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REVIEW

# Heterogeneity of Extracellular Vesicles and Particles: Molecular Voxels in the Blood Borne "Hologram" of Organ Function, Dysfunction and Cancer

**Janusz Rak**[1] · **Leon Strzadala**[2]

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**Abstract**
Extracellular vesicles (EVs) and particles (EPs) serve as unique carriers of complex molecular information with increasingly recognized roles in health and disease. Individual EVs/EPs collectively contribute to the molecular fingerprint of their producing cell, reflecting its identity, state, function and phenotype. This property is of particular interest in cancer where enormous heterogeneity of cancer cells is compounded by the presence of altered stromal, vascular and immune cell populations, which is further complicated by systemic responses elicited by the disease in individual patients. These diverse and interacting cellular compartments are dynamically represented by myriads of EVs/EPs released into the circulating biofluids (blood) during cancer progression and treatment. Current approaches of liquid biopsy seek to follow specific elements of the EV/EP cargo that may have diagnostic utility (as biomarkers), such as cancer cell-derived mutant oncoproteins or nucleic acids. However, with emerging technologies enabling high-throughput EV/EP analysis at a single particle level, a more holistic approach may be on the horizon. Indeed, each EV/EP carries multidimensional information (molecular "voxel") that could be integrated across thousands of particles into a larger and unbiased landscape (EV/EP "hologram") reflecting the true cellular complexity of the disease, along with cellular interactions, systemic responses and effects of treatment. Thus, the longitudinal molecular mapping of EV/EP populations may add a new dimension to crucial aspects of cancer biology, personalized diagnostics, and therapy.

**Keywords** Cancer · Extracellular vesicles · Heterogeneity · Oncogenes · Complexity · Liquid biopsy

## Introduction—Cellular Communication Networks in Cancer

The defining feature of multicellular organisms is the ability of constituent cells to communicate. Therein lie homeostatic mechanisms, growth control switches, maintenance of structure and function, as well as paths to their aberrations accompanying disease, including cancer. While the traditional preoccupation with biochemistry of signalling pathways and intracellular mechanisms of oncogenesis have placed an almost exclusive emphasis on cellular "autonomy" (Bishop 1995), the conceptual and translational insufficiency of this notion gradually gave way to a more nimble definition of the neoplastic process (Hanahan and Weinberg 2011). Indeed, the advent of technologies such as high-resolution flow cytometry, single cell sequencing, or spatial gene expression profiling (Li et al. 2022) sparked a renewed interest in cancer cell heterogeneity, tumour complexity and intercellular interactions, concepts pioneered decades ago (Fidler 1973; Heppner 1983), now leading to more multidimensional and dynamic models of the disease progression (Neftel et al. 2019). There is a rekindled appreciation for the existence of diverse and plastic tumour cell subpopulations, including cancer stem cells and their progeny immersed within the cellular ecosystem of pathologically altered tumour stroma, vascular and immune cells (Adnani et al. 2022b; Goveia et al. 2020; Varn et al. 2022). This interest brought to the fore the long-standing question as to how do these complex cellular landscapes arise? What

✉ Janusz Rak
janusz.rak@mcgill.ca

1 Research Institute of the McGill University Health Centre, McGill University, Montreal, QC, Canada

2 Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland

regulates them? What are their vulnerabilities and therapeutic responses? What are their diagnostic representations?

An aspect that makes this complexity even more intriguing (and daunting) are the systemic events associated with cancer. This includes not only the overt metastatic process, but also more subtle changes, such as premetastatic niche formation (Wortzel et al. 2019), cancer-associated thrombosis (Khorana et al. 2022; Tawil et al. 2019), systemic immunosuppression (Whiteside 2016), paraneoplastic syndromes (e.g. cachexia) and other responses. In this context, the inescapable notion is that transforming molecular events transcend cellular boundaries and propagate in a form of cell–cell interactions throughout the affected, as well as ostensibly unaffected, tissues, creating a new multicellular reality. The underlying cellular interconnectedness is essential for the disease process to occur, as revealed by experiments demonstrating numerical thresholds of cancer cell engraftment (Al-Hajj et al. 2003; Revesz 1956), niche effects (including perivascular niche) (Calabrese et al. 2007), context-dependent penetrance of oncogenic mutations (Martincorena et al. 2015) and long-term dormancy of transformed cells at sites non-conducive for disease development (Aguirre-Ghiso 2007; Black and Welch 1993; Magnus et al. 2014). For these reasons, the determinants, mechanisms and mediators involved in intercellular communications are of paramount biological, diagnostic and therapeutic interest (Broekman et al. 2018; Rak 2013). In this commentary article, we would like to reflect on some of these questions. Rather than providing a comprehensive review of the entire rapidly expanding extracellular vesicles/extracellular particles (EV/EP) research field, we chose to discuss the possibility that analytical approaches to EV/EP complexity per se could be useful in addressing challenges arising from the complexity of cancer itself.

## Oncogenic Drivers and Cancer-Related Cellular "Miscommunication"

While altered cellular communication may be linked to cancer in an indirect or "unspecific" manner (e.g. as a result of the breakdown in tissue barriers), a considerable body of evidence suggests that oncogenic transformation itself entails deep realignments in cell–cell interaction mechanisms. Historically recognized manifestations of this notion involve mostly homotypic interactions, exemplified by the loss of contact inhibition or three-dimensional growth patterns (Hanahan and Weinberg 2011). However, subsequent studies revealed a much wider impact of oncogenic mutations on the communication apparatus of cancer cells, including their ability to trigger tumours angiogenesis (Dameron et al. 1994; Rak et al. 1995), affect immunoregulation (Sparmann and Bar-Sagi 2004), activate the coagulation system and platelets (Boccaccio et al. 2005; Rong et al. 2005; Tawil et al. 2021; Yu et al. 2005) or engage in other non-cell autonomous processes.

