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Vesicle-MaNiA: extracellular vesicles in liquid biopsy and cancer

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Normal and tumor cells shed vesicles to the environment. Within the large family of extracellular vesicles, exosomes and microvesicles have attracted much attention in the recent years. Their interest ranges from mediators of cancer progression, inflammation, immune regulation and metastatic niche regulation, to non-invasive biomarkers of disease. In this respect, the procedures to purify and analyze extracellular vesicles have quickly evolved and represent a source of variability for data integration in the field. In this review, we provide an updated view of the potential of exosomes and microvesicles as biomarkers and the available technologies for their isolation.

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Introduction

Extracellular vesicles (EVs) encompass membrane vesicles that are released by most cells into the surrounding microenvironment, and mediate inter-cellular communication at both paracrine and systemic level [1,2,3••,4–10,11•]. EVs are a complex group of vesicles. Indeed, extensive efforts from the scientific community have been done to provide names and classification criteria to the different subtypes of EVs. EV preparations are constituted by exosomes, microvesicles (including ectosomes and microparticles) and apoptotic bodies. These vesicles originate from distinct sub-cellular compartments and exist in different proportions depending on the physiological state and cell type of origin. Although no consensus on marker classification has been established to differentiate EVs [12,13], exosomes are defined as endosome-originated membrane vesicles with a diameter of 40–150 nm [14], microvesicles refer to plasma membrane shedding vesicles of 0.1–1 μm (ectosomes within this group range from 0.1 to 0.5 μm) [15,16] and apoptotic bodies are originated from cells undergoing apoptosis and generally present bigger size [16]. The differential origin of EVs determines their specific cargos, including proteins and nucleic acids [16,17]. The cargo will have both a passive and active impact on the functionality of EVs, and will constitute a molecular fingerprint representative of the cell of origin. To date, the majority of biological functions ascribed to EVs have been studied upon isolation from cell cultures or from biological fluids (blood, urine and saliva) [17–20]. Given the significant presence of EVs in most, if not all, bodily fluids, they have been postulated as new potential biomarkers for a wide range of diseases, including cancer [21–23,24••,25•,26]. Cancer-derived vesicles isolated from liquid biopsies have the potential to be used as a novel clinical tool for refining cancer diagnosis, for therapeutic stratification as well as for monitoring therapy response and outcome prediction (metastasis). However, both the variety and technical complexity of methods used for vesicle isolation make the use of EVs in clinical practice a challenge.

In this review, we provide a perspective on the activities of EVs and discuss the improvement in isolation techniques as well as their potential use as cancer biomarkers.

EVs as non-invasive source for biomarker discovery

Cancer-derived EVs have inherited potential to be used as biomarkers because of their ubiquitous presence in biofluids [17,27–29]. The characterization of cancer-derived EVs, and in particular their molecular cargo, has emerged as source of circulating information to detect cancer and predict tumor progression and metastasis. Indeed, cancer-derived EVs have been reported as clinical markers

aiding the diagnosis of many cancer malignances. In ovarian and pancreatic cancer, the exosome pool found in circulation is increased [24••,30], whereas in prostate cancer a decrease in urine EVs has been observed when compared to benign hyperplasia specimens [25•]. *In vitro* and *in vivo* pre-clinical studies have incremented our understanding on how the tumor specific cargo of cancer-derived exosomes can provide information about the pathophysiological status of cancer patients, by representing a bioprint of the primary tumor [24••,25•,26] as well as a detection and monitorization tool [7,31••].

EVs are composed of a lipid bilayer and contain a cargo that includes all known molecular constituents of a cell: proteins, lipids, microRNA, mRNA and DNA [8,10,32,33]. Whereas membrane composition of cancer-derived EVs may offer unique insights, recent studies have highlighted the importance of the cargo (metabolites, proteins and nucleic acids) for this purpose. The differential presence of nucleic acids in cancer-derived exosomes is a relievable source of biomarkers for several cancers, such as glioblastoma, bladder, liver, colorectal, lung and prostate, as well as brain and melanoma metastasis [26,34–38]. Cancer-derived exosomes contain double-stranded DNA [32, 39–41] and tumor-specific mutations can be detected in circulating EVs isolated from cancer patients, both in isolated DNA [39,40], and RNA (EGFRvIII mutation in glioblastoma [26]).

mRNA from EVs recapitulates to a certain extent the transcriptional landscape of the tumor. We have shown that urinary EVs mRNA cargo can discriminate prostate cancer patients and differentiate them from patients with benign disease [25•]. We have observed that specific transcripts exhibit differential abundance in EVs isolated from urine. Some of these transcripts have differential abundance reminiscent of the prostate tumor. As an example, down-regulation of placental Cadherin (CDH3) in tumor tissue is recapitulated in mRNA from urine EVs [42•]. This observation could open a new avenue on non-invasive characterization of transcriptional alterations with prognostic or therapeutic implications. Indeed, urine exosome gene expression has been recently proposed as a novel non-invasive approach to differentiate patients with higher-grade prostate cancer among men with elevated PSA levels, thus reducing the number of unnecessary biopsies [43].

