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# Extracellular vesicles compartment in liquid biopsies: Clinical application

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ABSTRACT

Liquid biopsy is becoming a new source of biomarkers that complement and resolve some of the most important limitations of surgical biopsy, which are the accessibility to the diseased tissue and its heterogeneity, especially relevant for tumors. The diseased tissues release their molecule content to the bloodstream in free form, inside a cell or within extracellular vesicles (EVs). While the identification of molecular alterations in total DNA isolated from peripheral blood is already in use for some tumors that secrete large amounts of DNA, it is challenging to assay those secreting lower amounts of molecules as well as for many other non-tumoral pathologies like immunological and cardiovascular diseases. In this scenery, the compartment of diseased tissue-derived EVs will be one of the best alternatives for the detection and identification of current and new biomarkers and targets in the clinical management of these diseases. Here, we review the mechanisms of molecular internalization as well as the correlation of EV's cargo with clinical parameters in tumor and non-tumor diseases, with special emphasis in clinical application.



## 1. Introduction

The term "Liquid biopsy" encompasses three different sources of molecular information depending on whether it appears in the free form, inside a cell, or within extracellular vesicles (EVs) (Strotman et al., 2016). EVs are membranous cell-derived vesicles released from all cell types upon activation or apoptosis, and play an important role in paracrine and endocrine cell-to-cell communication by transporting their cargo between neighbouring cells or travel to reach distant cells (Tetta et al., 2013) through biological fluids where EVs can be detected, being blood and urine the most relevant and studied. Based on the size and biogenesis pathway, EVs are classified into Apoptotic Bodies (ABs), Shedding Microvesicles (MVs) and Exosomes (EXOs) (Kalra et al., 2012), and contain all types of nucleic acids, lipids, proteins and growth factors (Santiago-Dieppa et al., 2014), important for the management of several diseases. In this sense, the gold standard for many pathologies is to interrogate solid biopsy for the presence of specific molecular alterations or expression patterns. However, solid biopsy also has important limitations. Some tumors are unresectable or their location might be surgically inaccessible and, in most cases, the surgical specimen does not reflect the heterogeneous population characteristic in cancer (Nilsson et al., 2009). Likewise, in other diseases such as cardiovascular disease, current biomarkers, like cholesterol levels or Low-Density Lipoproteins (LDL), are only assessing the risk of developing disease, but cannot actually inform whether the process started (Ldl et al., 2000). Liquid biopsy can resolve some of these limitations. The approach to assay for specific biomarkers in total DNA isolated from peripheral blood is already in use for some tumors, like colon, lung, prostate and breast, that secrete large amounts of DNA to the bloodstream (Thompson et al., 2016) (Peeters et al., 2015). However, for those secreting lower amounts of molecules as well as for many other non-tumoral diseases, the compartment of tumor/diseased tissue-derived EVs might be the best alternative to increase the sensitivity of future tests for the detection and/or identification of new biomarkers and targets and, therefore, the possibility of translating it to clinical

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setting.

Here, we review the state-of-the-art in the internalization mechanisms of different molecules into EVs, as well as the correlation of EV's cargo in peripheral blood with clinical parameters for both tumor and non-tumor diseases.

## 2. Extracellular vesicles cargo and biosynthesis

In the last decade we have witnessed an increasing number of works demonstrating the presence of all types of macromolecules within EVs. However, the precise mechanisms by which these molecules enter the vesicles remain mostly uncovered. Understanding the process of such internalization offers unexpected opportunities for the identification of new biomarkers and targets for the management of different human pathologies.

Membrane lipid composition of Exosomes (EXOs) and Shedding microvesicles (MVs), such as phosphatidylserine, cholesterol and different long chain fatty acids (e.g. sphingomyelin), drive the preferential entry of the simple lipids opposed to phospholipids (Yáñez-Mó et al., 2015) (Fig. 1.1). Moreover, the presence of lipid raft domains in the EV membrane could be associated with the diffusion of proteins during EXO formation (Gassart et al., 2003) and even the lipid flux manages the type of proteins that could appear in EXO's membrane (Subra et al., 2007).

In the case of the EV protein composition, it appears not to be a random process. Specifically, the first sorting step in the EXO cargo starts with the endosomal-sorting complex required for transport (ESCRT), which is formed by four complexes; ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and several associated proteins, such as Vps4 or Alix. ESCRT-0, I and II recruit ubiquitinated membrane proteins into intraluminal vesicles (ILVs) while ESCRT-III promotes their scission (Colombo et al., 2011). Furthermore, other mechanisms of protein sorting into EVs could be found due to the presence of EXOs secreted by ESCRT-independent pathways (Babst, 2012), suggesting that ESCRT complex is not essential for EXOs secretion. In this sense, it is believed that the small GTPase ADP ribosylation factor 6 (ARF6) has a key role in the selective recruitment of proteins, since it participates in EXOs production by regulating the crossover of ILVs into multivesicular bodies (MVBs) (Machala and Zimmermann, 2014), and it has never been detected inside the cargo.

