Extracellular vesicles to diagnose and treat cancer

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SUMMARY

Extracellular vesicles transfer lipids, nucleic acids and membrane-associated as well as intraluminal proteins between cells to maintain homeostasis and regulate physiological functions. This communication system is hijacked in cancer. Tumour-derived extracellular vesicles enter the circulation and carry targeting motifs and unique messages for cell-type specific instruction of distant ecosystems to foster metastasis. In this review we focus on how extracellular vesicles provide new opportunities for the diagnosis and treatment of cancer. Quantification and characterisation of tumour-derived extracellular vesicles obtained by liquid biopsy may enable the diagnosis and prognosis of cancer patients. Interference with extracellular vesicle biogenesis and implementation of extracellular vesicles as cancer vaccines or drug delivery vehicles opens up therapeutic potential to treat cancer.

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INTRODUCTION

Cells at a distance from each other communicate by secreting soluble factors (e.g. hormones and growth factors) and complexes composed of proteins, lipids and/or nucleic acids (e.g. protein complexes, lipoproteins and extracellular vesicles (EVs)). EVs consist of a double layered lipid membrane, nucleic acids and membrane-associated as well as intraluminal proteins (*Figure 1a*). The lipid bilayer has a specific lipid composition and contains protective proteins against the complement pathway of the innate immune system. This makes EVs more stable in the extracellular environment compared to soluble proteins. EVs circulate in the extracellular environment close to their place of origin, although some EVs are able to diffuse away from this place of secretion and end up in biological fluids like blood, urine, saliva, sperm, breast milk, amniotic fluid, etc.[1]

Different types of EVs are secreted (*Figure 1b*). EVs can originate from direct budding from the plasma membrane, leading to microvesicles with a size between 50

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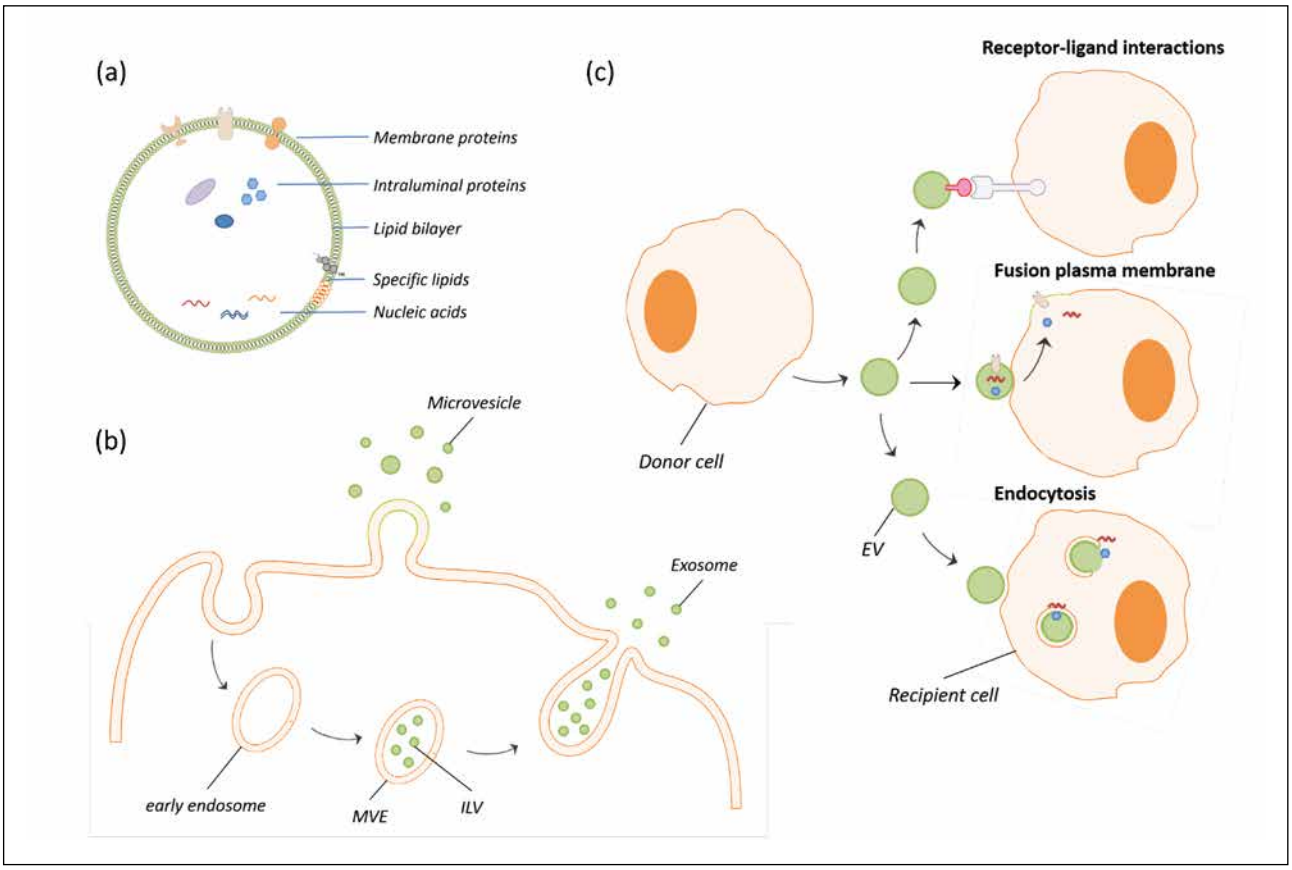

