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Extracellular vesicles as a novel source of biomarkers in liquid biopsies for monitoring cancer progression and drug resistance

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ABSTRACT

Cancer-derived extracellular vesicles (EVs) have been detected in the bloodstream and other biofluids of cancer patients. They carry various tumor-derived molecules such as mutated DNA and RNA fragments, oncoproteins as well as miRNA and protein signatures associated with various phenotypes. The molecular cargo of EVs partially reflects the intracellular status of their cellular origin, however various sorting mechanisms lead to the enrichment or depletion of EVs in specific nucleic acids, proteins or lipids. It is becoming increasingly clear that cancer-derived EVs act in a paracrine and systemic manner to promote cancer progression by transferring aggressive phenotypic traits and drug-resistant phenotypes to other cancer cells, modulating the anti-tumor immune response, as well as contributing to remodeling the tumor microenvironment and formation of pre-metastatic niches. These findings have raised the idea that cancer-derived EVs may serve as analytes in liquid biopsies for real-time monitoring of tumor burden and drug resistance. In this review, we have summarized recent longitudinal clinical studies describing promising EV-associated biomarkers for cancer progression and tracking cancer evolution as well as pre-clinical and clinical evidence on the relevance of EVs for monitoring the emergence or progression of drug resistance. Furthermore, we outlined the state-of-the-art in the development and commercialization of EV-based biomarkers and discussed the scientific and technological challenges that need to be met in order to translate EV research into clinically applicable tools for precision medicine.

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; AKT, RAC-α serine/threonine-protein kinase; ALIX, programmed cell death 6-interacting protein; AQP2, Aquaporin-2; AR-V7, androgen receptor splice variant 7; ATG12, autophagy related 12 protein; AUC, area under the curve; BMSCs, bone marrow stromal cells; CAF, cancer-associated fibroblast; CAGC, compound annual growth rate; CDC, complement-dependent lysis; CE-IVD, certified in vitro diagnostic medical device; CRC, colorectal cancer; CTCs, circulating tumor cells; ctDNA, circulating tumor DNA; ctRNA, circulating tumor RNA; DNMT1, DNA methyltransferase 1; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERK, extracellular-signal-regulated kinase; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; FasL, Fas ligand; GEM, gemcitabine; GPC1, glypican-1; GSTP1, Glutathione S-transferase P; HER2, human epidermal growth factor receptor 2; hnRNP, heterogeneous nuclear ribonucleoprotein; hnRNPA2B1, heterogeneous nuclear ribonucleoproteins A2/B1; HNSCC, head and neck squamous cell carcinoma; HSP90AB1, heat shock protein HSP 90-beta; IFN-γ, interferon- γ; ILV, intraluminal vesicle; IP, intellectual property; IPO, initial public offering; ISEV, International Society for Extracellular Vesicles; ISO, International Organization for Standardization; ITCH, E3 ubiquitin-protein ligase Itchy homolog; KRAS, Kirsten rat sarcoma viral oncogene homolog; LDT, laboratory development tests; lncRNA, long non-coding RNA; MAPK, mitogen activated protein kinase; MDR, multidrug resistance; mdr1, multidrug resistance protein 1 or P-gp1; MHC-II, major histocompatibility complex II; miRNA, micro RNA; mRNA, messenger RNA; MRP1, multidrug resistance-associated protein 1; MSCs, mesenchymal stem cells; MUC1, Mucin-1; MVB, multivesicular bodies; ncRNA, non-coding RNA; Ndfip1, NEDD4 family-interacting protein 1; NEDD4, E3 ubiquitin-protein ligase NEDD4; NK cells, natural killer cells; NSCLC, non-small cell lung cancer; OS, overall survival; PD-1, programmed death-1 receptor; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed death-ligand 1; PFS, progression-free survival; P-gp, P-glycoprotein; PI3K, phosphatidylinositol 3-kinase; PKM2, pyruvate kinase type M2; PR, progesterone receptor; PS, phosphatidylserine; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; PTEN, phosphatase and tensin homolog; PTMs, posttranslational modifications; R&D, research & development; RBP YBX1, RNA-binding protein Y-box protein 1; RBPs, RNA binding proteins; RISC, RNA induced silencing complex; TGF-β1, transforming growth factor beta 1; TME, tumor microenvironment; Treg, regulatory T cells; tRNA, transfer RNA; TRPC5, transient receptor potential channel 5; TSG101, tumor susceptibility gene 101 protein; UCH-L1, ubiquitin C-terminal hydrolase L1; XPO5, exportin-5

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1. Introduction

Resistance of cancer cells to chemotherapeutic agents, molecular targeted therapies or immunotherapy, may be either intrinsic or acquired (Gottesman, 2002; Gottesman et al., 2016; Kalbasi and Ribas, 2019; Kelderman et al., 2014; Assaraf et al., 2019) and is often ultimately responsible for cancer patient's reduced survival (Holohan et al., 2013). Some tumors present multidrug resistance (MDR), having cross-resistance to various anticancer drugs (Gottesman, 2002). Causes of drug resistance (Housman et al., 2014) may be categorized as host factors, tumor factors and tumor-host interactions (Alaoui-Jamali et al., 2004; Alfarouk et al., 2015; Assaraf et al., 2019). Host factors include genetic variants and drug-drug interactions (Mukerjee et al., 2018; Riechelmann and Del Giglio, 2009; Riechelmann et al., 2007). Tumor factors include mostly alterations in drug targets (Yaghmaie and Yeung, 2019), activation of prosurvival pathways (Indran et al., 2011), decreased intracellular drug concentration (caused by decreased drug influx, increased efflux, or drug sequestration in intracellular vesicles and compartments) (Goler-Baron and Assaraf, 2011; Gonen and Assaraf, 2012; Gong et al., 2013; Peetla et al., 2013; Robey et al., 2018), enhanced DNA damage repair (Buys et al., 2017; Sakthivel and Hariharan, 2017), epigenetic alterations (Brown et al., 2014; Kagohara et al., 2018) and/or deregulation of microRNAs (An et al., 2017), intratumor heterogeneity and dynamics (Burrell and Swanton, 2014; Turajlic et al., 2019), as well as the presence of cancer stem cells (Doherty et al., 2016; Mansoori et al., 2017; Najafi et al., 2019; Turdo et al., 2019). Tumor-host interactions refer to interactions of the tumor cells with the tumor microenvironment (TME) and the intracellular transfer of traits mediated by extracellular vesicles (EVs) (Junttila and de Sauvage, 2013; Namee and O'Driscoll, 2018; Samuel et al., 2017; Wu and Dai, 2017).

The ability to frequently monitor cancer progression and early diagnose tumor drug resistance could contribute to informed clinical decisions and personalized treatment of cancer. This may be possible with liquid biopsies, given that liquid biopsies might allow to survey tumor heterogeneity and clonal evolution (Babayan and Pantel, 2018; Liebs et al., 2019). The term "liquid biopsy" refers to a test performed on a sample of biofluid including blood or urine aiming to detect cancer cells or cancer-derived molecules (Babayan and Pantel, 2018). Liquid biopsies are obtained by non-invasive or minimally invasive means that allow serial sampling, therefore they have a potential utility for the detection of minimal residual disease (MRD) or recurrence, tracking tumor evolution and predicting the emergence of chemoresistance in solid tumors and hematological malignancies. Currently, the most widely studied analytes of liquid biopsies are circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and circulating tumor RNA (ctRNA) (Babayan and Pantel, 2018; Kawaguchi et al., 2016). Some of the CTC and ctDNA tests, such as CTC enumeration by CellSearch® technology or Cobas EGFR Mutation test have been approved by the regulatory authorities and are available for clinical use. However, there are many scientific, technological and regulatory challenges that still need to be overcome in order to introduce liquid biopsies into the standard clinical workflows (Ossandon et al., 2018; Reimers and Pantel, 2019).

The EVs have recently emerged as novel analytes for liquid biopsies. The term "EVs" refers to all kinds of particles naturally released from cells that are delimited by a lipid bilayer and cannot replicate (Thery et al., 2018). Various subtypes of EVs differ in their biogenesis, size and physical properties, molecular composition and functions in the body. In live cells, the main pathways for EV biogenesis are the release of the intraluminal vesicles of multivesicular endosomes in the extracellular space and direct budding from the cell surface. EVs of endosomal origin are called exosomes and the majority of them range between 30–150 nm in diameter, while vesicles derived by budding of plasma membrane may reach 1000 nm in diameter and have been referred to as microvesicles, ectosomes, shedding vesicles or microparticles (Colombo et al., 2014; Vestad et al., 2017). Apoptotic cells can also release a variety of EVs by blebbing of apoptotic membrane, formation of membrane protrusions such as microtubule spikes, apoptopodia, and beaded-apoptopodia. Apoptotic cell-derived EVs are commonly referred to as apoptotic bodies, and the majority of them are in size range from 1 to 5 μm in diameter, though the formation of smaller vesicles during the progression of apoptosis has also been reported (Caruso and Poon, 2018). Although the mean size of various EV subtypes is different, accurate separation of EV subtypes based on the size, biochemical properties or surface markers is currently not feasible. Therefore, the International Society for Extracellular Vesicles (ISEV) recommends using operational terms for EV subtypes such as size, density, marker profile etc., instead of using terms like exosomes or microvesicles, unless their origin is clearly established (Thery et al., 2018).

EVs are released by virtually all cell types in the body and they have been found in various biofluids, including blood, urine, saliva, bile and cerebrospinal fluid (Murillo et al., 2019; Yanez-Mo et al., 2015). EVs contain various lipids (Skotland et al., 2017), proteins (Vagner et al., 2019), metabolites (Royo et al., 2019), mRNA fragments and non-coding RNAs (Turchinovich et al., 2019) and even DNA fragments (Vagner et al., 2018). Importantly, EVs contain molecular signatures reminiscent of their cell of origin (Broggi et al., 2019; Platko et al., 2019). EVs isolated from cancer patients' biofluids have been shown to contain cancer-associated molecules such as amplified oncogenes, oncoproteins, specific miRNA signatures, and mutated mRNA or DNA fragments (Al-Nedawi et al., 2008; Broggi et al., 2019; Garcia-Silva et al., 2019; Lazaro-Ibanez et al., 2014). Moreover, it is increasingly recognized that cancer-derived EVs contribute to cancer progression via transferring phenotypic traits among cancer cells and mediating crosstalk with the TME, pre-metastatic niche and immune system (Becker et al., 2016; Whiteside, 2017b). These findings have raised the idea that the analysis of molecular content of EVs could inform about the presence, molecular profile and behavior of cancer and therefore they could serve as liquid biopsies. In this review, we summarize studies investigating EVs or their cargo as biomarkers for tracking cancer dynamics and predicting drug resistance and discuss the advantages, limitations and challenges of exploiting EVs as liquid biopsies of cancer.

