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# Extracellular Vesicles: A Mechanism to Reverse Metastatic Behaviour as a New Approach to Cancer Therapy

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Additional information is available at the end of the chapter

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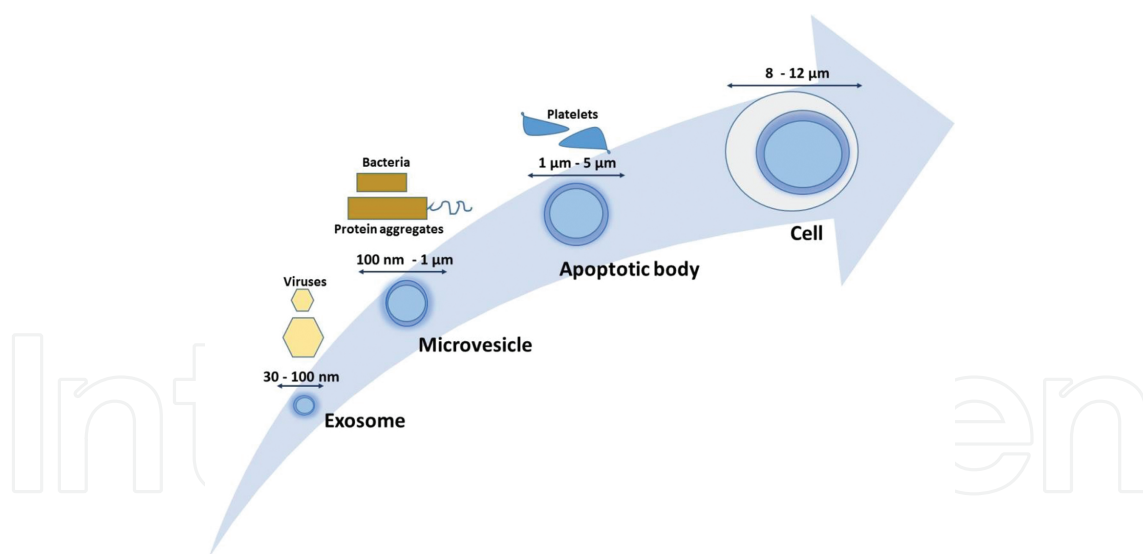
## Abstract

Extracellular vesicles (EVs) are membrane-bound particles shed from nearly all cell types into the extracellular environment. This collective term includes vesicles ranging in size from 30 nm to 5  $\mu$ m in diameter. Various isolation techniques are used in different studies to separate EVs with no consensus protocol. EVs are released from cells under normal physiological conditions as well as in stressful and pathological conditions. In malignancies, they have been shown to be useful circulating markers for risk assessment, early diagnosis, monitoring of therapeutic effectiveness and prognosis. In addition, they appear to influence cell death and growth, angiogenesis, immune surveillance, extracellular matrix degradation and metastasis. In this respect, EVs have generated considerable interest for their potential use in cancer therapeutics. Since they appear to be responsible for transference of cellular components between cells and thereby transfer of functional characteristics of the donor to the recipient, two strategies for their role in cancer therapeutics may be envisaged. The first would be to prevent formation and/or shedding of EVs to prevent communication to or from cancer cells. The second would be to utilize them as carriers to deliver inhibitory/toxic components into cancer cells to destroy or neutralize them. In this review, we discuss the current state of research on characterization of EVs and highlight possible strategies for their use in cancer therapy.

**Keywords:** cancer, microvesicles, EVs, exosomes, drug delivery

## 1. Introduction

Extracellular vesicles (EVs) are membrane-bound particles shed into the extracellular environment by many types of cell under different circumstances ranging from normal physiological conditions to pathological conditions like cancer. There are several ways of classifying EVs, including size and mode of biogenesis. Some authors use the designation EVs interchangeably with other terms like exosomes and microvesicles (MVs). This has led to some confusion and inconsistency as to the particles actually being studied. Therefore, we have included a section on the various isolation procedures to emphasise the importance of standardization. Indeed, in many studies, there is no or unconvincing characterization of preparations. In this chapter, we will use EVs as a broad term to encompass three categories: exosomes, microvesicles and apoptotic bodies [1]. Exosomes (30–100 nm diameter) are formed in multi-vesicular bodies (MVB) [2] and released upon MVB exocytosis [3]. They carry several kinds of cargo, depending on the surrounding physiological conditions prevailing at the time of their formation and this will determine their effect upon recipient cells. MVs (100 nm–1  $\mu\text{m}$ ) are produced by the outward blebbing and fission of the plasma membrane and appear to have possibly more selectively sorted cargo. MVs express surface receptors that differ depending on the membrane composition of the donor cell [2]. Apoptotic bodies (1–5  $\mu\text{m}$ ) are usually released by tumour cells undergoing apoptosis, are packaged indiscriminately and are often fragmented nuclei and cytoplasmic organelles [3, 4] **Figure 1**.



**Figure 1.** Schematic illustrating the relative sizes of the different classes of EVs (Adapted with permission from Ref. [3]).

