages has also been proposed (**Figure 3**) (73). Macrophages infected with intracellular pathogens such as *Mycobacterium tuberculosis*, *Mycobacterium bovis BCG*, *Salmonella typhimurium*, or *Toxoplasma gondii* release exosomes that contain pathogen-associated molecular patterns (PAMPs). These exosomes have immunomodulatory properties over naïve macrophages and neutrophils both *in vitro* and *in vivo* (74). Amoeboid prostate cancer cells have also been described to secrete miR-125a, which suppresses AKT1 expression and hence, the proliferation in recipient human peripheral blood mononuclear cells and macrophages (75). It was recently described that breast cancer-derived exosomes are capable of inducing an inflammatory response in macrophages through NF-κB activation, as seen in other pathologies (76), and that this may contribute to metastasis (77). Tumors may use exosome-mediated macrophage polarization to reinforce the escape of tumors from immune surveillance and in this sense, macrophage-derived exosomes regulate integrin β1 expression in endothelial cells *in vitro*, eventually promoting endothelial migration (78). Its contribution to vascular leakiness and tumor invasion is plausible but not determined yet. Macrophage-secreted exosomes may regulate the invasiveness of breast cancer cells through the delivery of oncogenic miRNAs. For example, miR-223 was detected within the exosomes promoting the invasion of breast cancer cells via the Mef2c-β-catenin pathway (73). These data suggest that exosome communication and transfer of information between tumor cells and macrophages could be actively involved in regulating tumor progression (**Figure 3**) (79). Although macrophage-secreted exososomes could play an active role regulating phagocytic events and degradation of foreign substances, there are a reduced number of evidence of their physiological function. However, their role in cancer is better defined, the continuous stimulation by the primary tumor normally overcome the macrophage-driven immunosurveillance. One potential consequence of the massive secretion of exosomes by macrophages in pathological scenarios would be the generation of an inflammatory microenvironment, which may ultimately result in an enhanced metastatic burden.

## EOSINOPHILS

Eosinophils are traditionally considered to be effector cells in allergic diseases and infections due to their cytotoxic potential (**Figure 2**). They are involved in the inflammatory response due to the production and release of a wide set of cytokines and mediators (80). Their granules contain highly cytotoxic proteins, such as major basic protein (MBP) or eosinophil peroxidase (EPO) (**Figure 2**), and they can present antigens, release other inflammatory mediators and participate in the regulation of the adaptive immune response. Like members of the innate immune system, eosinophils have been observed in the tumor-infiltrating area, and

their presence is correlated with good prognosis, irrespective of other prognostic factors such as stage, age, and histological grading (81). It is not fully understood what mechanisms drive eosinophil recruitment to the tumor site (82), although some tumor cells secrete IL-5 and IL-3 that modulate the differentiation and maturation of eosinophils and mast cells (83). A variety of studies indicate that eosinophils might be more than just an effector of tumor surveillance, indicating that they are capable of processing insults and of orchestrating a quick and selective response, mediated through the release of their contents (84).

Eosinophilic granules have been described intensely, and some of their features are similar to those features of exosomes (**Figure 2**) (85). Specific granules of human eosinophils are notable for their storage role for various proteins, such as EPO, MBP, and eosinophil-derived neurotoxin (**Figure 3**), all of them with potent cationic properties. In addition, these granules contain cytokines, chemokines, and growth factors, including IL-2, -3, -4, -5, -6, -10, -12, and -13, IFN-γ, TNF-α, NGF, GM-CSF, SCF, TGF-α, RANTES (CCL5), eotaxin-1 (CCL11), and CXCL5 (86), many of which are present in other immune secreted vesicles. In contrast to exosomes, eosinophil granules are larger in size (500–1000 nm) and express lysosome-associated membrane glycoproteins such as LAMP-2 (CD107b) and LAMP-3 but vesicle-associated membrane protein-2 is not expressed on human eosinophil granules (**Figure 2**) (87). Despite the selective and well-controlled secretion of cytokines and cationic proteins, there is no evidence that cell-free eosinophilic granules can function as antigen-presenting structures or express MHC class II molecules. Little is known at present about eosinophil-derived exosomes or secreted vesicles, and even less regarding their immunomodulatory functions. The pro-inflammatory and cytotoxic properties of eosinophils make them an interesting target for cancer immune surveillance. Indeed, if cancer cells could inactivate eosinophils or take advantage of other leukocytes recruitment that eosinophils promote, they might have a means to elude one of the main effector cells of the innate immune system. While the role of eosinophil-secreted exosomes is yet to be defined, they may have an important role in the pathogenesis of several diseased such as asthma and tumor progression. Their physiological role executing cytotoxic effects could be likely modulated by pro-inflammatory factors. Moreover, the release of their cargo together with mast cells could be considered as a future biomarker of chronic pathologies characterized by an inflammatory microenvironment.

### NK CELLS

Natural killer cells are among the most powerful effectors lymphocytes in the immune system: 15% of all circulating lymphocytes are NKs, and they are also found in many peripheral tissues. Different factors recruit NK cells to the peripheral tissues, such as IL-12, IL-15, IL-2, IFN-α, or β, and their role in innate immunity and in anti-tumor defense has been studied (88). Recent evidence shows that NK cells isolated from the blood of healthy donors release exosomes expressing proteins such as CD56 and that they contain the killer protein perforin (**Figure 2**) (89). Exosomes released by NK cells may exert cytotoxic activity against tumor target cells, pointing to an important role of NK cell-derived exosomes in immune surveillance (**Figure 3**) (89). NK-derived exosomes contain FasL, a traditional pro-apoptotic ligand (90) that has been implied in tumor tolerance (91), and perforin, a granzyme implicated in tumor and immune homeostasis (92), which lead to literary name NK-derived exosomes as "nanobullets" (93). The proliferation and cytotoxic activities of NK cells is impaired by tumor-derived exosomes, both *ex vivo* and *in vitro*, and this effect has been correlated to the growth of the tumor (94). In fact, exosomes released by human pancreas and colon carcinoma cells in culture express high levels of Hsp70, and they can stimulate NK cell activity, inducing their migration and cytolytic activity (95). Other reports described that tumor-derived exosomes can also promote tumor immune evasion by impairing NK cells effector functions in human breast cancer, mesothelioma, and various types of blood cancers (96). NKG2D is one of the most potent activating receptors expressed on the surface of NK cells, and it plays an important role in activating the anti-cancer immune response through an interaction with stress-inducible NKG2D ligands (NKG2DL) on transformed cells (97). However, cancer cells have developed numerous mechanisms to evade the immune system, NKG2D ligand-bearing exosomes downregulate NKG2D receptor-mediated cytotoxicity *in vitro* (98). Current tumor immunotherapies may produce severe side effects, although a Cbl-b-knockout mouse and mice treated with a TAM blocker do not exert severe signs of inflammation or autoimmunity, representing a therapeutic window for Cbl-b/TAM receptor inhibition to mediate tumor rejection without provoking serious cytotoxicity (99). The most recent data in this field suggest that NK cell-derived exosomes play an active role in the regulation of immune surveillance and homeostasis. Moreover, due to their cytotoxic activity, NK-derived exosomes are probably the best example so far of "nanobullets" that could possibly be used in future therapeutic approaches against different pathologies, including tumors.

## REINTERPRETING CANCER, LESSONS LEARNED FROM PARASITE WORLD

When parasites infect an organism, they infiltrate its tissues and organs, trying to avoid the host's defense systems. Eventually, they will find their niche, proliferate, and develop a full infection if the immune response is insufficient. This behavior resembles the colonization of metastatic sites by cancer cells and their adaptation to the metastatic niche, suggesting that both processes may share similar mechanisms (26). Both microorganisms and tumor cells need to interact with the innate immune system and evade leukocyte surveillance in order to achieve successful colonization. A comprehensive study of the tapeworm genome identified evolutionary adaptations that may influence such colonization, affecting metabolism, detoxification mechanisms, loss of homeobox genes, empowered stemness, and altered cadherins (100). As such, some enzymes involved in these adaptations may be relevant targets for anti-parasitic drug development and could also possibly be used as anti-cancer therapies. For example, anti-parasitic agents such as mebendazalone have been described as a treatment of chemotherapy-resistant malignant melanoma (101) and likewise, different classes of anti-malarial drugs (artemisinins, synthetic peroxides, and DHFR [dihydrofolate reductase] inhibitors) have strong anti-proliferative activity on cancer cells (102). These observations have encouraged further research into the possible

anti-cancer applications of new small compounds with anti-parasitic activity. Importantly, there are cases in which parasitic infections are the cause of cancer, expanding our perception of the relationship between parasites and cancer (103, 104). While such a relationship of carcinogenesis is not fully understood, it may be related to aberrant innate immunity and chronic inflammation.

Parasites secrete exosomes that interact with the innate and adaptive immune systems (105). Exosomes from *Leishmania donovani* modulate the monocyte response to IFN-γ, promoting IL-10 production and inhibiting TNF-α. *L. donovani* is the parasite responsible for the most severe leishmaniasis, and it infects the mononuclear phagocyte system, affecting macrophages in the spleen, liver, and bone marrow via a Rac1- and Arf6-dependent process (106). *M. tuberculosis* is another microbe that uses a strategy that cancer cells may mimic. Virulent *M. tuberculosis* evades innate immunity by inhibiting apoptosis and triggering necrosis of host macrophages, and escaping from adaptive immunity by delaying its initiation (107). This interaction with macrophages might me mimicked by cancer cells. Epigenetic modulation of the immune response has also been described in *Plasmodium falciparum*, the protozoan parasite responsible for the human malaria. *P. falciparum* has 60 *var* genes encoding distinct antigenic forms of the virulence protein PfEMP1 (*P. falciparum* erythrocyte membrane protein 1). The parasite expresses only one *var* gene at any time point during infection to avoid detection by the immune system, yet the mechanism controlling the silencing of the other 59 *var* genes is unknown (108). An epigenetic mechanism is thought to be involved in this silencing, whereby the histone lysine methyltransferase PfSETvs controls, which gene will be expressed and thus, enables the parasite to evade the immune system (108). Therefore, the transfer of epigenetic modulators through exosomes may participate in the avoidance of immune surveillance.

## THE ROLE OF INNATE IMMUNITY IN SPECIFIC PATHOLOGIES

Activation of the innate immune system, or the inhibition of pro-tumorigenic and inflammatory cells, can produce anti-tumoral effects in immunosuppressed cancer patients. Here, we discuss specific cancer types that provide evidence of the crucial role mast cells, macrophages, and NK cells could play in tumor formation and progression, as well as the targeted immunotherapy currently available in such cases.

### THE ROLE OF MAST CELLS AND MACROPHAGES IN NEUROFIBROMATOSIS-1 RELATED TUMORS

Although important roles of the tumor microenvironment and mast cells in the initiation and progression of neurofibroma have been described (109), their interactions remain poorly understood. The hypothesis that in neurofibroma, tumorigenic cells do not grow in isolation but rather the microenvironment contributes critically to their formation, suggests that exosomes may possibly mediate this process. So far, there is a lack of information of the role of secreted vesicles in the communication between tumor and stromal cells in neurofibroma progression, a complication of neurofibromatosis-1 (NF1) (110). Neurofibromas are formed in association with peripheral nerves and as well as containing large collagen deposits, they are composed of Schwann cells, fibroblasts, vascular cells, and mast cells. Tumor progression requires complex interactions between Schwann cells and NF1 heterozygous ($NF1^{+/-}$) cell lineages in the tumor microenvironment including innate immune system cells (111). Exposure of $NF1^{+/-}$ mast cells to conditioned media from Schwann cells and mast cells *in vitro* provoked hypersensitivity (112, 113), suggesting that activated mast cells can release inflammatory cytokines, growth factors, and other components such as extracellular vesicles, which may stimulate tumorigenic Schwann cell transformation and support the recruitment of macrophages, fibroblasts, and blood vessels. In addition, mast cells can secrete VEGF, a potent stimulant for Schwann cell proliferation and survival. VEGF inhibits the differentiation and functional maturation of DCs by suppressing NF-κB in hematopoietic stem cells, and by downregulating the anti-tumor response (114). VEGF has been demonstrated to be carried in tumor-shed vescicles and to be released in a bioactive form (115). Therefore, mast cell-derived exosomes are good candidates to mediate tumoral transformation. Several studies have tested the hypothesis that NF1 heterozygotes enhance angiogenesis and may promote neurofibroma formation (116, 117); nevertheless, none of these studies showed so far the role of secreted vesicles in the communication between different cell types. We postulate that tumor-secreted exosomes could play a novel role in cell–cell communication with mast and endothelial cells during neurofibroma progression.

Currently, many cancer therapies targeting innate immunity have been studied in the context of NF1 and some such trials are still ongoing (**Table 1**). Some of these drugs may target not only tumor molecules or soluble factors but also tumor-shed vesicles. NF1 patients with symptomatic plexiform neurofibromas had decreased tumor volumes in a phase 2 study after treatment with kinase inhibitor imatinib mesylate, which targets the c-Kit receptor, ABL, BCR-ABL, and PDGFRα (118). Based on the hypothesis that mast cell secretions contribute to the growth and associated symptoms of neurofibromas, ketotifen (a mast cell granule stabilizer) was shown to reduce neurofibroma-associated symptoms and tumor growth. A reduction in exosomes release might be associated to the treatment with granule stabilizers. A subsequent multiphase trial confirmed the symptomatic control with this agent, yet without neurofibroma reduction (119). As malignancy develops, macrophages are recruited at higher densities to peripheral nerves and neurofibromas in mice and human beings when NF1 is inactivated in Schwann cells (120). PLX3397 (Plexxikon) is a novel small molecule that selectively inhibits CSF1R, c-Kit, and mutant FLT3 kinases, by targeting key components of both the tumor and its microenvironment (such as macrophages, osteoclasts, and mast cells). The effects of PLX3397 in neurofibroma formation indicated that macrophage infiltration seems to have both anti- and pro-tumorigenic roles depending on the disease stage (120). Currently, the lack of efficient treatment options for neurofibroma patients is due to the lack of understanding the mechanisms how it progresses. Defining if exosomes play a role in neurofibroma-MPNST transformation will help in identifying individuals who may be at a high risk of progression. Early diagnosis of malignant transformation is key to design therapeutic approaches in NF1 patients, and exosomes could be the missing biomarker for neurofibromatosis.

**Table 1 | Current clinical trials in cancer involving innate immunity modulation.**

| Agents | Molecular targets | Target cells (innate immunity) | Tumor | Phase | Reference |
|---|---|---|---|---|---|
| Imatinib | c-KIT, ABL, BCR-ABL, PDGFRA | Mast cells | PN NF1 | 2 | (118) |
| Ketotifen fumarate | Blocking of histamine binding (H1 receptor), inhibition calcium-dependent vesicle degranulation of activated mast cell | Mast cells | Neurofibromas NF1 | Controlled multiphase trial | (119) |
| PLX3397 | CSF1R, KIT, FLT3-ITD | Machrophages Mast cells | PN NF1 (Nf1flox/flox mouse model) | P (*in vivo*) | (120) |
| L-MTP-PE, Mifamurtide Mepact | NOD2, NLRP3 (NF-kB, MAPKs activation) | Macrophages | Osteosarcoma | 3 | (121, 122) |
| Haploidentical NK cells + IL-2 + CT | Cytolytic activity | NK | Neuroblastoma | 1 | clinicaltrials.gov (NCT00698009) |
| Allogeneic NK cells + 3F8 + CT | Cytolytic activity | NK | Neuroblastoma | 1 | clinicaltrials.gov (NCT00877110) |
| Humanized Anti-GD2 (hu14.18K322A) ± NK cells + CT | Cytolytic activity | NK | Neuroblastoma | 1 | clinicaltrials.gov (NCT01576692) |
| Humanized anti-GD2 (Hu3F8) + GM-CSF | Disialoganglioside GD2 + GM-CSFR | NK cells, monocytes/ macrophages | Neuroblastoma | 1 | clinicaltrials.gov (NCT01757626) |
| Rhu-GM-CSF + CT | GM-CSFR | Monocytes/macrophages, CD4-T,NK, DCs | Colorectal cancer | 2 | (123) |
| Dendritic cells + PANVAC or PANVAC + GM-CSF | Carcinoembryonic antigen and mucin-1 | Tumor antigen specific T cells | Colorectal cancer | 2 | (124) |

*PN, plexiform neurofibroma; P, preclinical; MPNST, malignant peripheral nerve sheath tumor; PVNS, pigmented villonodular synovitis; GBM, glioblastoma; TMZ, temozolomide; RT, radiotherapy; CT, chemotherapy.*

## THE ROLE OF TUMOR-INFILTRATING MACROPHAGES IN OSTEOSARCOMA

Osteosarcoma is the most common primary malignant tumor arising in the bone, and it occurs most frequently in adolescents. In patients with osteosarcoma, the lungs are the most common site of distant metastasis (125) and the most common site for recurrence.

The immune system is likely to play an important role in osteosarcoma progression, and indeed, the presence of infiltrating macrophages is associated with improved survival and decreased incidence of metastasis. However, the role of vesicles in the osteosarcoma/immune system interaction has not been demonstrated yet. Thus, strategies that target innate immunity seem to represent a promising approach to treat this tumor (**Table 1**). Mifamurtide (liposomal muramyl tripeptide phosphatidyl ethanolamine: L-MTP-PE, Mepact), is a modulator of innate immunity that stimulates the anti-tumoral effect of monocytes and macrophages (121). The results of an US randomized phase III trial (INT 0133) of L-MTP-PE associated to chemotherapy to treat osteosarcoma reported a significant interaction of the combination L-MTP-PE/ifosfamide (122). M1 and M2 macrophages appear to inhibit growth of osteosarcoma cells *in vitro* and, in particular, the M1 subpopulation is activated by L-MTP-PE when associated with IFNγ and the M2 macrophages with IL-10 in the presence of anti-EGFR cetuximab involved in antibody-dependent phagocytosis (126). Osteosarcoma cell secrete exosomes, and they might interact with macrophages and other immune cells, as has been described above. We postulate a role of exosomes as mediators of immune response in osteosarcoma, and other studies will be needed to develop new drugs that enhance the innate immune system in these patients.