2. EV levels in biofluids – a marker on its own?

There is conflicting evidence regarding the levels of EVs in cancer patients as opposed to healthy controls (Cappello et al., 2017; Xu et al., 2018). Some studies show that cancer patients have increased EV levels in their blood (Alegre et al., 2016; Caivano et al., 2015; Duijvesz et al., 2015; Logozzi et al., 2009; Matsumoto et al., 2016; Nawaz et al., 2014; Ogorevc et al., 2013; Rodriguez Zorrilla et al., 2019). Moreover, several studies have shown that levels of plasma EVs may be associated with residual disease, disease progression, therapy failure (Konig et al., 2017), or with prognosis and/or survival (Silva et al., 2012). On the contrary, other studies show that there is no statistical difference in EV plasma levels between cancer patients and healthy individuals (Menck et al., 2017; Peinado et al., 2012). These conflicting results may be due to the lack of technical standardization between laboratories, including differences in EVs isolation and quantification protocols.

Furthermore, it is not clear what is the cellular source of EVs found in cancer patients' blood. Some studies have shown that the levels of circulating EVs were significantly reduced in the postoperative compared with preoperative plasma samples suggesting that the presence of tumor was at least partially responsible for the increased levels of EVs in patients' plasma (Osti et al., 2019; Rodriguez Zorrilla et al., 2019). However, the increased levels of EVs found in some studies may result from a systemic response to the disease and not to the disease itself or from a systemic response to treatment. Indeed, it has been shown that photodynamic therapy or chemotherapy increased the release of EVs both *in vitro* and *in vivo* in tumor-bearing mice (Aubertin et al., 2016) and that radiotherapy increased the release of EVs in cancer cell lines

(Mutschelknaus et al., 2016). It is also known that plasma has many EVs released from platelets, whose levels vary in many diseases, and therefore platelet-derived EVs may be interfering with the interpretation of plasma EV levels (Heijnen et al., 1999; Tao et al., 2017). Moreover, increased EV levels in the blood have been found in patients with various other diseases including ischemic stroke (Chiva-Blanch et al., 2016), atrial fibrillation (Mork et al., 2019), coronary heart disease (Cui et al., 2013), diabetes (Ogata et al., 2005) and diabetic kidney disease (Rodrigues et al., 2018), pre-eclampsia (Dragovic et al., 2013) and active Crohn's disease (Leonetti et al., 2013), as well as in healthy individuals during exercise (Brahmer et al., 2019; Chaar et al., 2011) or pregnancy (Dragovic et al., 2013; Sarker et al., 2014). Thus, increased release of EVs appears to be a common feature of many diseases and is likely to be triggered by common stress factors.

One of the factors that is well known to stimulate EV release from various cell types including cancer cells is hypoxia (Lowry and O'Driscoll, 2018). For example, hypoxia-resistant multiple myeloma cells released more EVs than their parental cells under normoxia (Umezu et al., 2014). Exposure of breast (King et al., 2012; Wang et al., 2014b), ovarian (Dorayappan et al., 2018) and colorectal cancer cells (Endzelins et al., 2018) as well as cardiomyocytes (Chistiakov et al., 2016) and mesenchymal stem cells (MSCs) (Bian et al., 2014) to hypoxia increased the release of EVs, suggesting that the release of EVs is a common hypoxia response, but its biological significance is likely to be different in various contexts.

Other factors that have been associated with increased EVs release are endoplasmic reticulum stress (Kanemoto et al., 2016), increase in intracellular Ca^{2+} levels (Savina et al., 2003), autophagy (Bhattacharya et al., 2014; Kumar et al., 2015), thermal and oxidative stress (Hedlund et al., 2011), tumor acidity (Logozzi et al., 2018; Parolini et al., 2009) or treatment with chemotherapy of photodynamic therapy (Aubertin et al., 2016). The release of EVs by cancer cells may also be regulated by cellular intrinsic mechanisms, such as activation of oncogenic pathways including EGFRvIII or H-RASV12 (Al-Nedawi et al., 2008; Lee et al., 2014), or by pyruvate kinase type M2 (PKM2), an enzyme typically associated with tumor cell dependence on aerobic glycolysis (Wei et al., 2017b).

Collectively, these data show that increased levels of plasma EVs is not a cancer-specific phenomenon and suggest that many cell types release EVs in response to various stress cues. However, it is yet unclear whether different stimuli induce the release of distinct EV subpopulations. Thus, further research to characterize EV subpopulations released by various cell types upon different stimuli and novel technologies for the isolation of cancer-derived EVs from the pool of EVs present in human biofluids are needed, in order to clarify the value of EV levels as cancer biomarkers.

3. Sorting of molecular cargo into EVs

One of the main features that makes EVs particularly attractive as analytes for liquid biopsies is that tumor-derived EV's cargo bares a strong pathological resemblance to the intracellular status of the cancer cell of origin (Baran et al., 2010; Rabinowits et al., 2009; Szajnik et al., 2013; Tanaka et al., 2013). However, several recent studies suggest that the sorting of molecular cargo into EVs is a regulated process leading to the enrichment or depletion of EVs in specific nucleic acids, proteins or lipids (Cha et al., 2015; Gangoda et al., 2017). Conceivably, these sorting mechanisms modulate the physiological and pathological effects elicited by EVs both in recipient cells and parental cells. Moreover, the existence of specific sorting mechanisms implies that the levels of some molecules in the cells and their EVs are not directly related. Thus, some biomarkers that are highly expressed in tissues may not be detectable in EVs and *vice versa* – deregulation of cargo sorting mechanisms in cancer cells may lead to the enrichment of EVs with molecules that are not overexpressed in cancer cells, thus rendering them useful biomarkers. Here, we will discuss the current understanding of mechanisms of sorting of molecular cargo into EVs.

3.1. Sorting of proteins

ExoCarta and Vesiclepedia databases show that the total number of different proteins identified in EVs represents almost half of the human proteome. Typically, the protein cargo of EVs is representative of the proteome of the cell of origin (Boukouris and Mathivanan, 2015), however, in some cases it can be distinct. Moreover, different types of EVs are enriched in different sets of proteins. For example, small EVs are enriched in proteins that are involved in exosome biogenesis, such as tetraspanins CD9, CD63 and CD81, ESCRT (endosomal sorting complex required for transport) proteins like TSG101 as well as syntenin-1 and ADAM10, while large EVs were found to be enriched in actinin-4 and mitofilin (Kowal et al., 2016). Several recent studies have identified various posttranslational modifications (PTMs) that are involved in protein sorting into EVs. One of the most studied PTMs that is essential for sorting protein cargo into exosomes is ubiquitination. A ubiquitin tag is recognized by ESCRT machinery and serves as a signal for the sorting of proteins into intraluminal vesicles (Foot et al., 2017). Ubiquitination is responsible for sorting of many proteins, including major histocompatibility complex II (MHC-II) and oncogenic protein EGFR (Gauvreau et al., 2009; Schmidt and Teis, 2012; Trajkovic et al., 2008). Another PTM known to alter protein localization in cells is SUMOylation (Gareau and Lima, 2010). For example, SUMOylation is required for loading RNA binding proteins (RBPs) (Villarroya-Beltri et al., 2013) and α-Synuclein (a neurotoxic protein that plays a role in the pathogenesis of Parkinson's disease) into EVs (Kunadt et al., 2015). Several other studies suggested that phosphorylation may play a role in loading specific proteins into EVs (Gonzales et al., 2009; Liem et al., 2017; Valapala and Vishwanatha, 2011; Zuccato et al., 2007). For example, phosphorylation of Tyr23 is required for incorporation of Annexin A2 into the membrane of EVs (Valapala and Vishwanatha, 2011), while phosphorylation of FasL at the proline-rich domain is required for sorting into the EVs (Zuccato et al., 2007). Furthermore, there is some evidence that oxidation could play a role in EV cargo sorting. For example, γ-Synuclein oxidized in Met38 and Tyr39 positions are sorted into EVs, while NADPH oxidase stimulation facilitates the release of galectin 3-containing EVs from human monocytes EVs in atherosclerosis patients (Madrigal-Matute et al., 2014; Surgucheva et al., 2012). However, more studies are needed to confirm these findings since the oxidation of proteins can occur in sample processing. Another study demonstrated that microvesicles are enriched with mannose, polylactosamine and other complex *N*-linked glycans compared to the cells of origin, suggesting that microvesicles emerge from specific plasma membrane microdomains and glycosylation may play a role in protein sorting into microvesicles by oligomerization of specific glycoproteins (Batista et al., 2011). Several studies have found citrullinated (a PTM that modifies a protein by altering their positively charged amino acid arginine into citrulline which is devoid of charge) proteins including fibronectin, α2-microglobulin, and fibrinogen fragment D in EVs isolated from synovial fluid of rheumatoid arthritis patients (Cloutier et al., 2013; Skriner et al., 2006), however, it remains unclear whether citrullination is required for sorting of these proteins into EVs.

Apart from PTMs, several protein domains have been implicated in the sorting of proteins into EVs. For example, Ndfip1 recruits WW domain-containing proteins like Nedd4, and Nedd4–2 into EVs. Importantly, Ndfip1 was found to be required for packaging cancer-related protein PTEN (Putz et al., 2012). Another study reported that EVs derived from colorectal cancer cell line LIM1215 were significantly enriched in coiled-coil domain-containing proteins, however, additional functional studies are needed to confirm the role of this domain in protein sorting (Mathivanan et al., 2010).

3.2. Sorting of RNA

It is well known that EVs contain different types of RNAs that are functionally active in the recipient cells, thus mediating intercellular communication and that RNA sorting is a regulated process (Alexander et al., 2015; Challagundla et al., 2015; Mittelbrunn et al., 2011; Ratajczak et al., 2006; Valadi et al., 2007). One of the proposed RNA sorting mechanisms involves RNA induced silencing complex (RISC). RISC proteins, including AGO2 and GW182, are located in MVBs and are involved in MVBs turnover, suggesting that these proteins could be involved in miRNA sorting (Gibbings et al., 2009). Another proposed mechanism involves ceramide pathway. Inhibition of neutral sphingomyelinase-2 (nMase-2) - a protein involved in ceramide pathway-dependent EVs biogenesis in HEK293 cells, reduced not only EVs production but also miRNA levels in EVs even after miRNA normalization against EVs quantity, suggesting that ceramide pathway is involved in the loading of miRNAs into EVs (Kosaka et al., 2010). Furthermore, analysis of RNA profiles in macrophages and their EVs showed that miRNA sorting is dependent on the amount of miRNAs and their target sequences in the cytoplasm. The downregulation of individual miRNAs promoted the miRNA relocation from the MVBs to the cytoplasm and the downregulation of the target mRNA promoted the corresponding miRNA relocation from the cytoplasm to MVB (Squadrito et al., 2014). Recently, several studies have shown that RBPs are involved in RNA sorting into EVs. For example, Villarroya-Beltri et al., discovered that 75% of miRNAs present in Jurkat cell-derived EVs had GAGG sequence motif at their 3′ end. This study confirmed that this motif is necessary for miRNA sorting into EVs and hnRNPA2B1 binds to miRNAs in a GAGG motif- dependent manner (Villarroya-Beltri et al., 2013). A similar sorting mechanism also exists for other RNA species like mRNA, Y-RNA, tRNA and Vault RNA (Shurtleff et al., 2017; Statello et al., 2018; Szostak et al., 2014). There is also some evidence that non-template terminal nucleotide additions may play a role in RNA sorting. For example, EVs produced by human B cells are enriched with 3′ uridinylated miRNAs and Y-RNAs (Koppers-Lalic et al., 2014). Finally, another study showed that colorectal cancer cell lines that differ only in *KRAS* mutation status had distinct EV miRNA profile, suggesting that KRAS regulates the composition of EV miRNA content (Cha et al., 2015).