When first discovered, the release of EVs from cells was thought to be a mechanism for removal of waste and harmful substances from the cell. Nowadays, they are viewed as mediators of intercellular communication through the transfer of biologically active molecules from donor to recipient cells where they can modulate the phenotype and function of those recipient cells [1]. EVs can interact through their surface proteins with receptors on the target cell, triggering

intracellular pathways, or by direct membrane fusion or endocytosis thereby releasing their cargo into the recipient cell [5]. Furthermore, EVs could transfer paracrine oncogenic features locally between different cells and endocrine signals to distal cells of any type through body fluids, usually blood [6].

The importance of exosomes may include antigen presentation and immune-stimulatory and inhibitory functions. Several key roles of MVs have been suggested to include contribution to the proinvasive character of tumours, induction of oncogenic cellular transformation, procoagulant activity and fetomaternal communication [3].

## 2. Techniques used for collection, measurement of concentration and preservation of EVs

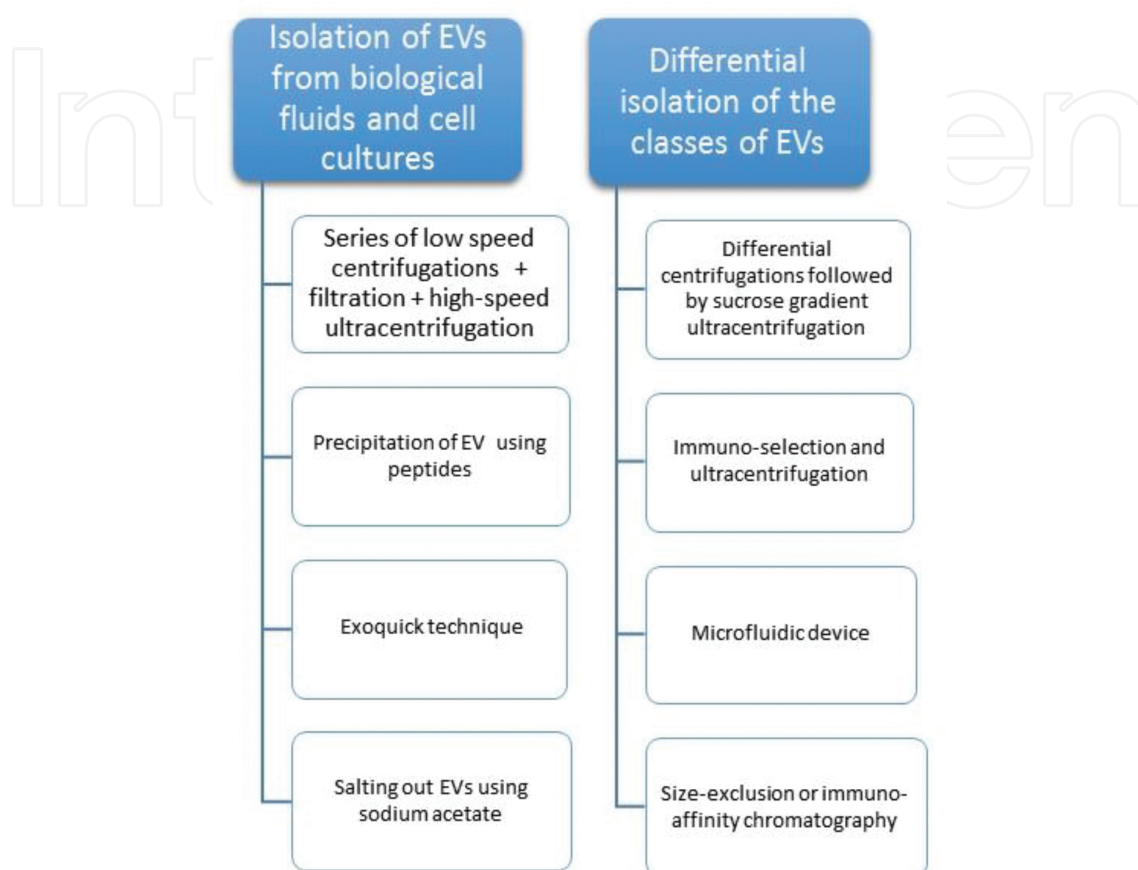
Because of the heterogeneity of EVs, a method of collecting a specific population of EVs of interest must be established. Moreover, the methods of efficient collection of EVs have been investigated in different studies. Previous reports have used two main methods: the ultracentrifugation method [7, 8] and FACS methods [9] for collecting EVs. Many studies have compared the efficiency of these collection methods and evaluated the effect of *in vivo* biodistribution of EVs [10, 11]. These observations suggested that bulk EVs are heterogeneous populations and that there is a need to collect a specific population of interest. In the following section, we will compare different methods for isolating EVs and different techniques used for collection and preservation of EVs.

### 2.1. Isolation techniques

In general, the isolation of EVs from biological fluids and cell cultures requires a series of standard differential centrifugation steps [22]. These sequentially remove dead cells and large cellular debris and then larger intracellular organelles, prior to obtaining a pellet from the cleared cell supernatant. Several modifications/elaborations of the basic procedure have been introduced to purify EVs as well as fractionate them further into discrete size groups; these are illustrated in **Figure 2**.