In the recent years it has been extensively reported the presence of specific microRNAs (miRs) in EVs, which are informative for the diagnosis and monitoring of cancer progression. miRs are small double-stranded RNAs with strong regulatory potential [44] and its differential abundance in cancer-derived EVs have been associated with the presence and aggressiveness of squamous cell carcinoma, prostate and bladder cancer, among others [22,30, 34–36,45–49,50•,51–55]

EVs can carry protein in their membrane or in the lumen, representing the tumor proteomic cargo. Differences in EV-protein content from cancer patients have been described in several tumor types [3••,7,24••,31••,56,57]. The diagnostic potential of EV-protein content is well-illustrated in pancreatic ductal adenocarcinoma (PDAC) and melanoma. The expression of the surface proteoglycan glypican-1 (GPC-1) in cancer-derived exosomes is ascribed to the cancerous state and can discriminate patients with PDAC from those with benign pancreatic disease [24••]. In melanoma, the abundance of macrophage migration inhibitory factor (MIF) and its phosphorylated form are increased in cancer-derived exosomes when compared with healthy donors [7]. More recently, exosomal tumor-secreted integrins have been postulated as identifiers of the metastatic organotropism [31••]. Lyden and colleagues have demonstrated that the enrichment of specific integrin heterodimers in circulating exosomes could predict metastatic organotropism in breast cancer and pancreatic patients. In this work, exosomal integrins α6β4 and α6β1 were associated with lung metastasis, while exosomal integrin αvβ5 was linked to liver metastasis. This data represents a novel strategy to predict metastasis in liquid biopsies [31••].

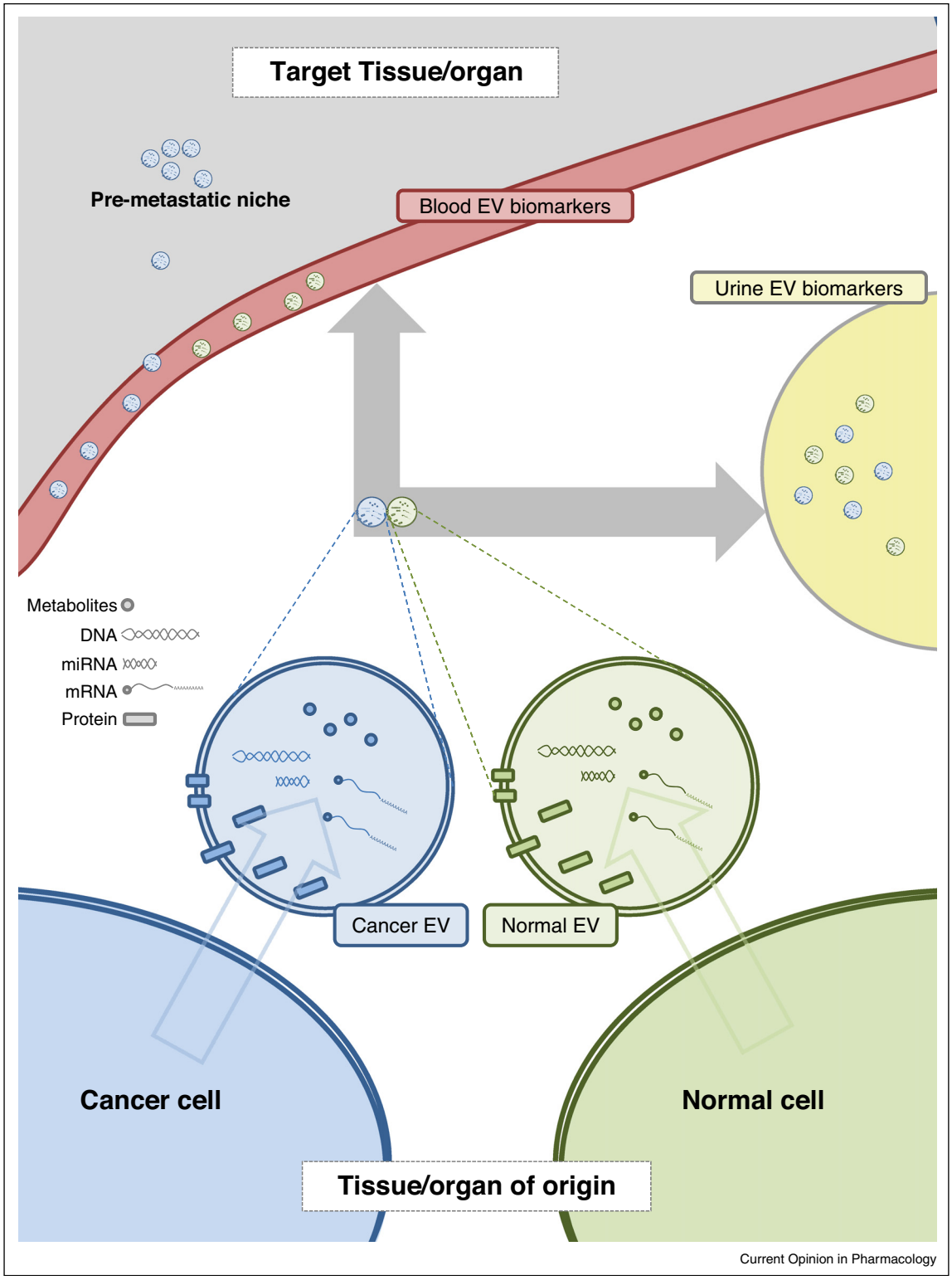
The characterization of EV cargo is still in its infancy. EVs research has shifted our expectations of liquid biopsy as a source of biomarkers [58]. With the advent of high-resolution/high-sensitivity genomics, transcriptomics, proteomics and metabolomics technologies, we envision that the next decade will consolidate non-invasive cell-free biomarkers as the *tour de force* of cancer diagnosis (Figure 1).