A variety of other pathways could also be involved in EV targeting, since mass spectrometry analysis revealed the presence of different post-translational modifications in proteins isolated from EVs, such as glycosylation, phosphorylation, addition of ubiquitin groups or small ubiquitin-related modifications (SUMOylation). Other authors suggest that the acylation of the N-terminal domain could also drive proteins into EVs (Shen et al., 2011). In addition, proteins related to the biogenesis process of EVs, such as tetraspanins, lectins, transmembrane proteins, GTPases, or those involved in the microvesicle bodies production, like Alix or TSG101, have a critical function in the EV protein content (Yáñez-Mó et al., 2015) (Fig. 1.2). Further studies on these specific changes in the protein content of EVs will represent a potential source of information to increase our knowledge of the biological process of the diseases but, more importantly, it will provide new targets for therapeutic intervention.

The presence of different types of nucleic acids, such as mitochondrial DNA (mtDNA) (Guescini et al., 2010), single-stranded DNA (ssDNA) (Balaj et al., 2011), double-stranded (dsDNA) (Thakur et al., 2014), RNA (Skog et al., 2008) and micro RNA (miRNA) (Manterola et al., 2014) inside the three types of EVs has been widely reported in the bibliography, though little is known about their uptake mechanism. In this regard, it is known that cell death and mitophagy drives mtDNA presence in the bloodstream, and allows it to be found into EVs (Ding and Yin, 2012). Interestingly, ssDNA fragments might emerge from replication errors occurring in late G1 phase of the cell cycle since the use of mimosine, an inhibitor of that phase, decreases the levels of ssDNA (Balaj et al., 2011). The presence of cDNA in EVs could be also due to the reverse transcription of RNA (Balaj et al., 2011). Furthermore, fragmented cellular DNA is detected inside ABs, which might be a consequence of the chromatin hypersegmentation that undergoes in apoptotic cells (Collins et al., 1997).

In spite of all this, the presence of double-stranded gDNA fragments of at least 10 kb found in EXOs, or the gDNA spanning all chromosomes shown in prostate patients (Lázaro-Ibáñez et al., 2014; Thakur et al., 2014) is yet to be explained, and further investigations are needed to understand the mechanisms by which DNA is introduced into EVs (Fig. 1.3).

Regarding the RNA molecules, the selective package of coding and non-coding RNA within EVs has been recently described (van der Vos et al., 2015). Indeed, a conserved sequence of 25 nucleotides with a CTGCC core has been found in the 3' Untranslated Region (UTR) of the mRNA, which drives its uploading inside the EVs. Furthermore, this conserved sequence has a binding site for a specific miRNA (miR-1289), which contributes to mRNA enrichment in EVs (Bolukbasi et al., 2012) (Fig. 1.4).

Similarly, post-transcriptional modifications in miRNAs have been shown to drive the sorting of miRNA into EVs both *in vitro* and *in vivo*. Specifically, adding poli-Uraciles, rather than Adenines, to the 3′-end targets miRNAs into the EVs (Koppers-Lalic et al., 2014) (Fig. 1.5). Interestingly, miRNA biogenesis and EV production might be potentially associated, as it has been shown that the

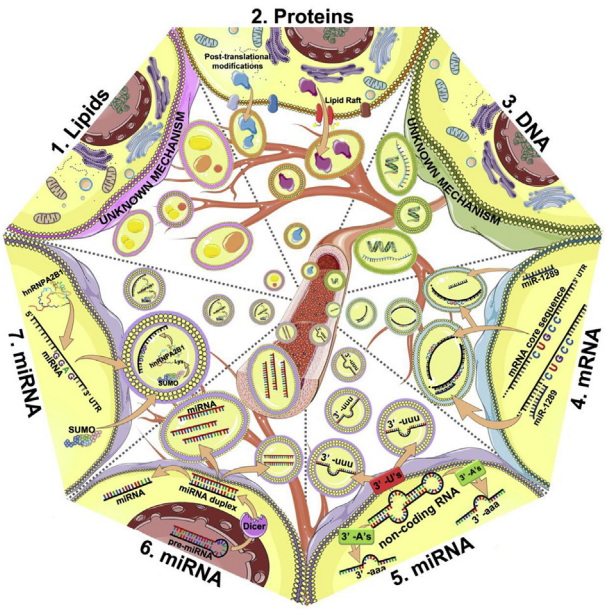


**Fig. 1. Internalization mechanisms of macromolecules into EVs.** The picture represents the current knowledge on the internalization mechanism of macromolecules into EVs. **1.1 Lipids**: The Lipids sorting mechanisms are still unknown, although some authors have reported the preferential entry of simple lipids (Yañez-Mó et al., 2015). **1.2 Proteins:** Proteins uptake into EVs is not exactly described. It is believed that post-translational modifications could modulate the entry of specific proteins inside EVs. **1.3 DNA:** Different types of DNA appear as EVs cargo via unknown mechanisms. **1.4 mRNA:** A conserved sequence of 25 nucleotides flanking a CTGCC core in the 3′ UTR, drives the mRNA into EVs supported by a binding site for miR-1289. **1.5 miRNA**: Adding Uraciles to the miRNA 3′-end as a post-transcriptional modification point them inside EVs. **1.6 miRNA**: One strand of the miRNA duplex is incorporated to the cell, while the complementary strand appears inside EVs, suggesting a specific sorting process. **1.7 miRNA**: Lysine residues of hnRNPA2B1 are recognized by SUMO proteins and form a complex that binds to GGAG core in the 3′UTR, this association could drive the miRNA into EVs.