In addition to genomic alterations, the properties of cancer cells and the architecture of their populations are strongly affected by the epigenome (Zhao et al. 2021). While changes in the epigenome may be a part of the overt oncogenic process (Schwartzentruber et al. 2012), they may also act as regulatory constraints upon cancer cell populations through lineage commitment and differentiation programmes, which may be either intrinsic, or triggered in response to microenvironmental cues. Indeed, cancer cells harbouring the same oncogenic mutations may be driven to either proliferative, differentiated, invasive or quiescent states, as a result of these external influences. Consequently, the formation of multicellular equilibria, stem and non-stem cell compartments, migratory cell cohorts, or growth promoting multicellular "units" often involves feedback mechanisms from adjacent cancer cells, microenvironment, vasculature and other sources (Adnani et al. 2022a; Calabrese et al. 2007; Koch et al. 2014; Lu et al. 2013; Rak 1989; Wang et al. 2018).

Thus, the interplay between hardwired cancer-causing mutations, epigenetic modulation and intercellular communication pathways emerges as a defining force in shaping the biological nature and the complexity of cancer. For example, in glioblastoma, genetic drivers create directional biases in the preponderance of cancer cell subsets harbouring certain gene expression profiles. In this setting, mutations of the *NF1* tumour suppressor gene are associated with more prevalent (albeit not uniform) mesenchymal phenotype of cancer cells and ample inflammatory infiltration, while oncogenic *EGFR* expression triggers a bias towards the "classical" tumour subtype and a more astrocytic gene expression profile (Neftel et al. 2019).

How do cancer cell populations "know" how many stem, differentiated or phenotypically committed cells to produce? There are experimental indications that clues as to the extent of these phenotypic shifts are often taken by cancer cells from their neighbours (proximal or distant) whether transformed (Koch et al. 2014; Wang et al. 2018) or stromal (Adnani et al. 2022a; Lu et al. 2013). Based on this logic, genes that control cell–cell and cell-microenvironmental communication may play a unique role in the biology and evolution of the underlying disease (Bonavia et al. 2011; Inda et al. 2010). This realization propelled the recent interest in mediators of cell–cell communication in cancer with ample literature covering soluble factors (cytokines, chemokines, metabolites) (Quail and Joyce 2017), specialized cell membrane structures (tunnelling nanotubes, microtubes, junctions) (Osswald et al. 2015) and mobile extracellular cell fragments often referred to as extracellular vesicles (EVs) or particles (EPs) (Broekman et al. 2018).

Our remaining comments will revolve around the latter class of mediators.

## Particulate Secretome of Cancer Cells

A considerable proportion of the molecular material released by normal and transformed cells into their microenvironment consists of sedimentable, multimolecular structures (particulate secretome) including EVs and non-vesicular extracellular particles (EPs or NVEPs) (Théry et al. 2018). These terms are intended to reflect the notion that EVs are formed by encapsulating cellular content within the lipid bilayer of cellular membranes either originating at the cell surface or within intracellular membraneous structures, especially the endosome (Fig. 1) (van Niel et al. 2018). In contrast, EPs are multimolecular structures largely devoid of the plasma membrane, but enriched in protein and nucleoid acid complexes of presently unknown subcellular origin (Zhang et al. 2018, 2021). While initially neglected as "cell debris", the various constituents of the particulate secretome proved to be extremely revealing, as a reflection of the phenotypic and functional complexity of EV/EP producing cells, their heterogeneity and interconnectedness (Rak 2013).

Indeed, cells possess highly conserved molecular programmes to remove parts of themselves and release this material into the pericellular milieu, biofluids, blood and secretions (e.g. milk), as myriads of EVs/EPs. Upon release,