3.3. Sorting of DNA

Unexpectedly, EVs also have been shown to carry fragments of genomic and mitochondrial DNA. Depending on the experimental conditions and cell type, single or double-stranded DNA fragments have been found either in the lumen or on the surface of EVs (Balaj et al., 2011; Guescini et al., 2010; Lazaro-Ibanez et al., 2014; Nemeth et al., 2017; Sansone et al., 2017; Thakur et al., 2014). Importantly, EVs have been shown to carry DNA fragments with tumor-derived somatic mutations that can be detected in patients' circulation (Kahlert et al., 2014; Lee et al., 2018). Surface-bound DNA has been shown to facilitate binding of EVs to the extracellular matrix protein fibronectin (Nemeth et al., 2017), while luminal DNA can be transferred to recipient cells and expressed into proteins (Cai et al., 2013; Lee et al., 2014). While the functional significance of EV-enclosed DNA has started to emerge, there is very little and controversial information about DNA sorting into EVs. Several studies have found that cancer cell line-derived EVs contain DNA from all the chromosomes (Kahlert et al., 2014; Lazaro-Ibanez et al., 2014; Thakur et al., 2014). Takahashi et al., recently published that knockdown of EVs production in cancer cells resulted in an increased amount of damaged DNA in the cell cytoplasm (Takahashi et al., 2017). These data suggest that DNA sequence-specific sorting mechanisms do not exist, and EVs play a role in cellular homeostasis by removing damaged DNA from cell cytoplasm in a non-selective manner. On the other hand, Lazaro-Ibanez et al., reported that different EV subpopulations share a unique DNA sequence motif, thus suggesting that specific DNA sorting signal sequence may exist (Lazaro-Ibanez et al., 2014).

3.4. Sorting of lipids

Lipidomic analysis of EVs produced by different cell lines (PC3, U87, Huh7 and MSCs) have highlighted that structural plasma membrane lipids, including phosphatidylcholine, phosphatidylinositol, phosphatidylglycol, and phosphatidylethanolamine were less represented in EVs while phosphatidylserine was enriched in EVs (Haraszti et al., 2016; Llorente et al., 2013). Currently, there is very little information about the sorting of lipids into EVs. However, Haraszti et al., found that lipids are sorted in a cell type-specific manner but protein and lipid sorting into EVs are most likely independent of each other (Haraszti et al., 2016).

In summary, a number of recent studies have identified several sorting mechanisms that contribute to selective enrichment of EVs with specific proteins and RNA molecules, while the process of loading DNA fragments, lipids and metabolites into EVs is less clear. Nevertheless, the ways how cancer cells dysregulate the sorting mechanisms are still poorly understood. Gaining a deeper understanding of the generation of selective molecular cargo is of paramount importance for exploiting EVs as liquid biopsies and therapeutic targets of cancer.

4. EVs as analytes of liquid biopsies

A growing body of evidence suggests that cancer-derived EVs propagate drug-resistant phenotypes and facilitate cancer progression by transferring aggressive phenotypic traits to other cancer cells, modulating the anti-tumor immune response, remodeling the TME and promoting the formation of pre-metastatic niches (Fig. 1). These effects can be triggered by internalization of EVs and release of their cargo into recipient cells, where they initiate various intracellular signaling events, or by binding to cell surface receptors and modulating gene expression via cell surface signaling (French et al., 2017; Whiteside, 2017a).

EVs produced by highly aggressive cancer cells have been shown to promote cancer cell survival, invasion, migration, epithelial-mesenchymal transition (EMT) and acquisition of stem cell-like properties *in vitro* and *in vivo* (Li et al., 2019, 2018; Schillaci et al., 2017; Zeng et al., 2018). Similarly, EVs produced by drug-resistant cancer cells can transfer their phenotype to drug-sensitive cancer cells, thus possibly conferring drug-resistant traits upon the whole tumor (Milman et al., 2019). Multiple studies have shown that EVs secreted by hypoxic cancer cells promote the migration, invasion and spheroid-forming ability of hypoxia-naïve cancer cells and induce angiogenesis and M2 macrophage polarization (Endzelins et al., 2018; Huang and Feng, 2017; Li et al., 2016; Ramteke et al., 2013; Wang et al., 2018b). Exposure of fibroblasts to cancer-derived EVs have been shown to promote the acquisition of cancer-associated fibroblast (CAF) phenotype (Vu et al., 2019; Wang et al., 2018a), induce the secretion of various chemokines, thus contributing to the CAF heterogeneity (Naito et al., 2019) or even induce transformation of repair-defective fibroblasts into cancer cells (Abdouh et al., 2019). Importantly, cancer-derived EVs can promote metastasis not only by reprogramming recipient cell functions locally within the TME, but also systemically by helping to establish pre-metastatic niches in lymph nodes and organ-specific distal sites (Nogues et al., 2018). Organ-specific metastasis is at least partially guided by surface adhesion molecules, such as integrins, carried on the surface of EVs that determine preferential uptake of EVs by specific cell types. For example, integrins α6β4 and α6β1 were associated with lung metastasis, while integrin αvβ5 - with liver metastasis (Hoshino et al., 2015). Hence, it seems likely that the analysis of surface molecules of circulating cancer-derived EVs may help to predict the presence and localization of metastases (Hoshino et al., 2015). Regarding the immune modulation, in some contexts, EVs can deliver activating signals

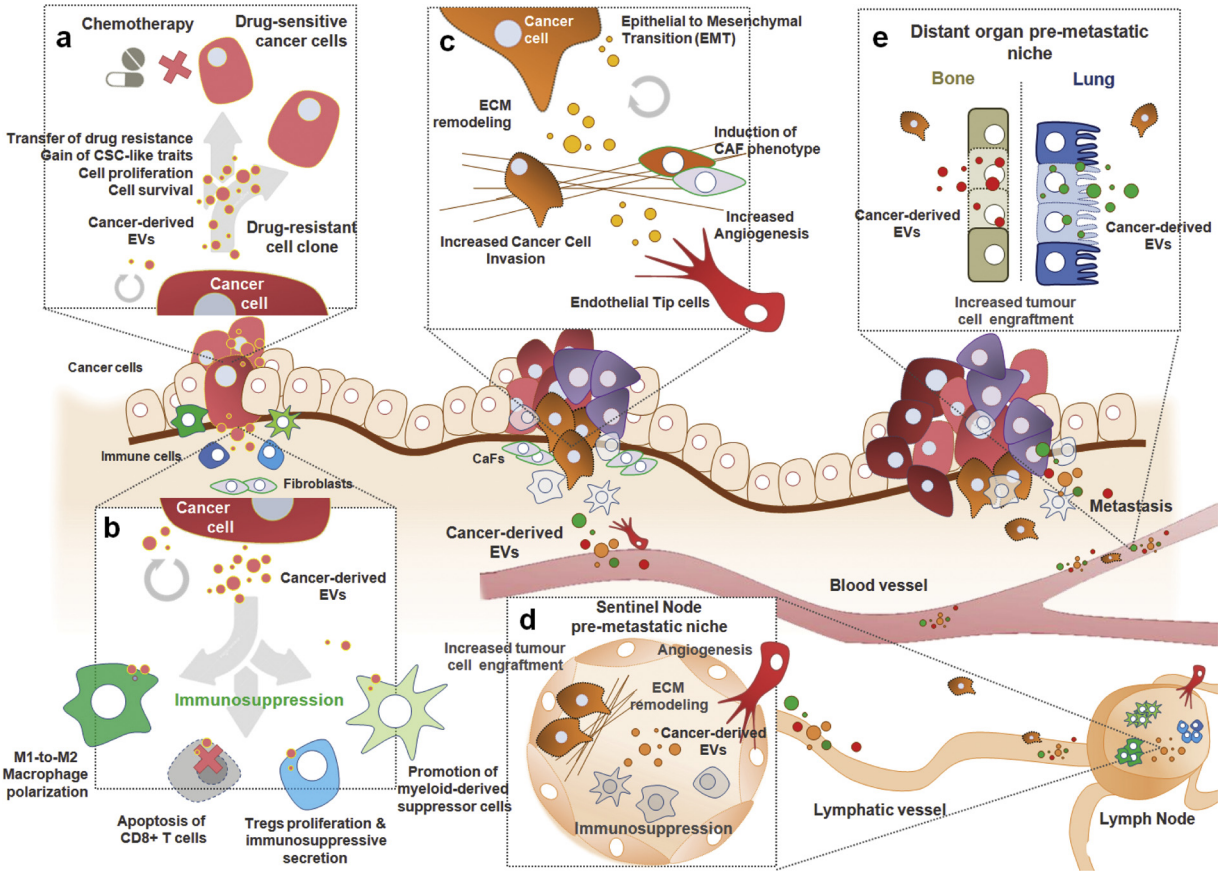

Fig. 1. Contribution of cancer-derived EVs to cancer progression and drug resistance. (a) Transfer of aggressive phenotype from drug-resistant cancer cells to neighboring cells via EV shedding. Aggressive cancer cells release EVs that will induce an aggressive phenotype in the neighboring drug-sensitive cancer cells including increased cell proliferation, resistance to cell death, gain of cancer stem cell traits and MDR. For successful cancer progression, cancer cells must interact with the TME and suppress the incoming anti-tumor immune cells that infiltrate the tumor mass. (b) Cancer-derived EVs can modulate the local immune and/or inflammatory response towards a pro-tumoral one by inducing a M1-to-M2 macrophage polarization, suppressing CD8 + T cell activation and proliferation and/or inducing CD8 + T cell apoptosis, promoting Treg proliferation and secretion of immunosuppressive cytokines and/or enhancing the action of myeloid-derived suppressor cells (MDSCs). (c) EV-mediated tumor-promoting microenvironment will grant the tumor cells the ability to evade the immune system, transform the secretion profile of the underlying fibroblasts into CAFs or even transform them into cancer cells, promote local angiogenesis and acquire an epithelial-to-mesenchymal transition (EMT) phenotype. In turn, this will enhance cancer cells ability to invade adjacent structures and metastasize. As the tumor progresses, a mixture of cancer-derived EVs circulate in the lymphatic's and in the bloodstream (cancer-derived EVs illustrated by different colors). Importantly, these circulating EVs are selectively taken up in the (d) lymph nodes and/or at (e) distant organs such as the lungs or bone, accordingly to their EV-surface integrin specificity (color coded). The cancer-derived EVs carry various factors (including, MMPs, LOX, IL-6) that upon uptake at the corresponding target organs, induce the pre-metastatic niche formation. This is a dynamic process where cancer-derived EVs arrive first and educate the cells in the target organs, thus granting an enhanced engraftment of circulating tumor cells later on.

to T or B lymphocytes, transfer antigens and facilitate DC maturation (Lindenbergh et al., 2019; Ma et al., 2018; Tkach et al., 2017; Xu et al., 2016). However, in the TME, cancer-derived EVs mostly mediate immunosuppression. Cancer-derived EVs have been shown to suppress proliferation and activation of T cells, induce apoptosis in activated CD8 + T cells, modulate the cytokine and chemokine profile secreted by immune cells and promote Treg and myeloid-derived suppressor cell proliferation and suppressor functions (Whiteside, 2017a).

