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Review

Extracellular Vesicles as Biomarkers and Therapeutic Targets in Breast Cancer

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Abstract. *Cancer-derived extracellular vesicles (EVs) contain various cancer-associated molecules, such as mutated or overexpressed oncoproteins, glycoproteins, mRNAs, various non-coding RNAs and DNA fragments. They have been shown to propagate phenotypic traits, such as drug resistance, increased proliferation rate, invasiveness and stemness across cancer cells and to mediate cancer-induced immunosuppression. Therefore, cancer-derived EVs have gained increasing attention as cancer biomarkers and therapeutic targets. Unlike circulating tumor cells they are highly abundant in biofluids and, on the contrary to single-molecule circulating biomarkers, they protect their molecular cargo against degradation and may carry molecular signatures associated with specific phenotypes. Herein, we summarize studies investigating EVs as biomarkers in breast cancer and propose scenarios for various clinical applications of EV-based biomarkers in the management of breast cancer. Furthermore, we provide an overview of recent findings regarding the cancer-promoting effects of breast cancer-derived EVs and discuss opportunities for blocking EV-mediated signaling as a therapeutic strategy for breast cancer.*

According to GLOBOCAN data, breast cancer (BC) is the most frequent cancer type among women worldwide, with an estimated 1.67 million new cases and 522,000 deaths in 2012 (1). Advances in diagnostic imaging technologies and BC awareness campaigns have improved early detection rates of BC in the developed countries, however, still over 33% and 5% of patients present with regionally disseminated disease or distant metastases, respectively (2, 3). Due to diverse mutational profiles and gene expression patterns among individuals and within tumors, BC is a profoundly heterogeneous disease with respect to its biology, clinical course and response to treatment (4). In routine clinical practice, treatment decisions are mostly based on disease stage and status of estrogen (ER) and progesterone receptors (PR), epidermal growth factor 2 receptor (HER2) expression and Ki-67 levels (5, 6). These sub-groups, however, show highly variable responses to therapy. Lately, gene expression studies have established five intrinsic molecular sub-types; Luminal A, Luminal B, HER-2-enriched, Basal-like and Claudin-low BC (7). These sub-types differ in response to treatment and survival and to some extent explain the inter-tumor heterogeneity of BC, thus providing a basis for new classification of BC that could potentially be used for patient stratification (4, 7), however, due to its cost and technical challenges, it is not yet widely introduced into routine diagnostic examinations.

In the past decade, extracellular vesicles (EVs) have emerged as important mediators of intercellular communication and are increasingly recognised as a potential "liquid biopsy" of cancer. EVs are a heterogeneous group of membrane-contained vesicles released in the extracellular space and biofluids by a variety of normal and cancerous cells. They transfer lipids, proteins and nucleic acids from a cell of origin to recipient cells, where they can trigger diverse physiological and pathological responses (8-10). Their molecular content reflects, at least partially, that of the cell of origin and cancer-derived EVs have been shown to contain tumor-specific molecules (11-16), hence the analysis of EV cargo might reveal the genetic make-up and molecular alterations in cancer cells. Furthermore, cancer-derived EVs have been shown to promote cancer development by mediating cancer cell cross-talk, contributing to the formation of pre-metastatic niche, promoting angiogenesis, modulating tumor stroma and interfering with anti-tumor immune response (17). Therefore, the inhibition of their release or uptake, or modulation of their

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cargo could represent a novel therapeutic option for the treatment of cancer.

In the current review, we summarize studies showing relevance of BC-derived EVs for detecting or monitoring BC and suggest clinical scenarios, on how the application of EV-based biomarkers could improve the management of BC. Furthermore, we provide an overview of biological functions of BC-derived EVs and discuss opportunities for the therapeutic targeting of EVs.

Areas of Unmet Needs in Management of Breast Cancer

ER-positive and HER2-negative BC is the largest and most diverse group accounting for approximately 65-70% of all BC cases (18). The treatment options for ER^+ cancers include surgery, radiation therapy, endocrine therapy and chemotherapy. It has been estimated that approximately 15-30% of patients with early-stage ER^+ BC will develop distant metastases within 10 years and therefore could benefit from adjuvant chemotherapy (19, 20). Hence, there is a clear need for a reliable predictive tool that could identify patients, who require chemotherapy, and spare from chemotherapy those, who can be successfully managed with endocrine therapy alone. Currently, several gene expression-based tests, such as Oncotype DX® and MammaPrint®, are available for such purpose for women with early-stage BC and have been shown to have an impact on clinical decision-making (21, 22). However, these tests require tumor tissue specimens that may not always be available and their cost limits a widespread application of these tests, particularly in lower income countries. Furthermore, during the disease progression, cancer cells acquire new mutations, undergo epigenetic alterations and changes in gene expression and are subjected to selection pressure favouring expansion of drug-resistant subclones (18). Therefore, novel methods alternative to single biopsy analysis allowing tracking tumor evolution and assessing intratumoral heterogeneity during the course of the disease are required.