FIGURE 1. (a) Extracellular vesicles (EVs) consist of a double layered lipid membrane, some specific lipids, nucleic acids and membrane-associated as well as intraluminal proteins. (b) Different types of EVs are secreted by cells. Exosomes have a specific intracellular origin. Intraluminal vesicles (ILV) are formed by budding of the limiting membrane of early endosomes which leads to the formation of multivesicular endosomes (MVEs). When the MVEs fuse with the plasma membrane the ILVs are released into the environment and from that moment they are called exosomes. Microvesicles don't have this specific intracellular origin and bud at the plasma membrane. Exosomes have a size of 50 to 100 nm while microvesicles have a size between 50 and 1000 nm. (c) EVs have different ways to interact with cells. They can initiate signal transduction through receptor-ligand interaction, they can fuse with the plasma membrane of the recipient cell or they can be endocytosed after which the membrane of the endosome can fuse with the lipid bilayer of the EV.

and 1000 nm. Another group of EVs is formed within multivesicular endosomes (MVEs). These MVEs can fuse with the plasma membrane, resulting in the release of the intraluminal vesicles (ILVs), from that moment called exosomes, in the extracellular environment. Exosomes have a size of 50 to 100 nm.

Different hypotheses were proposed regarding the mechanism of interaction between EVs and the recipient cell (*Figure 1c*). EVs can initiate the signal transduction through receptor-ligand interactions. This is similar to what happens when two cells interact but without the need for direct cell-cell contact. EVs can also be endocytosed after which they can fuse with the limiting membrane of the endosomes in the recipient cell. A third possibility is a direct fusion of the EVs with the membrane of the recipient cell. In this way, the content of the EVs can be delivered into the cytosol of the recipient cell and the membrane of the recipient cell can carry new surface molecules, which can lead to new characteristics.[2,3]

In the literature, many different methods are described to isolate EVs. To date it is impossible to discern different types of EVs on the basis of intrinsic properties such as size, structure, buoyant density or protein composition. Different isolation protocols will each lead to a different subset of isolated EVs and some methods will even co-isolate non-EV entities.[4,5] This is why, in this review, we will only use the term 'exosome' when the subcellular origin was ascertained and 'EVs' when the subcellular origin was not ascertained.