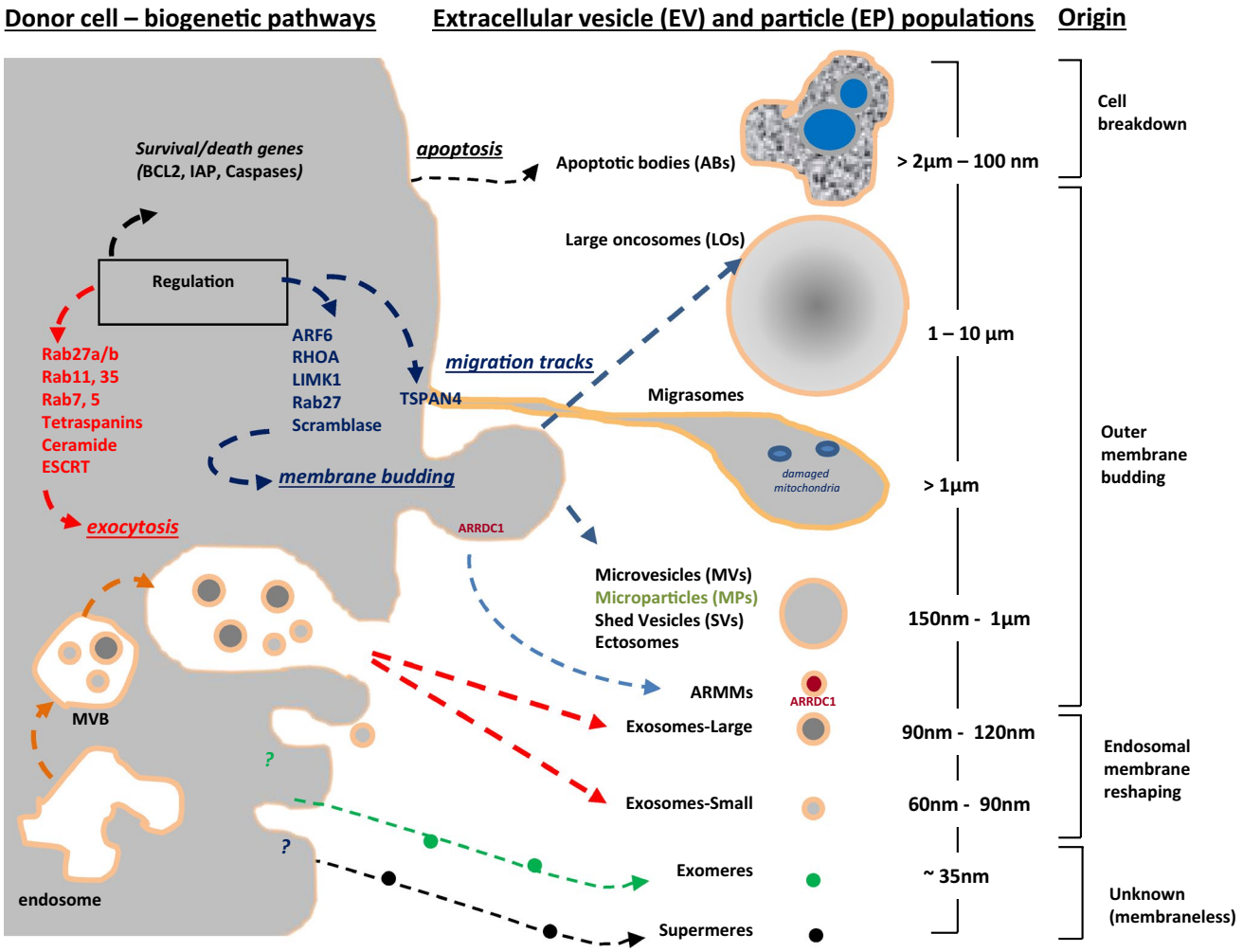


**Fig. 1** Subtypes of extracellular vesicles and particles and their biogenetic pathways. Extracellular vesicles (EVs) may emerge through several processes involving cellular plasma membrane. Apoptotic programmes of cellular demise may lead to formation of apoptotic bodies/vesicles (top). Membrane blebbing processes result in formation of several types of ectosomes/microvesicles from viable cells. This includes both small (ARMMs) and large (large oncosomes) EVs. Migrasomes form from migratory tracks of moving cells. In contrast, late endosome system structures known as multivesicular bodies (MVBs) can translocate to the plasma membrane and release intraluminal vesicles from within the cell into the extracellular microenvironment. Small membrane-less extracellular particles (EPs), such as exomeres and supermeres, are released through currently unknown mechanisms (see text).

these structures are either eliminated and/or may come into contact with, and are often internalised by, other cells, within and beyond their parental tissue, organ, or organism (e.g. during inter-kingdom interactions) (van Niel et al. 2018). The EV/EP release process is currently regarded as serving four fundamental functions: (i) *en masse* elimination of molecular constituents or organelles (e.g. damaged mitochondria) by EV/EP producing viable cells (Pan and Johnstone 1983; Yu and Yu 2021); (ii) intercellular communication through transfer of complex molecular messages contained in the cargo of EVs/EPs and involving their interactions with target cells (surface contact, membrane fusion, endocytosis, phagocytosis, macropinocytosis) (Mulcahy et al. 2014); (iii) modification of the acellular microenvironment by bioactive EV cargo molecules (enzymatic degradation, deposition of guiding cues, blood clotting) (Barman et al. 2022; Geddings and Mackman 2013; Hendrix et al. 2010); (iv) packaging of the content of apoptotic cells in a form of EVs (apoptotic bodies), which facilitates their phagocytic bioelimination (Gyorgy et al. 2011; Li et al. 2020), or serves distinctive signalling functions (Dieudé et al. 2020).

## Heterogeneity of Extracellular Vesicles and Particles

The multiplicity of biological functions is achieved through a remarkable diversity of EVs and EPs. Their landscapes are shaped by several factors including the properties of their parental cells, pathways involved in biogenesis of different EV/EP subtypes (Zijlstra and Di Vizio 2018) (Fig. 1) and variation in molecular composition of individual EVs/EPs (Fabbiano et al. 2020; Greening and Simpson 2018; Rak 2013; Roy et al. 2019; Willms et al. 2018; Yokoi and Ochiya 2021).