The *ERBB2* gene encoding HER2 is amplified and/or overexpressed in approximately 15-22% of BC cases (23, 24). HER2 is a receptor tyrosine kinase that activates proliferation and survival signaling in epithelial cells *via* Ras/Raf/MAPK, PI3K/AKT and mTOR pathways and its overexpression has been shown to confer a more aggressive behaviour of the disease, resistance to tamoxifen and shorter overall survival (23, 25, 26). On the other hand, therapeutic targeting of HER2 using monoclonal antibodies, such as trastuzumab, has demonstrated remarkable success in improving outcomes both in patients with early and metastatic BC and currently is standard treatment for patients with HER2-postive BC (23, 26). Its mechanisms of action are related to the down-regulation of signaling *via* MAPK and PI3K pathways and the induction of antibody-dependent cell-mediated cytotoxicity (27). Unfortunately, both primary and acquired resistance to trastuzumab is common – only about 30-40% of patients with HER2-positive BC respond to trastuzumab monotherapy, and about 70% of patients who initially responded tend to develop secondary resistance within one or two years (26, 28). Mechanisms of resistance involve generation of a truncated form of the HER2 receptor with constitutive active kinase activity (29), overexpression of other receptor tyrosine kinases, such as HER3 or EGRF, loss of PTEN and p21 expression and activation of PI3K/AKT/mTOR pathway and failure to induce immune response (23, 26, 27, 30, 31). Therapeutic strategies to overcome trastuzumab resistance include combining trastuzumab with chemotherapy, PI3K/mTOR inhibitors, small-molecule HER2 inhibitors, such as lapatinib and neratinib, that inhibit the intracellular domain of HER2, or monoclonal antibodies, such as pertuzumab, that inhibit HER2/HER3 dimerization, or using antibody-drug conjugates, such as T-DM1, that is composed of trastuzumab conjugated with a fungal toxin maytansine (23, 32). However, to date there exist no other validated biomarkers, apart from HER2 expression that can predict patients' response to HER2 targeted therapy (33). Moreover, little information on the *in vivo* molecular alterations that lead to development of secondary resistance is currently available. Hence, both additional biomarkers that could predict patients' primary response to HER2 targeted therapy and blood-based biomarkers that could allow monitoring the genetic alterations in the tumor during therapy and reveal the mechanism of acquired resistance are required for better management of HER2-positive BC.

A triple-negative breast cancer (TNBC) accounts for 10-20% of all BCs and is the most aggressive type of BC having a distinct pattern of metastasis and poor survival rates (34, 35). TNBC is defined by the lack of ER and PR expression and absence of HER2 overexpression or gene amplification, however it is a clinically and molecularly very heterogeneous disease (35-37). Recently, gene expression analyses identified six TNBC sub-types – two basal-like, an immunomodulatory, a mesenchymal, a mesenchymal stem-like, and a luminal androgen receptor subtype, which differ in their biology and show different sensitivities to various therapeutic agents, and in the future might serve as a key for individualised therapy selection (38). However, as TNBC cannot be treated with hormonal therapy or HER2 targeted therapy and efforts to define novel therapeutic targets in TNBC, so far, have not resulted in highly efficient targeted therapies, currently, the treatment options of this sub-type are limited to cytotoxic chemotherapy (37, 39). In most cases, patients with locally advanced or unresectable TNBC receive neoadjuvant chemotherapy consisting of taxanes and antracyclines. Up to 25% of patients achieve a pathological complete response (pCR) after neoadjuvant therapy and the survival rates in

these patients are similar to those in other BC sub-types, while the prognosis in the rest of the patients is much poorer (39). Although some promising predictive biomarkers have recently been identified (40, 41), they still need to be validated in independent clinical trials, and so far no biomarker assays for predicting probability of achieving pCR are available for routine use. Thus, defining robust biomarkers that could help select patients who are likely to benefit from chemotherapy and to stratify patients for clinical trials of targeted therapies as well as development of novel molecularly targeted treatment strategies is still a major unmet clinical need.