Heat shock proteins (HSPs), usually associated with cytoprotective functions or as receptor chaperones, are often associated with the cell surface of cancer cells. This feature has been exploited for the separation of EVs from biological fluids and conditioned cell culture growth media. Synthetic peptides called venteremin (Vn), with specific affinity to HSPs, have been used to precipitate out EVs expressing these proteins, in a procedure [16] that has advantages of speed and simplicity over those methods using standard ultracentrifugation. Further refinement of such a strategy, utilizing targets with an exclusively cell surface localization, could be used to distinguish the intracellularly generated exosomes from particles that are formed from the plasma membrane. Other techniques for separating EVs include a salting out process using sodium acetate, and the exoquick technique marketed as a kit by several companies. The latter is also based on selective precipitation of EVs but uses a commercial

agglutinating agent as well as two centrifugation steps. Exoquick has been claimed to produce the highest concentration of EVs when compared to differential centrifugation or salting out methods [14].



**Figure 2.** Summary of procedures for isolation of EVs from biological fluids or cell cultures.

Currently, there is no standard isolation protocol for clearly discriminating the different classes of EVs whether by size, density, morphology of the particles or molecular markers [2]. Various procedures have been described in an attempt to separate them. Among the different groups of EVs, the isolation of exosomes is the one most frequently reported in the literature [13]. Separation of exosomes from MVs usually involves a combination of low-speed differential centrifugation steps followed by sucrose gradient ultracentrifugation [21]. Apoptotic bodies can be collected at low-speed centrifugation of approximately 2,000 g. Microvesicles need a higher speed ranging from 10,000 to 20,000 g. Exosomes are pelleted by ultracentrifugation above 100,000 g for 1 hour or more [12]. Alternatively, immune selection of MVs can be performed instead of the differential centrifugation step. This involves the adherence of MVs to magnetic beads bearing antibodies against tumour-associated markers found on the surface of MVs. Ultracentrifugation would still be needed following this immunoselection in order to recover the exosomes in the eluate from the magnetic beads. Apoptotic bodies, on the other hand, can be separated from exosomes by flotation on a continuous sucrose gradient. Separation

tion of exosomes from biological fluids and other EVs through the steps mentioned above takes approximately 4–6 hours.

Another procedure devised to shorten the time for preparation is based on the use of a microfluidic device, which is said to allow extraction and purification of exosomal RNA from 100 to 400  $\mu$ l serum samples in an hour. This device relies on immunoaffinity isolation of exosomes from cell-free supernatant or serum samples. The sample is allowed to flow inside a microchannel coated with IgG against CD63 (which is highly expressed in exosomes from all cell origins). Specificity was demonstrated by showing that fluorescence intensity was higher in the microchannel coated with anti-CD63 antibodies compared to that coated with (control) anti-CD4 antibodies. As opposed to magnetic bead-based systems, the microfluidic device extracts exosomes directly from the serum in a single step. It does not require incubation, washing or centrifugation. This technique is not only faster compared to other methods of separation but is also cheaper, requires smaller volumes of samples, and fewer reagents [22].

Another method commonly used for isolation is size exclusion chromatography, which relies on size differences to separate EVs. Immunoaffinity chromatography is also an option for capturing exosomes with antibodies that recognize a marker on the surface of exosomes [13].

### 3. EVs in health and disease

#### 3.1. Biological roles of EVs

EVs that are derived from healthy cells transfer signals to other cells, which are needed to maintain their physiological homeostasis and biological functions such as growth, differentiation and apoptotic death. They exert their effects through multiple pathways, directly activating cell surface receptors through bioactive lipid ligands and proteins, integrating their membrane contents into the recipient cell plasma membrane and delivering effectors, such as transcription factors, oncogenes, small and large non-coding regulatory RNAs, mRNAs and infectious particles into recipient cells. Consequently, EVs contribute to the maintenance of normal physiology [12].

The following are some examples of the role of EVs in maintaining a wide range of cellular and biological functions:

##### I. Regulation of immune responses.

EVs might trigger adaptive immune responses or suppress inflammation in a tolerogenic manner [13]. They have been shown to implement immune suppression by several mechanisms, such as enhancing the function of regulatory T cells, suppressing natural killer (NK) cells, and inhibiting monocyte differentiation [12].

##### II. The nervous system.

The secretion of EVs can contribute to a range of neurobiological functions. For example, increased release of EVs containing neurotransmitter receptors from cortical neurons following enhanced glutamatergic activity [14].

### III. Embryonic development.

EVs are likely to be involved in the regulation of embryonic development, including maintenance of morphogen gradients, collective cell migration and tissue polarity. However, this still remains an emerging field with many unanswered questions, which need further investigation [15].

### IV. Tissue repair.

EVs derived from human adult mesenchymal stem cells (MSCs) have been found to prevent ischaemia-reperfusion kidney injury and improve survival in a model of lethal acute kidney injury [16]. MSC-derived EVs are reported to modify the expression of miR29c and miR150 and upregulate the expression of SDF-1, CXCR4, CXCR7, CCL2 and ANGPTL4, which are known to play essential roles in acute and chronic wounding [17].

### V. Liver homeostasis.

A comprehensive study of hepatocyte-derived EVs showed the presence of several members of cytochrome P450, uridinediphosphate-glucuronosyl-transferase (UGTs) and glutathione S-transferase (GST) protein families, supporting a role of these vesicles in the metabolism of endogenous and xenobiotic compounds [18]. Recently, it has been shown that EVs from hepatocytes were able to activate stellate cells to mediate a response to liver damage [19] and many studies support an important role of these vesicles in maintaining liver homeostasis.