### Exosomes

EVs originate mainly at two subcellular sites, namely at the outer plasma membrane (ectosomes) or within the intracellular endosome (exosomes) (Mathieu et al. 2019; van Niel et al. 2018; Xu et al. 2018). In the latter case, the late endosome evolves into structures known as multivesicular bodies (MVBs) filled with secondary intraluminal vesicles (ILVs), which compartmentalize the modified (e.g. ubiquitinated) and pre-packaged molecular content (cargo). MVBs may be either transported to the lysosome for degradation, or to the plasma membrane, where ILVs are released into the cell exterior as exosomes (van Niel et al. 2018). While exosomes are usually small in size (50–150 nm) and share several molecular features, such as enrichment in tetraspanins (CD9, CD63, CD81) (Koch et al. 2014), or high content of syntenin (Kugeratski et al. 2021; Luck et al. 2020), they are formed through at least two different molecular processes one involving the multimolecular endosomal complex required for transport (ESCRT), the other being ESCRT-independent and driven by tetraspanins, neutral sphinogomyelinase (SMPD3), ceramide and other factors (van Niel et al. 2018). Studies involving asymmetric flow fluid flow fractionation (AF4) suggested the existence of large (80–120 nm) and small (60–80 nm) exosome populations (Zhang et al. 2018). Further diversity of exosome-like EV subsets may stem from their release from the basolateral or apical domains of polarized cells (Lakkaraju and Rodriguez-Boulan 2008; Wang et al. 2021), or following epithelial-to-mesenchymal transition (Garnier et al. 2012; Tauro et al. 2013). Density fractionation revealed exosome-like EVs with either low or high floatation density, the latter being enriched in RNA (Barman et al. 2022). In many of these instances, the origin of exosome-like EVs cannot be unambiguously assigned to MVBs, and therefore, they are often referred to as exosome-like (rather than exosomes) or simply as small EVs (sEVs) (Théry et al. 2018). Nonetheless, even within the relatively uniform category of sEVs, notable heterogeneity appears to exist and reflect various aspects of the originating (donor) cells.

### Apoptotic Vesicles

Formation of EVs at the cellular plasma membrane results in astonishing diversity of vesicular structures. On one extreme of this spectrum are apoptotic bodies (ABs), or apoptotic vesicles (AVs), that may range from 50 nm to > 2 um in diameter and emerge due to packaging of the dying cell content (including nuclear material and organelles) into the remaining plasma membranes often enriched in outer phospholipids, such as phosphatidylserine (PS) (Gyorgy et al. 2011; Jeppesen et al. 2019). Indeed, circulating ABs/AVs could contribute to phospholipid and ceramide signal detectable in blood following brain injury (Fiedorowicz et al. 2019) and cancer (Huang et al. 2020). Treatment of cancer cells with inhibitors of oncogenic epidermal growth factor receptor (EGFR) gives rise to apoptotic particles (chromatimers) containing genomic chromatin, as well as EGFR antigen embedded in the remaining cellular membranes (Choi et al. 2019; Montermini et al. 2015). Likewise, endothelial cell damage may result in the release of DNA containing exosome-like EVs, referred to as ApoExo (Dieudé et al. 2020).

### Ectosomes

Viable cells also give rise to a wide range of EVs (van Niel et al. 2018) largely through external membrane protrusion leading to scission and release of ectosomes (also

referred to as shed vesicles, microvesicles or microparticles). They range in size between 150 to > 1000 nm and often carry tertraspanins, PS and annexin A1 on their surfaces (Jeppesen et al. 2019; Piccin et al. 2007). Mechanistically, this process may involve calcium fluxes that signal changes in membrane phospholipid asymmetry, impact activity of scramblases, are coupled with activation of ARF6, ROCK, and engage other factors including some ECSRT-related proteins, such as TSG101 (Li et al. 2012; Muralidharan-Chari et al. 2009; van Niel et al. 2018). Unique small ectosome-like EVs (~ 100 nm), referred to as ARMMs, form at the plasma membrane sites occupied by arrestin domain-containing protein 1 and are often enriched in NOTCH (Leidal and Debnath 2020). EV generating sites of the plasma membrane also include natural protrusions, such as cilia, that may shed their tips as small vesicles (Luxmi and King 2022). On the other side of the size spectrum, large ectosome-like structures (2–10 um) known as large oncosomes are generated during ameboid cell movement of metastatic cancer cells (Di Vizio et al. 2009). Another complex vesiculation process entails formation of migrasomes, large EVs (> 1 um) often containing damaged mitochondria and generated within migratory tracks from membranes laid down by moving cells (Yu and Yu 2021).

## Cell Type-Specific Extracellular Vesicles

Apart from general biogenetic mechanisms, different EV subpopulations may also originate from specific cell types performing distinct functions. For example, prostasomes are EVs associated with prostate cell secretion (Aalberts et al. 2014), prominosomes are related to their content of stem cell marker prominin 1 (Mittal et al. 2020), argosomes are involved in morphogenesis (Greco et al. 2001) and oncosomes (to be distinguished from large oncosomes) are defined by their content of cancer-specific, mutant oncogenic proteins and nucleic acids (Al-Nedawi et al. 2008; Meehan et al. 2016). It should be noted that different EV subtypes may be released by the same type of cells. For example, clonal glioma cells driven by oncogenic EGFRvIII receptor release EVs of which only a fraction contains EGFRvIII (oncosomes), while their majority does not (Choi et al. 2018). Elongated neutrophil-derived structures represent another type of EV-like structures unique to these cells (Marki and Ley 2022). However, neutrophils produce a range of other vesicular and particulate structures (migrasomes, mitosomes, ectosomes, exosomes), as well as cytoplasts and other particulate products associated with neutrophil extracellular trap formation processes (Marki and Ley 2022).

## Extracellular Particles

In addition to membraneous EVs, the particulate secretome is populated by often much more numerous, but membrane-less structures referred to as EPs, or NVEPs (Théry et al. 2018). These particles may be of complex and uncertain origin, as well as poorly understood content and function (Jeppesen et al. 2019; Zhang et al. 2021). For example, AF4 fractionation and ultra-high-speed centrifugation of EV-depleted cell culture supernatants revealed the existence of membrane-less EPs known as exomeres (Zhang et al. 2018) and supermeres (Zhang et al. 2021), the latter enriched in microRNA. Cell-free, DNA-containing nucleosomes may co-purify with fractions of EVs and EPs (Jeppesen et al. 2019). T cells may produce cytotoxic supramolecular attack particles, multiprotein complexes encapsulated in a glycoprotein coat (Bálint et al. 2020). Various cells and platelets are known to release free mitochondria in the absence of plasma membrane (Puhm et al. 2019). Biofluids may also contain other EP-like structures including lipoproteins, nucleoproteins and protein aggregates with currently unknown content, function or diagnostic significance.