Types, Biogenesis and Content of Extracellular Vesicles

The term "extracellular vesicles" comprises of several types of vesicles that differ in their cellular origin, mode of biogenesis, molecular cargo and membrane composition, size and physical properties. These parameters are highly heterogeneous and dynamic, therefore vesicle classification is difficult and presently there is no agreement in the scientific community regarding a unified nomenclature of EVs (42). EVs have been classified based on their cellular origin (*e.g.* prostasomes (43), oncosomes (44) *etc.*), specific functions (*e.g.* tolerosomes (45), vexosomes (46) *etc.*) or biogenesis. Based on the mode of biogenesis, three main types of EVs have been defined: (i) exosomes, (ii) microvesicles (also called ectosomes or microparticles) and (iii) apoptotic bodies (8, 42, 47, 48). Exosomes are the smallest EVs ranging between 30-100 nm in diameter (49, 50). They are derived from the endolysosomal pathway *via* the fusion of multivesicular bodies (MVB) with plasma membrane and the release of intra-lumenal vesicles into the extracellular space (51). Microvesicles (MVs) are larger EVs ranging from 50 to 1,000 nm in diameter and are generated by budding from the plasma membrane (48, 52). Apoptotic bodies are produced by membrane blebbing of cells undergoing programmed cell death and their main physiological role is to prevent leakage of cellular content in the extracellular space, thus preventing inflammation, autoimmune reactions and tissue damage (53, 54). They vary greatly in size ranging from 50 nm to 5 μm in diameter (48). The formation of apoptotic bodies is commonly considered a stochastic process leading to cell fragmentation and release of membrane-bound vesicles containing organelles and condensed chromatin that are promptly cleared by phagocytes. Recent studies, however, suggest that this might be a highly regulated multi-step process with substantial differences in certain cell types (55, 56). Furthermore, defects in the apoptotic cell clearance have been implicated in the induction of sterile inflammation (57) and the development of autoimmune diseases (58).

Still, the classification of EVs is inconsistent in the recent literature. Moreover, there exist substantial differences in the EV biogenesis in various cell types (59) and most of the current techniques for the fractionation of EVs do not allow isolating pure exosome or MV fractions. Hence, most of the EV preparations are likely to contain a mixture of various EV subtypes. Therefore, we will use the term "EVs" to designate all types of vesicles throughout this review unless the identity of a given EV subpopulation is thoroughly characterised and is of particular importance in the given context.

The common denominator of all EVs is that they are enclosed by a lipid bilayer and contain a wide variety of proteins and nucleic acids. The membrane composition and molecular content, however, differs in various types of EVs, and is regulated in a cell-type specific manner (8). Exosomal membranes are derived from endosomal intralumenal vesicles and are enriched in tetraspanins such as CD63, CD81 and CD9 (60-63) and proteins associated with the endosomal sorting complex required for transport (ESCRT), such as TSG101 and ALIX (59, 61). The composition of MV membranes is similar to that of the cellular plasma membranes, yet the distribution of lipids between the two leaflets of the membrane is altered, resulting in exposure of phosphatidylethanolamine on the surface of MVs (64). To the best of our knowledge, no protein markers that are specific for MVs have been so far identified. Common features of apoptotic bodies are surface markers such as N-acetylglucosamine, calreticulin and phosphatidylserine that facilitate their recognition and clearance by phagocytes (53, 65) and the content of fragmented DNA and histones (53, 57). However, a recent study described a novel mechanism of generating apoptotic bodies *via* the formation of "beaded apoptopodia" that facilitates sorting of cargo into apoptotic bodies and results in the exclusion of nuclear contents from apoptotic bodies (55).

EVs produced by certain cell types have been shown to express cell-type-specific markers. For example, prostate cancer cells release EVs carrying prostate-specific membrane antigen and androgen receptors (66), various epithelial cells – EpCAM, while intestinal epithelium cells – A33 (67). The identification of such markers could enable the isolation of tissue-specific EVs from biofluids containing complex mixture of vesicles.

EVs contain various RNAs, including mRNAs, miRNAs, rRNAs, lncRNAs, tRNAs, piRNAs, vault RNAs and Y-RNAs (68-72), however their proportions and repertoire seem to differ in various EV sub-types and depends on the type and physiological state of the cell. Exosomes derived from various cell sources have been found to be enriched in small RNA species with minor amounts of full-length rRNAs (71-74), apoptotic bodies contain large amounts of intact rRNAs, while the relative abundance of various RNA species in MVs seem to vary greatly depending on cellular source (73). In a