The refinement in EVs isolation methods

As discussed above, EVs are carriers of tumoral molecular information. However, a confounding factor in these studies is the heterogeneity of isolation procedures and the lack of consensus, which impacts on the reproducibility, yield or types of EVs that are isolated in each study. In order to select or develop an EVs isolation procedure for a specific application, several factors should be considered, such as sample nature (cell culture vs biological fluids), sample volume, the desired degree of purity, and the final use intended for the isolated vesicles.

In 2006, Thery and collaborators published a compendium of guidelines to isolate and characterize EVs from cell culture supernatants and biological fluids [59]. The protocols included purification routines that ranged from differential ultracentrifugation coupled to sucrose gradients, to immunocapture using antibodies against exosomal membrane proteins [59]. However, due to the exponential increase of the field in the recent years, new technical solutions have emerged to overcome the intrinsic limitations in the study of EVs.

Figure 1



Schematic representation of extracellular vesicles (EVs) as biomarkers in liquid biopsy. The distinct composition of EVs and its cargo in normal and cancer cells is indicated by differential coloring. Shedding of EVs to blood and urine is depicted (note that urine accessibility will be organ-dependent). The potential of cancer-EVs to educate the pre-metastatic niche is represented as the accumulation of vesicles in target organs.

Differential ultracentrifugation

Differential ultracentrifugation (coupled or not to density gradients) is the most extended and standardized procedure. This method is compatible with processing large sample volumes and allows obtaining preparations enriched in big EVs (mostly microvesicles) or small EVs (mostly exosomes) based on the use of 10 000 × *g* and 100 000 × *g* centrifugation forces, respectively [16,46]. In addition, when ultracentrifugation is performed on sucrose or idioxanol density gradients, further separation of the different subpopulations of vesicles is achieved. Despite the fact that differential ultracentrifugation could cause some 'damage' to the EV integrity in terms of vesicles breakage, fusion or aggregation, so far this method is the most commonly employed for 'omics'-based molecular and functional analyses. However, from a clinical point of view, ultracentrifugation presents several technical limitations for its practical implementation. In that sense, several alternatives have reached the market with distinct strength and weaknesses:

Polymer-based isolation systems

Most of these products are based on polymers adapted from virus-based studies [60,61]. Although these methods are not suitable to produce pure preparations of EVs, from a diagnostic perspective they are acceptable to analyze molecules that have been previously associated to extracellular vesicles. Among these, Exoquick (System Biosciences) and Total Exosome Isolation kits (Life technologies) have cornered the market. However, a new contender, Urine Exosome RNA Isolation Kit (NORGEN, Biotek Corp.), offers high-resolution and sensitivity [62,63]. It is worth noting that NORGEN kit allows the purification of proteins, as recently shown by our group [25•]. The introduction of these new methods have led to the concern of a bias in the type of EVs that are enriched with each approach, which could increase the inconsistency among different studies [25•,64]. Importantly, the presence of polymers could interfere with some of the analysis downstream, such as LC/MS-based techniques [65,66] or functional studies as well as carry soluble factor contaminants that should be determined and characterized in every model tested.