## NK CELLS IN NEUROBLASTOMA TREATMENT AND PROGRESSION

Natural killer cells recognize and eliminate transformed cells that downregulate the expression of human leukocyte antigen (HLA) class I molecules (127). The efficiency of NKG2D in NK cell-mediated cytotoxicity is strictly correlated with the expression and surface density of the MHC class I-related chain (MICA or NKG2D ligand) on target tumor cell (128). Neuroblastoma is a neuroendocrine tumor, arising from neural crest element of the sympathetic nervous system and represents the most common extracranial solid cancer in childhood. Patients with neuroblastoma show high serum soluble MICA (sMICA)associated with the downregulation of surface NKG2D in normal peripheral blood CD8+ cells, decreased NK-mediated killing of MICA+ neuroblastoma cells, HLA class I antigen-deficiency and defects in antigen processing (129). Furthermore, neuroblastoma may evade the immune system by downregulating activating ligands for the immunoreceptor NKG2D expressed by cytotoxic T lymphocytes and NK cells (130). It is reported that prostate carcinoma cell line secrete MICA in association with exosomes, and this may contribute to immune escape mechanism of different tumoral cells (131). Tumor-derived exosomes can both stimulate NK cell

activity or promote tumor immune evasion by impairing NK cells effector (132). Neuroblastoma cell lines are known to release exosomes (133), and their role in immune surveillance should be investigated, particularly as they might represent useful targets for immunotherapeutic approaches (134). NK-derived exosomes increase NK cytotoxic potential over the tumor. NK cells have been successfully used in adoptive immune cell infusions to cure various advanced or metastatic tumors inducing a graft-versus-tumor (GVT) effect (135), so NK-derived exosomes could be used as drugs to reduce neuroblastoma tumor size. At the moment, different trials are ongoing to evaluate the efficacy of NK cell infusion in patients with relapsed or refractory neuroblastoma, in conjunction with the use of anti-GD2 antibodies (**Table 1**, clinicaltrials.gov).

### INNATE IMMUNITY AND COLORECTAL CANCER

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. The risk of developing CRC is influenced by both environmental and genetic factors and patients with inflammatory bowel diseases (IBDs), among which the most common forms are Crohn's disease and ulcerative colitis, present a higher risk of developing colitis-associated CRC (136). We postulate that the continuous secretion of exosomes and recruitment of stromal cells promoted by the constant inflammation in IBD patients may be a risk factor in the development of CRC. However, the majority of CRCs develops without any apparent pre-existing inflammatory pathology. Commensal microorganisms contribute to host defenses, controlling intestinal inflammation, and maintaining intestinal homeostasis through crosstalk with the innate immune system. The benefits of targeting innate immunity in colon cancer has been studied, although a phase 2 study of recombinant human GM-CSF administrated preoperatively to colon cancer patients failed to find an association with beneficial immune function (123). Another trial compared the effectiveness of two different vaccines (DCs modified with PANVAC or PANVAC plus GM-CSF) in the treatment of patients with CRC (124) showed that DCs and poxvector vaccines have similar activity but that the survival of vaccinated patients was longer than for unvaccinated patients. Patients with CRC present higher serum exosomal levels if compared with controls, but their role in the innate immunity is not yet described (137). Exosomes (or tolerosomes) have been shown to contribute to maintaining tolerance to food antigens in the gastrointestinal tract (138). Therefore, exosomes released by immune cells not only play an immunostimulatory effect but they are also involved in the maintenance of immunological tolerance, which may be lost in inflammatory diseases, thereby contributing to cancer development. Furthermore, a study showed significantly higher levels of exosomal miRNAs in the serum of patients with colon cancer than in healthy controls, levels that were substantially downregulated after surgical resection of the tumors (137). Inflammation and cancer progression are very closely related in CRC progression, and exosomes are key players in this interplay.

## CONCLUSION

The immune system performs tumor immune surveillance to inhibit tumorigenesis and prevent the establishment of a premetastatic niche. In this context, it remains unclear which are the effector molecules that allow cancer cells to evade immune surveillance and that drive immunoediting and tumor promoting inflammation through interactions in the tumor microenvironment. In this sense, it would be of interest to determine *how immune activity can be harnessed for clinical benefit.* There is new evidence focusing on the interaction between the innate immune system and effector molecules secreted by tumor cells. However, to date, there is limited data about as to how tumor-secreted vesicles can act as "first messengers" to prepare the metastatic niche and how innate immunity reacts to that threat. One critical point in this field is that the majority of the studies up to date involves normally *ex vivo* manipulations on extracellular vesicles that do not necessarily reflect the physiological situation. Similarly, in most of the studies, it is hard to determine the biological role of extracellular vesicles *in vivo a*nd difficult to interpret. Furthermore, very often the exact source of extracellular vesicles is not well known, and the assays performed made the mechanistic analysis difficult to interpret. There is scarce information yet regarding the secreted vesicles actions in physiological conditions although progression rates in the field are promising. A better characterization of the source and more mechanistic assays would be required to define the specific role of extracellular vesicles secreted by each cell type. *Do tumor-secreted vesicles regulate the behavior of the innate immune system during metastasis*? *Can they act as an invisible threat to orchestrate metastatic niche formation*? *Can they be stopped*? Further research is needed to define the role of secreted vesicles in metastasis and how this information "educates" the tumor microenvironment.

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# Extracellular Vesicles – powerful markers of cancer EVolution

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Solid tumors are likely derived from the co-evolution of neoplastic cells, stromal components, vasculature, and immune cells (1). As tumors evolve and progress, an array of molecular changes accumulate giving rise to multiple cell subpopulations, each with the ability to divide and mutate further. In addition, neoplastic cell populations are able to modulate the behavior of other types of cells in their microenvironment, converting their intrinsic anti-tumoral into pro-tumoral activity (2, 3). Therefore, a malignant tumor is composed not only of neoplastic cells that are heterogeneous in terms of genetic and phenotypic features, but also by different pro-tumoral cells and a particular extracellular matrix that supports cancer evolution and progression.

Tumor heterogeneity has been recognized as one of the main factors for cancer therapy failure, and has just started to be dissected using next-generation sequencing (NGS) approaches (4). While whole genome sequencing, and particularly, exome sequencing have provided the molecular basis for several complex traits, RNA and bisulfite sequencing have been important to disclose expression regulatory mechanisms. However, NGS-derived studies have often been conducted using single fragments/biopsies of primary tumors, and therefore fail to reflect the global tumor heterogeneity, dynamics, and drug sensitivities, likely to change during tumor evolution and treatment. For these reasons, there is the need to develop strategies that may accurately capture the entire landscape and allow following clonal evolution of tumor populations.

Scientific evidence supports that most types of cells secrete small vesicles (exosomes and microvesicles) into the extracellular milieu (5), and that tumor cells in particular produce at least threefold more of these small vesicles than normal cells (6–8). These so-called extracellular vesicles (EVs) are emerging mediators of intercellular communication and orchestrators of health and disease, and contain a repertoire of genetic information (incorporated in DNA, RNA, microRNAs, and proteins), which may be a fingerprint of the releasing cell type (9, 10). EVs can be easily detected in biological fluids such as plasma, serum, ascites, or urine, and provide excellent minimally invasive biomarker candidates to monitor cancer patients' progression, prognosis, and treatment efficacy (10, 11). In fact, tumor-derived exosomes in patients' bloodstream were shown to contain fractions of tumor genome, transcriptome, and proteome such as *KRAS, TP53* mutations in pancreatic and colon cancer (12); mutant/variant *EGFRvIII* mRNAs in glioblastoma (13); microRNAs in ovarian cancer (14); MET in melanoma (15); and HER2 in breast cancer (16). Further, double-stranded DNA (exoDNA) representing the entire genome and reflecting the mutational status of parental tumor cells [e.g., *BRAF*(V600E) and *EGFR* exon 19 deletion — del19], was found in EVs from melanoma and non-small cell lung cancer cell lines (17). From a clinical perspective, molecules enclosed in EVs harbor potential usefulness as circulating biomarkers with impact in early detection and during cancer progression. Apart from carrying specific molecular signatures and disease effectors, EVs also contribute to horizontal cellular transformation and phenotypic reprograming, both locally and systemically (8, 10, 15, 18).

The identification of specific EV features has allowed developing isolation and characterization methodologies that have been used in numerous studies (19–21). Most of these have been focused on the characterization of the cargo of EVs in different types of cancer, using either conditioned media of cancer cell lines, or unique samples from cancer patients' body fluids. Cancer cell line studies have provided markers of EVs for different types of cancers, however, lack the representativeness of cancer as a heterogeneous cell population. Unique samples from cancer patients' body fluids have highlighted potential markers for cancer diagnosis and prognosis in cross-sectional studies, although fail to deliver useful information to monitor tumor heterogeneity and dynamics, and to allow therapy response and recurrence assessment.

Longitudinal studies of cancer patients, from whom samples are repeatedly collected along diagnosis, treatment, and follow-up, have been rarely reported, and may be the most adequate tools to address the abovementioned limitations. One possible longitudinal approach should enclose the molecular profiling of the following patient-derived samples: (1) biopsy/surgical tumor specimens; (2) body fluid-derived EVs collected prior/at surgery; (3) body fluid-derived EVs collected immediately after surgery; (4) body fluid-derived EVs collected along therapy cycles, and; (5) body fluid-derived EVs collected after disease remission (if possible).

The comparative analysis of data derived from each of these datasets will shed light into cancer-specific signatures that become represented in tumor-derived EVs (biomarker candidates), and that should be used to monitor tumor evolution, dynamics, and therapy response, as well as predict disease recurrence. This type of studies raises the need for close collaborations between clinical and basic research teams, to set-up effective study designs and ethically approved protocols, to allow collection of multiple samples and clinically relevant information from each individual patient. The power of this approach is the possibility of providing information useful for the design of precision-medicine approaches, with impact in clinical practice.

In summary, in this article, we discuss the impact of performing longitudinal studies through the analysis of EVs from cancer patients, to improve our understanding of tumor heterogeneity/evolution, and to identify minimally invasive markers, potentially useful for disease management of cancer patients. We further present a workflow that may be useful to consider when designing longitudinal studies involving cancer patients. Despite lengthy and labor-intensive, such studies will certainly provide answers for currently unsolved questions in cancer research.

At this point, it is clear that EVs have a tremendous potential to be used as a "liquid biopsy" for cancer patients, which would be less invasive compared to surgery and may provide diagnostic information, aid in therapeutic decisions, and monitoring of disease over time, on a personalized basis.

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# Urinary extracellular vesicles as source of biomarkers in kidney diseases

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Most cells physiologically release vesicles as way of intercellular communication. The so-called Extracellular Vesicles (EVs) include exosomes, ectosomes, and apoptotic bodies, which basically differ in their composition and subcellular origin. Specifically, EVs found in urine reflect the state of the urinary system, from podocytes to renal-tubular cells, thus making them an excellent source of samples for the study of kidney physiology and pathology. Several groups have focused on defining biomarkers of kidney-related disorders, from graft rejection to metabolic syndromes. So far, the lack of a standard protocol for EVs isolation precludes the possibility of a proper comparison among the different biomarkers proposed in the literature, stressing the need for validation of these biomarkers not only in larger cohorts of patients but also considering the different methods for EVs isolation. In this review, we aim to gather the current knowledge about EVs-related biomarkers in kidney diseases, with a special emphasis in the methods used to date for EVs enrichment, and discussing the need for more specific protocols of EV isolation in clinical practice.

**Keywords: urinary extracellular vesicles, kidney disease, biomarker, therapy, isolation**

## INTRODUCTION

Extracellular Vesicles (EVs) have awakened the interest of the scientific community due to their different roles in intercellular communication, pathogenesis, drug, and gene vector delivery, and as possible reservoirs of biomarkers (1–3). These vesicles can be released from different kind of cells, from platelets to cells of the immune system and neurons, among others (4), and can be found in several body fluids, including plasma, urine, and saliva (5, 6).

The term EVs includes different types of vesicles, mainly exosomes (EXs), ectosomes, and apoptotic bodies. These distinct types of vesicles differ in several aspects (7, 8). EXs are 30–150 nm diameter vesicles derived from the inward budding of endosomal membranes, resulting in the progressive accumulation of intraluminal vesicles (known as EXs) within large multivesicular bodies (MVBs). These EXs are released to the milieu by fusion with the plasma membrane (8, 9). Ectosomes, also referred as Microvesicles (MVs) are bigger than EXs (100–1000 nm) and they are produced by the direct budding of the plasma membrane (10). Finally, dying cells also shed membranous vesicles, called apoptotic blebs, with heterogeneous shape and size (8). Hence, the different subcellular origin of EVs accounts for their specific composition and function. In this sense, EVs contain a specific subset of common proteins related to biogenesis and trafficking and also a specific signature from their cell or tissue of origin (8, 11), including protein and nucleic acids (4, 12, 13). Therefore, the study of the proteome and the nucleic acid content of EVs may provide information about the cell or tissue of origin and, importantly, their physiological state. Three public online databases including all gathered information about EV content are available: EVpedia, ExoCarta, and Vesiclepedia (2, 14–16).

Urine is a body waste fluid, which can be easily obtained, being therefore an ideal fluid for biomarker determination and analysis. But urine is a complex mixture of filtered and secreted proteins, salts, urea, and metabolites that may vary, not only in physiological situations but specially in diseases associated with renal involvement (17, 18). In addition, the protein composition of the urine is highly dependent on the glomerular filtration rate (GFR), tubular metabolism, tubular reabsorption, diet, and hydration status of the patient among others. The variation in the concentration of a certain protein could be the result of any tissue disorder or pathophysiological alteration. It is estimated that only around 3% of urine proteins are contained in urinary EVs. Therefore, potential interesting biomarkers contained in EVs may be just undetected due to their dilution in whole urine. Thus, the determination of biomarkers specifically related to urinary EVs may be of interest to uncover alterations on the renal system (18).

## ISOLATION TECHNIQUES AND METHODS TO OBTAIN URINARY EXTRACELLULAR VESICLES

The complexity of body fluids, and in this case urine, hampers the study of the specific molecular content of EVs.

A previous step to EVs isolation is the management of the sample. Based on the studies of Zhou et al. (19), which focused in the protein content of EVs, it is recommended the use of protease inhibitors in the collection containers to preserve the sample. In addition, an extensive vortex is required to recover the highest amount vesicles, as EVs could remain attached to the plastic. Samples can be either processed immediately or storage at −80°C. Due to the lack of consensus in the published data, the international society of EVs has recently published a position paper, in which

they recommend further studies on this issue (6). In addition, as a normal procedure in urine samples, bacterial contamination of the physiologic micro-environment should be avoided (6, 20).

Many studies conducted to date have enriched urinary EVs using methods that do not exclude the presence of non-EV contaminants. However, a gold-standard technique for isolating EVs in the clinical practice is still missing. In this section, we summarize and discuss the different methods used to isolate urinary EVs.

When initiating the isolation of urinary EVs, a first consideration has to be paid to the presence of the Tamm–Horsfall protein (THP)-also known as uromodulin-, a common component of urine samples in both physiological and pathological conditions. THP, among others proteins (such as albumin) that are increased in urine under several kidney pathologies, do interfere with urinary EVs-related biomarkers research and discovery. Therefore, pre-isolation techniques to reduce or eliminate their presence before any EVs isolation/determination are recommended (6, 21). DTT-treatment has been the most widely solution to avoid EV-entrapment by THP (22). An alternative to DTT is the use of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic (CHAPS), a mild detergent also proposed to solubilize THP. The main advantage of CHAPS is the preservation of the protein conformation and the enzymatic activity (23). This may be important in experimental designs involving functional activities of urinary EVs. However, it has been also mentioned that CHAPS treatment is more time consuming than DTT-treatment (24). Thus, the use of each method would be directed by the final use of the sample.

Once these major contaminants are reduced, the sample is prepared for enrichment and isolation of EVs. This could be achieved following different methods.

#### ULTRACENTRIFUGATION

Ultracentrifugation was first used to isolate EVs by Jonhstone and Stahl (25, 26), and since then it has been widely used for EVs isolation regardless the origin of the sample. Thus far, most studies found in the literature carry out a two-step differential ultracentrifugation (UC) to isolate EVs: a first low-speed centrifugation at 10,000–17,000 $\times$ *g* to eliminate cellular debris, apoptotic bodies, and larger EVs, and a second step at higher speed – ranging 100,000–200,000 $\times$ *g* depending on the study-, to pellet smaller EVs. In some cases, this centrifugation method could be complemented or replace with a size filtration step to remove protein aggregates and cell debris. However, some authors consider that filtration step may fracture larger EVs in smaller ones (6).

Under pathological conditions, when glomeruli filtration is compromised, there is a massive loss of protein in urine. These proteins, together with THP and their aggregates could be co-isolated with urinary EVs. To obtain a more purified sample, the pelleted EVs by the differential UC can be processed and resolved by sucrose density gradients to remove contaminants (27). Other density gradients such as potassium bromide or iodixanol (Optiprep™) have been recently proposed to isolate EVs reducing protein contaminations in plasma (28, 29).

Although UC is the technique of reference for basic research in EVs, major drawbacks undermine their applicability in the clinical setting, including the high operator-dependent variability, the lack of a universal protocol, the expensive equipment required, and the low throughput (30).

#### FILTRATION

Filtration is based on passing the sample through a nanomembrane (in general polyethersulfone or PVDF membranes) in a short low-speed centrifugation. This method avoids UC, and can be used with low-volume samples. Cheruvanky et al. analyzed this method as a new option for isolating urinary EVs. They detected that some proteins classically related to EVs were retained by the nanomembrane, thus making necessary an extra-washing step to recover the total amount of EVs (31). In addition, Rood et al. found a low recovery of EVs markers together with co-isolation of proteins i.e., albumin within the urinary EVs pellet (21). Thus far, the protein adherence to the nanomembrane and the high protein retention are important disadvantages for isolating urinary EVs from proteinuric patients using this method. Other options, such as filtration applying vacuum or ultrafiltration, which use a low protein binding membrane, can be used to purify urinary EVs from low-volume samples (32).

#### IMMUNO-AFFINITY AND PEPTIDE-BASED ISOLATION

Extracellular vesicles are characterized by the presence of several surface proteins, such as the tetraspanin family. Taking advantage of this proteins expression, several affinity-based methods have been developed to isolate EVs. These methods use antibodies (attached to magnetic beads or other supports) to specific EV proteins (4, 33). Immuno-isolation of EVs still requires a low-speed centrifugation step or magnetic techniques to concentrate vesicles (6, 24).

Ghosh et al. proposed a method for isolating EVs using a synthetic peptide with specific affinity for heat shock proteins, which are described as EVs markers (16). Similar to antibodies, this technique takes advantage of the membrane proteins present on the surface of EVs. The so-called Vn-96 peptide seems to be able to capture EVs from plasma, urine, and cell culture supernatant (34).