Taken together, these findings have raised the idea that cancer-derived EVs may serve as a source of biomarkers for monitoring cancer progression and predicting drug resistance. The main purpose of cancer progression biomarkers is to detect disease recurrence, progression in metastatic disease and tracking tumor evolution, while drug resistance biomarkers should reflect the emergence or progression of drug resistance during cancer treatment. Indeed, EVs may have some advantages over other analytes exploited in liquid biopsies (Fig. 2).

At first, EVs are highly abundant in various human biofluids and therefore could be expected to represent intratumor heterogeneity better than CTCs. Using EVQuant technology, it has been estimated that cancer cells, on average, produce ~2000 EVs per cell and per hour (G. Jenster, personal communication). Nanoparticle tracking analysis of EVs isolated from human plasma shows that the mean concentration ranges from 1×10^9 to 3×10^{12} particles per ml (Eitan et al., 2017; Endzelins et al., 2017; Kuravi et al., 2017), in urine from 2×10^8 to 1×10^9 particles per ml (Gheinani et al., 2018), while in lymph, from 1×10^{11} to 2×10^{12} particles per ml (Broggi et al., 2019). These numbers, however, are greatly affected by the efficacy of a given EV isolation method, whereas direct quantification of EVs in biofluids is still a technically challenging issue.

Secondly, EVs contain molecular signatures reminiscent of their parental cells and protect their cargo from degradation. Therefore, the analysis of EV-enclosed RNA and DNA may be more informative and reproducible than the analysis of vesicle-free ctRNA or ctDNA. For instance, we compared the diagnostic potential of prostate cancer-associated miRNAs in whole plasma versus EVs isolated from the same sample of plasma and showed that, although only a small fraction of the total cell-free miRNA could be recovered from the EVs, some miRNA biomarkers performed better when analyzed in EVs than in the whole plasma (Endzelins et al., 2017). Interestingly, several studies have also shown that EV-associated DNA is more sensitive source of DNA for the

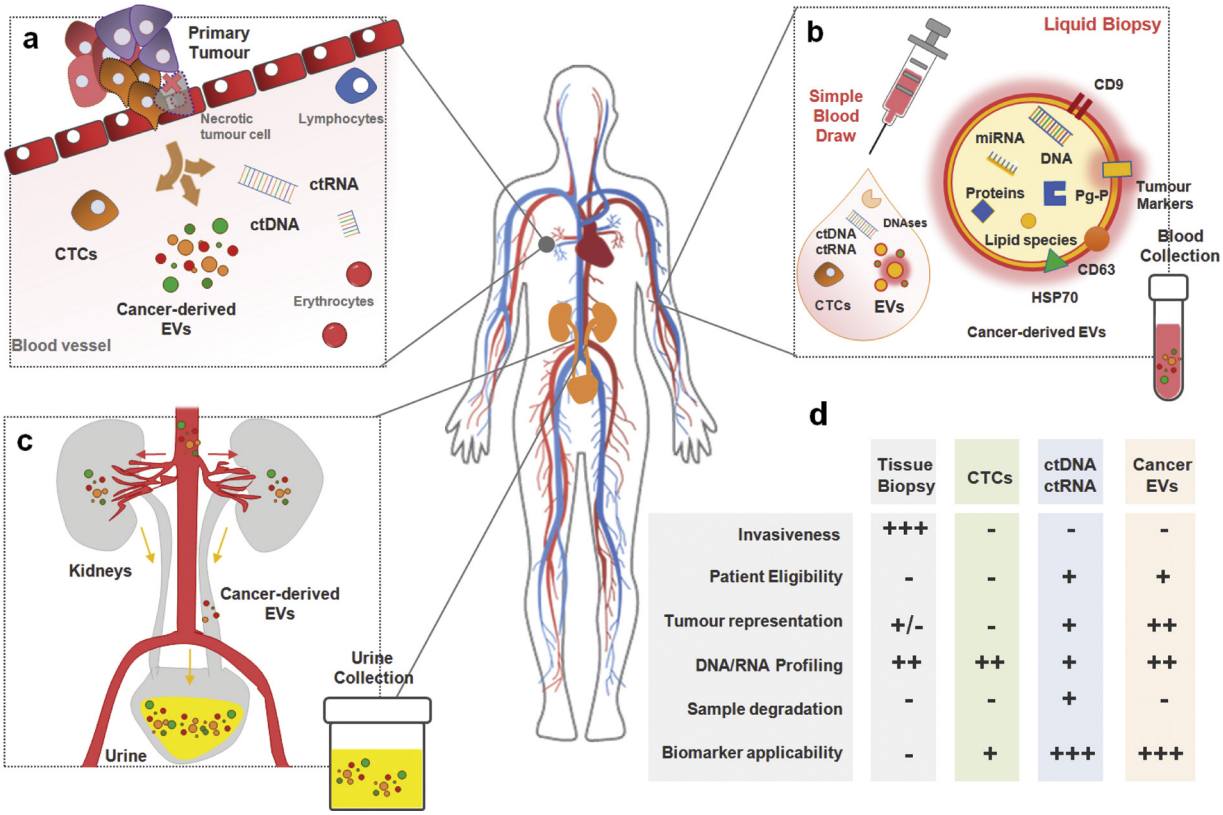

Fig. 2. EV-based analytes of liquid biopsies. (a) At the primary tumor site, living cancer cells release high amounts of EVs into the bloodstream and/or invade into adjacent blood vessels becoming circulating tumor cells (CTCs). Additionally, dying/necrotic cancer cells may release their DNA or RNA content into the bloodstream which will circulate freely in the blood (circulating tumor DNA/RNA, ctDNA/RNA). Importantly, all of these tumor-derived analytes can be easily accessed in liquid biopsies through multiple longitudinal sampling of (b) peripheral blood or (c) urine to interrogate the tumor landscape. In particular, EVs are an advantageous source of cancer biomarkers since they are much more abundant in biofluids than CTCs, protect their molecular cargo against degradation and carry molecular signatures associated with specific cancer phenotypes. (d) Comparative table illustrating the advantages of liquid biopsies (CTCs, ctDNA/RNA and cancer-derived EVs) over the traditional tissue biopsy.

detection of tumor mutations than total circulating cell-free DNA (Allenson et al., 2017; Garcia-Silva et al., 2019; San Lucas et al., 2016). Moreover, several recent studies have demonstrated that cancer-derived EVs carry miRNA and protein signatures associated with specific phenotypes. For example, EVs isolated from the lymphatic exudate of patients with metastatic melanoma were shown to be enriched in a 66 miRNA signature known to be associated with metastatic melanoma and harbor a proteomic signature associated with advanced melanoma (Broggi et al., 2019). Likewise, EVs isolated from lymphatic drainage were enriched in proteins resembling melanoma progression (Garcia-Silva et al., 2019), while EVs isolated from plasma of glioblastoma patients carried a set of proteins involved in inflammation and immune response, growth, survival, migration, as well as regulation of metabolism (Osti et al., 2019).

5. Clinical relevance of EVs for monitoring cancer progression

Despite overwhelming evidence on the contribution of EVs to cancer progression, so far only a limited number of clinical studies have reported EV-associated biomarkers for monitoring cancer progression and tracking cancer evolution. As preclinical studies demonstrating the role of EVs in cancer progression have been recently reviewed in several excellent recent articles (Kikuchi et al., 2019; Milman et al., 2019; Zhang and Yu, 2019), here we focused on longitudinal clinical studies reporting promising EV-associated biomarkers that are derived from tumor tissues and whose dynamics reflect tumor burden or clinical events. The main findings are summarized in Table 1 and discussed below.

5.1. Pancreatic cancer

In 2015, Melo et al., discovered that glypican-1 (GPC1) is a highly specific marker of cancer-derived EVs and the presence of GPC1-positive EVs in sera could distinguish patients with pancreatic ductal adenocarcinoma (PDAC) from healthy controls or patients with benign pancreas diseases with 100% sensitivity and specificity. The levels of GPC1-positive EVs were significantly decreased following surgical resection of the tumor in all patients analyzed (n = 29), correlated with overall survival (OS) and were significantly higher in patients with distant metastasis compared to patients with lymph node metastasis only or no metastases (Melo et al., 2015). To the best of our knowledge, this is one of the first studies demonstrating the relevance of circulating EVs for monitoring tumor burden. Subsequently, an independent study validated the fall in GPC1-positive EV levels in plasma of PDAC patients after surgical resection and demonstrated a correlation between the GPC1 expression in tumor tissues and the level of GPC1 in circulating EVs, while it failed to validate the diagnostic value of GPC1-positive EVs for distinguishing PDAC from benign pancreatic diseases (Frampton et al., 2018).

Giampieri et al., measured the levels of EpCAM-positive EVs in PDAC patients (n = 19) before chemotherapy and after 3 months. This study showed that higher EpCAM-positive EV levels before chemotherapy were associated with shorter progression-free survival (PFS) and OS, whereas the increase in EpCAM-EV levels during treatment was associated with better PFS (Giampieri et al., 2019).

Another interesting paper reported the detection of *KRAS* mutations in circulating EVs in a cohort of 263 PDAC patients and controls.

Table 1
EV-associated biomarkers of cancer progression validated in clinical studies.