## Extracellular Vesicle/Particle Cargo

The biogenesis of EPs and of several EV subsets remains presently uncertain and so too are the mechanisms of their cargo assembly. However, for subsets of more defined sEVs, some illuminating inroads have already been made in determining the pathways (van Niel et al. 2018) and protein–protein interaction hubs (Luck et al. 2020) that dictate their proteome (Greening et al. 2016; Hoshino et al. 2020), RNA content (Barman et al. 2022; Leidal and Debnath 2020; Villarroya-Beltri et al. 2014) and the presence of genomic DNA (Chennakrishnaiah et al. 2020). In the case of cancer cells, these features often depend on oncogenic transformation (Al-Nedawi et al. 2008; Choi et al. 2018; Kilinc et al. 2021; Lee et al. 2014), molecular subtype and dynamic changes in the cellular differentiation status (Garnier et al. 2012; Spinelli et al. 2018; Tauro et al. 2013). Thus, various cell types, states and responses may translate into correspondingly diverse landscapes of EV/EPs released into the microenvironment and biofluids. In some cases, it is possible to use computational deconvolution algorithms to predict the number of "carriers" (e.g. EVs) from the profiles of extracellular RNA found in biological material (Lucero et al. 2020), but several methods of direct detection and characterization of particulate content are still needed and continue being developed (Shao et al. 2018).

## Beyond Extracellular Vesicle/Particle Subtypes

The "granularity" of the cellular representation by EV/EP landscapes is likely greater than the composition of known EV/EP subtypes. This notion stems from the analysis of bulk sEV proteomes that can be isolated from relatively uniform, clonal cancer cells in culture. In such experiments, it is not uncommon to detect peptide annotation of 1,000 to upward of 4,000 proteins. A vesicle in the range of a sEV (e.g. synaptic vesicle) is estimated to sterically accommodate up to 200 proteins (Takamori et al. 2006), with abundant proteins being represented at 1–13 copies per vesicle on the average (Mutch et al. 2011). The extrapolation of these numbers to proteins identified in the cancer sEV proteome predicts the existence of 5–20 non-overlapping hypothetical protein sets (sEV subsets) (Choi et al. 2019, 2018). Considering that even vastly different EV subtypes harbour multiple common proteins (tetraspanins, integrins), the bulk mass spectrometry profile predicts dozens (if not more) of sEV subpopulations being generated from cultured clonal cells that are maintained under conditions of relatively high homogeneity. This estimate far exceeds the known range of EV subtypes and suggests that each cell may be represented by multiple distinctive EV phenotypes or their continuum (Choi et al. 2019). Inclusion of larger EVs (ectosomes) and EPs into this analysis is likely to further increase this dimensionality. In the other words, the apparent EV/EP heterogeneity far exceeds that of their producing cells (to the extent, this is known) suggesting that EV/EP landscapes may offer a relatively high-resolution molecular (and cellular) 'picture' of complex organs, tissues and their related alterations including cancers. Given the impact of malignant transformation on the molecular repertoire of EV/Eps, it could be postulated that the degree of their heterogeneity (diversity factor) could, in itself, be informative as to the underlying pathology in addition to specific molecular signals that EVs/EPs may carry.

## Liquid Biopsy

The representation of complex cellular traits is of great interest in the context of the emerging diagnostic applications of EVs/EPs as liquid biopsy analytes in cancer and beyond (Hoshino et al. 2020; Rak 2013; Skog et al. 2008). In recent decades, liquid biopsy in cancer has evolved from an intriguing exploratory concept to a clinically applicable diagnostic method with a solid analytical foundation and considerable practical advantages (Barault et al. 2015; Ignatiadis et al. 2021). The realization that cancer-specific signals can be recovered from blood and other biofluids containing circulating tumour DNA (ctDNA), circulating tumour cells (CTCs) or EVs (or EPs) brought about the prospect of unprecedented access to salient features of the disease that were hitherto unknown. Unlike tissue biopsy or surgical specimen collection, liquid biopsy offers a repeated, minimally invasive access to the source of cancer-related molecular information from all tumour sites that communicate with the relevant biofluid space, from which samples could be collected, such as blood. This approach may overcome the sampling errors, challenges associated with tumour heterogeneity, inaccessibility of tissue samples due to multifocal metastatic disease, anatomical factors, ethical constraints or medical contraindications, as long as means exist to extract the actionable information with required sensitivity, specificity and reproducibility (Ignatiadis et al. 2021). Therein lie many challenges that still remain. However, several platforms of liquid biopsy diagnostics are already in clinical use, while others are under rapid development (Ignatiadis et al. 2021).