Nevertheless, due the lack of unequivocally specific EVs pan-markers, EVs obtained by these methods would render biased samples depending on the antibody or peptide used (9).

#### AGGREGATING AGENTS

Several commercial precipitation reagents have been introduced in the last few years. These reagents such as ExoQuick-TC™, Total EX isolation reagent from Invitrogen™, Exospin™, and miRCURY™ EX isolation kit from cells, urine and CSF, among others, are based on aggregating agents followed by a low-speed centrifugation. The advantage of these methods is their easy application and faster performance, with a huge applicability in diagnostic laboratories and low operator variability (35, 36). On the other hand, their main drawback is the co-isolation of a complex mixture of EVs together with protein aggregates (37) that may interfere with further EV-related marker determination and analyses. Thus, these methods still require of specific pre-treatments to eliminate larger EVs and aggregates (4, 6, 24, 29).

#### SIZE-EXCLUSION CHROMATOGRAPHY

In the last years, size-exclusion chromatography (SEC) has been applied to fractionate complex biologic samples, such as urine and

plasma, and isolate EVs excluding the main contaminants, such as protein aggregates or lipoproteins usually co-isolated by UC. Rood et al. used UC followed SEC to optimize the purity of the urinary EVs. Importantly, this group demonstrated that non-EV-related contaminants could mask the presence of relevant EV markers (21), thus emphasizing the importance of eliminating as much contaminants as possible.

More recently, Muller et al. used this technique for isolating morphologically intact EXs without protein contamination from human plasma samples (38, 39). In our laboratory, we have obtained similar results using urine samples. Thus, SEC can be applied to highly enrich EVs in an efficient manner. In addition, SEC is a medium throughput technique susceptible to be implemented in the clinical setting.

#### MICROFLUIDIC-BASED METHODS

Microfluidic-based methods have been recently developed to isolate EVs. Exochip™ is a microfluidic-based platform based on a polydimethylsiloxane matrix covered with Abs against CD63 (classical exosomal marker) developed for the immuno-affinity isolation of circulating EVs. This method does not require a separation step for quantification of EVs, as the vesicles are labeled with a fluorescent dye allowing their quantification using a plate reader. In addition, RNA extraction or proteomic studies can be carried out as well (40).

Recently, Santana et al. have developed a microfluidic platform to isolate EVs based on their diameter and the deterministic lateral displacement. First assays carried out have shown an efficient separation among EVs subpopulations without altering their biology and morphology. However, the ability to separate EVs aggregates should be deeply analyzed in further experiments. In addition, this technology should also be tested on complex biological samples in which the density of the sample is highly variable (41).

#### HYDROSTATIC DIALYSIS

Musante et al. have recently presented hydrostatic dialysis to isolate urinary EVs without need of UC and as a possible solution to highly diluted samples. This method is based in a dialysis membrane with molecular weight cut-off of 1000 kDa, removing all possible contaminants from EVs samples (36).

In summary, although different isolation methods for EVs have been developed, a standard consensus – with special relevance in the clinical settings – is still missing. This is of special importance due to the variable results obtained when comparing different techniques (37) or even when using different RNA extraction methods, in which the presence of high amounts of RNA could be indicative of non-EV contaminating RNAs (42).

## URINARY EVs AS A SOURCE OF BIOMARKERS FOR KIDNEY-RELATED DISEASES

Chronic kidney diseases are a public health issue as they cause important morbidity and mortality and impose high economic burden. Several studies suggested that the chronic kidney diseases (CKD) are an independent predictor of mortality risk in the general population (43). In addition, associations between reduced GFR and the risk of death, cardiovascular events, and hospitalization were observed (44).

The replacement therapy for patients with irreversible chronic kidney failure includes hemodialysis or peritoneal dialysis, being the renal transplantation (RTx) by far the modality of choice for those patients (45). Many factors can affect long time graft survival, and despite remarkable progress has been made in the last years, immunologic rejection and adverse effects of immunosuppressive agents still have significant negative long-term consequences.

Currently, kidney function is indirectly monitored by measuring the GFR, creatinine clearance, serum creatinine, and proteinuria. However, these markers are usually a late sign of kidney damage that indicates – rather than predicts – renal dysfunction. Moreover, the unique gold-standard technique to diagnose kidney diseases is renal biopsy. Needless to say that needle biopsies are an invasive non-reproducible technique, and may be associated with patient morbidity. Thus, there is an additional need to find non-invasive alternatives to kidney biopsies.

In the case of kidney failure and/or renal diseases, urine is the perfect source of biomarkers for developing new diagnostic tools to identify and stratify patients. Different research programs have been conducted to biomarkers discovery. Initially, efforts were focused to the analyses of total urine, while lately, the study of urine derived EVs have gained interest. Particularly, urinary EVs are secreted from all cell types that face the urinary space (glomerular structures, renal tubule cells, and the cells lining the urinary tract) as represented in **Figure 1**. It is thus considered that urinary EVs may be a non-invasive image of the physiological state of the renal-tubular system (18, 46). Therefore, an increasing amount of research groups have focused their interest in the study of urinary EVs in kidney diseases. To date, several proteomic studies on EVs have identified proteins that could be associated to kidney diseases (47, 48). **Table 1** shows a summary of the current state of biomarkers discovery related to kidney disorders, which are also detailed below.

#### ACUTE KIDNEY INJURY

Acute kidney injury is a disorder characterized by a rapid decline in the GFR and retention of nitrogenous waste products (71).

Acute kidney injury can be divided in: pre-renal, intrinsic, and post-renal. Pre-renal acute kidney injury (AKI) is the most common cause of AKI and is an appropriate physiologic response to renal hypo-perfusion. The causes of intrinsic acute renal failure (ARF) can be categorized into the following: diseases involving large vessels, diseases of renal microvasculature and glomeruli, ischemic, and nephrotoxic acute tubular necrosis, all processes involving tubulointerstium. Post-renal AKI is due to urinary tract obstruction and accounts for <5% of cases of AKI (72).

Acute kidney injury may be one of the best examples in which serum creatinine concentration (Scr) is used as biomarker of kidney failure. Nonetheless, its concentrations vary widely, and it is not informative in asymptomatic stages and cannot predict the outcome of the disease. It is known that substantial loss of GFR may not manifest with elevations in Scr for several days, and creatinine based estimated GFR is not accurate.

Serum creatinine concentration concentration increases when renal filtration is decreased at least 30%. Moreover, it has been shown that high levels of Scr may not be associated to renal-tubular

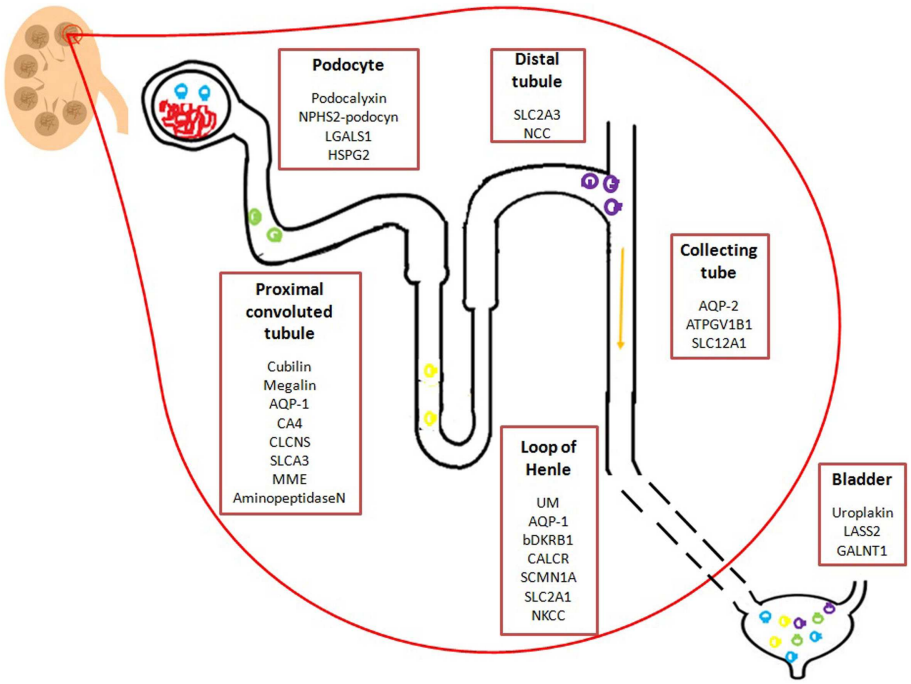


**FIGURE 1 | Schematic representation of potential urinary EVs biomarkers from the urinary tract**. Potential biomarkers identified in urinary EVs from different regions of the nephron, renal tubule, and the bladder. Mentioned molecules are hypothetically related to a specific region of the renal system. *Podocyte:* LGALS1: lectin galactoside-binding soluble 1; HSPG2 heparan sulfate proteoglycan 2. *Distal convoluted tubule*: SLC2A3: solute carrier family 2; NCC: Na-Cl co-transporter; *Proximal convoluted tubule:* AQP-1: Aquaporin-1; CA4: carbonic anhydrase 4; CLCNS: chloride channels; SLCA3: solute carrier family 3; MME: membrane metallo-endopeptidas; *Loop of Henle*: UM: uromodulin; bDKRB1: bradykinin receptor B1; CALCR: calcitonin receptor; SLC2A1: solute carrier family 2; NKCC: $Na^+/K^+/2Cl^-$ co-transporter; *Collecting tube:* AQP2: aquaporin-2; ATP6V1B1: TPase, H transporting, lysosomal 56/58 kD, V1 subunit B; SLC12A1: Sodium potassium chloride co-transporter 2; *Bladder:* LASS2: ceramide synthase 2; GALNT1 polypeptide N-acetylgalactosaminyltransferase 1.

injury or kidney injury (73). These reasons clearly reflect the need for new more sensitive biomarkers.

Kidney injury molecule-1, neutrophil gelatinase-associated lipocalin (NGAL), or interleukin-18 have been proposed as potential biomarkers for the early diagnosis of AKI in total urine (4, 74). Besides, other groups have recently reported Heat Sock Protein 72 (Hsp72) as putative biomarker, demonstrating an increase in mRNA levels after ischemia and kidney injury (73, 75). Despite these new approaches in biomarkers research, the "classical" parameters already mentioned continue to be used.

As mentioned before, one of the possible causes of acute renal damage is *renal ischemia-reperfusion.* During the ischemia, the absence of nutrients and oxygen creates a hypoxic condition, which promotes the reactive oxidative species formation and inflammatory response once the blood flux is restored. Related to EVs, Zhou et al. identified EVs containing Fenituin-A as potential biomarker after renal injury using an AKI rat model. These EVs were detected not only before any increase in Scr levels but also before any structural change could be detected by kidney biopsy. In addition, this marker was in the same way detected in total urine (76). A few years later, the same group identified transcription factor 3 (ATF3) as an additional EVs biomarker for AKI. Importantly, these biomarkers were only detected on EVs coming from patients and were not present in healthy volunteers (49).

Also, in a rat model for ischemia-reperfusion, Sonoda et al. reported the decrease of exosomal aquaporin-1. Similar results were confirmed in a transplant group of patients (50). The most interesting feature of this biomarker is that they could not find this decrease in EVs in a rat model of nephrotic syndrome, suggesting that this marker could be specific for this pathological state.

## GLOMERULAR DISEASES

Podocytes are specialized epithelial cells forming the glomerular filtration barrier together with the glomerular basement membrane and endothelial cells. The damage of this structure leads to a loss of proteins and blood cells (77). In glomerular diseases, podocytes are the main cells affected being therefore considered, which podocyte-derived EVs may be a promising source of biomarkers.

### *Diabetic nephropathy*

One of the main causes of glomerular disorders is diabetic nephropathy. Related to it, Barutta et al. described a differential expression of 22 exosomal miRNAs between normo and micro-albuminuric patients. Interestingly, the levels of miR-145 and miR-130a in urinary EVs were increased in diabetic and micro-albuminuric patients compared to normo-albuminuric controls. In contrast, miR-155 and miR-424 levels were lower

**Table 1 | Summary of EVs biomarkers related to kidney disease**.

| Pathology | Sample | Isolation method | Biomarker | Reference | Model |
|---|---|---|---|---|---|
| **ACUTE INJURE** | | | | | |
| AKI | Spot | DC/200000g | Fetuin A | (76) | Rat and human |
| | 10–16 ml | DC/200000g | ATF3 | (49) | Rat and human |
| I/R | 5–8 ml | DC/200000g | Aquaporin-1 | (50) | Rat and human |
| | 10–16 ml | DC/200000g | Transcription factor 3 | (49) | Rat and human |
| **GLOMERULAR INJURY** | | | | | |
| FSGS | 10–16 ml | DC/200000g | Wilm's tumor 1 | (49, 51) | Rat and human |
| | 45 ml | DC/200000g + SEC | PODXL | (21) | Human |
| GKD | 15 ml | DC | ADAM10 | (52) | Cell line and human |
| DN | 450 ml | DC/200000g | miR-130, miR-145, miR-155, and miR-424 | (53) | Mice, cell line, and human |
| | – | Filtra-centrifugation | DPP IV | (54) | Human |
| | 100 ml | DC/175000g | AMBP, MLL3, and VDAC1 | (55) | Human |
| LN | – | DC/200000g | miR-26a, ADAM10 | (56) | Mice and human |
| IgAN | 30 ml | SGC | Ceruloplasmin and miR-26a | (57) | Human |
| TBN | 30 ml | SGC | Aminopeptidase A and vasorin | | |
| **FIBROSIS** | | | | | |
| RF | – | DC/2000000 | miR-29c and miR-200 | (58) | Human |
| GF | – | DC/200000g | CD2AP, synaptodin mRNA | (59) | Human |
| **OTHER RENAL DISORDERS** | | | | | |
| CKD | 25 ml | Ultrafiltration | mRNA Il-18, NGAL | (60) | Human |
| Tx | 10 ml | DC/200000g | NGAL | (61) | Human |
| | 200 ml | DC/100000g | CD133 | (62) | Human |
| PKD | – | DC/150000g | PKD1, PKD2, PKHD1 | (63) | Human |
| **CANCER** | | | | | |
| MPC | 5 ml | DC/100000g | ATGB1 | (64) | Cell line and human |
| | | | ITGA3 | | Cell line and human |
| | – | DC/110000g | miR-34a | (65) | Cell line and human |
| | 200 ml | DC/100000g | PSA, PSMA | (66) | Cell line and human |
| BC | – | SGC | EDIL-3 | (67) | Cell line and human |
| | – | DC/100000g | LASS2, GALNT1 | (68) | Human |
| | 50 ml | DC/100000g | TACSTD2 | (69) | Human |
| RCC | 50 ml | DC/200000g | MMP-9, DKP4, EMMPRIN, PODXL | (70) | Human |

*Methods: DC, differential centrifugation; SGC, sucrose gradient centrifugation.*

*Disease: AKI, acute kidney injury; I/R, isquemia reperfusion injury; FSGS, focal segmentary glomerulosclerosis; GKD, glomerural kidney disease; DN, diabetic nephropathy; LN, lupus nephritis; IgAN, IgA nephropathy; TBN, thin basement nephropathy; RF, renal fibrosis; GF, glomerulofibrosis; CKD, chronic kidney disease; RTx, renal transplantation; PKD, polycystic kidney disease; MPC, metastatic prostate cancer; BC, bladder cancer; RCC, renal cell cancer.*

*Biomarkers: PODXL, podocalyxin-like; AMBP, α-microglobulin/bikunin precursor; MLL3, histone-lysine N-methyltransferase; VDAC1, voltage-dependent anion-selective channel protein 1; CD2AP, CD2-associated protein; NGAL, neutrophile gelatinase-associated lipocalin; ATGB1, GTP-binding protein 1; ITGA3, integrin, alpha 3; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; EDIL-3, EGF-like repeats and discoidin I-like domain-; LASS2, ceramide synthase 2; GALNT1 polypeptide N-acetylgalactosaminyltransferase 1; MMP-9, matrix metalloproteinase 9; DKP4, Dickkopf related protein 4; EMMPRIN, extracellular matrix metalloproteinase inducer; PKD1, polycystin-1; PKD2, polycystin-2; and PKHD, 1 polyductin.*

in micro-albuminuric patients whilst normo-albuminuric and healthy controls showed similar levels (53). Then, in mesangial cells cultures, they demonstrated that hyperglycemic conditions induce miR-145 expression. Thus, this miRNA profile represents a novel approach in the search of EVs biomarkers, although further studies with larger cohort of patients are required.

Another interesting putative biomarker for diabetic nephropathy is dipeptidyl-peptidase. This enzyme, which plays a role in T-cell activation, is known to be over-expressed in the plasma of diabetic patients. Its high expression in the kidney took Sun et al. to analyze the vesicular content in a subset of patients and found over-expression of this protein in urinary EVs compared to controls (54).

The quantitative analysis of urinary EVs proteome from diabetic patients carried out by Zubiri et al. showed interesting differences compared to controls. Interestingly, 65% of the proteins detected were identified only in one of the experimental groups. Among those, α-microglobulin/bikunin precursor (AMBP), histone-lysine N-methyltransferase (MLL3) were increased in patients, whilst voltage-dependent anion-selective

channel protein 1 (VDAC1) were decreased (55). Hence, these proteins are suggested as promising biomarkers, although more studies are needed for verification.

#### *Glomerulonephritis*

IgA nephropathy is the most common glomerular lesion of glomerulonephritis whose clinical presentation may vary from hematuria to rapidly progressive glomerulonephritis. Specifically, IgA nephropathy (IgAN) is induced by: aberrant glycosylation of IgA1, synthesis of antibodies directed against galactose-deficient IgA1, binding of the galactose-deficient IgA1 by the anti-glycan/glycopeptide antibodies to form immune complexes and accumulation of these complexes in the glomerular mesangium. Moreover, each step of this pathological process has susceptibility locis proposed by genome-wide association studies but DNA-based test have not been developed (78).

Purification of urinary EVs from IgA nephropathy patients was carried out by Moon et al. (57). These authors identified ceruloplasmin (CP) as biomarker in this disease. In this way, CP concentration appeared higher in IgA patients than in controls; though deepest studies must be perform to confirm these results.

Another important glomerulopathy characterized by podocyte injury is the focal segmental glomerulosclerosis. This pathology could be autoimmune or secondary to obesity and drugs among others. Initial studies by Zhou et al. (49) demonstrated the overexpression of the Wilm's Tumor 1 protein, a transcription factor required for normal kidney development, in urinary EVs from a mice model with podocyte injury and in humans with focal segmental glomerulosclerosis (49). Some years later, the results were re-evaluated and confirmed by the same group in a larger cohort of patients (51).