Cancer Type	EV biomarkers	Key findings/clinical significance	References
Pancreatic cancer	Protein-based: GPC1-positive EV levels	Decrease after surgical resection, correlated with OS and presence of distant metastases	Melo et al. (2015); Frampton et al. (2018)
	DNA-based: mutant *KRAS*	Drop in KRAS mutation detection rate after surgical resection	Allenson et al. (2017)
		Correlation with response to neoadjuvant therapy	Bernard et al. (2019)
	Protein-based: EpCAM-positive EV levels	Correlation with response to palliative chemotherapy	Giampieri et al. (2019)
Head and neck squamous cell carcinoma	Protein-based: total EV proteins and various cancer-derived and T cell-derived EV subpopulations	Changes during the course of therapy correlating with response to a combinatorial therapy	Theodoraki et al. (2019)
Prostate cancer	Protein-based: PSMA-positive EV levels	Decrease after prostatectomy	Biggs et al. (2016)
	Total EV levels	Increase after radiation therapy	Malla et al. (2018)
	RNA-based:hsa-let-7a-5p	Increase after radiation therapy	
	RNA-based: 57 miRNAs	Significantly altered after carbon ion radiotherapy	Yu et al. (2018)
	RNA-based: miR-654-3p; miR-379-5p	Predict efficacy of radiotherapy	
	Metabolite-based: adenosine, glucuronate, isobutyryl-L-carnitine, D-ribose 5-phosphate	Lower levels in pre- *vs* post-prostatectomy urinary EVs	Puhka et al. (2017)
Breast cancer	Protein-based: TRPC5-positive EV levels	Correlation with response to chemotherapy	Wang et al., 2016
	RNA-based: lncRNA HOTAIR	Decrease after surgery	Tang et al. (2019)
	RNA-based: miR-27a, miR-155, miR-376a, and miR-376c	Decrease after neoadjuvant chemotherapy	Stevic et al. (2018)
	RNA-based: miR-21	Associated with metastatic disease; no correlation with response to neoadjuvant chemotherapy	Rodriguez-Martinez et al. (2019)
Osteosarcoma	RNA-based: mutation burden	Increase in metastatic *vs* primary tumor	Bao et al. (2018)
	RNA-based: transcriptomic profile	Metastasis-associated signature	
Glioblastoma	Total EV levels	Decrease after surgery	Osti et al. (2019)
Melanoma	RNA-based: PD-L1 mRNA	Decrease in responders and increase in non-responders to anti-PD-1 antibodies.	Del Re et al. (2018)
	RNA-based: hsa-let-7g-5p	Increased levels during therapy associated with better response to MAPK inhibitors	Svedman et al. (2018)
	RNA-based: miR-497-5p	High levels during treatment with MAPK inhibitors associated with prolonged PFS	
Non-small cell lung cancer	RNA-based: PD-L1 mRNA	Decrease in responders and increase in non-responders to anti-PD-1 antibodies	Del Re et al. (2018)
Colorectal cancer	RNA-based: lncRNA CRNDE-p	Decrease after chemotherapy	Yu et al. (2017a, b)
	RNA-based: miR-217	Increase after chemotherapy	

Mutations were detected in 7.4%, 66.7%, 80%, and 85% of age-matched controls, localized, locally advanced, and metastatic PDAC patients, respectively. Importantly, in patients with localized PDAC undergoing surgical resection (n = 20), mutant *KRAS* detection rate dropped from 66.7% before surgery to 5% after surgery, suggesting that EV-associated *KRAS* mutations could serve as biomarkers for real-time assessment of response to therapy and tumor burden (Allenson et al., 2017). Indeed, a subsequent study demonstrated that the kinetics of EV-associated *KRAS* mutant allele frequency (MAF) was significantly associated with the response to neoadjuvant chemotherapy - 71% of patients with the absence of disease progression showed a decrease in *KRAS* MAF from baseline values, while 94% of patients who experienced disease progression showed an increase or no change in *KRAS* MAF (Bernard et al., 2019).

5.2. Head and neck squamous cell carcinoma (HNSCC)

A recent study from Theresa L. Whiteside's group demonstrated for the first time, the use of cancer-derived and T cell-derived EVs in the monitoring of HNSCC patients' responses to a combination therapy consisting of surgery followed by cetuximab, ipilimumab and radiation therapy. Total EVs and various EV subsets were isolated from patients' plasma by immunocapture and studied before surgery and at weeks 5 and 14 of therapy. In patients who experienced recurrence (n = 5), total EV protein levels as well as the ratio of cancer-derived to total EVs increased during the therapy, while in patients who remained disease-free (n = 13) in the 2-year follow-up period, the levels decreased relative to pre-therapy levels. The baseline levels and dynamics of various T cell-derived EV subsets – $CD3^+$ EVs, $CD3^+$ $CD15s^+$ EVs (Treg-derived EVs), $CD3^+PDL\text{-}L1^+$ EVs and $CD3^+CTLA4^+$ EVs differed in patients who later responded to therapy and those who recurred, suggesting that the levels of these EV subsets reflect patients' immune response to tumor and could be used for real-time monitoring of response to immunotherapy (Theodoraki et al., 2019).

5.3. Prostate cancer

Biggs et al., analyzed the concentrations of PSMA-positive EVs in plasma of prostate cancer patients (n = 25) collected before and 3 weeks after radical prostatectomy using nanoscale flow cytometry and showed that the EV levels were significantly decreased in the post-operative samples in the majority of patients excluding two patients in which EV levels increased after surgery. Hence, this study demonstrated that the quantification of PSMA-positive EVs could be used for monitoring tumor burden and the effectiveness of resection, however, the clinical significance of the increased EV levels remained unclear (Biggs et al., 2016). Two other studies focused on the changes in EV levels and EV-associated miRNAs during radiation therapy. Malla et al., reported that the total EV concentration in serum of patients undergoing radiation therapy (n = 11) tended to increase after treatment and the level of hsa-let-7a-5p significantly increased in high-risk patients after radiation (Malla et al., 2018). Yu et al., performed miRNA profiling in plasma EVs of 8 patients undergoing carbon ion radiotherapy and showed that the levels of 57 miRNAs were significantly altered after therapy, and two of them - miR-654-3p and miR-379-5p were associated with the efficacy of therapy (Yu et al., 2018). Another interesting study demonstrated that the metabolite content of urinary EVs is changed after prostatectomy and identified 4 metabolites - adenosine, glucuronate, isobutyryl-L-carnitine and D-ribose 5-phosphate, whose levels in the pre-operation samples were 2.5–26.6-fold lower than in the post-operation urine samples (Puhka et al., 2017).

5.4. Breast cancer

Wang et al., found that circulating EVs in peripheral blood from breast cancer patients carried TRPC5 – a protein shown to be essential for the chemoresistance of breast cancer cells. The level of TRPC5-positive EVs before chemotherapy was negatively correlated with chemotherapy outcome – responders had significantly lower levels than non-responders. Moreover, increasing levels during therapy predicted shorter PFS, thus indicating that examining the levels of TRPC5-positive EVs could be used for real-time monitoring of chemoresistance (Wang et al., 2017).

Several recent studies have investigated EV-associated RNA biomarkers in patients undergoing neoadjuvant chemotherapy. One of them studied HOTAIR – a lncRNA that is overexpressed in a variety of cancers and promotes cancer cell proliferation, invasion and migration. The levels of EV-associated HOTAIR were significantly higher in sera from breast cancer patients than healthy individuals and high pre-treatment levels correlated with a poor response to neoadjuvant chemotherapy and tamoxifen hormone therapy. Importantly, the levels of EV-associated HOTAIR markedly decreased in all patients studied (n = 15) three months after surgery as compared with the levels before surgery, suggesting that the source of serum HOTAIR is the tumor tissue and its level is associated with tumor burden and aggressiveness of the disease (Tang et al., 2019). Stevic et al., showed that the levels of 4 EV-associated miRNAs - miR-27a, miR-155, miR-376a, and miR-376c significantly changed after neoadjuvant therapy, however, the sample size (n = 9) was too small to evaluate their association with response to therapy (Stevic et al., 2018). Another study showed that the pre-treatment levels of EV-associated miR-21 and miR-105 were higher in metastatic *vs* non-metastatic breast cancer patients and miR-21 levels correlated with tumor size, however its level during therapy was not significantly associated with the response (Rodriguez-Martinez et al., 2019).

5.5. Osteosarcoma

RNA sequencing of paired EV samples isolated from plasma of three osteosarcoma patients before surgery when only primary tumor was present and upon progression into metastatic disease, revealed a drastic increase of mutation burden in metastatic samples. Metastatic EV samples contained a higher number of point mutations, fusion transcripts and alternative splicing events than non-metastatic samples. Moreover, clustering of gene expression profiles revealed a gene expression signature associated with metastatic disease that was further validated in a public dataset and could predict 5-year survival. Hence, this study demonstrated that plasma EVs carry sufficient amount of mRNAs for tracking transcriptomic alterations during the progression of the disease (Bao et al., 2018).

5.6. Glioblastoma

Osti et al., assessed the clinical significance of EV levels in plasma of glioblastoma patients and showed that the concentration of total EVs was higher in patients with glioma than healthy controls and the EV concentration decreased after surgery in all patients analyzed (n = 14), suggesting that EV levels could be used for monitoring tumor burden (Osti et al., 2019).

5.7. Melanoma and non-small cell lung cancer (NSCLC)

Del Re et al., evaluated PD-L1 mRNA expression levels in plasma EVs in melanoma (n = 18) and NSCLC (n = 8) patients undergoing nivolumab and pembrolizumab therapy. After 2 months of treatment, PD-L1 mRNA copy numbers significantly decreased in patients with complete or partial response, remained unchanged in patients with stable disease and increased in patients with progression of the disease as compared to the baseline level. Hence, this study demonstrated that EV-associated PD-L1 mRNA level could be used for real-time monitoring of patients' response to anti-PD-1 antibody therapy (Del Re et al., 2018).

Another study investigated EV-associated miRNA levels in patients with metastatic melanoma (n = 28) before, during and after treatment with MAPK inhibitors. Results showed that increased levels of hsa-let-7g-5p compared to the baseline levels were associated with better disease control and could distinguish responders from non-responders with AUC of 0.95, p = 0.001, whereas higher levels of miR-497-5p during treatment were significantly correlated with prolonged PFS (Svedman et al., 2018).

5.8. Colorectal cancer (CRC)

Yu et al., investigated the diagnostic potential of EV-associated miR-217 and CRNDE-p – a lncRNA that is overexpressed in CRC tissues and promotes growth and invasion of CRC cells (Yu et al., 2017b). High levels of CRNDE-p and low levels of miR-217 in the pre-treatment serum samples were associated with advanced clinical stage and presence of lymph node and distant metastasis. Comparison of pre- and post-chemotherapy samples from 10 CRC patients showed that CRNDE-p levels decreased in all except one patient studied, while miR-217 levels increased in all patients studied (Yu et al., 2017a). The clinical significance of these changes, however, remained unknown.

6. Pre-clinical evidence on the relevance of EVs for the prediction of drug resistance

The role of EVs shed by drug-resistant tumor cells on the protection of recipient cancer cells upon drug treatment has been reported in different cancer cell types (Table 2). For example, in CRC, EVs derived from cetuximab-resistant cell lines induced resistance in sensitive cell lines, by downregulating PTEN expression and increasing the phosphorylation of Akt levels (Zhang et al., 2017). Moreover, EVs released by mesenchymal NSCLC cell lines transferred their MDR phenotype to the parental epithelial NSCLC cell lines, through ZEB1 mRNA present in the cargo of EVs (Lobb et al., 2017). Other studies demonstrated that EVs released by HER2-overexpressing breast cancer cell lines contained HER2 molecules, which were able to modulate sensitivity to Trastuzumab (Ciravolo et al., 2012). Moreover, the expression of DNA methyltransferase 1 (DNMT1) in EVs shed by ovarian cancer cell lines mediated cisplatin resistance (Cao et al., 2017). Also, TP73 gene-derived isoform, DeltaNp73, which induces the expression of genes responsible for oncogenic potential, was found in EVs released by colon cancer cell lines that survive after oxaliplatin treatment (Soldevilla et al., 2014).