## 3.2. Pathological actions of EVs

Given their essential role in regulating biological processes, it is not surprising that EVs have a significant influence in disease pathogenesis. This has been most extensively studied in tumour biology. Several reports have indicated that EVs may be an important means of driving the formation of a pre-metastatic tumour [12, 20]. EVs can promote proliferation of their target cells, stimulate angiogenesis, induce metastasis and promote immune escape by modulating T-cell activity [21–24].

Prior to the discovery of EVs, it was known that the vesicles secreted by tumour cells retained procoagulant activity, linking cancer progression with EV-induced thrombosis [25–27]. In addition, a direct link between EVs and tumour invasion of healthy tissues was reported in 2008 [28]. It was shown that the mRNA expression of an activated mutated epidermal growth factor receptor (EGFRvII) in glioma cells can enhance vesiculation significantly and intercellular transfer of this oncoprotein to adjacent tumour cells, leading to the production of angiogenic mediators such as vascular endothelial growth factor (VEGF) [28].

Similar results were reported in another study by Skog et al. [22] showing that various miRNAs that stimulate tumour growth and angiogenesis in addition to EGFR can be transferred by human primary glioblastoma cell-derived EVs. Moreover, EVs derived from tumour cells were shown to transfer activated EGFR to endothelial cells, inducing VEGF expression and resulting in VEGF receptor activation to stimulate angiogenesis [29]. Many of the previously mentioned

studies suggested that EVs can trigger tumour growth by stimulating the proliferation of cancer cells and by stimulating angiogenesis in the adjacent normal endothelial cells.

Additional data also support the association of tumour-secreted EVs in the promotion of metastasis and tumour invasion; for example, transfer of the EMMPRIN transmembrane glycoprotein, which stimulates matrix metalloproteinase (MMP) expression in fibroblasts and remodelling of the ECM [30]. Recently, it was shown that EVs derived from melanoma cells directed bone marrow cells towards a prometastatic phenotype, mediating the communication between tumour cells and normal cells [31, 32].

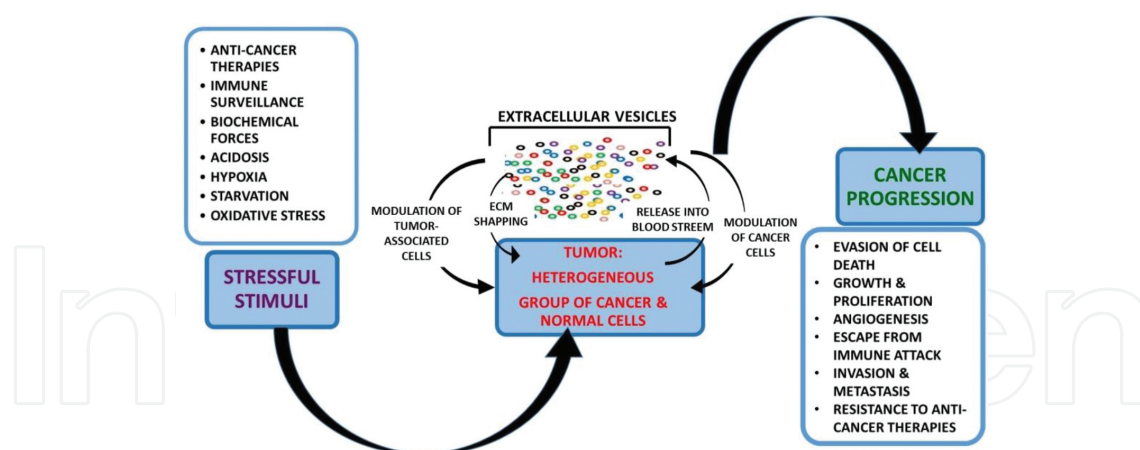
Furthermore, tumour-associated macrophages can secrete EVs, which contain certain miRNAs that can promote breast cancer cell invasion [33]. In addition to their role in cancer, EVs have been associated with various pathogens, including HIV-1, Epstein-Barr virus (EBV) and prions [34–36].

#### 4. Involvement of EVs in cancer

Tumour EVs (oncosomes) are associated with many types of cancers [37–39], with elevated concentration in the plasma of cancer patients compared to healthy controls [40]; this can be up to 10-fold more than the approximately  $10^{11}$  MVs per ml of serum measured in healthy individuals [41, 42]. Tumour EVs contain lipids and proteins as well as RNAs, genomic DNA and cellular metabolites, which can be transferred between cells [43], thus regulating the bioactivities of recipient cells. Production of EVs seems to be highly regulated. Several studies have characterised tumour EV components to identify useful cancer biomarkers [44]. For example, in two breast cancer cell lines, MCF-7 and MDA-MB 231, the cell-derived EVs show different profiles; 59 proteins were identified in MCF-7-derived EVs and 88 in EVs from MDA-MB 231, with 27 proteins common between the two exosome-like vesicle types [45]. Among all of these molecules that can be transferred from one cell to another through EVs, miRNAs have attracted most attention because of their newly recognised regulatory role in modulating gene expression. As some profiling studies have shown, miRNAs are not randomly incorporated into exosomes. According to previous studies, there exists a class of miRNAs that are preferentially sorted into exosomes, such as miR-320 and miR-150. Members of the miR-320 family are widely distributed in exosomes derived from both normal tissue and tumours [22, 46, 47]. Moreover, some reports have shown that exosomal miRNA expression levels are altered under different physiological conditions. Exosomal miR-105 released from the breast cancer cell line MDA-MB-231 reduced *ZO-1* gene expression in endothelial cells and enhanced metastases to the lung and brain [48]. Exosomal miR-214, derived from the human microvascular endothelial cell line HMEC-1, stimulated migration and angiogenesis in neighbouring HMEC-1 cells [49]. Thus, it is attractive to speculate that EVs may 'export' the ability of the producer cells to metastasise, to other cells.