recent study, Lunavat *et al*. compared the small RNA cargo in exosomes, MVs and apoptotic bodies released by melanoma cells by deep sequencing, that revealed substantial differences in the RNA repertoire in different sub-types of EVs (75). Similarly, Ji *et al*. compared miRNA profiles in three sub-types of EVs (MVs, A33-positive exosomes and EpCAM-positive exosomes) released from colon cancer cells that resulted in the identification of a subset of miRNAs common to all EV sub-types and a number of other miRNAs that were selectively represented in a specific EV subtype (67). Although multiple previous studies have explored miRNA content in EVs, Chevillet *et al*. for the first time performed a stoichiometric analysis of miRNA abundance in exosomes isolated from various sources (76). Surprisingly, that study showed that, on average, there were 0.00825 molecules of a given miRNA per exosome. Therefore, the authors questioned the concept that exosomes contain specific miRNA signatures and may serve as vehicles for intercellular miRNA transfer, and proposed two stoichiometric models for exosomal miRNA content – one suggesting that a small fraction of exosomes carries a low concentration of miRNAs and another – that only very rare exosomes carry many copies of a given miRNA.

It has been known for long that apoptotic bodies contain fragments of DNA and subsequent studies have demonstrated that these fragments can be horizontally transferred by the uptake of apoptotic bodies (77, 78). Several recent articles, however, reported the presence of DNA in exosomes and MVs, too (11, 16, 79). Thakur *et al*. showed that exosomes released by various cancer cell lines contain dsDNA fragments ranging in size from 10 kb to 100 bp and comparative genomic hybridisation analysis revealed the entire genome coverage of exosomal DNA, however only a subset (approximately 10%) of exosomes contained DNA (16). A study by Lazaro-Ibanez *et al*. showed that all sub-types of EVs released by prostate cancer cells contain dsDNA fragments harbouring specific mutations (79). These findings suggest that cancer-derived EVs could serve as "liquid biopsies" of parental tumor allowing for detection of the whole mutation spectrum without a need to sample multiple biopsies and monitoring of genetic alterations in evolving tumor cell clones during the course of disease. In comparison with the total cell-free DNA, EV-enclosed DNA is protected from degradation by plasma DNases and is likely to be enriched with tumor DNA. However, the processes of DNA fragmentation, sorting and packaging into EVs are poorly understood so far.

EVs as Biomarkers for Diagnosis and Monitoring of Breast Cancer

An elevated number of EVs has been found in the peripheral blood of patients with various cancers (80, 81), including BC (82-85), thus suggesting that the measurement of EV levels *per se* could serve as a diagnostic tool. The studies investigating EVs as biomarkers of BC are summarised in Table I. Although various methods have been used for the isolation and quantification of EVs, these studies consistently report that the level of EVs is increased in the blood and other biofluids of BC patients compared to cancer-free healthy controls. A study by Galindo-Hernandez *et al*. showed that increased number of plasma EVs correlate with tumor size (83), while none of the studies have found an association between EV number and disease stage or BC sub-type. However, an increased number of EVs in the blood has been observed in a number of non-cancer related diseases, such as coronary heart disease (86), pre-eclampsia (87), active Crohn's disease (88) and diabetes (89) or physiological states, such as pregnancy (87, 90), hence showing that the number of EVs is not a highly specific diagnostic criterion. These findings also raise a question about the cellular origin of EVs found in the blood of cancer patients. Clearly, a proportion of them are released by cancer cells as they contain cancer-associated molecules, such as amplified oncogenes and oncoproteins (11, 83, 84, 91). On the other hand, in inflammatory conditions, the main sources of EVs are platelets, lymphocytes, leucocytes and endothelium (86, 87), therefore it is possible that at least a part of the EVs found in the blood of BC patients are released by immune cells. In fact, a study by Toth *et al*. showed that BC patients have increased levels of leukocyte-derived EVs, while endothelial cell-derived EV levels were similar to those in the controls (92). Thus, it remains to be determined, which are the main cellular sources of EVs and what are the stimuli inducing the EV release in cancer patients.

Alternatively to EV count, their molecular cargo could serve as a BC biomarker. EVs isolated from the peripheral blood of BC patients have been shown to contain various BC-associated molecules – oncogenic proteins such as EGFR, FAK, survivin, EMMPRIN (83, 84, 93), and various miRNAs (94). The analysis of EV-enclosed proteins or small RNAs could potentially yield higher sensitivity and specificity in comparison to whole-blood analysis, as cancer-derived EVs are likely to be enriched in diagnostically-relevant molecules and they protect their molecular cargo from degradation. For example, the expression pattern of EV-enclosed survivin and its splice variants has been shown to mimic that in the breast cancer tissue (84). Eichelser *et al*. compared serum levels of cell-free and EV-enclosed miRNAs in BC patients and found that EVs were enriched in specific miRNAs that were overexpressed in BC sera compared to healthy controls. Moreover, high EV-enclosed but not cell-free miR-373 level could distinguish TNBC and luminal BC sub-types and was associated with estrogen- and progesterone receptor-negative cancers (94). This finding suggests that EV-enclosed miRNA profiles have a potential to discriminate between