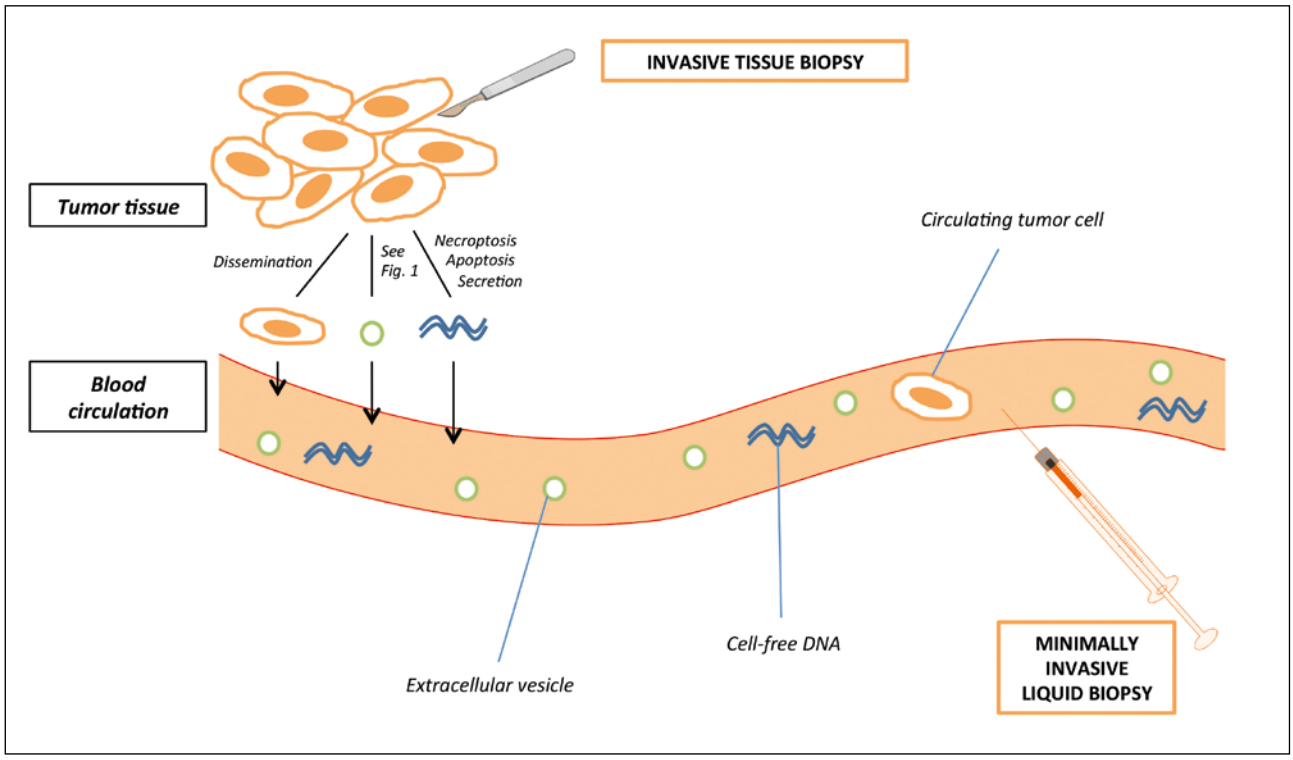

FIGURE 2. In contrast to an invasive tissue biopsy, liquid biopsy is a minimally invasive way to get valuable information about the tumour. Circulating tumour cells, cancer-specific cell-free DNA and extracellular vesicles are directly derived from the tumour and can be found in many body fluids (blood, saliva, urine, ascites, pleural effusion, etc.). Liquid biopsy provides an easily accessible source of predictive and prognostic biomarkers which underlie the clinical usefulness of this method.

Due to the capacity of EVs to transfer proteins, lipids and nucleic acids, they can influence various physiological and pathological functions. EVs play an important role in vascular biology (e.g. coagulation, reticulocyte maturation, etc.), immune responses and regulation, embryogenesis, tissue repair, etc. Besides their physiological function, EVs also play an important role in tumourigenesis, auto immune diseases, etc.[6] Over the last decades the role of EVs in pathology has gained a lot of interest. In this review we will discuss the possible use of EVs as a biomarker for prognosis or diagnosis of cancer and as a therapeutic target, agent or vector.

EVS AS A POWERFUL BIOMARKER

At all times, every droplet of human blood contains more than ten billion EVs.[7–9] In other words, more than ten billion unique sources of biological information about a possible tumour are in the body. It is becoming increasingly clear that EVs play an important role in the communication between distant cells, which is essential for the development of metastasis. If tumour cells want to survive, progress and metastasise, they have to interact with each other and host cells by exchanging nucleic acids, proteins and lipids. EVs seem to be the perfect vehicle to carry all this valuable information and that is why they can be used as a powerful biomarker.

By using more and more powerful biomarkers, researchers should be able to move to an era where cancer will be discovered more accurately in the earliest stage due to the high sensitivity and specificity of these markers. Besides this diagnostic utility, the ideal biomarker should also have a prognostic value. The illustration of this search for novel markers to improve clinical outcome can be exemplified by prostate cancer biomarkers. Several studies demonstrated a lack of sensitivity and specificity for serum prostate-specific antigen (PSA), utilized for over twenty years.[10] In response to the urgent need for new useful biomarkers, researchers discovered biomarkers by using advanced genomic and proteomic technologies. Although some markers like the TMPRSS2-ERG gene fusion test and prostate cancer antigen 3 (PCA3) show promise, none seems poised to replace PSA. Combining TMPRSS2-ERG and PCA3 improves the sensitivity for prostate cancer diagnosis to about 73%, without losing specificity.[11–14] In the search to finding even more powerful prostate cancer biomarkers, but

also biomarkers for other cancers, EVs gained interest. Tumour-derived EVs can be a source of new biomarkers. In addition, these EVs might increase the sensitivity of several biomarkers by solving the dynamic range problem. This problem is known by the fact that a few high-abundance proteins (e.g. albumin, immunoglobulins, transferrin, complement factors, fibrinogen) make up 97% of body fluids, in contrast to the most promising candidates for biomarker discovery, which are the low-abundance proteins.[15] In fact, tumour-derived EVs provide for a specific enrichment of valuable biological molecules coming from the tissue of interest. Referring to the search of a powerful prostate cancer biomarker, the markers PCA3 and TMPRSS2-ERG were already detected in EVs.[16] More research is needed to investigate whether the advantages of EVs result in an improvement of sensitivity and specificity of these biomarkers. Recently, Exosome Diagnostics Inc. (Cambridge, MA, USA) developed ***ExoDx® Prostate (IntelliScore),*** a urine-based test which analyses the EV-RNA values of ERG (including TMPRSS2-ERG), PCA3 and SPDEF, and is now available for clinical use in the United States. Although further validation is needed, researchers found that the ***ExoDx® Prostate (IntelliScore)*** is predictive for High Grade (Gleason Score ≥ 7) prostate cancer with a sensitivity of 92% and specificity of 34%.[17] EVs seem to be an ideal candidate for a non-invasive biomarker for either diagnosis or prognosis. As stated before, EVs play an important role in cell-cell communication through the transport of nucleic acids, proteins and lipids, and each of these classes of molecules provide a completely different way of using EVs as biomarkers.