Stressful stimuli, such as hypoxia, acidosis, oxidative stress, radiation and cytotoxic drugs, activate signalling pathways that can trigger exosome production and secretion [50]. The p-53-regulated gene product, TSAP6 [51], as well as ceramide [52] have been documented as





**Figure 3.** Extracellular vesicles (EVs) are potential carriers of stress-mediated tumour progression (Adapted with permission from Ref. [50]).

triggers. Stressful conditions can change both the molecular content and function of EVs, allowing for cancer progression through any of the processes displayed in **Figure 3**. For example, thermal and oxidative stress on leukemia/lymphoma T and B cells has been shown to induce the release of exosomes rich in Natural Killer Group 2 and member D (NKG2D) ligands that confer immunosuppressive properties to the exosomes. In addition, aggressive B-cell lymphoma cells that have been exposed to rituximab, which is an anti-CD20 chimeric antibody, started secreting CD20-positive exosomes that protected the lymphoma cells from antibody and complement-dependent cytotoxicity. It is known that the phenotype of metastatic cells is a result of an accumulation of stress conditions on tumour cells. It has also been reported that while EVs derived from primary tumour cells can contain cell-adhesive proteins, those from metastatic cells are loaded with proteins that are responsible for cancer progression, invasion, metastasis and multidrug resistance. Thus, EVs can act as conveyors of stress-mediated tumour progression. Like cancer cells, stromal cells could release EVs with modulated function upon exposure to stress. As an example, mesenchymal stem cells exposed to hypoxic conditions released microvesicles with angiogenic effects [50]. The horizontal transfer of bioactive molecules by EVs can influence the different aspects of tumour progression, which include angiogenesis, decrease of immune surveillance, ECM degradation, metastasis and chemoresistance. The following sections discuss the influence of EVs on the processes that are vital for tumour progression, through horizontal transfer of bioactive molecules.

### I. Neoangiogenesis.

Fibrin, the end product of the coagulation process, plays an important role in tumour growth as tumour cells can be coated with fibrin to escape immune surveillance; at the same time, the fibrin matrix enhances the outgrowth of new blood vessels. In several studies, it has been shown that EVs support coagulation through various mechanisms. They expose negatively charged phospholipids, which enable binding of coagulation factors and hence formation of prothrombinase complexes [53, 54]. In cancer, tissue factor vesicles are present in the peripheral blood [27, 55]. A part of these MVs originates from cancer cells and usually participate in

thrombus formation equally to leukocyte-derived vesicles. Those MV-exposed tissue factors can promote coagulation by adhering at the site of vascular damage [56, 57].

In addition, tissue factor also plays a more direct role in angiogenesis, which is induced through cytoplasmic domain phosphorylation of the tissue factor and subsequent downstream signalling events. Consequently, thrombin will be generated through the activation of coagulation by tissue factor, which cleaves several protease-activated receptors (PARs), in order to initiate angiogenesis [58].

Besides, platelet-derived vesicles stimulate mRNA expression of angiogenic factors in cancer cells and then cancer cell-derived vesicles will contain mRNA for growth factors, such as VEGF and hepatocyte growth factor [59]. It has been showed that such vesicles fuse with monocytes, conveying their nucleic acids content and altering their biologic activity [60]. It is believed that cancer cell-derived MVs transfer mRNA to other cancer cells, enhancing their malignant potential, and it has been reported, as mentioned previously, that intercellular transfer of oncogenic growth factor receptor by cancer cell-derived EVs modify the phenotype of these cells [28].

## II. Escape from immune surveillance.

Collective data suggest a relationship between stressful conditions due to the tumour environment and immunological tolerance of tumours [50]. There are many mechanisms, either direct or indirect, that have been suggested which can facilitate escape from immune surveillance. For example, cancer cells may employ vesiculation as a means to efficiently deceive the immune system and survive [13]. Another study also showed that under the pressure of oxidative stress, tumour cells release NKG2DL-expressing tumour exosomes, which facilitate tumour escape from cytotoxic immune attack [61]. Further, exosomes from various cancer cells were shown to expose Fas ligand (FasL, CD95L) of the death receptor Fas (CD95), to trigger T-cell death and to diminish the function of adaptive immune cells [62].

Tumour-associated EVs may also enhance the function of regulatory T ( $T_{Reg}$ ) cells, weaken natural cytotoxic responses mediated by natural killer cells, downregulate dendritic cell differentiation from monocytes and turn these cells into immunosuppressive cells [24, 63, 64]. In addition, cancer cells can integrate with EVs derived from non-cancer cells, for example, platelets, by this means receiving lipids and transmembrane proteins, which would protect them from immune surveillance [59]. Additionally, cancer cells may hide from the immune system by mimicking the host environment.