Various studies reported an intercellular transfer of MDR efflux pumps mediated by EVs, from drug-resistant to drug-sensitive cancer cell lines. For instance, the intercellular transfer of functional P-glycoprotein (P-gp/ABCB1) mediated by EVs was observed between drug-resistant leukemia cells and drug-sensitive leukemia cells (Bebawy et al., 2009). Similarly, EVs from doxorubicin-resistant (Ning et al., 2017) or docetaxel-resistant (Lv et al., 2014) breast cancer cell lines transferred chemoresistance to recipient cancer cells, via P-gp present in the cargo of the EVs. Moreover, the transfer of drug resistance from paclitaxel-resistant ovarian cancer cells to paclitaxel-sensitive cancer cells, was also caused by the transfer of functional P-gp mediated by EVs (Zhang et al., 2014). EVs derived from docetaxel-resistant prostate cancer cell lines also conferred resistance to drug-sensitive cancer cell lines, through the intercellular transfer of P-gp (Corcoran et al., 2012). Furthermore, in a pilot study, EVs isolated from the serum of prostate cancer patients that did not respond to docetaxel treatment protected prostate cancer cell lines from the effects of docetaxel (Corcoran et al., 2012). In addition, MDR osteosarcoma cell lines transferred doxorubicin-resistance to sensitive cancer cell lines, through EVs carrying P-

Table 2
Potential EV-enclosed biomarkers of drug resistance.

Cancer Type	EVs biomarkers	Drug-mediated resistance	References
Pre-Clinical Studies (Cell and Animal Studies)			
Colorectal cancer	–	Cetuximab	Zhang et al. (2017)
	RNA-based: mRNA DeltaNp73	Oxaliplatin	Soldevilla et al. (2014)
Lung cancer	RNA-based: ZEB1 mRNA	Multidrug	Lobb et al. (2017)
	RNA-based: lncRNA H19	Gefitinib	Lei et al. (2018)
	RNA-based: lncRNA RP11-838N2.4	Erlotinib	Zhang et al. (2018a)
	RNA-based: miR-222-3p	Gemcitabine	Wei et al. (2017a)
	RNA-based: miR-96	Cisplatin	Wu et al. (2017)
	RNA-based: miR-214	Gefitinib	Zhang et al. (2018b)
	RNA-based: miR-100-5p	Cisplatin	Qin et al. (2017)
Breast cancer	Protein-based: HER2	Trastuzumab	Ciravolo et al. (2012)
	Protein-based: P-gp	Adriamycin	Ning et al. (2017)
	Protein-based: P-gp	Docetaxel	Lv et al. (2014)
	RNA-based: lncRNA SNHG14	Trastuzumab	Dong et al. (2018)
	RNA-based: miR-155	Doxorubicin and Paclitaxel	Santos et al. (2018)
Ovarian cancer	DNA-based: gene DNMT1	Cisplatin	Cao et al. (2017)
	Protein-based: P-gp	Paclitaxel	Zhang et al. (2014)
	RNA-based: mirR-1246	Paclitaxel	Kanlikilicer et al. (2018)
Leukemia	Protein-based: P-gp	Multidrug	Bebawy et al. (2009)
	Protein-based: MRP1	Multidrug	Bouvy et al. (2017) and Lu et al. (2013)
	RNA-based: miR-365	Imatinib	Min et al. (2018)
	RNA-based:miR-19b and miR-20a	Multidrug	Bouvy et al. (2017)
Prostate cancer	Protein-based: P-gp	Docetaxel	Corcoran et al. (2012)
Osteosarcoma	Protein-based: P-gp and RNA-based: mRNA P-gp	Doxorubicin	Torreggiani et al. (2016)
Esophageal cancer	RNA-based: lncRNAlinc-VLDLR	Adriamycin	Chen et al. (2019)
	RNA-based: lncRNA PART1	Gefitinib	Kang et al. (2018)
Hepatocellular cancer	RNA-based: lnc-VLDLR	Multidrug	Takahashi et al. (2014)
	RNA-based: miR-32-5p	Multidrug	Fu et al. (2018)
Glioblastoma	RNA-based: lncRNA SBF2-AS1	Temozolomide	Zhang et al. (2019)
Gastric cancer	RNA-based: miR-155-5p	Paclitaxel	Wang et al. (2019)
Oral cancer	RNA-based: miR-21	Cisplatin	Liu et al. (2017)
Synovial sarcoma	RNA-based: miR-761	Pazopanib	Shiozawa et al. (2018)
Clinical Studies			
Breast Cancer	RNA-based: mRNAs TrpC5, mdr1, MUC1 and flotillin2	Anthracycline/taxane-based neoadjuvant	Ma et al. (2014)
	RNA-based: mRNA GSTP1	N = 29 Anthracycline/taxane-based neoadjuvant chemotherapy N = 30	Yang et al. (2017)
	RNA-based: mRNA UCH-L1	Anthracycline/taxane-based neoadjuvant chemotherapy N = 93	Ning et al. (2017)
	Protein-based: HER2	Trastuzumab monoclonal antibody therapy N = 11	Ciravolo et al. (2012)
	Protein-based: TGF-β1	Trastuzumab and lapatinib monoclonal antibody therapy N = 26	Martinez et al. (2017)
Prostate Cancer	RNA-based: mRNA AR- V7	Hormonal Therapy	Del Re et al. (2017)
	RNA-based: mRNA ΔNp73β	N = 36 Oxaliplatin, N = 69	Soldevilla et al. (2014)
Pancreatic Cancer	RNA-based: miRNA-155	Gemcitabine, N = 45	Mikamori et al. (2017)
Melanoma	Protein-based: PD-L1	Pembrolizumab N = 44	Chen et al. (2018)
Lung Cancer	RNA-based: miRNA-222-3p	Gemcitabine,	Wei et al. (2017a,b)
	RNA-based: miRNA-146a-5p	N = 50 Cisplatine, N = 12	Yuwen et al. (2017)
B-cell lymphoma	Protein-based: exosomal CD20	Rituximab, N = 5	Aung et al. (2011)
Multiple Myeloma	Mechanism not described (patients' exosomes in *in vitro* experiments)	Bortezomib, N = 3	Wang et al. (2014a,b)

gp (Torreggiani et al., 2016). Moreover, EVs released by multidrug-resistant leukemia cell lines transferred functional multidrug resistance-associated protein 1 (MRP1/ABCC1) to drug sensitive recipient cell lines, inducing a MDR phenotype in recipient cells (Bouvy et al., 2017; Lu et al., 2013).

The relevance of lncRNAs carried by EVs in the transfer of drug resistance has been extensively reported. For instance, EVs released by esophageal cancer cells contained the lncRNAlinc-VLDLR responsible for sustaining doxorubicin resistance (Chen et al., 2019), or harbored the lncRNA PART1 inducing gefitinib resistance in sensitive cells (Kang et al., 2018). On the other hand, under gefitinib treatment, NSCLC cell lines released EVs that transferred their drug resistance phenotype to non-resistant cells through lncRNA H19 (Lei et al., 2018). Another study reported that NSCLC cell lines resistant to erlotinib disseminated their resistance through EVs containing the lncRNA RP11-838N2.4 (Zhang et al., 2018a). Also, EVs released by breast cancer cell lines contained high levels of the lncRNA SNHG14, which were responsible for transmitting trastuzumab resistance to sensitive cell lines (Dong et al., 2018). Interestingly, EVs released by hepatocellular cancer cell lines following treatment with different anticancer agents, presented high amounts of lnc-VLDLR, responsible for promoting chemoresistance in recipient cells (Takahashi et al., 2014). In glioblastoma, an upregulation of lncRNA SBF2-AS1 was found in EVs released by temozolomide-resistant cancer cell lines, which was responsible for spreading temozolomide resistance to recipient chemoresponsive glioblastoma cells. The same study demonstrated that xenografts injected with EVs containing lncRNA SBF2-AS1 displayed significantly advanced tumor growth in response to temozolomide, being in contrast to mice injected with EVs-depleted of SBF2-AS1, which showed a tumor regression after temozolomide treatment (Zhang et al., 2019).

The contribution of miRNAs, present in EVs' cargo, to the dissemination of drug resistance was evaluated in several cancer cell lines. For instance, the induction of a drug resistance phenotype in sensitive lung cancer cell lines was possible by co-culturing sensitive cells with EVs released by cisplatin-resistant cells carrying miR-96 (Wu et al., 2017) or with EVs released by gefitinib-resistant cells carrying mir-214 (Zhang et al., 2018b). *in vivo* data confirmed the involvement of EVs carrying miR-214 on drug resistance, via the suppression of tumor growth in mice treated with gefitinib and injected with EVs containing an antagomir for miR-214, when compared with the controls (Zhang et al., 2018b). On the other hand, downregulation of miR-100-5p in EVs from cisplatin-resistance lung cancer cell lines induced resistance in recipient lung cancer cells. This effect was confirmed *in vivo* by the slower growth of tumors from nude mice treated with cisplatin and injected with EVs carrying a mimic of miR100-5p, when compared with controls (Qin et al., 2017). In addition, EVs released by ovarian and gastric cancer cell lines contained mirR-1246 (Kanlikilicer et al., 2018) and miR-155-5p (Wang et al., 2019) in their cargo, respectively, were responsible for inducing paclitaxel resistance in recipient drug-sensitive cancer cell lines. Similarly, in chronic myeloid leukemia cells, EVs mediated the horizontal transfer of imatinib-resistant traits by delivering miR-365 to recipient drug-sensitive cell lines (Min et al., 2018). The miR-222-3p present on the cargo of EVs secreted by NSCLC cell lines promoted gemcitabine resistance in recipient drug-sensitive cancer cells (Wei et al., 2017a). In oral squamous cell carcinoma, cisplatin-resistant cell lines transmitted miR-21 in the cargo of EVs, leading to dissemination of drug resistance to drug sensitive cell lines by targeting PTEN and PDCD4 in recipient cells. *in vivo,* the injection of EVs derived from a drug resistant oral squamous cancer cell line in a xenograft mouse model treated with cisplatin, caused tumor growth and enhanced cisplatin resistance, when compared with EVs derived from a sensitive cell line (Liu et al., 2017). Furthermore, miR-155 present in the cargo of EVs shed by breast cancer cell lines, provoked doxorubicin and paclitaxel resistance in drug sensitive recipient cancer cell lines (Santos et al., 2018). Similarly, miR-761 present in the cargo of EVs secreted by synovial sarcoma cells was responsible for pazopanib resistance in recipient cancer cells (Shiozawa et al., 2018). In addition, EVs released by hepatocellular carcinoma MDR cell lines delivered miR-32-5p to drug sensitive cells inducing MDR in recipient cells (Fu et al., 2018). Also, EVs shed by MDR acute myeloid leukemia cell lines transferred miR-19b and miR-20a to sensitive cells, shifting their phenotype to MDR (Bouvy et al., 2017). Interestingly, miR-106a/b present in EVs released by cisplatin-resistant hepatocellular carcinoma cell lines were transferred to cervical cancer cells, which became resistant to cisplatin, thus demonstrating the horizontal transfer of drug-resistant traits from one cell type to another (Raji et al., 2017).