In 2007, Valadi *et al.* discovered that EVs can transfer microRNA (miRNA) between cells. These small, non-coding RNA molecules can promote tumourigenesis by altering the behaviour of recipient tumour and stromal cells.[18,19] Researchers are convinced that EV-derived miRNA (EV-miRNA) could be a potential cancer biomarker. An aberrant expression of cellular miRNA has been observed in cancer and there is evidence that EV-derived miRNA expression is also altered.[20,21] As with more conventional tumour markers, sensitivity and specificity is low for several reasons (e.g. tumour-specific EV-miRNA signals can be masked by EV-miRNA secreted by other cell types). It is clear that these EV-derived biomarkers would gain sensitivity and specificity by isolating only tumour-derived EVs or by profiling multiple EV-miRNA markers.[22] Similar to the struggle to find the 'perfect' prostate cancer biomarker, in the ovarian cancer research field, the best prospects for further improvement in survival resides in early diagnosis.[23] One year after the discovery of EV-miRNA, miRNA signatures of tumour-derived EVs were described for the first time as diagnostic biomarkers of ovarian cancer.[21] Tumour-specific EVs were isolated using anti-EpCAM antibodies. Ovarian cancer patients could be discriminated from patients with benign disease by detecting 8 miRNAs, suggesting a possibility of high specificity.

As indicated in the introduction, EVs also contain cytosolic and membrane proteins and some of them seem to be cancer-specific and are described as a potential cancer biomarker in literature. A wide variety of biologically important proteins is identified and these molecules reflect the cellular origin of the EV. An example of such an EV-protein cancer biomarker is the cell surface proteoglycan Glypican1 (GPC1), specifically enriched on tumour-derived EVs. In 2015, Melo *et al.* gave a proof of concept that the search for a powerful biomarker with the highest sensitivity and specificity can lead to the use of EVs.[7] In this paper, a good biomarker for early diagnosis of pancreatic cancer was sought. In the absence of good biomarkers, 80-90% of pancreatic ductal adenocarcinoma cases are diagnosed too late for surgical resection to be an effective option.[24,25] Interestingly, measurement of the level of GPC1$^+$ EVs strongly correlates with pancreatic cancer (carcinoma-in-situ, stage I as well as stages II–IV) as a result of a high sensitivity. The level of GPC1$^+$ EVs could also distinguish patients with validated pancreatic cancer precursor lesions from healthy individuals and patients with benign pancreatic disease, suggesting a high specificity. In this paper, the added value of EVs as biomarker is again emphasised by discovering a remarkable decrease in sensitivity and specificity if the level of circulating GPC1 in serum is measured instead of measuring the level of GPC1$^+$ EVs. Although the findings of this research remain preliminary at this point, and the data needs reproduction and validation by other research groups, it's clear that several companies like Codiak Biosciences, Inc. (Woburn, MA, USA) and Exosome Diagnostics, Inc. (Cambridge, MA, USA) are making the step towards maturity for the fledgling science of exosome biology.[26]

Similar to the potential use of GPC1$^+$EVs as a cancer biomarker, Leca *et al.* (2016) found that the presence of annexin A6 (ANXA6) in EVs represents a potential biomarker for pancreatic ductal adenocarcinoma (PDA).[27] They suggest that crosstalk between cancer-associated fibroblasts and tumour cells, supported by ANXA6$^+$ EVs, is predictive of PDA aggressiveness, highlighting a ther-

apeutic target and potential biomarker for PDA.

Lyden *et al.* (2015) showed that a specific repertoire of integrins expressed on EVs dictates EV adhesion to specific cell types and extracellular matrix-molecules in particular organs. In this way, tumour-derived EVs will reach organ-specific cells and will prepare a pre-metastatic niche which might lead to organotropic metastasis. The integrin expression profiles of circulating tumour-derived EVs may be useful to predict metastatic propensity and to determine organ sites of future metastasis.[28]

Although lipidomic analysis of EVs is not common, some research groups tried to find a lipid cancer biomarker.[29] The study by Llorente *et al.* (2013) identified potential biomarkers in EVs derived from metastatic prostate cancer cell lines and found that glycosphingolipids were highly enriched in EVs compared to their cells of origin. These lipids, detected on the outer leaflets of the EV-membrane, are accessible to specific antibodies and may be used as novel potential biomarkers.

COMPARISON OF EVS AND OTHER LIQUID BIOPSY BIOMARKERS

Next to the use of EVs, also circulating tumour cells (CTCs) and circulating nucleic acids are described as promising cancer biomarkers (*Figure 2*). They also contain valuable information about the tumour tissue and can be used sequentially for liquid biopsy in order to avoid invasive biopsies. If a local tumour starts to metastasise, tumour cells enter the blood circulation and lodge themselves in new tissues to form metastases. By detection, monitoring and molecular investigation of these CTCs, they can be used in terms of diagnosis, genomic alteration determination, treatment response and finally prognosis prediction. Although several studies demonstrated that CTCs are valuable predictors of progression-free and overall survival, more research is needed to determine whether a patient's tumour burden correlates with the number of CTCs.[30,31] Also further determination of latter's content (e.g. genetic mutations that CTCs carry) is needed before CTCs can be used clinically as a surrogate biomarker for tumour progression. An average metastatic carcinoma patient has between 5 and 50 CTCs for every 7.5 mL of blood.[32–35] Although the exact number of clinically valuable EVs in a metastatic carcinoma patient is not known, it can be deduced from literature that for every CTC, more than 30 billion EVs are present in the blood circulation.[36] This small number of CTCs in blood places technical limitations and is the main reason why current CTC detection techniques lack sensitivity.