Table I. *Studies investigating EVs as biomarkers of BC.*

Marker	Study design and main findings	Reference
EV protein level	Various biofluids from patients with OC (n=24), LC (n=6) and BC (n=96) and HC (n=14). EVs were isolated by sucrose density gradient and quantified by Bradford protein quantification kit. EV protein levels are significantly higher in BC sera than in HC sera and in BC pleural effusions and OC ascites than in LC ascites. No significant differences in serum EV level between patients with localised and metastatic BC. EVs isolated from ascites and pleural effusions but not serum from BC and OC patients are EpCAM and CD24 positive.	Rupp *et al*., 2011 (82)
Number of EVs	Plasma from BC patients (n=50) and HC (n=31). Platelet-free EVs were isolated by differential centrifugation and quantified by FC. Number of EVs is higher in plasma from BC patients than HCs ($p<0.05$) and *in situ* BC; number of EVs correlates with tumor size ($p<0.0001$) but no differences between BC stages. FAK-positive EVs found in stage I-IV and *in situ* BC patients, undetectable in HCs. EGFR-positive EVs found in stage I and *in situ* BC patients, undetectable in stage II and III BC and HCs.	Galindo-Hernandez *et al*., 2013 (83)
Number of EVs	Serum from patients with BC (n=40) and HCs (n=10). EVs were isolated using ExoQuick and quantified by acetylcholinesterase assay. EV amounts were significantly higher in BC than HC serum ($p<0.01$). BC serum EVs contain Survivin protein and its splice variants, Survivin 2B and ΔEx3.	Khan *et al*., 2014 (84)
Number of EVs	Serum from patients with BC (n=11) and HCs (n=8). EVs were isolated by sequential centrifugation and filtration steps and quantified by NanoSight. EV levels were significantly higher in BC than HC serum ($p=0.012$). BC associated EVs contain Dicer, AGO2 and TRBP and have a cell-independent capacity to process precursor miRNAs.	Melo *et al*., 2014 (85)
miR-373, miR-101, miR-372	Compared cell-free *vs*. EV-enclosed miRNAs in serum of patients with BC (n=50) and HC (n=12). EVs were isolated using ExoQuick and miRNA levels measured by qRT-PCR. miR-101, miR-372 and miR-373 were enriched in EVs relatively to the cell-free miRNAs. miR-101 and miR-372 levels were higher in BC EVs than HC EVs. EV-enclosed miR-373 levels (but not cell-free) were higher in EVs from TNBC patients than luminal BC and HCs and could discriminate ER^+ *vs*. ER^-, and PR^+ *vs*. PR^- BC.	Eichelser *et al*., 2014 (94)
EMMPRIN-positive MVs	Serum from 15 metastatic BC patients and 16 HCs. MVs were isolated by centrifugation at 14,000 ×g and quantified by FC. Levels of EMMPRIN-positive MVs were significantly higher in sera from BC patients than HCs ($p=0.0075$). EMMPRIN-positive MVs (but not exosomes) induce invasion of tumor cells.	Menck *et al*., 2015 (93)
GPC1-positive EVs	Identified GPC1 as a specific marker for cancer-derived EVs. Serum from patients with BC (n=32), PDAC (n=190) and HCs (n=100). EVs were isolated by sequential filtration and centrifugation steps and quantified by NanoSight and FC. 75% of BC patients and 100% of PDAC patients had higher levels of GPC1-positive EVs than HCs ($p<0.0001$). No correlation with BC subtypes.	Melo *et al*., 2015 (95)

FC, Flow cytometry; HCs, healthy controls; LC, liver cirrhosis; OC, ovarian cancer; PDAC, pancreatic ductal adenocarcinoma.

various BC sub-types that may offer a chance to define the sub-type in cases where the tumor tissue is not available for gene expression analyses.