## III. Environmental degradation.

It has been shown that degradation of the ECM is needed for tumour growth [65]. EVs expose and contain several proteases, including matrix metalloproteinase (MMP)-2 and MMP-9 and urokinase-type plasminogen activator (uPA). uPA catalyzes the conversion of plasminogen into plasmin, whereas MMPs degrade basement membrane collagens. Plasmin, which is a serine protease, degrades numerous components of the ECM, including fibrin, and activates various MMP zymogens [66].

When Ginestra et al. [67] analyzed vesicle content in ascites fluids from 33 women with different gynaecologic pathologies, they found that malignant tumour fluids contained higher amounts of vesicles compared to benign proliferative cells. Moreover, they showed that the EVs from benign serous cysts had only minimal lytic activity, whereas those from cancer ascites contained active metalloproteases [67]. Furthermore, a link was found between the malignant potential of tumours and the MV-associated MMP-2 activity [68]. Another study reported an increase in numbers of vesicles in late stage ovarian cancer ascites and showed that MMP-2, MMP-9 and uPA activities were mainly concentrated within the MVs. Further, the MMP-2, MMP-9 or uPA inhibition using antibodies almost eliminated the ability of these MVs to enhance tumour invasion capacity, which highlights the significance of this pathway [69].

#### IV. Metastasis.

Metastasis necessitates an increase in cellular survival and invasiveness, which are both enhanced by MVs. Some evidence suggests that MVs may favour lymphogenous and haematological spread as the expression of Fas ligand by cancer cell-derived MVs plays a role in lymph node infiltration [70]. Furthermore, as mentioned above, activation of platelets by tissue factor-derived vesicles supports the haematological spread of cancer cells. Since the cancer cells will be surrounded by platelets, this would afford them some protection from immune surveillance and enhance their attachment to the vessel wall [59]. In addition, the procoagulant properties of cancer cell-derived MVs further support intravascular fibrin formation, which in turn facilitates adherence of cancer cells to the vessel wall [27].

### 4.1. EVs in cancer therapeutics

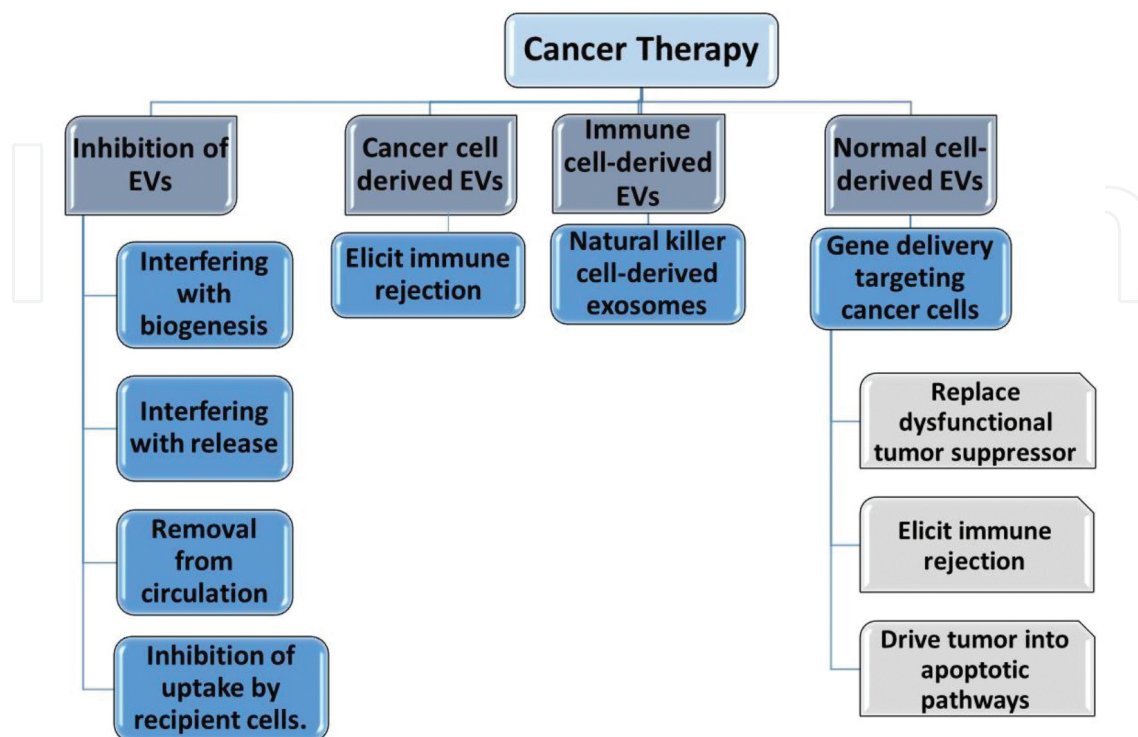
Since MVs appear to contribute significantly to cancer development, it is not surprising that much effort is being focused on trying to find ways of utilizing them in therapy as well. There are at least four strategies that could potentially be used to oppose EV-driven disease by inhibiting various aspects of EV function; these are summarised in **Figure 4**. The most obvious approach is to get rid of them and this can be achieved by blocking their biogenesis, by interfering with their release from the cell, removing them from the circulation or inhibiting their uptake by recipient cells [1].