Due to apoptotic and necrotic processes typical of tumour cells with a high cellular turnover, patients with cancer contain higher levels of cell-free DNA (cfDNA) in their plasma compared with healthy controls.[37–40] Although cfDNA originates from both healthy and tumour cells, indicating the levels of cfDNA might also reflect pathological and physiological processes that are not tumour-specific, it may have clinical relevance for the diagnosis and prognosis of cancer.[41] To illustrate, high cfDNA concentrations in cancer patients indicate a poor outcome in terms of disease-free interval and survival.[42–46] Also in terms of response to therapy, persistently increasing cfDNA levels after surgery can be an indication of an incomplete response to treatment or developed systemic disease.[47] Additionally to this quantification, cfDNA can also be characterised by genomic analysis and can be used to examine microsatellite instability, loss of heterozygosity, mutations, polymorphisms, methylation, and DNA integrity.[48] Similar to CTCs, cfDNA is often proposed as a biomarker for disease monitoring, prediction of prognosis and of treatment response.[49] Although cfDNA only represent one class of biomolecules, compared to EVs, it is likely to play an important role in the upcoming implementation of precision oncology and has the clinical potential to be a more specific tumour marker. Different cfDNA isolation kits are available and, similar to EV isolation kits, the decisive parameter is efficiency and standardisation of the isolation.

Finally, also the analysis of tumour-educated blood platelets (TEPs) can play an important role in cancer diagnosis. It's well-known that blood platelets interact with tumour cells and sequentially affect tumour growth and dissemination by altering the expression of relevant genes.[50] Researchers also discovered that this interaction via transfer of tumour-associated biomolecules ('education') alters the RNA profile of blood platelets, which allows cancer diagnostics. By RNA sequencing, Best *et al.* (2015) could distinguish cancer patients from healthy individuals with a high accuracy.[51]

EVS AS PROMISING THERAPY

EVS AS THERAPEUTIC TARGET

As stated before, EVs play an important role in cancer progression by acting as a vehicle that provides communication between cells. Additionally, EVs can stimulate angiogenesis, invasion, metastasis, resistance to chemo-

therapeutics, etc., which makes them potential targets for cancer therapy. Different strategies can be used to inhibit the communication between cells by means of EVs. The EV biogenesis as well as the release of the EVs can be blocked, the circulating EVs can be captured and EV uptake by the recipient cell can be prevented (*Figure 3*).[52,53]

The first possibility to reduce the secretion of EVs is to inhibit the biogenesis. Different factors are known to play an important role in the biogenesis of EVs and blocking these simultaneously could be a potential strategy to reduce EV secretion. Exosomes are secreted when MVEs fuse with the plasma membrane and the ILVs are released into the environment. These ILVs are formed by inward budding of the limiting membrane of the MVEs. As exosomes carry a specific set of proteins, nucleic acids and lipids, a sorting machinery is necessary. There is an endosomal sorting complex required for transport (ESCRT)-dependent and an ESCRT-independent mechanism. The ESCRT consists of four complexes. ESCRT-0 recognises the ubiquitinylated proteins on the limiting membrane of the endosome. The ubiquitinylated proteins are passed to ESCRT-I and subsequently ESCRT-II which will induce inward membrane budding. ESCRT-III will cause the fusion of the two neck regions of the inward budding vesicle.[54] In the simultaneous absence of key subunits of all ESCRTs, a reduction in EV secretion is observed. Nonetheless, EV production is not totally inhibited and MVEs can still be formed, suggesting that also an ESCRT-independent pathway must exist.[55–57] Proteins can also be sorted through association with lipid rafts rich in cholesterol and sphingolipids. Exosomes were shown to be enriched in ceramide, which was proposed as a trigger for exosome formation. Ceramide is formed from the sphingolipid sphingomyelin by hydrolysis with sphingomyelinases (SMases). Inhibiting the neutral SMase showed a marked decrease in EV secretion.[58] Also syndecan together with syntenin and ALIX was shown to play an important role in the biogenesis of exosomes. Disrupting the heparin sulphate structures of syndecan, for example by heparitinase, was shown to reduce the level of EVs released.[59]

Besides the biogenesis, the release of EVs can also be inhibited. Rab GTPases were shown to regulate - together with their effector molecules - exocytic and endocytic trafficking. Rab GTPases are molecular switches that oscillate between an active GTP-bound and an inactive GDP-bound state. Ostrowski *et al.* (2010) showed that knockdown of five Rab proteins (Rab2B, Rab5A, Rab9A, Rab27A, and Rab27B) resulted in a reduced exosome secretion in HeLa cells.[60] Rab5 and Rab9 are important in the movement of respectively early and late endosomes, while Rab2 is known to support transport between the endoplasmatic reticulum (ER) and the Golgi apparatus.[61] Inhibition of Rab27A and Rab27B caused a decrease in the amount of exosomes secreted, but did not alter their composition, suggesting that Rab27A and Rab27B do not participate in exosomal cargo sorting but in the release of exosomes.[60] In two murine mammary adenocarcinoma models, Rab27A was shown to be required for the secretion of exosomes and for the secretion of non-exosome-associated pro-metastatic matrix metalloproteinase 9 (MMP9). Rab27A inhibition led to a decrease in exosome secretion of 50% and a reduction in growth and metastasis in one, but not in the other cell line.[62]