Using proteomic analysis of BC cells, fibroblasts and non-tumorigenic cells, Melo *et al*. identified glypican-1 (GPC1), a cell surface proteoglycan, as a specific marker of cancer-derived EVs (95). Increased levels of GPC1-positive EVs were found in 100% of patients with pancreatic cancer and 75% of patients with BC compared to healthy controls. This finding has numerous potential clinical applications. At first, it demonstrated that cancer-derived EVs can serve as highly specific diagnostic markers that, at least in pancreatic cancer, outperform previously known blood-based biomarkers, such as CA19-9 and other previously defined biomarker panels (96). Although the diagnostic sensitivity of GPC1-postive EVs as an individual biomarker for BC is not sufficiently high for an immediate application, it can probably be improved by combining it with other BC-associated EV surface markers. Furthermore, more importantly, this allows isolating cancer-derived EV populations from biofluids and dissecting their molecular content that could be of paramount importance for detecting the presence of drug targets, monitoring of treatment response and detecting evolving cancer cell clones that have acquired novel mutations or lost the expression of therapeutic targets. For example, EVs released by *ERBB2*-overexpressing BC cell lines have been shown to be enriched in HER2 protein (97, 98). Hence assessing the HER2 status in EVs present in patients' blood, could potentially serve as a tool for monitoring the efficacy and predicting resistance to HER2 targeted therapy. However, an accurate quantification of HER2 and other tyrosine kinase receptors associated with the resistance in the complex mixture of plasma EVs is unlikely to be achieved. Whereas the isolation of cancer-derived EVs from patients' blood prior to analysis of the receptor status would allow assessing the proportions between EVs expressing full-length HER2, HER3, EGFR, truncated HER2 and HER2-negative EVs, thereby helping to evaluate the intratumoral heterogeneity and detect the presence of resistant cancer cell clones.

Extracellular Vesicles as Therapeutic Targets in Breast Cancer

Growing evidence shows that cancer-derived EVs promote cancer development and progression, and interfere with anti-tumor immune response both, in a paracrine and systemic manner. EVs released by cancer cells can be taken-up by other cancer cells, stroma cells such as cancer-associated fibroblasts, normal epithelial cells, endothelial cells and tumor-infiltrated immune cells, and transmitted *via* the bloodstream or lymphatic system to distant organs throughout the body (17).

The functional effects elicited by cancer-derived EVs in BC are summarised in Figure 1. Briefly, several studies have demonstrated that EVs released from highly metastatic BC cell lines promote cell proliferation, induce epithelial-to-mesenchymal transition (EMT), migration and invasiveness and inhibit apoptosis, when internalised by non-invasive or non-malignant cells (93, 97, 99-101). These effects were, at least partially, mediated by transfer of miR-10b that suppressed the protein levels of its target genes in the recipient cells (101) or activation of p38/MAPK signaling pathway by EMMPRIN (93). Furthermore, EVs were shown to promote angiogenesis (99) and increase vascular permeability *via* the transfer of miR-105 to endothelial cells, where it down-regulated tight junctions by targeting ZO-1 (102). Uptake of miR-105-containing EVs promoted the metastatic potential of poorly-metastatic BC cells *in vivo* by enhancing tumor cell invasion and destroying the vascular endothelial barriers (102). Likewise, Tominaga *et al*. showed that miR-181c-containig EVs released by TNBC cells were able to trigger the breakdown of blood-brain barrier and promote brain metastasis (103). miR-200-expressing EVs were shown to alter the gene expression profile and induce mesenchymal-to-epithelial transition (MET) in non-metastatic cells, thus conferring an increased metastatic capability to these cells (104). Besides, BC-derived EVs have been shown to be internalised by cancer-associated fibroblasts (105) and normal mammary epithelial cells (106), where they induced reactive oxygen species and autophagy and triggered the production of cancer cell growth-promoting factors, thus contributing to the creation of tumor permissive microenvironment. A recent study by Fong *et al*. showed that BC-released EVs that carried high levels of miR-122 were able to re-program energy metabolism in lung fibroblasts, brain astrocytes and neurons by down-regulating the glycolytic enzyme pyruvate kinase in these cells thus contributing to the formation of a premetastatic niche in BC (107).

Furthermore, EVs released by aggressive TNBC cells were found to express high levels of functional tissue factor (TF) that is responsible for cancer-associated thrombosis. TF-bearing EVs could transfer TF to less aggressive BC cells and propagate a TF-associated aggressive phenotype (108).

On the same note, EVs released by docetaxel-resistant cells were capable of transferring resistance to the drug-sensitive cells, probably *via* transfer or induction of P-glycoprotein (109), while tamoxifen-resistant ER-positive cells could transfer tamoxifen resistance to the drug-sensitive cells *via* miR-221/222 carrying EVs (110). Furthermore, packaging of various drugs into the lumen of EVs thus sequestering them away from their cellular targets was recently identified as a novel mechanism of multidrug resistance (111). Whereas HER2-overexpressing EVs have been shown to bind to trastuzumab, thus sequestering the drug and reducing its efficacy (98).