EVs/EPs occupy a unique place in the space of liquid biopsy, for several reasons. Numerically, the content of EVs ($10^9$–$10^{12}$ per mL), or exomeres ($>10^{12}$/mL) in the circulation exceeds that of CTC (< 10/mL) by orders of magnitude (Killingsworth et al. 2021). This is important not only because of technicalities associated with the sampling process, but also as a reflection of cellular heterogeneity of the underlying cancer that may consist of numerous different cellular subpopulations that circulating EVs may reflect more accurately than small numbers of accessible CTCs. While circulating EVs contain a large majority of host cell-derived "normal" vesicles (> 90%) emanating from platelets, blood cells and other sources (Arraud et al. 2014), in many instances, the content of tumour EVs and their associated diagnostic molecular cargo is amenable to biomarker exploitation. The turnover of EVs in blood is relatively rapid (minutes to hours) (Lázaro-Ibáñez et al. 2021) resulting in dynamic changes in their composition that may reflect disease progression, or effects of therapy (e.g. surgical debulking) (Chennakrishnaiah et al. 2018). Moreover, the external lipid bilayer enables protection of the intraluminal EV cargo (e.g. RNA), a factor that may improve sample storage and analytical stability. Unlike ctDNA, which can often be traced to breakdown of dying cancer cells, EV/EPs originate mostly from viable cells, and they reflect their molecular composition and function in a more direct and comprehensive fashion. Each EV contains several macromolecules of potential diagnostic interest including proteins, lipids, metabolites and nucleic acids (RNA biotypes, genomic or mitochondrial DNA). This molecular complexity fundamentally separates EVs from other traditional molecular biomarkers in cancer,

such as PSA, CEA, CA125 or CA19-9. Indeed, each EV non-randomly combines several molecular traits potentially informative as to the identity, state, oncogenic status and function of the originating cell.

Of particular interest is the ability of EVs to carry specific indicator molecules with a causal role in cancer progression. This may be exemplified by the oncogenic EGFRvIII in glioblastoma (Al-Nedawi et al. 2008), MET receptor in melanoma (Peinado et al. 2012), and multiple other oncogenic and mutant cancer-specific molecular entities (Choi et al. 2017). Multiplexing these traits by inclusion of stem cell markers, effectors of metastasis (Hoshino et al. 2015), drug resistance indicators (Garnier et al. 2018), or other informative sets of proteins and nucleic acids offers a considerable analytical and diagnostic depth (Shao et al. 2012, 2015, 2018). Moreover, some of the functionally meaningful dynamic switches in the EV/EP profiles, as exemplified by those observed during epithelial-to-mesenchymal transition (Garnier et al. 2012) or induced differentiation (Spinelli et al. 2018) in culture, may also, at least in theory, offer valuable insights as to the corresponding processes in vivo. Thus, the unique biological properties of EVs/EPs may offer significant advantages in the context of liquid biopsy in cancer (Liang et al. 2021), at the time when the field enters the realm of clinical trials and possible large-scale translation (Hanjani et al. 2022; Margolis et al. 2022).

## Towards Molecular "Hologram" of Cancer

In spite of the growing enthusiasm that currently surrounds EVs/EPs studies, relatively few candidate biomarkers have emerged in this domain over the past decade (Al-Nedawi et al. 2008; Graner et al. 2009; Hanjani et al. 2022; Liang et al. 2021; Margolis et al. 2022; Skog et al. 2008). In fact, studies aiming at the exploitation of circulating EVs/EPs as carriers of pre-determined or correlatively defined molecular signatures of cancer cells were often confronted with limitations of sensitivity, specificity and reproducibility of the respective assays (Hanjani et al. 2022). These hurdles may be potentially linked to pre-analytical variables and the fluctuation of the signal itself (Coumans et al. 2017). However, at least some of these obstacles could also be attributed to an intrinsic difficulty associated with detection, or purification of desired EV subsets, or EV-associated specific signals, often present at low abundance and 'diluted' in the background noise of myriads of "irrelevant" EVs/EPs present in complex biological fluids, such as blood.

As a corollary to this point, it should be mentioned that the isolation methods currently in use to purify EVs/EPs are based on empirical experiences and/or theoretical assumptions that are not free from certain biases impacting the nature of the resulting isolates (Clos-Sansalvador et al. 2022; Royo et al. 2020; Sidhom et al. 2020). These variations may affect downstream analysis of the molecular cargo, diagnostic utility and functional properties of EV/EP preparations and may lead to misplaced generalizations and distorted subpopulation patterns. For example, ultracentrifugation may co-purify EVs, EPs and molecular aggregates (Jeppesen et al. 2019), while size exclusion chromatography restricts the isolates to a presumed relevant range of EV sizes, to the exclusion (!) of other EV subsets (Sidhom et al. 2020). Density separation may favour buoyant EVs over solid EPs, while chemical precipitation combines EVs, EPs and impurities (Royo et al. 2020). An in-depth analysis of these important questions can be found in the recent literature (Clos-Sansalvador et al. 2022; Jeppesen et al. 2019; Kowal et al. 2016; Royo et al. 2020) including community guidelines (Théry et al. 2018; Witwer et al. 2021).

While efforts are underway to improve the detection of specific EV/EP subsets, alternative "philosophies" could also be considered. For example, instead of separating the desired EVs/EPs from their "irrelevant" counterparts, one might ask whether a more holistic landscapes of EV/EPs carry additional informative (e.g. diagnostic) value. This is because, as discussed in prior sections, the malignant process engulfs multiple tissues and affects multiple non-transformed cellular populations, both locally and systemically. Single cell sequencing datasets reveal multiple changes in gene expression profiles in stromal, immune, endothelial, and other cells associated with cancer, a notion of emerging and considerable significance (Goveia et al. 2020; Wang et al. 2017). Indeed, paraneoplastic alterations of platelets, blood cells, adipose tissue, nervous system cells and other compartments are increasingly appreciated as functionally relevant components of cancer-related pathology (Pelosof and Gerber 2010). It could be speculated that EVs/EPs harbouring fingerprints of these wide-spread alterations may form unique patterns accessible in biofluids, where intrinsic molecular complexities of individual EVs/EPs could act as "voxels" in a larger "hologram" of the disease, its cellular constituents and driving processes (Fig. 2).