Lupus nephritis is a frequent and potentially serious complication among patient with systemic lupus erythematosus whose clinical manifestations are varied being the most common manifestations proteinuria (commonly leading to nephrotic syndrome), microscopic hematuria, and reduced GFR. The histopathology of lupus nephritis (LN) is pleomorphic; based on light microscopic (LM), immunofluorescence (IF), and electron microscopic (EM) findings it can be classified into six classes (79).

Some differences in the levels of several miRNAs have been reported between controls and LN and IgAN patients. Among those, miR-26a is normally found in the glomeruli of control samples, but their levels are decreased in glomeruli from patients. Interestingly, Ichii et al. (56) have reported a higher level of miR-26a in EVs from patients compared to controls, thus suggesting a mechanism for the reduced levels found in patient's glomeruli. The authors suggested this microRNA could be considered a direct and predictive biomarker of podocyte and glomerular injury.

Similarly, Gutwein et al. studied ADAM10 and its substrate L1, are expressed in differentiated podocytes. Moreover, ADAM10 could be found in urinary EVs and in total urine of LN and IgAN patients but it is absent on healthy donor urine or EVs (52). Although it is not clear the role of this molecule in the kidney or in the EVs, their differential expression suggests they could be considered as possible biomarkers of glomerular damage.

#### *Thin basement membrane nephropathy*

Thin basement membrane nephropathy is characterized by non-progressive hematuria, minimal proteinuria, and normal renal function due to a thinned glomerular basement membrane. This pathology is thought to be caused by mutation in collagen genes such as COL4 and COL5 (80).

Renal biopsy is the only technique to diagnose between IgAN and thin basement membrane nephropathy (TBMN). In urinary EXs from TBMN patients', aminopeptidase N, and vasorine precursors were increased compared with IgAN patients. Thus, these precursors could be used as biomarkers in hematuric patients to differentiate between TBMN and early IgAN (57).

### KIDNEY FIBROSIS

As mentioned before, CKD is a major death cause worldwide, associated with fibrosis leading to organ failure in the final stages, independently of the primary cause. Lv et al. (58) found a reduced level of urinary EVs miR-29 and miR-200 in a selected group of CKD patients compared to controls. Furthermore, they found that miR-9a and miR-29c could discriminate between mild and moderate-severe fibrosis. Similarly, the same group evaluated other molecules such as CD2AP, NPHS2, and synaptodin mRNA in urinary EVs. They reported an increase of synaptodin and a decrease in CD2AP mRNA levels in patients. As before, their results suggested that CD2AP mRNA levels could reflect tubule-interstitial fibrosis and the glomerulosclerosis degree (59). These results clearly support the idea of using RNA content from urinary EVs as non-invasive tools in the study of renal function and diseases progression.

### POLYCYSTIC KIDNEY DISEASE

Polycystic kidney disease (PKD) is a genetic disorder characterized by kidney cystic dilatations that may course with multiple organ involvement. This disease is caused by a dysregulation of PKD1, PKD2, or PKHD1 genes (81, 82). Proteomic studies in urinary EVs by several groups revealed that polycystin-1, polycystin-2, and polyductin (products of the mentioned genes) are easily detectable in patients' samples, and therefore could be considered for the analysis of the disease (18, 63).

### RENAL TRANSPLANTATION

Renal transplantation is the best option for end stage renal disease patients.

The use of urinary EVs as a source of biomarkers for kidney injury after RTx was probed by Alvarez et al. (61). In this study, the authors demonstrated the presence of NGAL in cellular fraction and in urinary EVs from patients. Urinary EVs NGAL detection differed between patients and controls. Interestingly, different quantities of NGAL were detected between deceased and living donors. Thus, suggesting that NGAL could be a biomarker of damage and delayed graft function (61).

Likewise, it has been recently published that other proteins classically associated to kidney injury in total urine could not be considered as EV biomarkers such as kidney injury molecule-1 (KIM-1) and cystatin. These molecules did not shown significant changes in urinary EVs while NGAL mRNA was decreased after transplantation but arose to normal levels in a few days (60); in

addition, EV NGAL did not correlate with creatinine reduction. Moreover, in this case, urinary levels of IL-18 and NGAL correlate better with creatinine reduction than EVs markers (60). NGAL, KIM-1, and IL-18 have been proposed as AKI biomarkers as well, but it seems that in the case of RTx, these EVs markers do not correlate with outcome or creatinine level. Thus, the patients' selection, the degree of the disease and the biomarkers approach play a critical role in the wide of results found between studies. One of the main goals in the search of urinary EVs biomarkers is the reproducibility of the results between different methods and different sample volumes, among others.

The presence of urinary EVs expressing the progenitor marker CD133 has been lately reported by Dimuccio et al. (62). In healthy donors, they could identify two EVs subpopulations based on CD133 expression with a different profile for classical and urinary specific markers. These $CD133^+$ EVs were detectable at high levels in urine from healthy donors but not in patients with end stage renal disease. In the case of transplanted patients, $CD133^+$ EVs levels were lower than in healthy donors but higher than in chronic patients. The authors considered $CD133^+$ EVs as possible biomarkers for tubular function and renal tissue regeneration after transplantation in order to detect possible graft rejection, kidney damage, or incomplete tissue regeneration.

### RENAL CELL CARCINOMA

Recently, the comparison of the protein profile of urinary EVs derived from renal cell carcinoma (RCC) patients with control subjects was carried out by Raimondo et al. (70). This study has shown a different protein profile between them. Specifically, in the case of matrix metalloproteinase 9 (MMP-9), Dickkopf related protein 4 and Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), all of them involved in matrix remodeling, and found over-expressed in RCC patients. Moreover, these proteins correlated with the disease progression and the metastatic potential (70).

### PROSTATE CANCER

Benign prostate hyperplasia is one of the most common diseases in men, and only a low proportion of prostate hyperplasias progress to an aggressive disease. The current diagnostic protocol of this tumor includes the serum determination of the prostate specific antigen (PSA), digital rectal examination, and biopsy. This combination could fail identifying properly between aggressive or non-aggressive cancer, leading to an over-treatment of patients (83). Despite urine would be apparently the best biological fluid to find new biomarkers in this pathology, a major effort has been focused in plasma samples (84). Regarding to urine studies, Mitchell et al. found the presence of prostate markers PSA and PSMA in EVs from patients' samples compared to EV's from healthy controls (66). Recently, ITGA3 and ITGB1 proteins have been identified significantly more abundant in urinary EVs from metastatic prostate cancer patients compared to benign prostate hyperplasia patients and patients without metastasis (64).

Corcoran et al. (65) described a panel of miRNAs that could be used as biomarker for metastatic prostatic cancer. This panel was set up from studies performed in cell lines in previous publications, and analyzed in human urine samples. The decreased expression of miR-34a is suggested to be a useful tool to discriminate between prostate cancer and benign hyperplasia. In addition, BCL-2, a well-known pro-apoptotic gene, is described as the target of this miRNA (65).

### BLADDER CANCER

Together with prostate and RCC, bladder cancer is another important disorder of the urinary tract. Recently, it was described that urinary EVs from bladder cancer patients promote tube formation on endothelial cell lines and cell migration; once again EVs are shown to be a vehicle to promote cancer progression. Chen et al. reported the diagnostic potential of the cancer-related protein TACSTD2 in a short cohort of bladder cancer patients (69). Moreover, EGF-like repeats and discoidin I-like domain-3 (EDIL-3) was found to be over-expressed on bladder cancer cell lines (67). Later, the presence of this protein was confirmed in patients' EVs (67).

Recently, Perez et al. described a different expression profile for four different EV mRNA encoding for LASS2, GALNT1 ARHGEF39, and FOXO3 (68). Briefly, ARHGE39 and FOXO3 were detected only in controls whilst GALNT1 and LASS2 were detected only in urinary EVs from patients. Further studies are required to assess these proteins as bladder cancer biomarkers.

## EVs AS A THERAPEUTIC APPROACH IN KIDNEY DISEASES

Research on EVs is not only focused on their potential role as source of biomarkers but also as new therapeutic tools. Taking into account the properties and functions of EVs, different clinical studies have been developed with the aim to use them in therapy (85).

In the context of AKI, only a few studies have tested different sources of EVs for their therapeutic potential. Cantaluppi et al. tested the effect of EVs from endothelial progenitor cells in a rat model for ischemia and reperfusion injury. The miRNA content of these vesicles seems to have a positive effect in tubular cells, reducing apoptosis and promoting cell proliferation (86, 87). Ischemia-reperfusion is characterized by the over-expression of the adhesion molecule MCP-1. During this process, transcriptional repressor activating transcription factor 3 (ATF3), which has an anti-apoptotic effect and inhibit inflammatory state, is induced. Chen and colleagues probed that injection of exosomal ATF3 into model mice, reduces I/R kidney injury (88). Similarly, the effect of liver stem cells EVs in the regeneration of renal tubule injury has been demonstrated in the last months. Sánchez et al. suggest the role of these vesicles in a paracrine mechanism, inhibiting apoptosis of renal-tubular cells in an AKI murine model (89).

## CONCLUSION

Kidney-related diseases, among others, might clearly benefit of research focused on urinary EVs. These vesicles may concentrate potential biomarkers – otherwise unnoticed by dilution in whole urine – that may reflect the physiological state of the renal system, and which may be relevant in several pathologies affecting the urinary tract, from the kidney to the bladder. However, some issues need to be solved before urinary EVs could be established as a source of biomarkers in the clinical setting. First, it is important to define whether these potential biomarkers should be determined

in whole urine or specifically associated to urinary EVs, as this could be related to each pathologic condition. It is considered that RNA and proteins are better preserved in EVs than in urine, being protected from the milieu. Indeed, EVs have the advantage of being directly derived from cells of the renal system, but the disadvantage of needing additional steps. Moreover, due to the different EV isolation procedures used – perhaps biasing the results – it is not clear whether some of the biomarkers identified so far are actually EV-related. Thus, a standard consensus on this subject would be desirable before blind studies on larger cohort of patients are performed to unequivocally identify urinary EV-related biomarkers for kidney diseases. Although most of the results so far are still preliminary, several of the proposed biomarkers have enlighten the way to a better understanding and diagnose of kidney diseases. Besides, as EVs can target and modify the behavior of specific cells, their potential use in therapeutic protocols will merit future research in kidney-related diseases.

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# Methods for extracellular vesicles isolation in a hospital setting

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The research in extracellular vesicles (EVs) has been rising during the last decade. However, there is no clear consensus on the most accurate protocol to isolate and analyze them. Besides, most of the current protocols are difficult to implement in a hospital setting due to being very time-consuming or to requirements of specific infrastructure. Thus, our aim is to compare five different protocols (comprising two different medium-speed differential centrifugation protocols; commercially polymeric precipitation – exoquick – acid precipitation; and ultracentrifugation) for blood and urine samples to determine the most suitable one for the isolation of EVs. Nanoparticle tracking analysis, flow cytometry, western blot (WB), electronic microscopy, and spectrophotometry were used to characterize basic aspects of EVs such as concentration, size distribution, cell-origin and transmembrane markers, and RNA concentration. The highest EV concentrations were obtained using the exoquick protocol, followed by both differential centrifugation protocols, while the ultracentrifugation and acid-precipitation protocols yielded considerably lower EV concentrations. The five protocols isolated EVs of similar characteristics regarding markers and RNA concentration; however, standard protocol recovered only small EVs. EV isolated with exoquick presented difficult to be analyzed with WB. The RNA concentrations obtained from urine-derived EVs were similar to those obtained from blood-derived ones, despite the urine EV concentration being 10–20 times lower. We consider that a medium-speed differential centrifugation could be suitable to be applied in a hospital setting as it requires the simplest infrastructure and recovers higher concentration of EV than standard protocol. A workflow from sampling to characterization of EVs is proposed.

**Keywords: extracellular vesicles, protocol standardization, clinical application, nanoparticle tracking analysis, flow cytometry, translational research, urine**

## INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound particles shed from almost all cell types, carrying components from the cell donor such as lipids, proteins, RNA, glycolipids, and metabolites (1). It has been suggested that they play several biological roles like, for example, antigen presentation without cell contact (2), microenvironment modification, and distant cell education (3), roles that have been encompassed under the term "cell-to-cell contact-free communication". In turn, their biological functions have been related to many pathophysiological processes, the most studied being cancer (4), immune-mediated diseases (5), and cardiovascular disorders (6).

A widespread concern in the biomedical research community is the gap between the basic research carried out in the laboratories and the clinical setting where the new biological information should have a direct impact. Many researchers have directed their efforts toward bridging that gap and look for ways to translate lab findings into clinical solutions, emerging therefore the translational research. The translational research on EVs is not foreign to this goal: the current knowledge about EVs, mostly developed *in vitro*, has been proposed to be applied in a daily hospital routine giving answers to specific health queries (7–13). This possible application ranges from diagnostic to therapeutic objectives, including disease monitoring and the search of prognostic biomarkers, among others. But are the hospitals technologically prepared to employ EVs studies routinely?

The main steps for studying EVs and applying the results involve sampling (blood, urine, saliva, cerebrospinal fluid, joint

fluid, breast milk, ascitic fluid, etc.) and isolation, to be afterward characterized and analyzed their cargo and, finally, give a potential clinical interpretation and application. Concerning the first steps, sampling and pre-analytical parameters have been widely studied and are close to reach a consensus (14, 15). However, isolation is still a critical step due to several reasons. First, the methods to isolate EVs are currently highly diverse [reviewed by Momen-Heravi et al. (16) and Witwer et al. (17)] and depending on which one is employed, the results can be considerably different, even having started from the same sample. At the moment, most of them are based on EV density, including differential centrifugation steps from low speeds ($1{,}500 \times g$) to ultracentrifugation ($>100{,}000 \times g$), combined or not with density gradient and/or filtration. Precipitation using polymers and immunoaffinity agglutination are also widely used. Recently, the size-exclusion chromatography (18) and chip devices (19) have been added to the rest of methods. All of them, either by themselves or in combination, yield a solution enriched in EVs in different extents. Finally, depending on several factors such as time consumption, cost, friendly use, and reproducibility, these methods are or not able to be applied in a daily clinical routine. Despite lots of important works shedding light on this field, there is still a lack of consensus (20) evidencing the urgent need of standardized protocols appropriate for hospitals.

Considering the problems and needs regarding the use of EVs in a clinical setting, we established the following objectives for the present study: to compare several protocols for EVs isolation and to analyze which of them could be the most suitable one to be used in daily clinical setting.

## MATERIAL AND METHODS

Blood and urine are the most widely used samples in a hospital setting, as they provide useful information and they are easy to obtain with minimally invasive techniques. Thus, we decided to isolate EVs from these biofluids as the starting point for EV isolation.

Samples were collected from 10 healthy individuals (5 males and 5 females; average age = 37 ± 8 years old) and stored according to the criteria of the Donostia node of the Basque Biobank. All subjects gave written informed consent and the study was approved by the Hospital Ethics Committee.

Donors underwent a questionnaire about recent exercising (within the last hour), drugs/medication intake, ovulatory cycle, acute illness, and sleeping hours.

The workflow followed in the present work is summarized in **Figure 1**.

### BLOOD

Peripheral blood samples were collected at the Donostia University Hospital at 8:30 a.m. on fasting and were processed separately (without pooling them) within the first hour. After discarding the first milliliter, blood collection was done by venipuncture with a

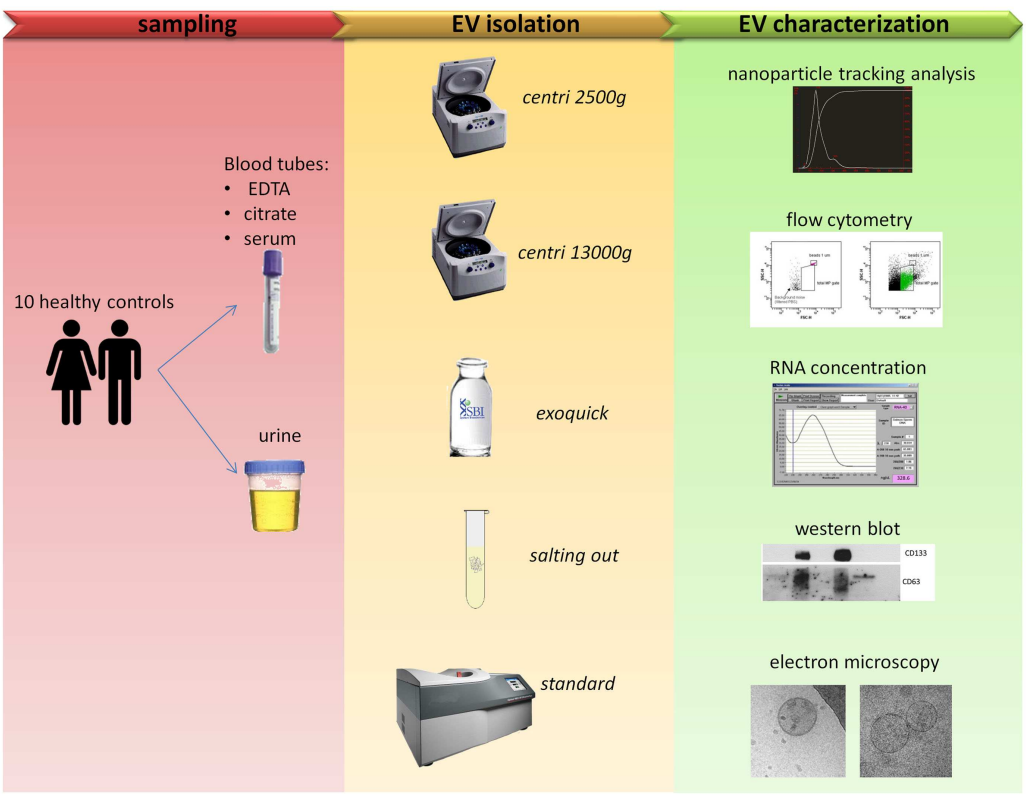


**FIGURE 1 | Comparison of five protocols for EVs isolation**. Blood and urine samples were isolated with five different protocols (see **Table 1** for more details) and then characterized with five outputs methods.

21-gage needle in a 10-ml EDTA tube, a 3.8-ml citrate tube, and a 8-ml serum tube [Vacutainer, Becton Dickinson (BD)], kept upright and centrifuged at 2,500 × *g* during 15 min. The supernatant was recovered to obtain platelet-poor plasma (PPP) or platelet-poor serum, being these samples the starting point for all the protocols. Besides, additional blood samples were collected in EDTA and serum tubes to perform a hemogram and obtain protein and lipid profiles in the core laboratory of the Hospital. The parameters tested are shown in **Table 1**.