Furthermore, BC-derived EVs have been shown to interfere with the anti-tumor immune responses in a variety of ways. EVs released by murine mammary tumor cells inhibited differentiation of bone marrow dendritic cells by inducing IL-6 production in the myeloid precursors (112). NKG2D-ligand-bearing EVs reduced the proportion of NKG2D-positive effector cells and impaired their cytotoxic functions (113). In macrophages, BC-derived EVs induced expression of Wnt 5a and production of WNT 5a-postive EVs that in turn stimulated invasion of cancer cells (114). Another study showed that BC-derived EVs triggered NF-κB signalling and stimulated production of pro-inflammatory cytokines, such as IL6, TNFα, GCSF and CCL2 by macrophages (115). Furthermore, EVs were shown to contain tumor-associated antigens that sequester tumor-reactive antibodies thus reducing the antibody-dependent cytotoxicity (116).

Collectively, these findings suggest that inhibition of EV secretion or uptake, or blocking specific EV components could suppress the cancer-promoting effects of BC-derived EVs and therefore EV targeting may represent a novel strategy for anticancer therapy. Results of the initial pre-clinical studies are promising and suggest that EV-targeting approaches may have a therapeutic benefit. For example, in the study by Tominaga *et al*. the EV secretion in the BC cell lines was inhibited by siRNAs against two proteins required for the EV biogenesis – neutral sphingomyelinase 2 and RAB27B. The results of *in vitro* blood-brain-barrier transmigration assay showed that these cells had a significantly reduced ability to pass through the blood-brain barrier and that extravasation was restored by adding BC-derived EVs (103). This finding is in line with a previous study in melanoma showing that knockdown of *Rab27a* in mice melanoma cell lines reduced primary tumor growth, lung colonisation and metastasis (15). In a lung cancer model, treatment of xenograft-bearing mice with diannexin, annexin V homodimer that prevents EV interactions with cellular surfaces, resulted in the inhibition of primary tumor growth and angiogenesis (117). In the study by Fong *et al*., systemic treatment of mice bearing miR-122-expressing BC xenografts with anti-miR-122 oligonucleotides significantly alleviated EV-induced metabolic re-programming in the brain and