#### 4.1.1. Inhibition of EVs

##### I. Inhibiting EV formation.

Various cellular components are known to be vital for EV formation but until now no clear inhibition strategy has been forthcoming although many are under investigation. However, some studies showed that inhibition of ceramide formation (which is essential for endosomal sorting and exosome biogenesis) using small molecule inhibitors of neutral sphingomyelinase or through treatment with the blood pressure-lowering drug amiloride (which decreases endocytic vesicle recycling) can reduce EV formation [52, 71]. Another interesting study emphasised the importance of syndecan proteoglycans and their cytoplasmic adaptor syntenin, in regulating exosome formation and release, directly interfering with this interaction either by RNA interference (RNAi) or using small molecule inhibitors [72].

## II. Inhibiting EV release.



**Figure 4.** Flowchart showing the variety of roles of EVs in cancer therapeutics.

Many proteins have been shown to be associated with the secretion of EVs, but again the exact mechanism of regulated EV release remains unclear and probably varies between different cells. However, it has been shown that some small GTPases in some tumour cells, such as RAB27A73, can be a promising therapeutic target (by RNAi) for reducing tumour exosome-mediated signalling to inhibit neutrophils that support tumour growth [73, 74]. This approach was used in two independent studies and it showed a significant reduction in the growth rate of primary metastatic carcinoma and in metastasis progression [73, 75]. Other GTPases such as RAB11 and RAB35 might serve as alternative targets for inhibiting the release of exosomes by weakening and loosening the docking and/or fusion of multi-vesicular bodies with the plasma membrane [74].

## III. Inhibiting EV uptake.

Several uptake mechanisms have been suggested for EVs, but there is insufficient information about the fundamental phases in EV trafficking and target specification. However, some studies showed that the uptake of EVs released from tumour cells can be reduced by diannexin, which can block phosphatidylserine, an important cell adhesion protein [76]. On the other hand, this concept can also be used in diseases other than cancer. For example, diffusion of HIV-1 to T cells could be reduced by targeting intercellular adhesion molecule 1 (ICAM1), which is exposed on EV-encapsulated viruses, thus preventing binding specifically to  $\beta$ 2 integrin [77]. Moreover, another suggested mechanism of HIV-1 diffusion to non-haemato-

poietic cells is by the horizontal transfer of chemokine receptors through EVs, which makes these vesicles valued targets for investigation [34].

#### IV. Blocking specific EV components.

Blocking specific signalling components of EVs was shown to have therapeutic significance. It was demonstrated that FASL-specific monoclonal antibodies targeting FASL1 displayed on EVs reduced tumour growth in a melanoma model [78]. However, this method may lack specificity and has negative impact on immune function. In the same way, the targeting of MET oncoprotein by RNAi to inhibit its active involvement into EVs was shown to be useful in reducing metastasis in late-stage melanoma [31].

All the above approaches highlight promising targets to develop small molecule therapeutics. Nevertheless, it is important to note that interfering with EV biogenesis could result in unwanted off-target effects, given that EVs are important for the regulation of normal core cellular processes and of course such approaches will need to be translated into a drug delivery system (DDS) that is capable of targeting specific EVs.

##### *4.1.2. Cancer cell-derived EVs*

Since it is known that EVs released from normal cells trigger positive effects and those released from cells under pathological conditions usually trigger undesirable effects, it might initially seem surprising that cancer cell-derived EVs could play a therapeutic role. Cancer cell-derived EVs carrying tumour antigens could actually help in initiating immune attacks by providing these antigens to antigen-presenting cells. These cells would then activate a T cell-dependent immune response against the tumour; their antigen content theoretically makes them ideal cancer vaccines. This has been reported in a number of studies of animal models of cancer [79, 80].

##### *4.1.3. Normal cell-derived EVs*

Normal cell-derived EVs can be used as drug delivery systems that transfer therapeutic nucleic acids or proteins. Unlike synthetic liposomes and viral vectors, EVs would be immunologically protected as 'self'. In addition to being sufficiently stable with a long tissue half-life, the small size of cell-derived EVs is suitable to allow them to penetrate through the target tissues [81] and cross biological barriers [2] and at the same time be large enough to carry sufficient payload. Moreover, EVs are capable of carrying a wide range of bioactive components, including mRNA, miRNA, DNA and proteins. In this regard, most studies have focused on the delivery of genes to cancerous cells to either replace dysfunctional tumour suppressor genes or to activate immune rejection or trigger cells into apoptotic pathways. Another potential advantage of EVs that makes them competitive in the pool of delivery vehicles is the suggestion that specific peptides could be introduced into EVs to provide them with targeting abilities toward a certain tissue.

#### 4.1.4. Immune cell-derived EVs

EVs that are produced by immune cells have been shown to have an important role in the regulation of immunity. They can mediate immune stimulation or suppression and they can drive inflammatory, autoimmune and infectious disease pathology. Therefore, EVs have the potential to be used as therapeutic agents to modulate the immune system. It has been found that EVs released by B cell lines carry MHC class II, co-stimulatory and adhesion molecules indicating that such vesicles could directly stimulate CD4<sup>+</sup> T cell clones [82]. This idea was further supported by the observation that the vaccination of mice with exosomes derived from tumour peptide-pulsed dendritic cells (DCs), enhanced tumour-specific cytotoxic T lymphocytes (CTLs) and inhibited tumour growth in a T cell-dependent manner [83]. Numerous studies have shown the direct effects of EVs in T cell activation. It has been demonstrated that immature DC-derived EVs express a low ratio of co-stimulatory molecules to co-regulatory molecules on their surface and therefore act as immunosuppressives [84].