In a similar manner, as single cell sequencing has opened unprecedented opportunities for grasping the cellular complexity of cancers in their tissue contexts, albeit as a single snapshot, so too multidimensional landscapes of individual EVs/EPs could open a window of opportunity for cancer diagnostics and monitoring, in real time and in real complexity. Arguably, two factors would determine the practicality of such approaches: (i) computational tools, including machine learning, to integrate vast amounts of data EV/EP profiling would potentially generate; (ii) data generation mechanisms involving efficient high-throughput multidimensional molecular profiling capabilities of EV/EP populations at the single particle level. The latter aspect is an area of considerable

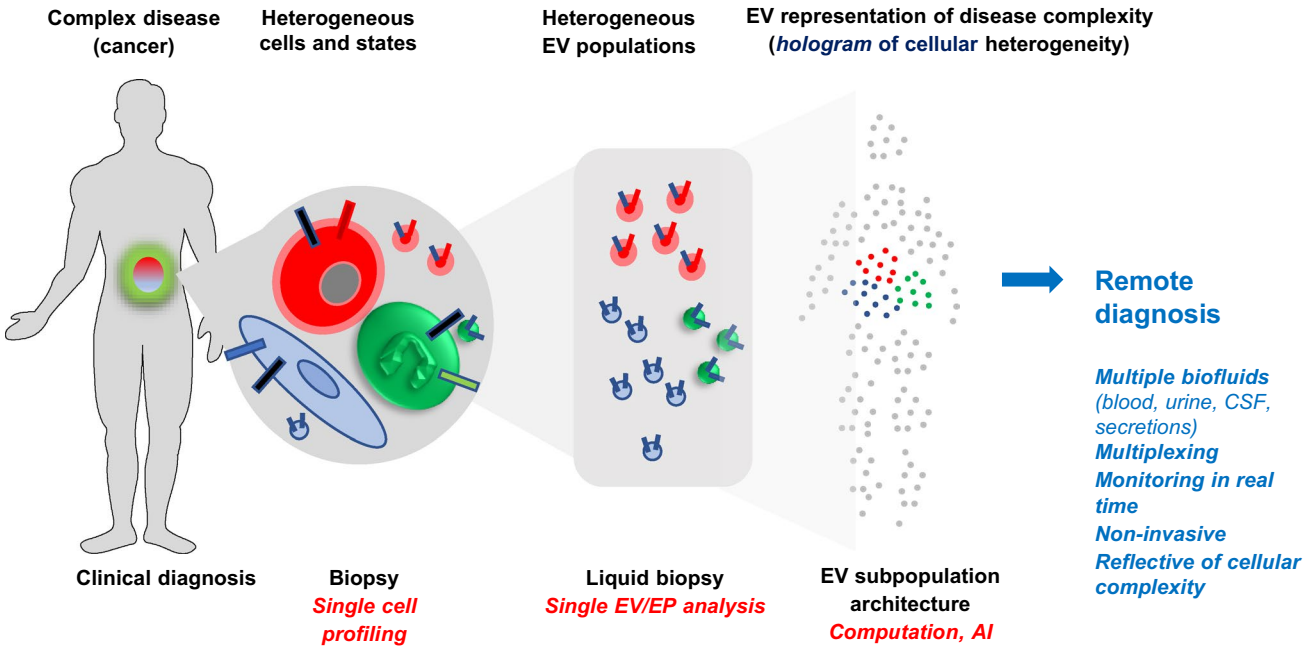


**Fig. 2** The holographic representation of disease complexity by circulating extracellular vesicles and particles. Complex disease states such as cancer comprise the involvement of heterogeneous transformed and non-transformed, but functionally altered cellular populations. These cells are sources of EVs and EPs that may enter the biofluids and could collectively reflect the underlying cellular processes. The intrinsic complexity of individual EVs/EPs positions them as rich sources (molecular 'voxels') of multidimensional information that can be integrated into a more holistic landscapes of the underlying disease process (molecular 'hologram') for biological analysis and diagnostic applications. *AI* artificial intelligence, *CSF* cerebrospinal fluid, *EV* extracellular vesicles, *EP* extracellular particles

interest and activity worthy of a brief commentary (Bordanaba-Florit et al. 2021).

## Single Particle Analysis Technologies

The advent of technologies enabling the analysis of EVs/EPs at the single particle level makes the comprehensive assembly of their landscapes more realistic than ever before. However, major challenges do remain, especially in areas of limited dimensionality, throughput and integration of various analytical read outs. While the various platforms of single vesicle analysis have been recently reviewed in considerable detail (Bordanaba-Florit et al. 2021; Liang et al. 2021; Shao et al. 2018; van der Pol et al. 2012), it may be useful to briefly reflect on some of the highlights that have emerged in this extremely active field.

Indeed, several methods to detect, measure and characterise single EVs either alone or in a population have become cornerstones of current EV/EP experimental work up (Théry et al. 2018). For example, nanoparticle tracking analysis, dynamic light scattering or resistive pulse sensing enable high-throughput recording of individual particles to compute their size distribution and numbers. However, these technologies generally do not capture the intrinsic complexity or molecular features of EVs/EPs. In contrast, transmission electron microscopy (TEM), scanning electron microscopy or cryo-TEM (including immunogold labelling), atomic force microscopy, and, increasingly, different modalities of super-resolution microscopy, such as TIRF, PALM, STORM or SIM, may reveal important structural and molecular features of individual EVs, but at a very low throughput, and with somewhat limited dimensionality (Bordanaba-Florit et al. 2021). In this case, achieving high resolution (voxels) compromises the opportunity to assemble a comprehensive landscape of EVs/EPs (hologram).