Although we collected plasma and serum from peripheral blood, the present work is focused on plasma. In this sense, we are going to refer only to plasma in all the sections of "Materials and Methods" for the sake of simplicity. The results and discussion of blood-derived EVs will be also centered on plasma and only relevant results will be presented in the case of serum.

## URINE

Sixty milliliter-first void of the day was collected in aseptic conditions by each individual at home, kept at 4°C until their processing with an average time of 2.7 h between collection and processing. Ten milliliters were sent to the core laboratory for the analysis of the most common urine parameters (**Table 1**). The rest was aliquoted in five tubes of 10 ml and centrifuged at 2,500 × *g* for 15 min in order to obtain cell-free urine (CFU).

### *EV isolation protocols*

The protocols described below are summarized in **Table 2**.

***Centri2500.*** This method is based on the protocol published by Lacroix and colleagues (14). Briefly, 1.3 ml of PPP or 9.5 ml of CFU obtained at the first centrifugation are centrifuged again at 2,500 × *g* during 15 min to get 1 ml of platelet-free plasma (PFP) or 9 ml of debris-free urine (DFU). Both PFP and DFU samples were stored at −80°C for later use. When needed, samples were thawed on ice and centrifuged once again at 20,000 × *g* during 20 min to pellet the EVs, discarding 900 μl of supernatant, following the protocols described by Ashcroft and colleagues (21) and Jayachandran and colleagues (22). The pellet containing the EVs was resuspended in 100 μl of PBS (GIBCO, Life Technologies) filtered twice through a 0.22 μm-pore filter.

***Centri13000.*** This method is based on the protocol published by Dey-Hazra and colleagues (23) and Dignat-George and colleagues (24). In brief, it is a modification of the previous method where the second centrifugation performed on the 1.3 ml of PPP was done at 13,000 during 2 min to obtain PFP or DFU. The rest of the protocol was the same as the previous one. To note, this protocol was not performed for urine samples.

***Exoquick.*** The basis for this method lays on the precipitation of EVs using a commercial agglutinating agent and was performed following the manufacturer's instructions. In summary, 63 μl or 2 ml of exoquick TC (System Biosciences) were added either to 250 μl of PPP or to 9.5 ml of CFU, respectively, and the mix was incubated overnight at 4°C with no rotation. Then, two centrifugation steps were performed at 1,500 × *g* for 30 and 5 min, respectively, to sediment the EVs and the pellet was resuspended in 200 μl of PBS. It needs to be noted that, although the first versions of the manufacturer's instructions included a filtering step using a 0.45 μm-pore filter, it was removed in the latest version and, thus, it has not been included in our protocol.

***Salting out.*** This method has been adapted from the protocol recently published by Brownlee and colleagues (25) and it is based on the precipitation of EVs through an aggregate of sodium acetate 1 M, ph 4.75. A centrifugation was performed on 1.3 ml of PPP or 9.5 ml of DFU at 13,000 × *g* for 30 min; we collected the supernatant (1 ml of PPP or 9 ml of DFU), added sodium acetate (dilution 1/10), and incubated on ice for 60 min and, subsequently at 37°C for 5 min. The dilution was then centrifuged at 5,000 × *g* during 10 min and the resulting pellet was washed with

**Table 1 | Lab parameters analyzed in blood and urine samples**.

| | | |
|---|---|---|
| Blood | Creatinine (mg/dl) | 0.9 ± 0.3 |
| | Total cholesterol (mg/dl) | 184.9 ± 37.7 |
| | HDL (mg/dl) | 69.9 ± 14.1 |
| | Triglycerides (mg/dl) | 65.7 ± 20.1 |
| | LDL (mg/dl) | 101.9 ± 37.5 |
| | Total proteins (g/dl) | 7.2 ± 0.6 |
| | Albumin (g/dl) | 4.3 ± 0.3 |
| | Hematocrit (%) | 41.7 ± 3.4 |
| | Leukocyte (10e3/μl) | 7.0 ± 2.5 |
| | Platelet count (10e3/μl) | 238.7 ± 56.7 |
| | Lymphocyte count (10e3/μl) | 1.9 ± 0.5 |
| Urine | Density (g/l) | 1019.4 ± 7.7 |
| | pH | 6.0 ± 0.9 |
| | Glomerular filtrate (mL/min/1.73 $m^2$) | 84.3 ± 15.5 |
| | Erythrocyte count (ery./μl) | Negative |
| | Leukocyte count (leu./μl) | Negative |
| | Epithelial cell count (cells/μl) | Negative |

*Values represent the mean ± SD from the 10 healthy controls.*

**Table 2 | EV isolation methods compared in this work**.

| Method | Isolation principle | Steps |
|---|---|---|
| *Centri2500* | Differential centrifugation | 2500 × *g* 15′ × 2 + 20,000 × *g* 20′ to pellet the EVs |
| *Centri13000* | Differential centrifugation | 2500 × *g* 15′ + 13,000 × *g* 2′ + 20,000 × *g* 20′ to pellet the EVs |
| *Exoquick* | Agglutination–precipitation | 2500 × *g* 15′ + agglutination with exoquick + 1500 × *g* 30′ and 5′ to pellet the EVs |
| *Salting out* | Precipitation | 2500 × *g* 15′ + 13000 × *g* 30′ + acid precipitation + 5000 × *g* 10′ to pellet the EVs |
| *Standard* | Differential centrifugation – size filtration – ultracentrifugation | 2500 × *g* 15′ + 0.22-μm filter + 10,000 × *g* 30′ + 1,00,000 × *g* 75′ to pellet the EVs |

a buffer with sodium acetate at 0.1 M to be finally resuspended in 200 μl of PBS.

***Standard.*** This is the method considered as the standard isolation protocol nowadays (26). The starting point was 1.3 ml of PPP or 9.5 ml of CFU that were filtered through a 0.22 μm-pore filter and centrifuged at 10,000 × *g* during 30 min to obtain either PFP or DFU. These were ultracentrifuged at 100,000 × *g* in an Optima MAX tabletop centrifuge (Beckman Coulter) during 75 min. The resulting EV pellet was resuspended in 200 μl of filtered PBS.

#### *EV detection and characterization methods*

***Nanoparticle tracking analysis.*** The size distribution and concentration of EVs were measured using a NanoSight LM10 machine (NanoSight). All the parameters of the analysis were set at the same values for all samples and 1 min-long videos were recorded in all cases. Background was measured by testing filtered PBS, which revealed no signal. The EVs obtained from PFP (5 μl) were diluted with filtered PBS to 1:150 and the ones obtained from DFU (5 μl) to 1:50. For each sample, two measurements were performed. It is necessary for a minimum of 200 tracks (movements of single particles recorded by a camera) to obtain valid results. The following parameters were measured: the mean and mode of the size distribution and the concentration of EVs (27).

***Flow cytometry.*** The labeling and gating of EVs were performed as described by Sáenz-Cuesta and colleagues (28). Briefly, 4 μl of CD61-PE (Cytgonos) or CD45-PE (BD) monoclonal antibodies were mixed with 40 μl of resuspended EVs and incubated for 20 min. Next, labeled EVs were washed once with 300 μl of filtered PBS, resuspended in further 200 μl of filtered PBS and acquired at low rate in a FACS Canto II flow cytometer (BD). Side and forward scatter were measured on a logarithmic scale with the threshold set at 300 for each parameter to avoid instrument noise (background signal). Then, the lower limit was defined with the exclusion of background noise given by the signal of PBS filtered twice. To define the upper limit of the total MP gate, 1-μm non-labeled polystyrene latex beads were used (Sigma-Aldrich). The events that appeared in this region were included in the total EV count and were further analyzed for specific labeling (positive for PE marker). We defined CD61+ EVs as platelet-derived EVs (PEV) and CD45+ EVs as leukocyte-derived EVs (LEV). The total and cellular origin-specific EV concentrations were obtained using Trucount™ tubes [BD; Ref. (28)].

***Western blot.*** Primary CD133 (Miltenyi Biotec S. L) and CD63 (Santa Cruz Biotechnology, Inc.) antibodies were used to study specific EV transmembrane markers. Mouse and rabbit HRP-conjugated antibodies (Cell Signaling) were employed as secondary antibodies. All protein procedures were done at non-reducing conditions. Samples (10 μl of PBS-resuspended EVs) were incubated at 95°C for 5 min, separated in SDS polyacrilamide gels, and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked for 1 h at room temperature with 5% milk (w/v) in TBS solution with 0.1% Tween-20 (T-TBS) and incubated in the same solution with primary antibodies overnight at 4°C. Primary antibodies were washed with the T-TBS solution and incubation with secondary HRP-conjugated antibodies was performed at room temperature for 1 h in the same solution used for the primary antibodies. After washing with T-TBS solution, the HRP signal was detected by a chemiluminiscent reaction with the SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific, Inc.).

***RNA isolation.*** A 185 μl-aliquot of resuspended EVs was used to extract total RNA with the miRNeasy serum/plasma kit (Qiagen). RNA concentration was measured with the nanodrop 1000 spectrophotometer (Thermo Scientific).

***Cryo-electron microscopy.*** The cryo-electron microscopy (EM) was performed following the protocol used by Perez and colleagues (29). Briefly, 10 μl of EV preparations were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL Micro Tools GmbH). Grids were blotted at 95% of humidity and rapidly plunged into liquid ethane with the aid of VITROBOT (Maastricht Instruments B). Vitrified samples were imaged at liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL) equipped with a field emission gun and operated at an acceleration voltage of 200 kV.

#### *Statistical analysis*

The statistical analysis was performed with PASW Statistics v18.0 (SPSS Inc.). Kolmogorov–Smirnov and Shapiro–Wilk tests were used to check normality of distributions. As all of the variables were shown to follow a normal distribution, *T*-tests were applied to assess differences between the groups. Pearson's *R* correlations were computed to explore the relations between lab parameters and some EV parameters. Both differences between groups and correlations between variables were considered significant when $p < 0.05$.

## RESULTS

### BLOOD

#### *EV concentration*

Extracellular vesicles concentration was measured using two independent methods: nanoparticle tracking analysis (NTA) and conventional flow cytometry (FC). It is to be noted that the lower detection limits are different, being 50 nm for NTA (27) and around 400 nm for FC (30).

#### *Nanoparticle tracking analysis*

When using NTA, the software requires a minimum of 200 tracks during the capture time of the video. In the case of the samples processed with the salting out and standard methods, only few of them reached that minimum. This was critical for NTA analysis causing a high variability on these samples (**Figures 2A,E**). The exoquick method yielded higher EV concentration values than any other method used. We obtained four times higher EV concentration with exoquick than with centri2500 ($p = 0.007$) and centri13000 ($p = 0.05$) and 23 times higher concentration values comparing to salting out ($p = 0.002$) and standard ($p = 0.002$) methods (**Figure 2A**). No significant differences have been found either between the EV concentrations obtained with the centri2500 and centri13000 methods, or between those yielded by the standard and the salting out methods. However, there are significant

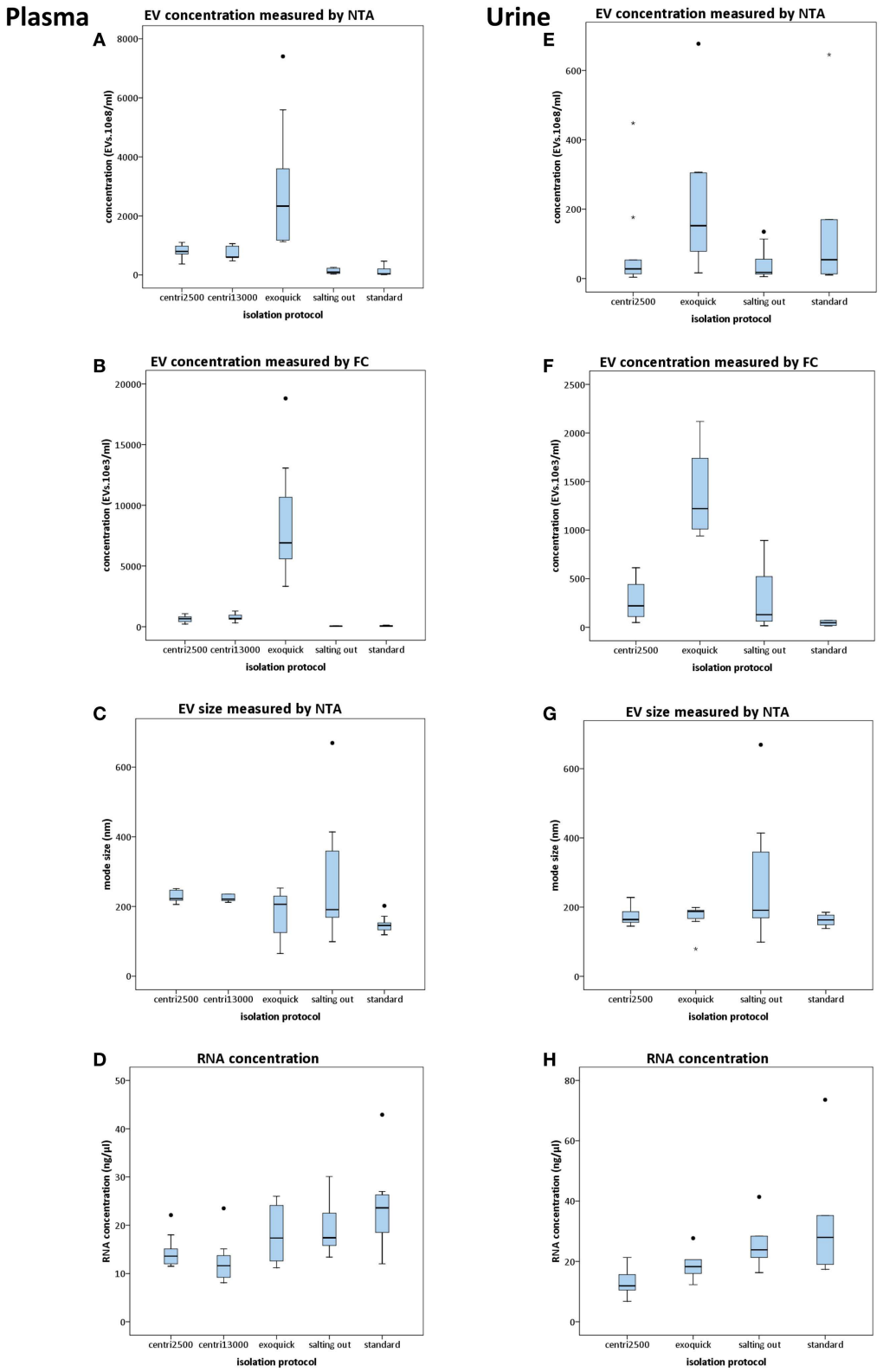


**FIGURE 2 | Results of the comparison of five protocols for isolation of EVs**. Box plots show EV concentration measured by nanoparticle tracking analysis [NTA; **(A,E)**] or conventional flow cytometry [FC; **(B,F)**], EV size distribution measured by NTA **(C,G)**, and concentration of RNA yielded from EVs **(D,H)**. In the left column, the results from plasma-derived EVs are shown and in the right column those from urine. For statistical significance, see text. All bars represent mean values with SD except for size plots **(C,G)** that bars indicate mode with SD.

differences between these two groups of methods (**Figure 2A**) obtaining a $p$ value of $<0.001$ for both centri2500 vs. salting out and standard and 0.002 and $<0.001$ for centri13000 vs. salting out and standard respectively. Regarding the EVs isolated from serum, they were obtained using the exoquick, centri13000, and standard protocols and we have observed that, as it happens with plasma, the exoquick method yields significantly higher EV concentrations than the other two methods. When we compared serum and plasma considering all isolation methods, 3.4 times higher EV concentrations have been observed for serum using exoquick and 1.3 times higher ones when using centri13000, but these comparison did not reach statistical significance (see Table S1 in Supplementary Material).

***Flow cytometry.*** Although the EV concentrations obtained using FC were lower than those obtained by NTA, there was good correlation between the concentration profiles yielded by each approach when using the averages for each isolation method for the comparison ($R = 0.99$; $p < 0.001$). Nevertheless, no significant correlations were observed when performing the analysis for each isolation method separately. Besides, significant differences were observed between all isolation methods except for *centri2500* vs. *centri13000* and *salting out* vs. *standard* (**Figure 2B**).

### *EV size*

There is great similarity between the modes of EV size obtained with the different isolation protocols ranging from 150 to 277 nm, average of mode size: 228 nm (**Figure 2C**). In accordance to these results, the EM images show EVs with a size between 100 and 200 nm (**Figure 3A**). Significant differences in size only exist between the EVs isolated with the *standard* method when compared to those obtained through the *centri2500* and *centri13000* methods, being the former the smallest of all at 158.7 nm. Besides, the EM images of the EVs obtained with *exoquick* present several filamentous aggregates and other globular structures not considered EVs.

### *RNA concentration in EVs*

Despite the *salting out* and the *standard* methods being the ones that yield the lowest EV concentrations, the highest RNA concentrations have been obtained through these methods ($19.5 \pm 5.7$ and $23.6 \pm 8.2$ ng/μl, respectively), higher than with the *centri2500* and *centri13000* methods ($14.5 \pm 3.3$ and $12.5 \pm 4.5$ ng/μl, respectively). It is remarkable that *exoquick* yields $18.1 \pm 6.0$ ng/μl of RNA, despite having isolated around 23 times more EVs than the *salting out* and *standard* methods (**Figure 2D**).

### *Western blot*

The detection of EV markers through western blot (WB) was used as a confirmation of the presence of EVs in the solutions obtained at the end of the isolation protocols. The objective was not to perform a detailed characterization of the markers, but to look for differences in their detection between the different methods.

Great inter-method variability has been observed. Briefly, CD63 detection is better in EVs isolated from plasma than in those obtained from serum. Among the plasma-derived EVs, CD133 showed better signal for *centri2500* and *centri13000* than for the *salting out* and the *standard* protocols. The detection of CD133 was also worse in serum-derived sample than in plasma-derived samples. Nonetheless, CD133 detection was better in urine-derived EVs than in the samples obtained from blood (**Figure 3B**). It is of note that the EVs isolated with *exoquick* could not be used for WB marker analysis using plasma samples due to be impossible to dissolve its pellet.