It was shown in maturing red blood cells that both Rab11 and calcium participate in the regulation of the exosome pathway. Rab11 and calcium are considered to be involved in a coordinated manner in the earlier steps of MVE biogenesis. Treatment with a calcium chelator, even when Rab11 was overexpressed, led to a reduced exosome secretion. Calcium plays an important role in homotypic MVE fusion and in fusion of MVEs with the plasma membrane.[63] In oligodendrocytes, inhibition of Rab35 leads to intracellular accumulation of endosomal vesicles and impairs exosome secretion.[64] ARF6 was shown to play an important role in the release of plasma membrane-derived microvesicles into the surrounding environment. ARF6 facilitated actomyosin-based contraction at vesicle necks and in this way, lead to microvesicle release. Inhibition of actin polymerisation and myosin function prevented microvesicle release.[65]

Circulating EVs from cancer cells can also be captured. Aethlon Medical, Inc. (San Diego, CA, USA) has developed a hemofiltration approach that can be integrated into standard dialysis units or continuous renal-replacement therapy machines. Patients' blood passes through a hollow-fibre plasma separator cartridge in which components smaller than 200 nm travel through porous fibers and interacts with the immobilised affinity agent(s). The target molecules are adsorbed while blood cells and non-bound serum components pass through the device. Antibodies and other affinity reagents, like protein ligands or lectins, can be used for capturing single or multiple targets. Antibodies that recognise tumour-specific proteins to capture only cancer EVs and not EVs coming from non-malignant cells could be used.[66] For example, when breast cancer cells are overexpressing

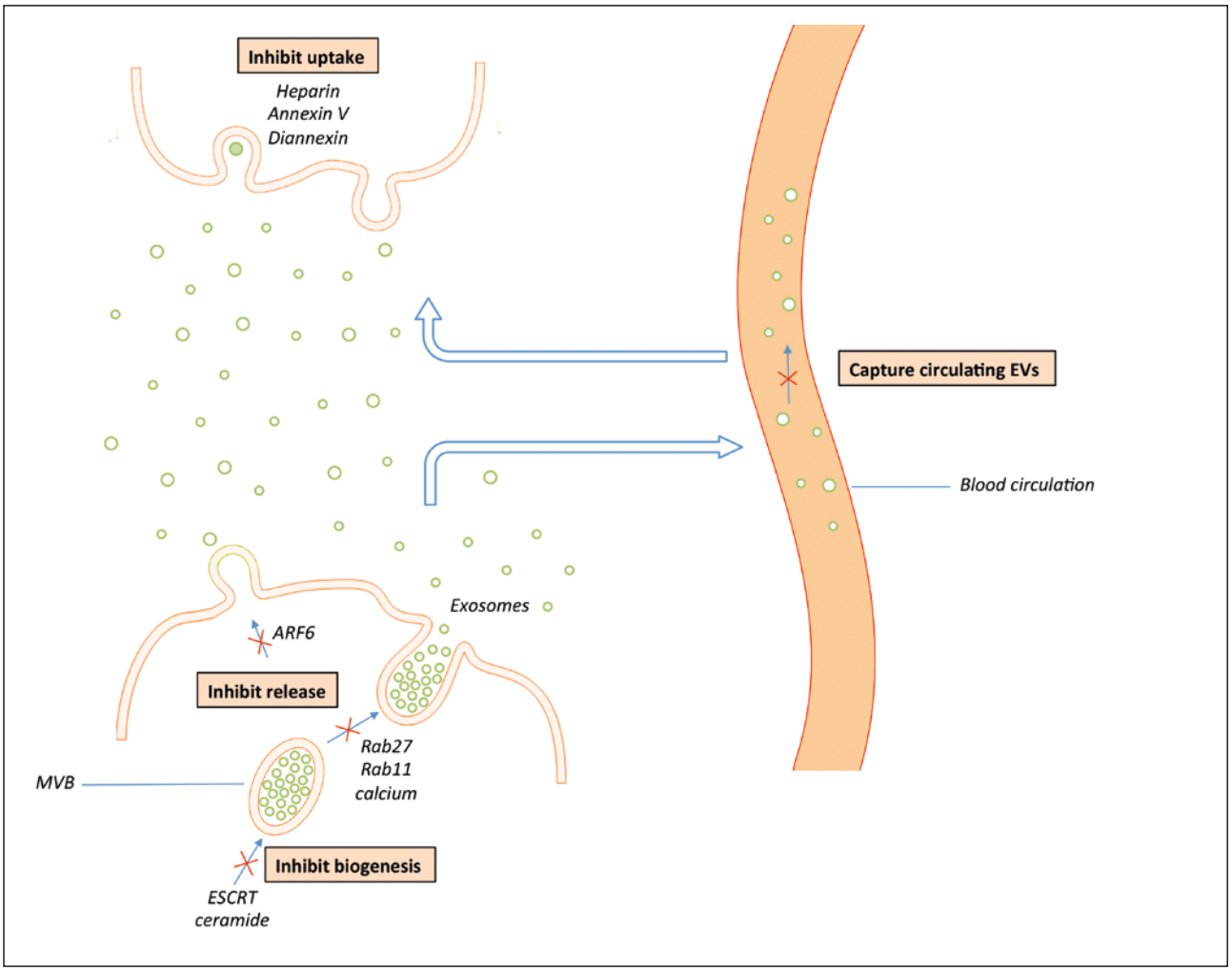

FIGURE 3. EVs can stimulate angiogenesis, invasion, metastasis, resistance to chemotherapeutics, etc. For that reason it could be interesting to use EVs as a therapeutic target. The biogenesis as well as the release of EVs can be inhibited, circulating EVs can be captured and the EV uptake by the recipient cell can also be blocked.