An encouraging compromise between these priorities is emerging through improvements in several traditional high-throughput technologies. For example, the increased accessibility of high-resolution nano-flow cytometry (nFC) (Stoner et al. 2016; van der Vlist et al. 2012; Welsh et al. 2020b) has enabled mapping the subpopulations of EVs and EPs as they are released from cancer cells in vitro. This entails simultaneous recording of EV/EP sizes and molecular phenotypes (surface receptors, DNA) (Choi et al. 2018, 2019). While tens of thousands of EVs/EPs can be profiled using nFC, and their preparative sorting has already been successfully attempted (Higginbotham et al. 2016), multiple limitations do remain. They include a relatively high electronic background of nFC instruments, noise related to fluorescent probes (e.g. antibody aggregates), drop in reliability of particle

detection below 80 nm–100 nm in size, detection artefacts (e.g. swarm effect), limited molecular dimensionality restricted to a handful of targets, and inaccessibility of structural information regarding individual EVs (Choi et al. 2019). While the latter challenge may be overcome by imaging flow cytometry (Droste et al. 2021), a major effort is still required to improve rigour, reproducibility, standardization and data reporting for these complex technologies (Welsh et al. 2020a).

Technologies predicated on highly efficient microfluidic workflows may offer new opportunities in multidimensional EV/EP profiling. During the past decade, platforms predicated on antibody-based capture of specific EV populations and their analysis for multiple protein and RNA signals have been explored (Shao et al. 2012, 2015), including platforms recently commercialized (NanoView). Single EV analysis (SEA) represents further improvement of this approach with multiple fluorescent probes being detected sequentially and integrated across the population of dispersed and immobilized EVs, resulting in maps of their distinctive subsets. In contrast, ExoScreen exploits the close distance interaction between nano-beads binding to individual EVs in suspension (Shao et al. 2018; Shao et al. 2015). Several non-fluorescent physical effects have been explored in the context of single EV detection, including electrochemical signals, surface plasmon resonance analysis and surface-enhanced Raman spectra (SERS) (Shao et al. 2018). SERS may utilize antibodies for signal enhancement, or act as a label-free platform that registers unique profiles of spectral peaks (Shao et al. 2018). These peaks form patterns corresponding to molecular traits of individual EVs and can separate fingerprints of cancer EVs vs those of normal cells (Jalali et al. 2021). In many of these instances, the use of patterned plasmonic materials, accurate size detection systems and enhanced data analysis using machine learning algorithms enables high-throughput definition of EV populations (Hosseini et al. 2021; Jalali et al. 2021). Again, it should be kept in mind that in addition to analytical improvements still underway also the pre-analytical/preparatory phase of EV/EP isolation is of considerable importance, as a potential source of biases resulting from the aforementioned peculiarities of currently used EV/EP isolation techniques (Clos-Sansalvador et al. 2022). Arguably, development of methods to analyse EVs/EPs in unprocessed biofluids may be a worthy objective on the path to recover complete EV/EP profiles in cancer. While multiple challenges still do exist, a combination of various platforms may, in a not too distant future, enable construction of data-rich comprehensive single EV/EP landscapes for biological research and diagnostic purposes in cancer and beyond.

## Perspective

As the new biology of EVs/EPs sweeps through the cancer research field, it is becoming increasingly clear that biologically relevant events extend beyond the cellular-to-soluble phase paradigm. Rather, relevant events occur, and information is contained and exchanged, via supramolecular structures that comprise the particulate secretome. This should come as no surprise, as processes central to life and disease occur in distinct subcellular physical microdomains (organelles, membranes, lipid rafts (Sezgin et al. 2017), liquid–liquid phase separation sites (Shin and Brangwynne 2017)) where the proximity of molecular players within the cell define their biochemical activity. This may, at least to some extent, be true outside of the cell, as well, and between the cells that trade large amounts of biological material during the exchange of EVs/EPs. This aspect has been largely overlooked in the past, as EVs/EPs were in the blind spot of conventional methodology, being too complex for biochemical purification and to small for standard microscopy to observe. This is beginning to change due to the advent of new technologies and methods of computation.

While comprehensive landscapes of EVs/EPs discussed here are of great diagnostic interests, as representations of multiple cells affected by the malignant process (albeit in different ways), their multidimensional 'shapes' may also have functional consequences. This is because, while some EVs/EPs may exert defined effects on specific cells with which they interact (e.g. transfer mutant oncogenes), the sum of weaker and diverse influences of other EV/EP populations, and impacting one or many cellular targets may also be biologically relevant.

Moreover, while some aspects of EV-EV competition are gradually emerging in the literature (Adnani et al. 2022a), such interactions (cooperation, competition, modulation) are likely to be more common. Furthermore, EV effects in the context of soluble factors are of considerable interest (Szabó et al. 2014) and may intersect with a larger EV/EP landscape, as a part of the web of communication between cellular populations in cancer. Indeed, there is still much to be learnt about the intercellular EV/EP regulatory network. As the field progresses, it could be expected that integrative analysis of the particulate milieu may shed new light on the important fundamental and biomedical questions in cancer and beyond, including the complexities reflected by myriads of molecular voxels the combined meaning of which is still to be grasped more fully.

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**Data availability** This article is a commentary and contains no data that fall under data availability policies.

## Declarations

**Conflict of Interest** Authors declare no competing interests.

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