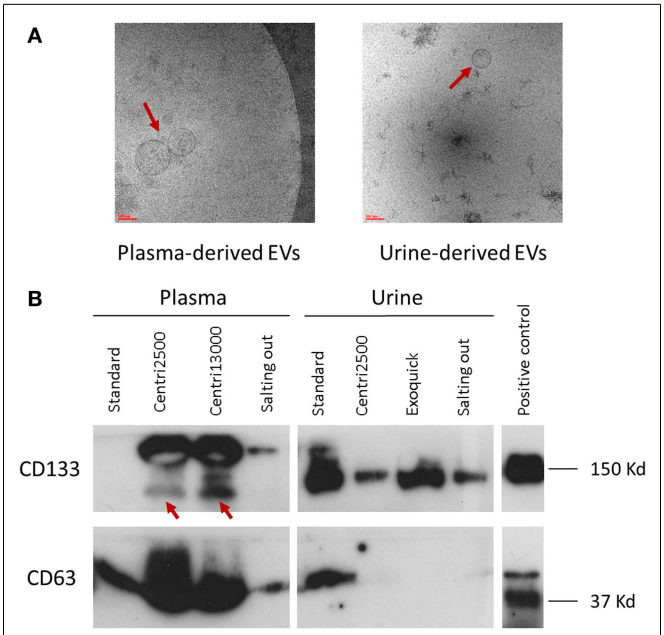


**FIGURE 3 | Characterization of EVs**. **(A)** Two images of electronic microscopy of EVs (pointed with red arrows) derived from plasma and urine. Scale bar represents 100 nm. To note, in the image of urine-derived EVs, it is observed contaminants not seen in the plasma-derived one. **(B)** Western blot analysis using the specific EV markers CD133 and CD63. In CD133 plasma samples, the specific bands are pointed with red arrows. Positive control was performed with NHC-2 p10 cell line.

## URINE

### *EV concentration*

In the NTA analysis of the EVs isolated from urine, we have observed that the concentrations were as low as the great majority of the samples have not reached the minimum track-count. Thus, once again the interpretation of NTA data from urine-derived EVs was carried out with caution. *Exoquick* was the method that yields the highest EV concentrations from urine using either NTA or FC, followed by the *standard* protocol. The *centri2500* and *salting out* protocols yielded very similar concentrations. **Figures 2E,F** summarizes the EV concentration results from the application of the different protocols to urine as measured by NTA and FC, respectively (for $p$ values, please see Table S1 in Supplementary Material). In summary, using both NTA and FC, we obtained a similar concentration profile to that of blood but with 10–20 times lower concentrations.

### *EVs size*

The modes of the size distributions obtained with the different protocols were similar and, the average of these modes (207 nm)

was similar to that achieved in blood samples (228 nm). As it happened with blood samples, the smaller EVs were isolated with the *standard* method (162.5 nm) comparing to the other protocols (**Figure 2G**). The results of the size assessed by NTA were, once again, consistent with the observations done using EM. In most of these urine samples, the number of contaminating particles that could not be considered EVs was higher than in blood (**Figure 3A**).

#### *RNA concentration in EVs*

Higher RNA concentrations were obtained from EVs isolated with the *standard* (33 ng/μl) and *salting out* (25.9 ng/μl) methods and even higher than the concentrations obtained from blood samples, where the EV concentration was from 10 to 20 times higher than in urine (**Figure 2H**).

#### *Western blot*

The EV samples isolated from urine using *exoquick*, in contrast to the ones isolated from plasma, can be used for marker analysis with WB (**Figure 3B**).

In brief, CD133 detection in urine-derived samples is better with precipitant agents (*exoquick* and *salting out* methods) than with centrifugation methods, although the *standard* shows better results in urine and serum than in plasma. On the opposite, CD63 signal is weak in urine-derived samples compared to that of plasma- or serum-derived samples for all methods.

### CORRELATION WITH LABORATORY PARAMETERS

We have also tested whether the EV concentration values obtained with different methods and the different types of samples (plasma, serum, or urine) could be reflected in some of the lab parameters measured in blood and urine, especially the ones that are related to the main components of EVs, i.e., lipids and proteins. Furthermore, the analysis of these correlations would serve to test a possible interference in EV quantification produced by these parameters, a phenomenon that has been previously described (31).

Interestingly, a significant correlation has been observed between the concentration, as measured by NTA, of the EVs isolated from plasma with the *centri13000* method and the total cholesterol ($R = 0.953$; $p = 0.003$) and LDL concentrations ($R = 0.935$; $p = 0.006$) in blood. We have also detected a significant correlation between the NTA-measured concentration of the plasma-derived EVs isolated using the *standard* method and the concentration of triglycerides in blood ($R = 0.789$; $p = 0.007$).

No significant correlation has been found between the concentrations of the EVs of specific cell-origins and the concentrations of the respective source cells in blood. The concentrations of CD61+ (platelet origin) or CD45+ (leukocyte origin) EVs are plotted in Figure S1 in Supplementary Material.

In regard to the EVs isolated from urine, the only significant correlation we have observed is the one between the density of urine and concentration of EVs, measured with both NTA and FC, isolated with the *salting out* protocol ($R = 0.841$; $p = 0.002$ for NTA and $R = 1.000$; $p = > 0.001$ for FC).

## DISCUSSION

In the present work, we have studied and compared several widely used methods for the isolation of EVs, including differential centrifugation, agglutination, precipitation, and the one considered the standard that includes ultracentrifugation (plus filter). All methods under study can be applied using relatively simple technology, with the exception of ultracentrifugation, which must be performed with an instrumentation that, even if it is easy to use, is not usually found in most hospital laboratories. The election of one or other method as the most suitable one to be used in a hospital setting greatly depends on the goals to be reached with the method, which could be, among others: to maximize the final EV concentration, to obtain high levels of purity as measured by markers and several classical characteristics of EVs, to select one of the three fundamental types of EVs (exosomes, microvesicles, and apoptotic bodies) or to get the less time and/or money consuming protocol. We have set the first two as preferential aims, leaving the rest out of the scope of this work.

### ISOLATING EVs

We have observed that, besides being the method that can be implemented most easily (it is quick and relies on very little technology), *exoquick* is also the method that yields, in a statistically significant manner, the highest concentration of EVs (as measured by NTA and FC) compared to the other four isolation protocols. The EV quantity is even higher when using serum as the starting sample. On top of that, to dissolve the pellet obtained using *exoquick* from serum-derived samples is notably easier than plasma-derived samples. Nonetheless, the considerably higher EV concentrations obtained with *exoquick* (23 times higher than those obtained with the *standard* protocol) could be linked to the aggregation and precipitation of other elements in suspension in the sample that are not necessarily EVs; as it can be observed in the images obtained by EM. Taylor and colleagues (32) demonstrated that using *exoquick* more EVs are isolated than using ultracentrifugation (*standard*), chromatography, and magnetic beads, and with a higher purity of exosomal RNA and proteins. Our results only partially coincide with the observations of Taylor and colleagues, as the RNA concentration we obtained with *exoquick* is lower than that yielded by the *standard* method. In another study that compared the *exoquick* method with the *standard* method, the authors concluded that a combination of these two methods is the protocol that yields the highest EV counts, although exosomes of higher quality were obtained combining the *standard* method with the sucrose density gradient (33). Yet, *Exoquick* is the most expensive of the methods used in the present work.

The differences in EV concentration between the two centrifugation methods (*centri2500* and *centri13000*) are not statistically significant. The sole differences are that the cluster of EVs observed by FC in the FSC/SSC dotplot shows less debris around in the case of *centri13000* and that the EVs isolated with this method also show a stronger labeling of CD63 in WB. The conclusions reached at the workshop of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis to promote the use of these protocols (15) and aimed to reducing the variability due to a resuspension of the pellet (24). Nonetheless, in our opinion, the main drawback of this proposal is that, as the EVs are not concentrated in a pellet–like, we performed with the final centrifugation at 20,000 × *g*, a pellet-washing step cannot be introduced and EVs are maintained in dissolution along with many other contaminating particles such as protein aggregates.

Regarding size, very similar EV sizes have been obtained with these two methods, even when measuring size on EM imagery. These suggest that the second centrifugation is probably not that critical and could vary, at least between 2,500 and 13,000 × *g*, with the objective of eliminating cell debris. Moreover, these methods collect six times higher EV concentrations than the *standard* protocols, what can be explained by the fact that they are less restrictive methods. Finally, the technical requirements for the use of these methods are usually met in most basic research laboratories and they are considerably less time-consuming than the *standard* method.

Ultracentrifugation is nowadays the "*gold standard*" method for the isolation of EVs, fundamentally exosomes. With the aim of finding alternative methods to this protocol, Brownlee and colleagues (25) have recently described a new method called *salting out*, based on the precipitation of EVs using the aggregate of acetic acid. In the present study, the *salting out* method yielded the lowest EV concentrations when compared to the other protocols, although showing similar values to those obtained with the *standard* method as the authors of the aforementioned article also pointed. It has to be noted, though, that Brownlee and colleagues isolated EVs from cell culture supernatants and, thus, comparisons with the present work must be done with caution.

Although out of the main objectives of this work, we have also compared the EVs isolated from three different types of samples: plasma, serum, and urine. We have observed that higher concentrations of EVs are obtained from serum than from plasma for all methods, and 10–20 times more, depending on the method, when comparing plasma with urine. As comparisons between serum and plasma have been performed by other authors (34, 35), we just present our results.

The EV size distributions that we have obtained with the *exoquick*, *salting out*, *centri2500,* and *centri13000* methods are very similar, being the EVs with a size below 200 nm the most abundant. Nevertheless, a cluster of EVs can be observed with a size around 500–600 nm that could represent the population of microparticles. On the contrary, the standard method isolates smaller EVs as it uses a 0.22 μm-pore filter leaving out the bigger EVs (microparticles and apoptotic bodies). We agree with Jy and colleagues (36) that the capacity of the first four methods to isolate the bigger EVs can be useful when applying these protocols in clinical practice.

In the case of urine, very low EV concentrations have been obtained with the five methods and, thus, we recommend not to dilute or to dilute very little urine-derived samples before analyzing them by NTA, FC, and WB. Once again, *exoquick* was the method that yields the highest concentrations according to other authors' results (37). Certainly, when using urine samples, it would be of great consequence to avoid contaminating proteins such as Tamm–Horsfall, which traps EVs but it can be removed with the simple addition of dithiothreitol and heat (38). Furthermore, Rood et al. (18) suggest that the most effective method in terms of purity for urine-derived EVs to undergo downstream proteomic analysis is the combination of ultracentrifugation followed by size-exclusion chromatography. The major disadvantage of this protocol would be that it is time-consuming and it requires of specific infrastructure that make it difficult to be compatible with clinical applications.

**DETECTING AND CHARACTERIZING EVs**

During the processing, after the centrifugation at 20,000 × *g* for 20 min, a fine lipidic layer could be observed in some of the samples. This corresponded to a FC image with a higher EV density (data not shown). Nevertheless, the presence of this layer did not show correlation with NTA results. It is well known that the density and size of the EVs can overlap with these of lipoproteins and this can produce artifactual results in FC analyses (31). Besides, we have found positive correlation between the LDL levels in blood and the concentration of EVs obtained with several methods, which suggests that, when isolating the EVs, some LDL particles are also dragged and counted as EVs. One approach to measure the purity of EVs is the EV/protein ratio (39), a method that is easy to use and yield reproducible results. However, it remains out of the scope of the present work.

The most widely used methods for the quantification of EVs are NTA and FC. According to our data, the results yielded by these two methods are not interchangeable, probably because the size ranges that they can analyze are different. The correlation between the two methods would be better studied using only the concentration of EVs larger than 400 nm, as this is the minimum size for the FC analysis. Nevertheless, we have looked for correlation using concentration results for EVs larger than 400 nm in another dataset (data not shown) and we have found none. Thus, we consider that these two quantification methods do not exclude each other but are complementary, as NTA gives more accurate counts but FC allows the characterization of distinct cellular origins.

From the comparison of the methods that we have used to study the size of EVs, we can conclude that, while the NTA, as it allows to recover information from individual particles, allows to obtain and compare size distributions, EM provides more robust information on the characteristics of EVs but size distributions cannot be obtained through EM imagery. Furthermore, NTA has the advantage of performing a multiple analysis in few minutes.

Tetraspanins have been widely used as general markers of EVs; however, during the last years, some works have provided evidence that not all vesicles express them at the same levels suggesting that different EV subsets could coexist in the same pellet (40, 41). In the case of urine-derived EVs, our results present low or undetectable levels of CD63 except for those obtained with the *standard* protocol (**Figure 3B**). Both the previously described lack of CD63 in urine-derived EVs larger than 100 nm (42) and its expression in EVs obtained with the *standard* protocol (43, 44) are congruent with our results. In the other hand, we found expression of CD133 with all the methods. In agreement with other authors, we concluded that the presence of CD133+/CD63− EVs demonstrate the recovery of the large ones that usually express this pattern of markers (45–47). Moreover, Bobrie and colleagues described the CD63 as a variable marker found only in a fraction of the sucrose gradient (40), which implies questioning the use of CD63 as a standard EV marker (48). Finally, the expression of CD63 is susceptible to SCORT regulation leading to the blockage of the budding of this EV subset (49) and this mechanism could hypothetically be more frequent in urine-derived EVs. Regarding to plasma-derived EVs, the detection of the opposite pattern (CD133−/CD63+) in the EVs obtained with *standard* protocol unravel the isolation of a specific EV fraction, being probably only exosomes (46).

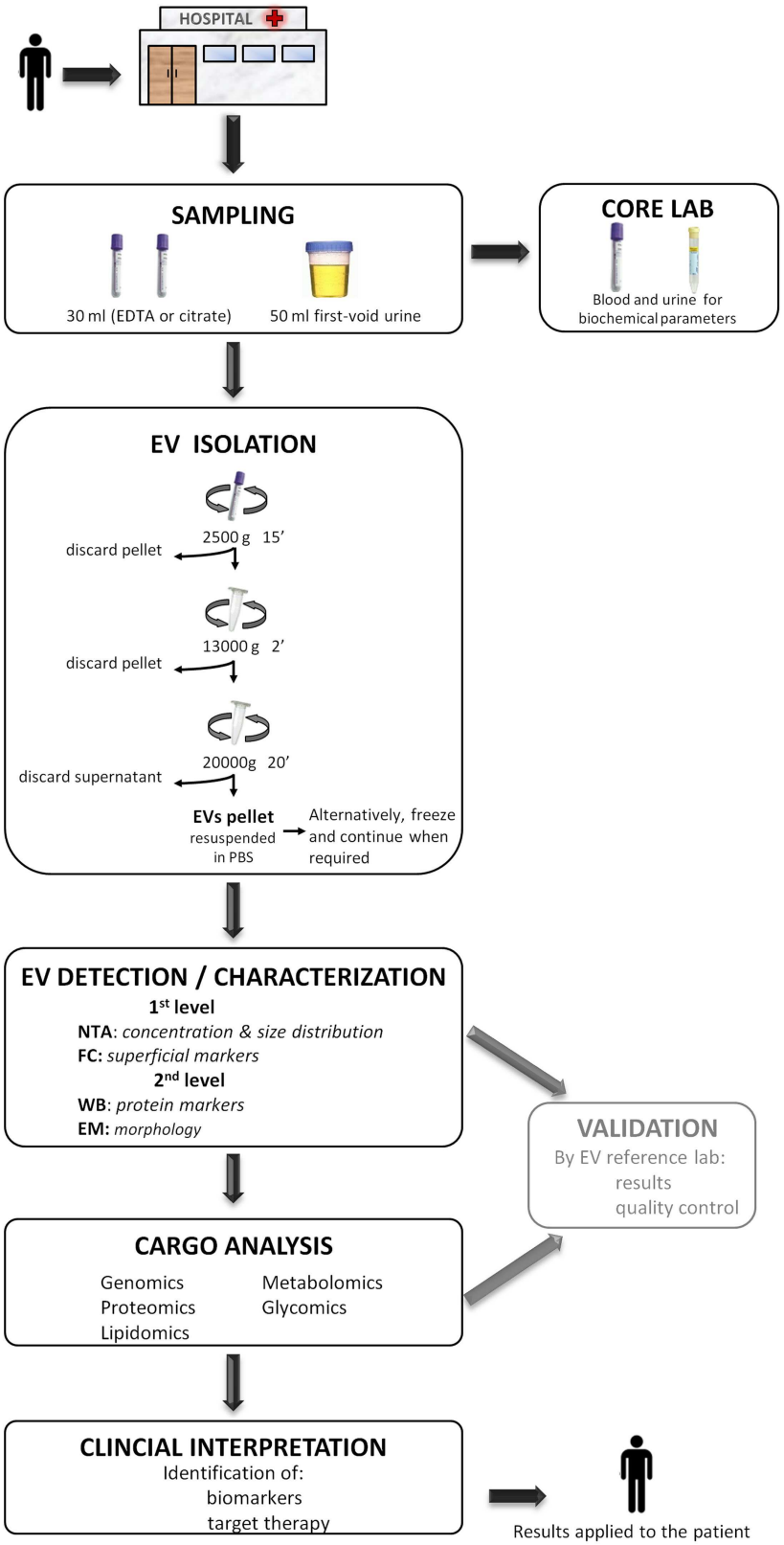


**FIGURE 4 | A proposed workflow for the study of extracellular vesicles (EVs) in a hospital setting**.

(*Continued*)

**FIGURE 4 | Continued**
Patients visited during the morning in the hospital, preferentially on fasting, undergo sample collection of 30 ml of blood (EDTA or citrate) and 50 ml of the first void urine. Immediately, 15 ml of blood and 40 ml of urine are destined to the EV isolation protocol to obtain a pellet and the rest 15 and 10 ml are sent to the core laboratory to analyze biochemical parameters. The obtained EV pellet resuspended in PBS could optionally be frozen at −80°C and continue when required. Next, the detection/characterization of EV is divided in two levels for quantification, size [nanoparticle tracking analysis (NTA)], and initial characterization with flow cytometry (FC) followed by an extensive description with western blot (WB) and electronic microscopy (EM). Subsequently, the analysis of EV cargo with several omics platforms allows the identification of specific compounds carried by EVs. EVs detection and their cargo analysis could optionally be referenced, at least during the initial setting of this workflow, to an expert EV laboratory in order to provide a validation of the results and pass a quality control test. Finally, the detected molecules are interpreted in the whole context of the patient with the aim of identifying biomarkers or a target for a putative therapy. The results provided by the study of EV are applied back to the patient improving the diagnosis or course of the disease.