human epidermal growth factor receptor 2 (HER2), also HER2 expressing EVs and soluble HER2 can be found in the blood stream. Treatment with Herceptin is frequently neutralised by HER2 on EVs and soluble HER2 leading to treatment resistance.[67] Removal of these EV-bound and soluble forms of HER2 could offer a new therapy for HER2 positive breast cancer patients.[66]

As a last possibility, the uptake of EVs in recipient cells can be blocked. It was shown that heparan sulfate proteoglycans (HSPGs) have an important role in EV uptake. HSPGs are a family of proteins substituted with glycosaminoglycan polysaccharides that are extensively modified by sulfation. Cell-surface HSPGs are required for efficient uptake of EVs while EV-associated HSPGs appear not to be involved in cellular uptake.[68] Heparin was shown to block transfer of brain tumour-derived EVs into recipient cells. Direct interaction between EVs and heparin as well as aggregation of EVs in the presence of heparin was observed. It has been suggested that EVs carry ligands that bind with heparin. In this way, these ligands won't be able to bind the receptor on the recipient cells (e.g. HSPGs).[69] Transfer of EVs often involves interaction between phosphatidylserine residues exposed on the EV surface and on the plasma membrane. This process can be blocked by annexin V and Diannexin, a homodimeric form of annexin V. Diannexin was shown to exert a noticeable anti-cancer and anti-angiogenic effect in vivo which was suggested to involve, at least in part, interference with EV exchange between tumour and endothelial cells.[70]

EVS AS THERAPEUTIC AGENT

In 1996, Raposo *et al.* first stated the presence of MHC class II molecules on EVs released by B cells. These

MHC class II molecules were in a functional, peptide bound state and could exert T cell stimulatory functions.[71] Zitvogel *et al.* showed two years later that EVs derived from dendritic cells (DCs) pulsed with tumour antigens induce a potent immune response resulting in tumour growth delay or complete tumour eradication in a T cell-dependent manner.[72] From these results the hypothesis that EVs play an active role in communication between different cells of the immune system gained interest. EVs were proposed as a new avenue for cancer vaccine development to prime the immune system to recognise and kill cancer cells. Ideally, a cancer vaccine should prime the immune system to recognise specific tumour antigens and it should lead to an immune response towards the cancer cells without damaging the healthy neighbouring cells. EVs could be a possible agent for that. EVs also have several possible applications in inflammation associated diseases. EVs were shown to be a promising tool for treatment of graft-versus-host disease. Mesenchymal stem cells (MSCs) were used as a first strategy, but the beneficial effects of MSCs were suggested to be derived from secreted factors. Treating patients with MSC-EV therapy improved the symptoms significantly, shortly after the start of the therapy.[73]

The use of DC-derived EVs (also called dexosomes or Dexs) as cancer immunotherapy has been explored extensively.[74–77] The outer membrane of Dexs contain a wide range of antigen presentation (MHC class I, class II), adhesion (ICAMs), costimulatory (CD8), and docking (integrins) molecules.[78] Compared to cell-based therapies involving DCs, Dexs have some advantages. DCs, for example, are difficult to store over long periods of time while Dexs can be stored at -80°C for six months.[79] To illustrate their clinical usefulness, Chaput *et al.* demonstrated that Dexs pulsed with tumour peptides are more efficient as a cancer immunotherapy compared to peptides alone and as efficient as mature DCs.[74]

So far, two phase I clinical trials (in France and the United States) and one phase II clinical trial (in France) using Dexs have been performed (*Table 1*).[78,80-81] The first phase I clinical trial was conducted to test the feasibility and safety of using autologous Dexs pulsed with MAGE 3 peptides for the immunisation of stage III/IV melanoma patients. MAGE antigens are one of the tumour-associated antigens that are capable of eliciting cytotoxic T cell responses and are among the most frequently expressed across many malignancies. From this phase I clinical trial, it was concluded that the large scale EV production and the safety of EV administration was warranted. One partial response and some tumour regressions at skin and lymph node sites were observed. No specific CD4+ or CD8+ T cells were generated by the EV vaccine but NK cell effector functions were enhanced in the blood of eight out of thirteen patients.[80]

Around the same time, a similar phase I clinical trial was performed with autologous Dexs loaded with MAGE tumour antigens in patients with advanced non-small cell lung cancer (NSCLC). Also in this clinical trial, immunotherapy was generally well tolerated with the most frequently reported adverse events being mild (Grade 1-2) in severity. Three out of nine patients who did not exhibit reactivity to MAGE prior to the immunisation showed a systemic immune response against MAGE after the last injection. A minimal increase in antigen-specific T cell activity was found. One of the hypotheses for this could be the influence of the regulatory T cells which were shown to be elevated in some patients following immunisation. An increase in NK activity was found in two out of four patients. This trial was able to observe stable disease in one out of two of the immunised patients with disease progression at study entry.[82]