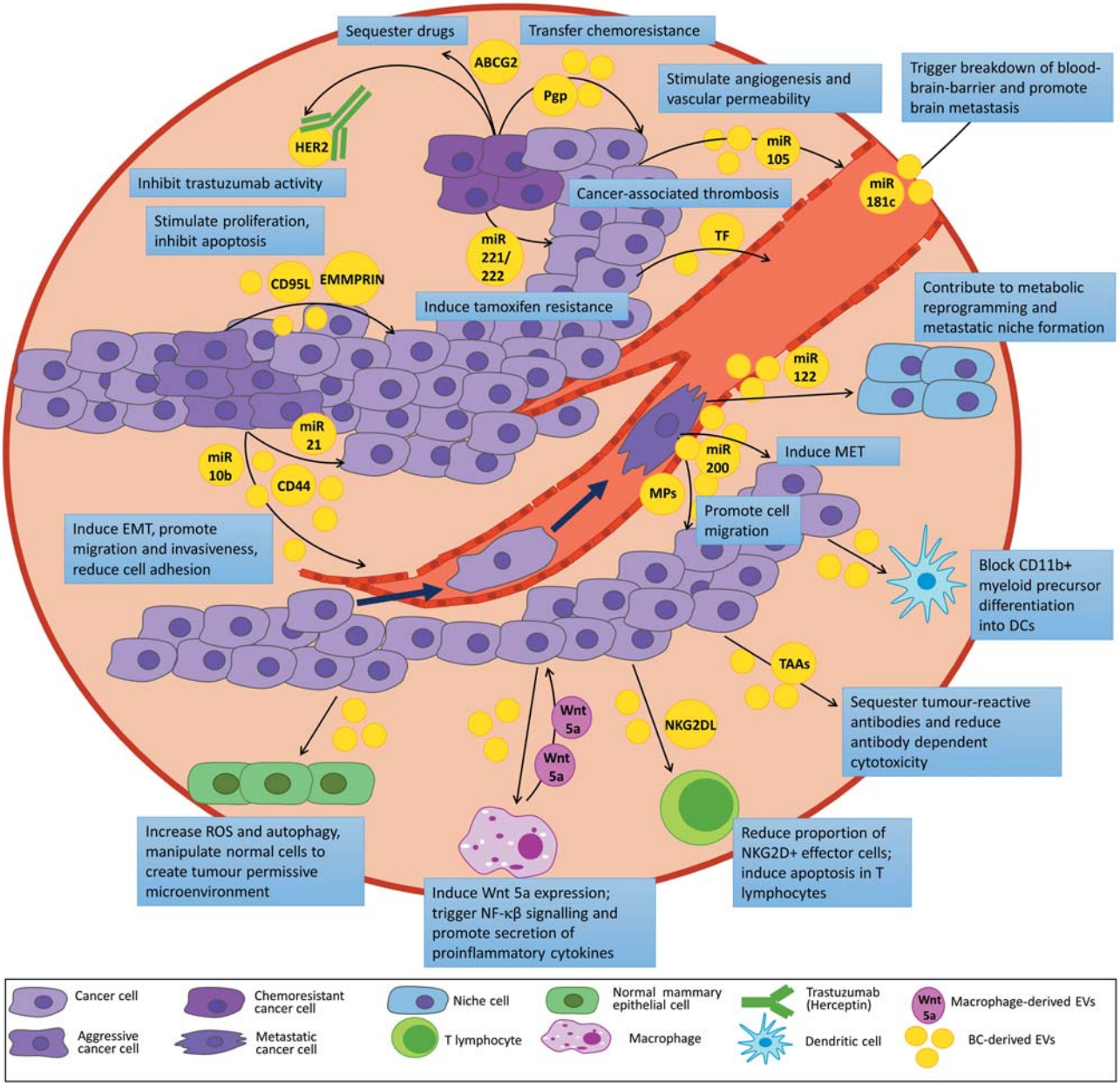

Figure 1. *Cancer-promoting and immunosuppressive effects elicited by BC-derived EVs. Pgp, P-glycoprotein; TF, tissue factor; TAAs, tumor-associated antigens; MPs metalloproteases; MET, mesenchymal to epithelial transition; ROS, reactive oxygen species; EMT, epithelial to mesenchymal transition.*

decreased the incidence of metastasis (107). In a similar experiment, the same group demonstrated that miR-105 inhibition reduced the volume of primary tumors and suppressed distant metastases by increasing ZO-1 expression and restoring the vascular integrity (102).

Marleau *et al*. proposed to exploit extracorporeal hemofiltration of EVs as a novel therapeutic approach (118). This technology is based on an affinity plasmapheresis platform allowing capturing of EVs from the circulation using various affinity agents, such as EV-binding lectins and antibodies. Theoretically, such an approach may have numerous clinical applications. For instance, the depletion of HER2-postive EVs may improve the therapeutic efficacy of trastuzumab (98), the removal of EMMPRIN-bearing EVs could decrease invasion of BC cells (93) while the depletion of GPC1-positive EVs might abrogate multiple cancer-

promoting and immunosuppressive effects. However, the efficiency of EV depletion and the clinical benefits of this approach still remain to be proved.

Concluding Remarks

Since the initial discoveries that EVs contain cancer-associated molecules and mediate numerous cancer-promoting and immunosuppressive effects, enormous efforts have been devoted to finding ways to exploit them as biomarkers and therapeutic targets. The potential advantages of EVs over other circulating biomarkers include relatively high abundance in biofluids, the capacity to protect their cargo against degradation in plasma and enrichment with rare cancer-specific molecules and molecular signatures associated with disease subtypes, prognosis and response to therapy. A number of initial studies have shown that the count and molecular content of EVs is altered in patients with BC compared to healthy controls, and therefore they hold promise as diagnostic markers of BC. However, the sample size in most of these studies was relatively small and the initial findings must be validated in larger cohorts of samples in order to establish their diagnostic value. Furthermore, to the best of our knowledge, none of the studies, so far, had reported the prognostic value of BC-derived EVs nor addressed longitudinal changes of EV-associated biomarkers and their correlation with the clinical events, which is of particularly high clinical significance.

Recent studies on the pathological roles of EVs have provided deeper insight into the ways on how EVs transfer phenotypic traits among cancer cells and mediate cancer-induced immunosuppression and prompted to explore possibilities to block the EV-mediated signaling. Thus far, the pre-clinical trials of EV targeting have shown that this has a capacity to delay the growth of primary tumor and reduce the metastatic potential but can't entirely stop the growth of cancer. Thus, it is not yet clear, if EV-targeting approaches have a potential to become stand-alone cancer therapeutics, yet they certainly have a potential to improve the therapeutic efficacy if combined with chemotherapeutics, immunotherapy or molecularly targeted drugs. However, it has become evident that cancers are very heterogeneous with respect to their biogenesis, molecular cargo and levels of EVs they produce. We believe, it is crucial to reach a deeper understanding of the causes and functional consequences of this heterogeneity in order to design rational therapeutic trials on EV targeting.

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