Regarding the RNA concentrations yielded by the different EV isolation methods, we have observed great variability. Although Taylor and colleagues conclude that *exoquick* isolates more than, among other methods, ultracentrifugation (32), we have observed, unexpectedly, that the RNA concentrations obtained with the different methods are very similar, despite the notable differences in EV concentrations. Surprisingly, high RNA concentrations were obtained from urine (especially when using the *salting out* and *standard* methods), concentrations similar to or even higher than those obtained from plasma and serum, regardless of EV concentrations being between 10 and 20 times lower. These results lead us to think that, as we have not used RNAses, we are measuring the concentration not only of the RNA contained in the EVs but of the free RNA. In a position paper of the International Society of Extracellular Vesicles, the authors suggest that the use of RNAses only removes the free RNA not specifically bound to EVs, while their use in combination with proteases also removes the nucleoproteic complexes (50). In any case, if the final objective is to use the RNA as a source of potential biomarker, we believe that it would be useful to preserve not only the RNA inside the EVs but the RNA stuck to their membrane.

### EVs FROM BENCH TO BEDSIDE

The importance of the study of EVs in a hospital setting to complement the diagnosis and prognosis of several diseases has been well demonstrated (51–53). Moreover, their application in therapeutic approaches has already been tested in clinical trials with promising results (54). Nonetheless, we believe that the workflows from the collection of the samples aimed at the isolation, processing, and characterization of EVs to yield significant results to be applied on patients need to be urgently standardized. Specifically, the different isolation method can yield different types of EVs and, thus, omics studies performed on them could give incomparable results. Besides, not all methods are applicable in a hospital setting.

With aim of contributing to this debate and in accordance to the results of the present work, we consider that the *centri13000* method is the most suitable one to be used in a hospital setting as (a) it requires a simple infrastructure (and does not require ultracentrifuge) that is available in any general laboratory, (b) isolates EVs with similar characteristics to the ones isolated with the *standard* method but in higher concentrations, (c) it recovers not only small EVs as standard does but also the largest, and (d) in analysis with FC and WB showed less contamination when comparing with *centri2500*. We concur with Deun and colleagues (55) in that it is necessary for a validation of the isolation procedure and we propose that this validation could be carried out in referent laboratories lead by group with great expertise in the study of EVs. The results obtained in the hospital setting should be compared to those obtained by the reference lab to assure a quality control. On the other, for the posterior detection and characterization of EVs, we recommend to analyze them with at least one quantification method (NTA or FC) and one characterization method (WB, EM, or FC) as they provide complementary information. **Figure 4** summarizes a proposed workflow based on the discussion above.

To conclude, the isolation of EVs, at least for plasma-derived ones, through differential centrifugation at medium speed (*centri13000*) and their posterior analysis with at least one quantification method (NTA, for example) and another characterization method (FC or WB, for example) could fit in a workflow that goes from the patient to lab and all the way back to the patient and would contribute to face several health problems.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Jounal/10.3389/fimmu.2015.00050/abstract

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# Should cell culture platforms move towards EV therapy requirements?

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Cells *talk* to each other, but do members of the scientific community communicate with each other? Do molecular and cellular biologists speak with clinicians? In the last few years, numerous research centers have been located close to hospitals leading toward a more fluid connection between clinical and basic researchers, and therefore, *translational medicine* is becoming increasingly prominent.

Progress in translational medicine crucially depends on close collaborations between three areas: technology development, research, and hospitals. This combination allows a research idea (or hypothesis) to be developed into a real concept (or product), thus producing substantial benefits to citizens. Such synergy between these areas is beginning to be found in health research institutes located in close vicinity to hospitals. These institutes usually house several core units, also known as *platforms* or *facilities*, which provide scientific support not only to their own researchers but also to hospitals and medtech companies.

Our cell culture platform participates in the three areas previously mentioned. On one hand, scientific, medical, or technological companies are constantly developing new products (such as cell culture dishes, factors, and antibodies) and advanced research tools. Prior to their commercialization, or in order to determine their efficacy, companies often need a network of scientific partners to test the products. The cell culture platform in our institute, for example, fostered by its physical proximity to technological companies, works as a service that tests those new products and tools.

On the other hand, research areas of the institutes require cell culture platform infrastructure in order to proceed with its research lines linked to cell cultures.

Hospitals are the third area related to the cell culture platform. Currently, the services offered by the cell culture platform are highly demanded by clinicians working in the hospital. For example, we assist in the expansion of clinical samples. Clinicians face difficulties to obtain large amounts of biological material (for example, blood samples of newborns, or amniotic liquid) in order to obtain DNA or RNA to perform a diagnosis. In undiagnosed newborns, pediatricians may decide to perform skin biopsies in order to establish cell cultures and this way, get enough DNA or RNA for the diagnosis. The cell culture platform offers all the basic equipment needed to process these biopsy samples, expand the cells *in vitro* and get enough genetic material for the genetic analysis. Moreover, these samples are deposited in the Biobank in order to be preserved for future diagnostic tests or for research purposes.

Additionally, increasing developments on cell transplantation-based therapies in hospitals is leading to hand-in-hand coworking between clinicians and scientists through cell culture platforms and is evoking an internal transformation. Currently, the therapeutic potential of several cell types is being tested in hospitals. Some examples include the injection of chondrocytes for bone regeneration, and the transplantation of human heart stem cells to treat myocardial infarctions.

Lately, innovative therapies based on the transplantation of extracellular vesicles (EVs) are emerging. In 2007, Valadi and colleagues (1) described a new mechanism of cells-to-cell communication consisting in the transfer of genetic material through EVs, which can be either intracellularly generated or extracellularly formed from the cell membrane. Actually, EVs have been detected in most body fluids, including blood and urine, and they have become a target to identify diagnostic biomarkers. During the last years, an increasing number of publications are demonstrating their importance, especially for exosomes (cell-derived vesicles of 30–100 nm of diameter) as prognostic markers or as therapeutic tools to treat cancer and vascular diseases (2, 3). EVs are optimal therapeutic vectors due to their ability to present MHC antigens, which prevents immune rejection when used in allogeneic transplantations (4). To date, three cell types – regulatory T-cells, mesenchymal stem cells (MSC), and dendritic cells – have been used as sources of EVs for transplantation. In order to standardize EV-based therapies, several parameters need to be controlled. These include, but are not limited to, EV extraction and processing protocols, EV identification protocols, purity assessment, route of administration, and doses.

From a clinical perspective, the performance of a clinical trial requires a quality control of the exosomes that are going to be injected in patients. This quality control should include at least a flow cytometry-based characterization, an ELISA-based quantification, and a nanoparticle tracking, besides purity assessment and exhaustive analysis to ensure the absence of mycoplasma, adventitious agents, and endotoxins (5).

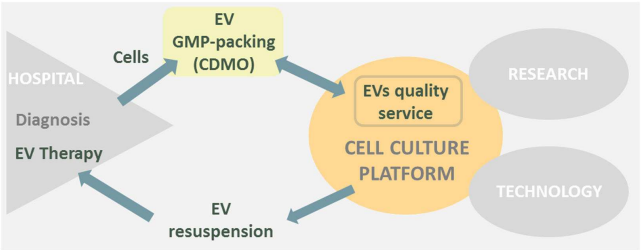


**FIGURE 1 | Cell culture platform is placed in between research, hospital, and technological areas**. EVs therapies derived from hospitals could transform part of the cell culture platform in a EVs sample quality service and simultaneously EV resuspension point prior to human injection.

The establishment of EV-based therapies will lead to standardized production and manipulation protocols following good manufacturing practices (GMPs), as well as quality controls to ensure the safety and efficacy of the treatments. Hence, how will these therapies impact cell culture platforms?

From my point of view, there could be two options for our platform, as cell extraction and culture, batch-production of EVs and packaging will have to be performed in GMP conditions: the first one is to adapt these requirements to a white room that will operate in line with GMP guidelines. That can be extremely costly. The second option will be to use the cell culture platform just as a service to perform EVs quality control whereas EVs obtaining from cells and packing is performed in a Contract Development and Manufacturing Organization (CDMO) specialized in the process development and GMP manufacture of cell therapy products from early stages up to clinical and commercial. In this case, the platform will only have to make a small investment to incorporate specific apparatus to determine EVs size and other EV-specific parameters.

In addition, even if the EVs production and packing is performed in an external CDMO, resuspension of the cryopreserved EVs can be performed in the platform in our biosecurity flow hoods. This way, the EVs delivery to the hospital, ready to inject, will be achieved a minimum time, as we are already operating with other cell therapies. This new challenge will have to be supported by specialized personnel in cell culture and EV related machinery as flow cytometry and EV related specific tools.

Bearing all this in mind if the cell culture platform would have to go toward EVs therapy, I would transform part of the platform in a "EVs sample quality service" (**Figure 1**). Otherwise, a big construction work with its high costs and complications would be difficult to face.

In conclusion, EVs therapies may have a great impact in the close future as new cell-based therapeutic approach; therefore, I will keep a sharp eye on EVs therapies future publications to envision the transformation of our cell culture platform toward a "EVs quality service."

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# Considerations for applying metabolomics to the analysis of extracellular vesicles

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Cell-derived extracellular vesicles (EVs) are involved in the development of different pathologies including inflammatory diseases and cancer (1) and have demonstrated a promising potential for human diagnostic (2) and therapeutic applications (3). Constitutive secretion of these vesicles has been described for platelets, reticulocytes, dendritic cells, lymphocytes, mast cells, intestinal epithelial cells, adipocytes, and hepatocytes among others (4). In addition, EVs have been isolated from many body fluids including bile (5), blood (6), and urine (7, 8), which indicates that they can be systemically disseminated, transferring their content/signals to cells physically separated from the secreting cell. Depending mainly on the vesicle's origin and the way of vesicle-discharge from the cells, at least two types of EVs have been described: the endosome-derived vesicles named "exosomes" and the plasma membrane shedding vesicles referred to as microparticles. Microparticles are a heterogeneous population of vesicles with a size up 1000 nm, this group consists of vesicles that are formed directly from plasma membrane by so-called reverse budding through membrane protrusion and fission (9). Exosomes, on the other hand, are a more homogeneous vesicle population with a size of 30–150 nm and an endocytic origin. They are formed by inward budding of the membrane of an endocytic organelle named multivesicular body and released to the extracellular space by fusion of this organelle with the plasma membrane (9).

The content of EVs and their biological function depend on the cell-type origin. In addition to lipids (10, 11) and proteins (10, 11), EVs give also refuge to mRNA, small RNAs including miRNA (12–17), mtDNA (18), and even genomic DNA (19). It is important to highlight that this material could be incorporated during the budding process of EVs in which a portion of cytosol is also engulfed into the vesicles (20) by controlled mechanisms (21, 22). In the process of their formation, cytosolic small molecules (metabolites) such as sugars, amino acids, nucleotides, different enzymatic cofactors, or redox regulatory molecules among others are also included into the vesicles. However, data regarding metabolites associated with EVs are still very limited and therefore extensive work in this is necessary in this area. This research effort is not only needed to map the metabolome of EVs from different origins but also to elucidate whether there are specific mechanisms at play for loading predestined metabolites into specific vesicles.

Extracellular vesicles are widely studied in order to better understand their physiological and pathological role as well as to identify potential non-invasive biomarkers (23). Recent research indicates that EVs have an important effect on the development and progression of diseases such as cancer (24) or immunological (25) diseases. Some of the mechanisms of action responsible for these effects are starting to be unraveled. Remarkably, some publications have demonstrated the involvement of EVs in the metabolic regulation of the extracellular space. In this context, EVs derived from cancer cells are able to modify and educate the microenvironment to facilitate tumor growth and the establishment of metastasis (26, 27). The importance of EVs in keeping the normal homeostasis in the neuronal environment is demonstrated by the implication of EVs in the development of Alzheimer and prion-related diseases (28). Clayton and collaborators have showed that EVs modify the extracellular adenosine levels, which has important implications for the activation of the immune system (29). Taking into account the fact that EVs from different cellular origins and with different activities can co-exist in a determined environment and condition, it is clear that these recent studies only reveal the "tip of the Iceberg." It is therefore important to elucidate the function of EVs in other cellular systems to understand the complex EV network, which will influence the final outcome of a determined stimulus or biological process. In the case of hepatocyte-derived EVs, a comprehensive proteomic analysis revealed the presence of proteins involved in metabolizing lipoproteins, endogenous compounds, and xenobiotics, which suggests a role of exosomes in the metabolism of these molecules (30). Our proteomics analysis identified more than 100 different proteins with the potential to modify the extracellular space. These include glycolytic enzymes, fatty acid modifying-enzymes, carboxylesterases, cytochrome P450 monooxygenases (CYPs), and uridine dinucleotide phosphate glucuronosyl transferases (UGTs), among others. The fact that hepatocytes are able to secrete EVs that contain a high number of enzymes

to extracellular compartments could also suggest that hepatocyte-derived EVs may play a role in the homeostasis of biofluids including blood and bile (31, 32).

To elucidate both the metabolome of EVs and the contribution of these vesicles to hepatic and extra-hepatic metabolism, comprehensive technological platforms are needed to integrate the final outcome of the various activities of these vesicles. In this context, the last-up – omic technology, referred as metabonomics or metabolomics, has opened new opportunities to provide a global view of mechanisms and pathways involved in normal physiological processes as well as in the development of diseases. Metabolomics comprises the qualitative and quantitative measurement of the metabolic response to physiological or pathological stimuli. It involves the extraction and measurement of hundreds to thousands of small molecules (<2000 Da) from cells, tissues, or biofluids to generate metabolic profiles (33). Comparisons of such profiles from different genotypes are being used to identify specific metabolic changes leading to the understanding of physiology, toxicology, and disease progression. The recent developments in spectroscopic and separation methods allow for quick and simultaneous measurements of all classes of metabolites in biological samples (33, 34). Advanced bioinformatics and biostatistics can then be used for data mining and modeling. Metabolomic profiling can be performed using a number of analytical techniques including high-field nuclear magnetic resonance (NMR), gas chromatography/mass spectrometry (GC/MS), and liquid chromatography/MS (LC/MS). Despite its excellent inter-lab reproducibility, GCMS requires that the majority of metabolites analyzed need derivatization to provide volatility and thermal stability prior to analysis (35). While NMR demonstrates its advantages in highly selective, quantitative and non-destructive analysis, the sensitivity and therefore the amount of detected metabolites is low compared to MS. LC/MS, while notoriously irreproducible, possesses much higher sensitivity toward most metabolic classes than NMR. Moreover, by using tandem MS in combination with high-resolution spectra, isotope distributions, and ion mobility, identification of unknown metabolites is facilitated. However, due to the before-mentioned irreproducibility chemical standards are normally necessary for absolute metabolite identification. The development of ultra-performance liquid chromatography (UPLC) has made it possible to achieve higher resolutions, higher sensitivities, and rapid separations as compared to those achieved using conventional LC (36).

Moreover, the combination of metabolomics and specialized software devoted to visualize cellular pathways offer a tool to integrate and identify the main mechanisms that trigger a specific biological process. Application of metabolomics is growing rapidly in an increasing range of fields such as biomarker discovery, clinical studies, diagnostics, plants, nutrition, and toxicology (37, 38). Furthermore, metabolic biomarkers are expected to be less species dependent than gene or protein markers, facilitating the direct comparison of animal models with human studies (39).

Form a practical point of view, it is important to highlight that for the characterization of the metabolome of EVs additional controls are required that are currently not included in the proteomics and transcriptomics analysis of these vesicles. This is exemplified by the UPLC-MS/MS analysis of exosomes-depleted media that has been incubated in the absence of cells during 72-h at 37°C, and subjected to the conventional ultracentrifugation method currently used to isolate EVs (40). In these control samples, substantial number of metabolites were detected using conventional solvents to extract metabolites (**Figure 1A**: $ClCH_3$:methanol, **Figure 1B**: methanol). These metabolites are derived from the tissue culture medium and need to

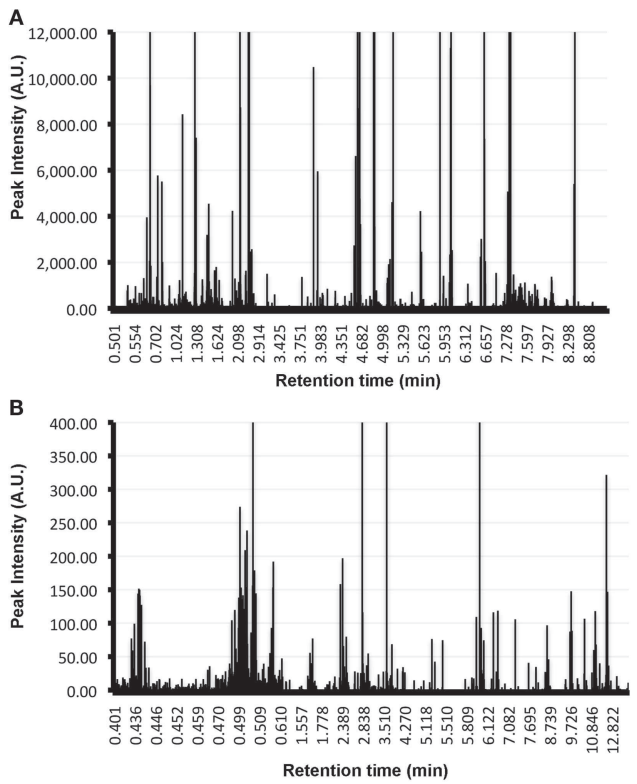


**FIGURE 1 | Metabolic background introduced by tissue culture media in EVs analysis**. Metabolites were extracted using $Cl_3Cl$:methanol **(A)** or methanol alone **(B)** from the pellet obtained by applying the conventional ultracentrifugation-based exosomal purification procedure to 300 ml of exosomes-depleted complete media incubated at 37°C during 72 h in absence of cells. Extracted metabolites were analyzed by UPLC-MS/MS using a C18 chromatographic column. Retention time of the metabolites along with their peak intensities are indicated in the plots. Note the elevated number of metabolites of different chemical nature that are isolated using the exosomal purification procedure and that need to be removed in order to determine the metabolome that is truly associated to EVs.

be taking into account and removed from the metabolites detected in the medium that has been conditioned by the cells in order to identify the metabolome truly associated with the EVs of interest. In the case of proteomics and transcriptomics analyses, the background introduced by the tissue culture media can be overcome given that currently tissue culture media used serum from bovine origin as major contributor to the background. Given the species-specific features of proteins and nucleic acids from the media, the contaminating material can be discriminated from the EVs secreted by the cells of interest. An exemption to this latter has to be made if a cell line of bovine origin is used for the production of EVs; in that case a cell free medium incubated under the same conditions is also recommended for proteomics and transcriptomics analyses.