7. Clinical evidence for the relevance of EVs in the prediction of drug resistance

An overwhelming amount of evidence, corroborated by multiple research groups, suggests that EVs derived from drug-resistant cancer cells and/or cells present in the TME, are causally involved in disseminating a drug-resistance phenotype in clinical context (Chen et al., 2018; Corcoran et al., 2012; Martinez et al., 2017; Mikamori et al., 2017; Ning et al., 2017; Soldevilla et al., 2014; Wang et al., 2014a; Wei et al., 2017a). Importantly, these alterations in circulating EVs cargo, responsible for the observed therapeutic resistance challenge, could serve as a biomarker for the presence of tumors that are either resistant to specific drugs or harbor the capacity to transfer this chemoresistance phenotype. This grants the unique opportunity to exploit circulating EVs to monitor in real-time, the progression or emergence of drug resistance during cancer treatment. Importantly, the clinical contribution of EVs for cancer drug resistance seems to be transversal to all solid tumors and hematological malignancies (Mikamori et al., 2017; Namee and O'Driscoll, 2018; Wang et al., 2014a; Yang et al., 2017). Indeed, several clinical studies reported the potential of EV-based biomarkers to predict patients' therapeutic outcomes in a plethora of cancer types and treatment regimens (Table 2), as detailed below.

7.1. Breast cancer

Regarding breast cancer, Xin Ma and colleagues profiled the mRNA cargo of circulating EVs from 29 breast cancer patients being treated with chemotherapy or not. Strikingly, these authors found that 4 mRNAs (includingTrpC5, mdr1, MUC1 and flotillin 2) were amplified in EVs isolated from patients that were being treated with chemotherapy (n = 17), but not in patients that did not receive chemotherapy (n = 12) (Ma et al., 2014). Interestingly, since the Ca^{2+}-permeable transient receptor potential channel 5 (TrpC5) is reported to regulate P-gp expression in target cells, it was hypothesized that the TrpC5-containing circulating EVs could support the development of cancer MDR in these patients (Ma et al., 2012). More recently, Yan et al., analyzed the levels of glutathione S-transferase P (GSTP1) mRNA, which encodes for an enzyme that plays an important role in cell detoxification, in the cargo of EVs, isolated from the serum of 30 breast cancer patients treated with anthracycline/taxane-based neoadjuvant chemotherapy. Importantly, these researchers observed that patients who had higher amounts of GSTP1 mRNA in the isolated circulating EVs, did not respond to this treatment regimen (n = 14), when compared to those that did respond to therapy (n = 16) (Yang et al., 2017).

Another EV-based prognosis biomarker of breast cancer is Ubiquitin C-terminal hydrolase L1 (UCH-L1). Again, UCH-L1overexpression has been reported to upregulate P-gp expression levels via the MAPK/ERK signaling pathway, enhancing a MDR phenotype in breast cancer. Indeed, it has been reported that breast tumors (n = 100) have significantly increased levels of UCH-L1 mRNA when compared to normal breast tissue (n = 24). Most importantly, this is a poor prognostic marker associated to ER-/PR- aggressive breast cancers subtypes (Miyoshi et al., 2006). Interestingly, Kuan Ning et al., reported that patients with UCH-L1 levels above average in circulating EVs, are also associated with poorer response to adjuvant anthracycline/taxane-based chemotherapy, when compared with those that do not have increased UCH-L1 levels (n = 93) (Ning et al., 2017). Additionally, in an *in vitro* setting, these authors further demonstrated that UCH-L1 + EVs produced by a breast cancer cell line can transfer chemoresistance to recipient cells, suggesting that EVs may be a useful non-invasive diagnostic biomarker for predicting chemoresistance in breast cancer patients.

Most importantly, EV-mediated drug resistance phenomena also seem to be relevant for biological drugs such as monoclonal antibody-based therapies. Indeed, Ciravolo et al., observed that 73% of advanced-stage breast cancer patients (n = 8/11) had HER2-positive EVs in circulation which effectively sequestered Trastuzumab monoclonal antibodies, thus hampering the corresponding therapeutic efficacy of this monoclonal antibody. Interestingly, only 18% of early stage breast cancer patients (n = 2/11) had HER2-positive EVs in the circulation. Importantly, this study demonstrated that HER2-positive EVs present in the serum of breast cancer patient's act as decoys and suggested that they can be used to predict patients' response to Trastuzumab therapeutic regimens (Ciravolo et al., 2012). Similarly, Dong et al., have shown that EVs isolated from advanced HER2-positive breast cancer patients that did not respond to trastuzumab treatment, presented higher expression of lncRNA SNHG14 when compared with those who did respond (Dong et al., 2018). Recently, the O'Driscoll' group reported that HER2 drug-resistant cells and their corresponding EVs carry in their cargo increased amounts of TGF-β1 immunosuppressive cytokines compared to their sensitive counterparts. Most importantly, in a recent neo-adjuvant clinical trial including trastuzumab and lapatinib, the same group has further demonstrated that HER2-overexpressing breast cancers patients that did not respond to HER2-targeted drug treatment (n = 4) had significantly higher amounts of TGF-β1 in the isolated

plasma-derived EVs compared to those who experienced a partial or complete response (n = 26). Despite the low robustness of this study due to the low number of enrolled patients, these preliminary results suggest a potential use for EV-TGF-β1 as a minimally-invasive predictive biomarker for breast cancer patients undergoing this therapeutic regimen (Martinez et al., 2017).

7.2. Prostate cancer

Considering prostate cancer, the O'Driscoll' group observed that EVs isolated from the blood of prostate cancer patients that did not respond to a docetaxel-based regimen (n = 2), protected both the 22Rv1 and the DU145 prostate cancer cell lines from the cytotoxic effect of docetaxel. On the other hand, circulating EVs isolated from the plasma of prostate cancer patients that did respond to therapy (n = 6) enhanced docetaxel cytotoxicity (Corcoran et al., 2012). Interestingly, this study supports the idea that the type and cargo of EVs in the circulation can modulate the individuals' response to therapy. In line with this notion, more recently De Re et al., reported a new method for androgen receptor splice variant7 (AR-V7) detection in RNA extracted from circulating cancer cell-derived EVs from castration-resistant prostate cancer patients (CRPC) (n = 36). Importantly, these authors showed that EV-AR-V7 mRNA levels predicted resistance to hormonal therapy, with overall survival being significantly shorter in patients with EVs containing AR-V7-mRNA compared with those that did not (3 months versus 20 months). Noteworthy, these data support the role of EV-AR-V7 as a predictive biomarker of resistance to hormonal therapy (Del Re et al., 2017).

7.3. Colon cancer

The ΔNp73, a TP73 gene-derived isoform, has been reported to be upregulated in colon cancer thus inhibiting the tumor suppressor function of TP53 or/and inducing a set of genes involved in tumorigenesis and drug resistance (Soldevilla et al., 2011). Consistent with this finding, Soldevilla et al., carried out a clinical study (n = 69) which reported that colon cancer patients with lower levels of EV-ΔNp73β prior to oxaliplatin-based therapy had an improved 5-year disease-free survival, when compared to those with high levels of EV-ΔNp73β (56% vs. 49%, respectively) (Soldevilla et al., 2014). Importantly, functional chemoresistance assays have further confirmed the potential prognostic value of EV-ΔNp73 in colon cancer patients.

7.4. Pancreatic cancer

Mikamori et al., showed that high expression of miRNA-155 in resected tumor tissue samples from pancreatic ductal adenocarcinoma (PDAC) patients treated with gemcitabine (GEM) correlates with a poorer prognosis, compared to patients with a lower level of miR-155. Importantly, this study described a positive correlation between the levels of miRNA-155 both in patients' pancreatic tissue and plasma-derived EVs (n = 45) (Mikamori et al., 2017). Interestingly, this study seems to support the notion that circulating EVs can reflect, to some extent, the tumor landscape being feasible to use them as a real-time monitoring tool for therapy resistance. Importantly, these authors have further demonstrated that an increase in miRNA-155 expression by pancreatic cancer cell lines leads to increased anti-apoptotic signaling and EV secretion *in vitro*. Most importantly, these authors further verified that the EVs released by this miRNA-155 over-expressing PDAC cell lines were able to deliver chemoresistance-related substances (including miR-155) to other cancer cells, and that the recipient cells subsequently developed chemoresistance to GEM *in vitro*.

7.5. Malignant melanoma

Tumor cells evade immune surveillance by upregulating the surface expression of programmed death-ligand 1 (PD-L1), dampening the immune checkpoint response mediated by programmed death-1 (PD-1) receptor on T cells. The disruption of the interaction of the checkpoint ligand (e.g. PD-L1) with the inhibitory checkpoint receptor (PD-1) on T cells, restores T cell function and anti-tumor immunity. Indeed, immunotherapy with anti-PD-1 antibodies has shown remarkable promise in treating different tumor types (Chen and Han, 2015). Nevertheless, for some patients the therapeutic response seems to be rather low (Ribas et al., 2016; Zaretsky et al., 2016). To tackle this important issue, Chen and colleagues have recently shown that circulating EVs display a major impact in rendering immunotherapy approaches ineffective for certain patients with melanoma. Remarkably, these authors reported that metastatic melanomas cancer cell lines release EVs that carry PD-L1 on their surface and that interferon-γ (IFN-γ) increases the amount of PD-L1 on these vesicles, which suppresses the function of $CD8^+$ T cells, facilitating tumor growth in experiments with human melanoma xenografts in nude mice. Strikingly, these authors showed that the level of circulating EVs that carry PD-L1 distinguishes clinical responders (n = 21) from non-responders (n = 23) to anti-PD-1 treatment (Pembrolizumab). Most importantly, this study provides a rationale for the application of PD-L1 positive EVs as a predictor for anti-PD-1 therapy in melanoma patients (Chen et al., 2018).

7.6. Lung cancer

Regarding the predictive ability of EV-based biomarkers in lung cancer, Wei et al., have recently reported that NSCLC patients with high levels of miR-222-3p in circulating EVs had a limited response to gemcitabine regimen (n = 50) (Wei et al., 2017a). Recently, Zhang et al., demonstrated that EVs isolated from serum samples of advanced NSCLC patients that did not respond to erlotinib treatment, had significantly higher amounts of IncRNA RP11-838N2.4, when compared with those that did respond (Zhang et al., 2018a). Additionally, Yuwen et al., demonstrated that patients with advanced NSCLC after cisplatin-based chemotherapy (n = 12) with lower levels miR-146a-5p in circulating EVs, were associated with shorter progression-free survival (PFS). The authors indicated that EV-miR-146a-5p targets ATG12 to inhibit autophagy, thus having the potential to predict the efficacy of cisplatin regiments and monitor drug resistance in these NSCLC patients (Yuwen et al., 2017).

7.7. B-cell lymphoma

Rituximab is a monoclonal antibody that targets the CD20 protein on the surface of B-cell lymphoma cells inducing their apoptosis via multiple mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent lysis (CDC) (Adams and Weiner, 2005). Importantly, EVs have also been reported to mediate the B-cell lymphoma drug resistance to immunotherapy with rituximab (Namee and O'Driscoll, 2018). Indeed, it has been reported that 3 h post-administration of rituximab, half of all plasma rituximab was actually bound to circulating EVs isolated from the plasma of B-cell lymphoma patients (n = 5) (Aung et al., 2011). This strongly suggests that patients' lymphoma-derived EVs may act as a decoy for CD20 receptors, reducing rituximab availability for therapeutic benefit.