## 5. EVs as drug delivery tool in cancer

Effective therapeutic agents have been extensively studied and tried for many decades to be developed in order to deliver an effective therapeutic agent to its target specifically with minimal side effects. It is well known that non-targeted drugs are inefficient and have side effects when they are delivered systemically. The purpose of a drug delivery system (DDS) is to deliver a drug efficiently, improve the effect of the drug and minimise its side effects [85, 86]. Many useful drug delivery tools and cargos have been developed, such as PEG, liposome, nanoparticles and cell penetrating peptides (CPPs) [87–89]. However, despite the persistent efforts of researchers, the delivery to specific organ and the side effect of DDS remain unsolved completely. DDSs are desirable for use in cancer therapy, and EVs have been recently proposed as promising natural drug delivery tool to serve different diseases [90, 91]. It has been noticed that EVs have a tropism to some organs or cells, and because of their biological significance, they have gained a potential benefit in drug delivery field to target organs or cells. Furthermore, EV-based DDSs are expected to have huge impact and revolution in drug delivery industry field because of their minimal side effects, as they are naturally occurring in the body, in addition to their ability to mediate tumour-selective drug delivery or to mediate organ-specific drug delivery.

### 5.1. Drug delivery techniques

#### 5.1.1. Encapsulation of drugs to EVs

Drugs should be conjugated or encapsulated in EVs in order to be used as DDSs cargo or vehicle. Several methods have been utilized for encapsulating existing drugs in EVs using methods, such as sonication, extrusion and electroporation [92]. One study investigated four different methods for incorporating catalase into EVs from a Raw 264.7 macrophage cell line, where incubation at room temperature, freeze-thaw cycles, sonication and extrusion were

applied [93]. Interestingly, it has been reported that melanoma cells treated with the anticancer drug cisplatin eliminated the cisplatin through EVs [94]. When human pancreatic adenocarcinoma CFPAC-1 cells were treated with EVs containing paclitaxel, this produced an anti-tumour effect [95].

### 5.1.2. Organ tropism of EVs

Another promising therapeutic application of EVs is through the delivery of molecules to certain organs or cells using a phenomenon known as EV tropism. There have been various attempts to use this pathway for treatment of brain disorders and cancer. For example, Alvarez-Erviti et al. [7] used EVs as part of a neuronal-specific delivery system to effect an siRNA-mediated knockdown of the  $\beta$ -site amyloid precursor protein cleaving enzyme 1 (BACE1), an initiating enzyme required for  $\beta$ -amyloid peptide synthesis. A significant reduction (60%) of the BACE1, at both mRNA and protein level, was achieved in the brain cortical tissue by this delivery system, indicating its utility for the treatment of Alzheimer's disease. Zhao et al. [96] showed that systemic administration of glial cell line-derived neurotrophic factor to a Parkinson's disease (PD) mouse model, significantly ameliorated both neurodegradation and neuroinflammation through the specific transmission of the neurotrophic factor by the released EVs into the target neurons. Also Zhuang et al. [97] showed that intranasal delivery of EVs containing curcumin or the STAT3 inhibitor JS1-124 to microglial cells in mice significantly reduced Lipopolysaccharide (LPS)-induced brain inflammation and delayed tumour growth in the GL26 tumour model. Furthermore, Pascucci et al. [95] showed a strong anti-proliferative activity of EVs delivered from mesenchymal stromal cells (MSCs) incorporated with paclitaxel, against the human pancreatic CFPAC-1 cell-line. These data suggest a more potent and specific cell target delivery system aiming to increase the anti-tumour efficiency of chemotherapeutic drugs. Skog et al. [22] demonstrated a future possibility to use EVs as a diagnostic tool for certain tumours, such as glioblastoma. These tumour cells are able to release their own EVs, which contain mRNA/miRNA and proteins into the blood stream. Various mRNA/miRNA characteristics of glioblastoma cells were detected in the blood in about one-third of the tested glioblastoma patients, suggesting its utility in diagnosis and for design of optimal treatment plans for each patient.

## 6. Future perspectives

EVs are endogenous carriers that facilitate intercellular communication. Although their existence has been known for a long time, they have attracted recent renewed interest because of their possible participation in the spread of particularly cancer initiating or metastasis promoting agents from tumour cells, which appear to produce them in excessive amounts. By the same token, EVs from normal cells may be able to reverse the malignant characteristics of cancer cells by transfer of tumour suppressors or pro-apoptotic molecules, providing more 'natural' therapy. In the drug delivery field, they are causing much excitement as potential therapeutics because of their efficient transfer of proteins, mRNA and miRNA, as well as existing drugs, into selective targets. They have obvious advantages over artificial liposomes

or other nanoparticles. However, this requires more knowledge of EV content and how they are released, their stability and how they target cells. There is also a need for clearer quantitative and qualitative analysis of EVs in terms of their classification and production from normal and cancerous cells.

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