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# Dendritic cell-derived exosomes may be a tool for cancer immunotherapy by converting tumor cells into immunogenic targets

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Dendritic cells (DCs) have been attracting attention in cancer immunotherapy because of their role in inducing and modulating effective immune responses. Besides the direct contact with other cell types and the secretion of cytokines, it is becoming clear that nanovesicles, such as exosomes (Exo), secreted by DCs also have a role in their function. Conversely, tumor-derived Exo carry antigens and have been used as a source of specific stimulus for the immune response against tumors. At the same time, several works have shown that different cells types incorporate DC-derived Exo (DC-Exo), resulting in modifications of their phenotype and function. Since DC-Exo carry many of the immune function-associated molecules of DCs, their incorporation by tumor cells could turn tumor cells into immunogenic targets. We have, therefore, treated human breast adenocarcinoma cells (SK-BR-3) with DCs-Exo and used these to stimulate previously SK-BR-3-primed $CD3^+$ T-cells. Sensitized T-cells cultured with DC-Exo-treated tumor cells showed a significantly higher percentage of IFN-γ-secreting cells (as measured by ELISPOT), when compared to the frequency of cells responding to non-DC-Exo-treated cells. These data show that the incorporation of DC-Exo by the tumor cells increased their ability to activate T-cells for a possibly more effective response, thus showing that DC-Exo may become another tool in cancer immunotherapy.

**Keywords: dendritic cells, exosomes, cancer immunotherapy, immunomodulation, tumor immune response, tumor cell immunogenicity**

## INTRODUCTION

Dendritic cells (DCs) are key players in the immune response; they are able to capture antigens with their pattern-recognition receptors, process and present them to *naïve* T-cells, inducing their activation (1, 2), thus, building an essential bridge between innate and adaptive responses. The central role that DCs play in the immune response, and the possibility of their *in vitro* generation has pathways for immunotherapy, in particular, for the treatment of cancer (3–8). However, the use of DCs outside clinical studies is hampered by the difficulties inherent to cell therapy strategies and, furthermore, in the case of DCs specifically against cancer, also by the compromised function of these cells in cancer patients (9–12). Not surprisingly, therefore, the general appraisal of DC-based strategies against cancer has been negative (10, 13). On the other hand, tumor cells do present potentially immunogenic antigens (14), which, when recognized by T-cells in immunotherapeutic approaches, seem to be associated with lasting tumor remissions (15). Therefore, strategies aimed at exposing tumor antigens to the immune system, bypassing the need for very active DCs, but in such a way that it leads to the establishment of T-cell responses, would be a potentially effective approach to harness the immune system to fight cancer.

In this context, therefore, it is relevant to note that, as most other cell types, DCs secrete nanovesicles, among which are the exosomes (Exo) (16–19). Exo are secreted vesicles that originate in the late endosomal compartment and result from the fusion of multivesicular bodies with the plasma membrane (20) and which can be acquired by other cells, at least in *ex vivo* cell cultures (21–24). These nanovesicles contain membrane proteins and genetic material, which, upon capture by other cells, contribute to the intercellular communication in the body (25–27). In fact, membrane traffic between DCs via Exo has been shown to occur (22), and Exo-carried antigens can be reprocessed for presentation or simply transferred directly to the membrane, in a process called cross-dressing (28). Furthermore, Exo transfer has been reported also to happen between cells of different types (25, 29, 30). Indeed, we demonstrated previously that Exo originated from DCs may be incorporated by tumor cells *in vitro* and that these tumor cells, after treatment with DC-derived Exo (DC-Exo), expressed molecules involved with antigen presentation, such as HLA-DR and CD86 (21).

Therefore, in this paper, we investigated if DC-Exo have the capacity to turn tumor cells into better targets for the immune system. We show that, indeed, DC-Exo treated tumor cells are able to induce tumor-sensitized T-cells to secrete higher levels of IFN-γ than non-DC-Exo-treated tumor cells. This observation supports our hypothesis and indicates that, as a minimum, DC-Exo used in cancer immunotherapy may act as a means to sensitize tumor

cells to other immune effectors, thus enhancing the effectiveness of different immunotherapeutic approaches.

## EXOSOMES FROM DENDRITIC CELLS AND THEIR ROLE IN ANTI-TUMOR RESPONSE

Raposo et al. (20) were the first to describe that Exo (originating from EBV-transformed B cells) contained functional MHC-II molecules, which carried peptides to which the cells were exposed and were able to stimulate peptide-specific $CD4^+$ T-cells. From this initial observation, many others indicated a role for Exo in immune response to various stimuli, including tumors. Actually, in a mouse model, DC-Exo, containing class I major histocompatibility antigens (MHC-I) complexed with tumor-derived peptides were shown to induce a cytotoxic T lymphocyte (CTL) response, which inhibited tumor growth and rejected established tumors (16, 19, 22), probably due to the incorporation of the Exo by host DCs *in vivo* (31). Also, in a clinical trial, ascites-derived Exo administered with GM-CSF induced CEA-specific-T lymphocytes (17), confirming the potential of Exo to carry and deliver effectively tumor antigens, as observed in various other settings (16, 18).

## EXOSOMES FROM DCs COULD TURN TOLEROGENIC TUMOR CELLS INTO IMMUNOGENIC TARGETS?

Though the generation of effective T lymphocytes responses is one of the main goals of cancer immunotherapy and the use of DC-Exo has been shown to be a means to achieve this goal, directly (19, 32, 33) or indirectly, by their incorporation by the recipients DCs (31, 33), the effectiveness of these immune effector cells depends on other factors. Actually, this is well illustrated by the fact that tumor-specific immune responses are frequently detected in patients who, nonetheless, have a progressive disease. This observation, essentially, has challenged for a long time, the potential of immunotherapy to control cancer. However, more recent experimental and clinical data have led to the realization that the immune system can, indeed, control and, probably, eliminate tumors, if "properly" engaged (34). In various clinical settings, this "proper" engagement of the immune system has been achieved, so far, either by the elimination of immune checkpoints, with monoclonal antibodies directed to immunoregulatory surface molecules (35), or by "arming" T lymphocytes with tumor-specific chimeric receptors, a strategy that, likewise, bypasses physiological controls of the immune response (36). However, these strategies depend, on one hand, on the existence of tumor cell targets already recognized by the immune effectors and, on the other hand, on the establishment of strong enough interactions between the immune effectors and the tumor targets.

In this perspective, our observation that tumor cells incorporate DC-Exo and, thereafter, express various surface molecules involved in the interaction between antigen-presenting cells and T lymphocytes (21) gains a new relevance. Not only could DC-Exo-treated tumor cells gain antigen-presenting capabilities, which might induce *de novo* anti-tumor responses, but also such cells could become better targets to any immune effector cell, either naturally induced by the presence of the tumor in the patient or induced by immunotherapeutic approaches (**Figure 1**).

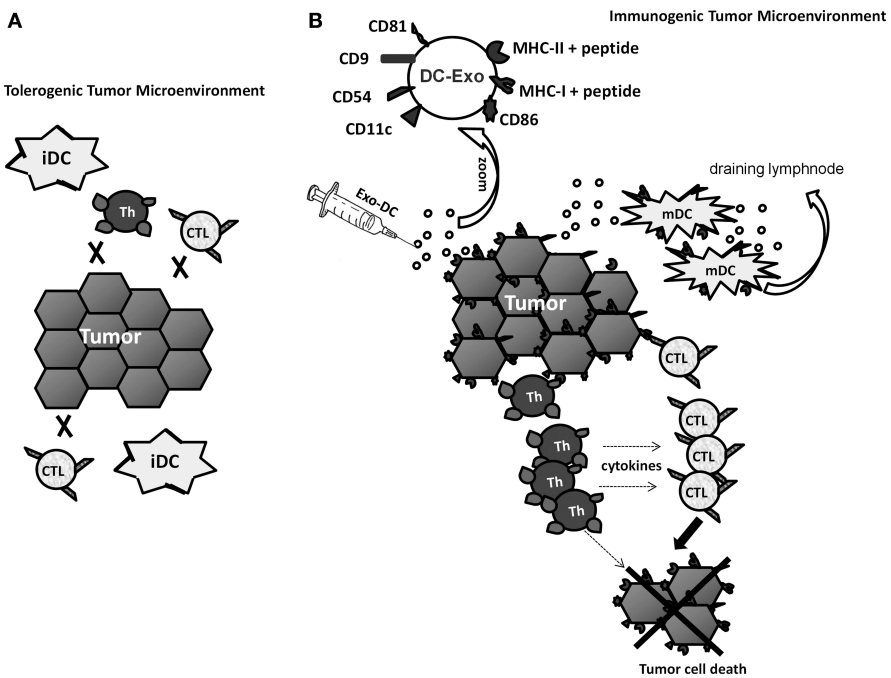


**FIGURE 1 | Hypothetical scheme of the tumor microenvironment changed by treatment with Exo from immunogenic DCs (DC-Exo).** **(A)** Low immunogenic tumor cells and immature DCs (iDCs) cannot induce specific-T lymphocyte response and, if such effectors do exist, they are not sufficiently stimulated by the tumor cells. **(B)** Enrichment of the tumor microenvironment with Exo-DC modifies tumor cells, enhances their immunogenic potential and turns them into better targets for any immune effector cell. Furthermore, immature DCs present in the tumor could also capture DC-Exo, acquire a mature phenotype and migrate to draining lymph nodes, where they could set up and amplify the immune response.

To test this hypothesis, we sensitized human $CD3^+$ T-cells against the breast carcinoma cell line SK-BR-3. Monocyte-derived DCs were pulsed with tumor cell Exo for 24 h, in the presence of IL-1, IL-6, TNF-α, and $PGE_2$. Tumor-Exo were used as antigen source, since it was shown that they induce anti-tumor responses more efficiently than irradiated tumor cells, apoptotic bodies, or tumor cells lysate (37). Next, these DCs were co-cultured with autologous T-cells, in the presence of IL-2 and IL-7 for 14 days. After that, tumor-Exo sensitized T-cells were exposed to SK-BR-3 cells that were treated or not with DCs-Exo (Exo Control in the figure) or Exo obtained from DCs exposed to tumor Exo (Exo Tex in the figure). After 2 days, the IFN-γ-producing T-cells were quantified by ELISPOT (**Figure 2**). It is interesting to note that SK-BR-3 cells, alone, seemed to induce some cytokine production by sensitized T-cells, but this production was not statistically different from that of T-cells cultured alone. However, the treatment of the tumor cells with Exo (both Exo control and Exo Tex) increased this response to a significant level. It is noteworthy that these responses were modest, but nevertheless, significant, thus in agreement with our hypothesis: tumor cells treated with Exo derived from DCs became better targets for an already existing immune effector cell population – those T-cells that had been sensitized *in vitro* against the tumor cells, but whose response was not strong enough to raise above the background. Furthermore, when the Exo were obtained from DCs that were treated with tumor cell exosomes (Tex), the enhancement of IFN-γ production seemed to be even higher. Though speculative, it is possible to recognize, here, an antigenic enrichment of the targets, since cells may load their Exo not only with their co-stimulatory molecules but also with antigen-loaded MHC complexes (20), which would be captured by the tumor cells, hence turning these into even better targets for the antigen-specific-T-cells.

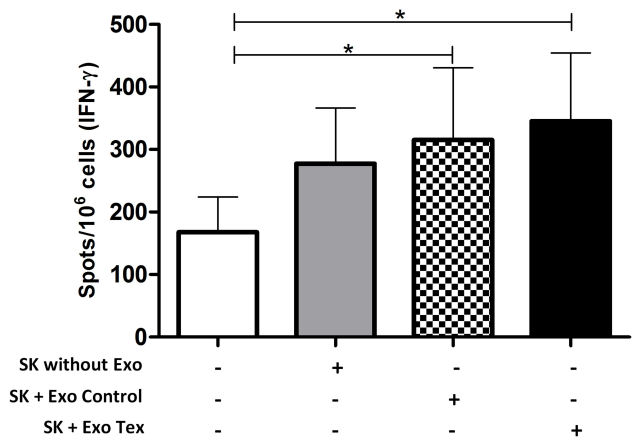


**FIGURE 2 | IFN-γ-production by tumor-sensitized T-cells is enhanced when tumor target cells are treated with Exo obtained from DCs**. Cells of the human breast adenocarcinoma cell line SK-BR-3 were pre-treated or not with Exo (130 μg/$10^6$ cells), obtained from control DCs (Exo Control) of from DCs exposed to tumor cell Exo (Exo Tex) and co-cultured for 2 days with previously sensitized $CD3^+$ T-cells (autologous to the DC source of the Exo). After that, the cells were harvested and the number of IFN-γ-producing cells was quantified by ELISPOT. T-cells pre-sensitization was obtained by co-culture with autologous tumor cell exosome-treated DCs, for 14 days, in presence of IL-2 and IL-7. Average and SD of the number of cytokine-producing cells are represented ($n = 6$; ANOVA $*p < 0.0028$, flowing Dunnett's Multiple Comparison Test).

## DISCUSSION

Tumor cells are poorly immunogenic and this has hampered the development of effective cancer immunotherapy. Yet, new insights on the role of different components of the immune response have broadened the field, so that, today, effective immune strategies to treat cancer are not longer seen as a remote possibility, but as a concrete breakthrough. However, though such approaches, as the use of checkpoint inhibitors or chimeric antigen receptor-transfected T-cells have reached astounding successes in some situations, the need for improvement is still pressing.

As depicted in **Figure 1**, even the presence (naturally or by transfer) of tumor-specific immune effectors within the tumor microenvironment may not be enough to hamper tumor growth and development. An immunosuppressive environment and targets that are poor in both antigens and co-stimulatory molecules may decrease significantly the effectiveness of tumor-specific responses. Therefore, any strategy that achieves the enhancement of tumor cell antigenicity or, even better, immunogenicity, should make it easier to control the disease in cancer patients. Such strategies could do so either by directly inducing tumor-specific responses, if the increase in tumor immunogenicity is strong enough, and, if not, by sensitizing tumors to borderline responses that, by themselves are not able to control the disease, but in face of more readily recognizable targets would do it.

Here, we tested the hypothesis that DC-Exo would perform such a role, increasing tumor cell antigenicity/immunogenicity. The transfer of information between cells via Exo is being recognized as a significant phenomenon for the body intercellular communication (38, 39), since Exo can interact with several cells types (26) and transfer information by means of fusion with the plasma membrane (40). Furthermore, this transfer seems not to be random, but controlled by molecules expressed by Exo and the "target cells," as we have shown for the incorporation of DC-Exo by tumor cells, where the incorporation of DC-Exo by tumor cells was directly proportional to the expression CD9 molecules by the latter (21). In other setting, Exo capture, by the monocytes lineage, THP-1, has been shown to depend on toll like receptors, TLR-2 and TLR-4, leading to activation of NF-κB and STAT3, and to cytokine secretion (41).

Since Exo carry several molecules of their cells of origin (27), they would be a possible way to load tumor cells with antigen presentation-associated molecules from DCs. Indeed, we did observe the transfer of HLA-DR and CD86 molecules from DC-Exo to tumor cells (21). Furthermore, when we analyzed tumor cells treated with DC-Exo, we noticed that these had an increased expression of ICAM-molecules, which could facilitate their interaction with T lymphocytes, since the interaction between ICAM-I and LFA on lymphocytes accentuates TCR/MHC/peptide interaction (32).

Thus, we tested if SK-BR-3 tumor cells treated with DC-Exo would be able to induce a T-cell response more effectively. Accordingly, we observed that treatment of the tumor cell line with

DC-Exo enhanced their ability to activate tumor-sensitized T-cells to secrete IFN-γ. We chose this cytokine because of its role in anti-tumor immune responses (42, 43) being associated with both CD8 and CD4 T-cell responses (44, 45), and essential for priming long-lived memory $CD8^+$ T-cells (46, 47). It must be recognized, however, that the response we obtained though significant, when compared to the non-stimulated T-cell response, was modest, showing just a tendency to statistical significance, when compared to the response induced by the non-Exo-treated cells. This is, actually, in agreement with our hypothesis, since the expected action of the nanovesicles would be to enhance just enough the sensitivity of the targets to already existing immune effector mechanisms – and not, necessarily, the induction of new responses. Another issue that should be noted is that the clinical application of such a strategy would be restricted to situations where tumors might be directly accessed, since the systemic inoculation of Exo would hardly deliver them to the tumor cells, but, rather, to other cells along their distribution through the body.

On the positive side, however, it is worth noting that this strategy, of treating tumor cells with DC-Exo, eliminates the need of specific tumor antigens identification, since it transfers to the patients' immune system this task. Through their association with DC-derived molecules, tumor antigens that would be poorly presented otherwise, would have their "visibility" increased, and, therefore, would be more likely to become effective targets of the patient's immune response. Furthermore, the local injection of these DC-Exo could allow them to be captured also by the patient's DCs in the tumor microenvironment. This would have a positive effect in the tumor immune response since in cancer patients, tumor-infiltrating DCs have functional deviations, maintaining an immature phenotype (9, 48–51). Thus, if these DCs incorporate Exo derived from immunogenic DCs, a phenomenon shown to occur (22), they might even become able to induce the response of tumor-specific *naïve* T lymphocytes.

Finally, even if the modifications of tumor cells immunogenicity caused by their incorporation of DC-Exo are not enough to give rise to *de novo* tumor-specific immune responses in the patients, they would, as we show here, turn the tumor cells into more effective targets to immune effectors induced by any other means. Thus, it is possible to suggest that treatment of tumors with DC-Exo could contribute to the effectiveness of any immunotherapeutic strategy, be it active, like vaccination protocols or passive, like the transfer of tumor-specific effectors. In face of this hypothesis and the data we obtained, we believe that this approach should receive consideration and be further investigated, in order to evaluate better its possible effectiveness, and to determine the most effective Exo doses, the length of its effect on tumor cells, and, mainly, the precise activation status of the DC, used as Exo source.

## AUTHOR CONTRIBUTIONS

Graziela Gorete Romagnoli: project development, participation in all experiments, analyses, and discussions. Bruna Barbosa Zelante: participation in the experiments, discussions, and drafting the article. Patrícia Argenta Toniolo: participation in the experiments, discussions, and drafting the article. Isabella Katz Migliori: participation in the experiments and discussions. José Alexandre M. Barbuto: project development, discussions, drafting, and revising the article.

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