7.8. Multiple myeloma

There is an intimate relationship between bone marrow stromal cells (BMSCs) and multiple myeloma (MM), for conferring a drug resistance phenotype upon the latter. Consistently, Wang et al., have further dissected the role EV shedding by the healthy bystander BMSCs in this process. Most interestingly, these authors showed that EVs isolated from both MM patients and healthy donors BMSCs (n = 3 each), induced MM cell proliferation *in vitro*, migration, survival and, most importantly resistance to bortezomib proteasome inhibitor therapy

regimen by nearly 25% (Wang et al., 2014a).

Taken together, there is certainly a great deal of evidence that pinpoints EVs as a major determinant for inducing/dissimilating a drug resistance phenotype in the clinical setting. Unfortunately, so far none of these promising clinical studies established the actual clinical value of these EV-based biomarkers of drug resistance for the interest of patients and also for economic benefit. Indeed, the accreditation of research laboratories under ISO standard 15189 would certainly improve the reproducibility and standardization of current EV-based biomarkers for clinical use. This will ultimately facilitate the implementation of randomized trials involving multicenter validation and clinical approval of these promising EV-based biomarkers of drug resistance. Only then, EV research may grant clinicians the opportunity to make better-informed therapeutic decisions and, eventually, target both cancer cells and the EV-mediated communication for improved therapeutic outcomes.

8. EVs in diagnostics and therapeutics: from the bench to bedside

With a plethora of EV-based technologies just over the horizon, it is of utmost importance to fulfil their potential into clinically useful enabling tools for diagnosis and treatment of cancer patients. Indeed, EVs-derived products have the potential to fulfil two major unmet clinical needs in a new era of precision medicine: their use as (i) biomarkers for diagnosis, prognosis, prediction of drug response and monitoring tumor evolution and as (ii) naturally engineered drug-delivery magic bullets for improved cancer therapy. As a result, much of the groundbreaking EV-based cancer research carried out in academia generated a significant amount of protectable intellectual property (IP) (Roy et al., 2018). These EV-based technologies are now being pushed forward to reach commercialization/clinical use by several startup companies with the strong support of industrial partnerships.

Indeed, there is a growing belief that the EV-based industry is poised for growth as observed by the recent market activity. Only last year, EV-based start-up companies have been the recipients of more than $386.2 million in investor funding (Hildreth, November 7, 2018). From these, CodiakBioSciences (USA, founded in 2015), Exosome Diagnostic, Inc. (USA, founded in 2008) and EVOX Therapeutics (UK, founded in 2016) alone, were able to secure nearly $168.5, $112, and $59.5Million, respectively, throughout Series A, B and C rounds of funding (Cambridge, December 15, 2017; Exosome Diagnostics, 2019; Therapeutics, September 3, 2018).

More importantly, most of these cancer EV-based startup companies became the focus of strategic acquisitions, partnerships, collaborations, and agreements by large industrial players between 2016 and 2018. Some examples include Lonza that in 2017 acquired HansaBioMed Life Sciences (Estonia, founded in 2007) and invested in Exosomics Siena (Italy, founded in 2011), Merck that in 2017 announced a deal with Exosome Diagnostics to use their EV-protein analysis equipment Shahky™ and other expertise in profiling exosomes in Merck's oncology-related drug development programs and Boehringer Ingelheim who established a research collaboration with EVOX Therapeutics to develop EV-mediated delivery of RNAs for specific diseases from the Boehringer Ingelheim' portfolio (Exosome Diagnostics, February 6, 2017; Lonza, May 16, 2017; Therapeutics, 2017). More recently (2018), Bio-Techne Corporation acquired Exosome Diagnostics for $250 million plus contingencies up to $325 million for achieving certain milestones, while Jazz Pharmaceuticals announced earlier this year a strategic deal with Codiak Biosciences to develop and commercialize engineered EVs for hard-to-treat cancers for $56 million upfront with up to $200 million in milestones (Exosome Diagnostics, June 25, 2018; Pharmaceuticals, January 03, 2019).

Most importantly, April 2019 represents an unprecedented financial landmark in the field of EV-based companies; CodiakBioSciences filed a prospectus for its initial public offering (IPO) with the U.S. Securities and Exchange Commission to secure an additional $86 million of investment (Keown, May 01, 2019). Therefore, it is with great expectation that the EV community will follow the markets' perception on the potential commercialization value of EV research for clinical use. Indeed, Codiaks' IPO represents the first of its kind and its' outcome will certainly steer the future market investment strategy for EV-based startup companies.

Currently, there are 120 clinical studies registered at https://clinicaltrials.gov/ involving the use of EVs for cancer diagnosis, prognosis or therapeutic purposes. Importantly, approximately 2/3 of these studies are related with cancer biomarkers, while a minor fraction represents EV-based therapeutics. From these studies, 15 are new and do not have any patients enrolled yet, 46 are active and recruiting, 18 are active but not recruiting and 25 are completed. Noteworthy, 8 studies were classified with unknown status (a study whose last known status was active but that has passed its completion date, and the status has not been last verified within the past 2 years), 5 were terminated and 3 studies were suspended.

Most importantly, despite the high investment in the R&D EV-based companies, there are still very few EV-products approved for clinical use and commercialization. Indeed, the first generation of EV-products with clinical intent includes: (i) ExoDx Prostate (IntelliScore), a urine-based test used in combination with PSA for prostate cancer (clinicaltrials.gov, January 25, 2017; Corporation, Jan. 31, 2019; Exosome Diagnostics, September 7, 2016; McKiernan et al., 2018, 2016) and (ii) ExoDx™ Lung(ALK), a plasma-based liquid biopsy for lung cancer (clinicaltrials.gov, August 2, 2017; De Rubis et al., 2019; Exosome Diagnostics, January 21, 2016), both commercialized as laboratory development tests (LDT) from Exosome Diagnostics, Inc (USA). In the European arena, Exosomic Siena commercialized the (iii) Cancer rE-Veal™ Kit Line, an ELISA-based assay for the quantification of cancer-derived EV subsets (Siena, June 10, 2019), and (iv) LB-SeleCTEV™ for EGFR Mutation Analysis, the latter as Certified In Vitro Diagnostic Medical Devices (CE-IVD) in Europe (Siena, Mar 21, 2019).

Taken together, between the observed market investment in EV technologies and the actual commercialization of EV products for cancer diagnosis, the EV global market worth's nearly $91 million in 2019 (Mr. Shelly Singh, 2018). Most importantly, this value is projected to increase to nearly $265 million in 2024 at an estimated 5-year Compound Annual Growth Rate (CAGC) of 23.8%. Strikingly, EV diagnostic-based biomarkers represent the largest sector of this market, expected to grow from $56.5 million in 2019 to $250 million in 2024 at a CAGR of 29.4% (Mr. Shelly Singh, 2018; Roy et al., 2018).

9. Perspectives and future challenges

Collectively, these studies have provided proof of concept that EVs can be used for real-time monitoring of tumor burden, tracking cancer evolution, predicting response to treatment and monitoring the emergence or progression of drug resistance. However, most of the clinical studies were based on very small sample size, therefore the identified biomarkers must be validated in large longitudinal studies before they can be translated into clinically applicable tools. Moreover, the future of EV-based biomarker assays depends on some scientific and technological challenges that still need to be met. Blood contains a complex mixture of EVs derived from various cell types. It is not known what fraction of blood EVs is derived from the tumor, but current evidence suggests it is small and highly variable among patients. Thus, cancer-derived biomarkers are highly diluted in blood. One of the solutions is the isolation of specific EV populations that are enriched in cancer-derived EVs, such as phosphatidylserine-externalized EVs (Matsumura et al., 2019), GPC1-positive EVs (Melo et al., 2015) or PSMA-positive EVs (Biggs et al., 2016). However, the main challenge of such an approach is the identification of highly specific and sufficiently sensitive EV surface markers, which is not met for the majority of cancer types. Moreover, it is possible that the levels of particular biomarkers might be enriched in specific subpopulations of EVs and the capacity to isolate

them would greatly enhance the sensitivity and specificity of the assays. Alternatively, other biofluids including lymph, urine or milk, that might be specifically enriched with EVs derived from tumors that are in contact with those biofluids, can be considered as a source of EVs. For instance, in melanoma patients, lymphatic exudate was found to be dramatically enriched with cancer-derived EVs as compared to plasma, and lymph vessels were shown to be the major route of EV transport from tumor tissue to the blood circulation (Broggi et al., 2019; Garcia-Silva et al., 2019). Similarly, tumor-draining pulmonary venous blood was shown to be enriched in cancer-derived EVs as compared to peripheral blood in patients with lung cancer (Navarro et al., 2019). However, collection of tumor-draining lymph or pulmonary venous blood could be technologically challenging. Whereas the main challenge for the development of assays based on urine or milk as a source of EVs, is the highly variable concentration of EVs in these biofluids and the lack of reliable normalization methods.

Thus, novel technological platforms capable of enriching cancer-derived EVs or specific EV subpopulations from human biofluids are urgently needed to move this field forward. In this regard, various nanotechnology and microfluidics-based platforms are rapidly evolving (Iliescu et al., 2019). Some of these devices are designed to collect and quantify EVs directly from biofluids without the need for prior EV isolation. For instance, a microfluidic ExoSearch chip that combines immunomagnetic isolation with an *in situ* multiplexed immunoassay allows quantification of CA-125, EpCAM and CD24-positive EVs using as little as 20 µl of plasma. The diagnostic value of this device was tested in a small set of patients with ovarian cancer (n = 15) and healthy controls (n = 5) and showed remarkable diagnostic accuracy with an AUC of 1 (Zhao et al., 2016). Other technologies combine immunomagnetic isolation of EVs with on-chip analysis of their RNA content. For example, Shao et al., developed a microfluidic platform termed immuno-magnetic exosome RNA (iMER) analysis, which integrates immunomagnetic selection, RNA collection and real-time PCR into a single microfluidic chip format and demonstrated its application for quantification of mRNAs that are associated with drug resistance in glioblastoma patients (Shao et al., 2015). Another interesting technology, termed NanoVilli Chip, is based on anti-EpCAM-grafted silicon nanowire arrays that are engineered to mimic intestinal microvilli, thus dramatically increasing surface area and enhancing the capture of EVs. RNA from the captured EVs can be recovered and analyzed by droplet digital PCR. This technology has been applied for monitoring changes of ROS1 rearrangements and EGFR T790 M mutation in NSCLC patients (Dong et al., 2019).

In summary, a growing body of preclinical and clinical evidence reveals that EVs hold great potential as a source of cancer biomarkers for liquid biopsies. The main scientific challenges that still need to be addressed to translate EV research into reliable tools for the clinical management of cancer patients are to find ways to identify cancer-derived EVs or EV subpopulations that are enriched in relevant biomarkers and decoding the “messages encrypted” in the molecular cargo of EVs. Several novel technologies for capturing and analyzing EVs have recently emerged, however they are still under development and warrant validation in a clinical setting.

Declaration of Competing Interest

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