Filtration systems

Ultra-filtration and gel-filtration chromatography (based on sepharose columns for size-exclusion chromatography (SEC)) have been reported to be efficient, quick and able to achieve results comparable to standard methods [67–69]. These techniques are effective in removing contaminant proteins, and they can be applied downstream of other methods. Remarkably, an increasing number of laboratories are incorporating the SEC procedure in their studies mainly due to the low level of contaminants obtained in the EVs preparations. In particular, the SEC-based procedure has been very successful for the analysis of plasma EVs [70]. However, this technique can only be performed with relatively small volumes. For big volumes, other filtration-based approaches have been developed including the hydrostatic dialysis, a technique that has been proposed to analyze and banking urine samples [71].

Affinity methods

Affinity methods specifically separate EVs by their surface proteins. Nowadays, there are a variety of commercial immunoprecipitation kits for a range of proteins, such as Cd81 or Cd63, which allows a more specific isolation of EVs subpopulations, with limitations in their discriminative capacity [59,72]. In addition, ELISA-based methods [73], Exosearch [74] and the Immunochip [75] allow an specific quantification of subpopulation of EVs for a large number of samples. Recently, high-resolution flow cytometry has been developed as an interesting alternative to characterize and quantify different subpopulations of EVs [76,77], and for sorting a subset of EVs based on specific surface molecules [78]. It is worth noting the potential applicability of lectin [79] or heparin-based [33] systems for detection and isolation of EVs based on surface protein glycosylation.

Towards the production of EVs for therapeutic purposes

Several studies support the use of EVs for delivery of molecular cargo and related signaling [80,81,82••]. These ideas have expanded since the description of key molecules, such as integrins, determining the organ-targeted distribution of tumor-secreted exosomes [31••]. Production of clinical-grade exosomes classically require well-established methods of microfiltration, ultrafiltration, and a rapid one-step ultracentrifugation into a discontinuous gradient consisting of 30% sucrose/deuterium oxide (98%) [83]. More recently, the use of EVs as a carrier of selected siRNAs has attracted the interest of researchers and a detailed protocol based on ultracentrifugation has been established [84]. As the field of exosome research grows, therapeutic applications and GMP-grade purification methods are expected to be refined. Thus, in order to progress towards clinical trials, several topics should be considered: EV source, EV characterization and storage strategies, pharmaceutical quality control requirements and *in vivo* analyses of EVs [82••]. One of the main limitations of this field is that the majority of studies reporting tissue and location-specific distribution of EVs are restricted to tumor-derived vesicles. Therefore, further research on EVs derived from normal tissues and their characteristics is warranted. Using tissue-derived EVs as a new field in regenerative medicine could be one of the main areas that will be developed during the next years.

In summary, most of the existing procedures harvest a mix of EVs. Due to the intrinsic heterogeneity of these vesicles, the isolation procedure needs to be carefully considered, since it could deeply impact on the final outcome of the study.

Concluding remarks

Our current knowledge on what EVs do and how they recruit their cargo is limited. We are still far from clinical use of these vesicles as biomarkers of disease. However, EVs, exosomes and microvesicles in particular, present features that make them ideal candidates for liquid biopsy-based biomarkers. On the one hand, they are tissue-specific, which is one of the essential characteristics of biomarkers. On the other hand, they carry and protect the cargo from their tissue of origin, hence representing a bioprint of both physiological and pathological scenarios. However, there are also challenges that the field needs to face. We need to understand and define the heterogeneity of EVs and their associated cargo, and develop specific and reliable methods to work with well-defined preparations. In addition, the EV scientific community is also in urgent need of reaching a consensus regarding the isolation procedures and characterization [85], so that the field can integrate the observations coming from different research groups. The last challenge is to define to which extent EVs are a reflection of the molecular landscape of cancer that can be applied to precision medicine. We learn as we grow, and we need further knowledge and technological development in the field of EVs. These light and shadows predict an exciting bright future for EVs and their applicability in liquid biopsy.

Conflict of interest

Nothing declared.

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