Because of the limited T cell response in the two described phase I clinical trials, EVs derived from interferon (IFN)-γ-maturated DCs were used in the phase II clinical trial conducted in advanced NSCLC patients. The 22 patients included in the trial first received four cycles of a first-line platinum-based chemotherapy. Three weeks after inclusion in the trial, patients received metronomic cyclophosphamide for a period of three weeks (to inhibit regulatory immune responses and to further promote the induction of effector T cell responses) followed by four intradermal Dex vaccinations at one-week intervals. After a two-week break, patients received six Dex vaccinations at two-week intervals followed by a two-week break and continued vaccination at three-week intervals until progression or Dex unavailability. Despite the intention behind the development of this 'second generation' Dex, it was not possible to observe T cell stimulation but rather a positive effect on NK cells. Unfortunately, it was not possible to observe at least 50% of patients with progression-free survival at four months after chemotherapy cessation, which was the primary endpoint. The median time to progression was 2,2 months and median overall survival was fifteen months. Seven patients experienced stabilisation of more than four months.[78]

Besides the use of Dexs, also EVs isolated from ascites (called Aexs) were used in a phase I clinical trial (in

TABLE 1. EVs in human clinical trials.

Phase	Disease	EV source	Tumour antigen	EV modification	Immunisation approach	Study size	Reference	Use
I	Metastatic Melanoma	moDC[1]	MAGE		4 immunisations, 1-week intervals	15	[80]	Therapeutic agent
I	Advanced NSCLC	moDC[1]	MAGE		4 immunisations, 1-week intervals	9	[82]	Therapeutic agent
I	Advanced Colorectal cancer	ascites	CEA (detected in Aexs)		4 immunisations, 1-week intervals	40	[83]	Therapeutic agent
II	Advanced NSCLC	IFN-γ-DC	MAGE A1, MAGE A3, NY-ESO-1, MelanA		4 immunisations, 1-week intervals. 6 immunisations, 2-week intervals followed by immunisations at 3-week intervals	22	[78] NCT01159288	Therapeutic agent
I	Colon cancer	plant		Curcumin loaded	7 tablets taken, daily	35	NCT01294072	Therapeutic vector
II	Malignant ascites and pleural effusion	Tumour cells		Chemo-therapeutic drug loaded	4 times a week	22	NCT01854866	Therapeutic vector

[1]moDC: monocyte-derived dendritic cells.

China). Forty patients with advanced colorectal cancer were randomly assigned to treatments with Aexs alone or Aexs together with the granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate anti-tumour DC activity. Both approaches were found to be safe and well tolerated, but the Aexs together with GM-CSF were able to induce tumour-specific anti-tumour cytotoxic T cell responses while Aexs alone could not. The presence of GM-CSF as an adjuvant could significantly promote the efficiency of the vaccine Aexs. Aexs contain tumour-associated carcinoembryonic antigen (CEA), MHC molecules and heat shock proteins (HSPs), which might be recognised by antigen-presenting cells, stimulating the activation of T cells.[83] We can conclude that EVs are able to stimulate immune responses and promote anti-tumour responses. In this way, they might operate as a novel anti-tumour strategy in the future.

EVS AS THERAPEUTIC VECTOR

Besides the use as a therapeutic target and agent, EVs can also be used as a drug delivery system. Because of the instability of newer drug candidates such as proteins and nucleic acids, and the urgent need for targeted therapies without side effects, researchers were motivated to find new advanced delivery systems. The aim was to keep drugs in the circulation for extended periods of time and deliver them to the right place of action (e.g. tumour tissue). EVs seem to be an ideal candidate because of their non-immunogenic character due to the similar composition as the body's own cells.[84] In the case of cancer therapy, EVs may achieve passive targeting to tumour cells via the enhanced permeation and retention effect (*Figure 4*). This unique effect is known as the phenomenon of macromolecules to accumulate more in tumour tissue compared to normal tissues, due to the 'low-quality', leaking tumour blood vessels. [85] Active targeting can be performed by the attachment of specific ligands to the surface of EVs to recognise and bind tumour cells.[85,86] As stated before, Lyden *et al.* (2015) showed that specific integrins expressed on EVs dictate homing of EVs to distant organ sites.[28] To illustrate the clinical usefulness of EVs as a therapeutic vector, Tian *et al.* (2014) intravenously injected in-

tegrin-targeted EVs loaded with the chemotherapeutic drug doxorubicin in mice with breast cancer.[85] They observed a significantly improved suppression of breast tumour growth compared to administration of the free drug. Additionally, a safety evaluation of the targeted EVs investigated cardiac damage, the most important dose-limiting side effect of doxorubicin, and concluded that the doxorubicin-loaded EVs are less cardiotoxic than the free drug.

A phase II clinical trial was conducted to test the efficacy of tumour cell-derived EVs, loaded with a chemotherapeutic drug, to treat malignant ascites and pleural effusion (NCT01854866). Another phase I clinical trial uses plant EVs to deliver curcumin to normal colon tissue and colon tumour cells (NCT01294072). Other non-human EVs are also being tested. For example, it was shown that animal milk-derived EVs act as an effective drug carrier.[87]

As mentioned earlier, next to the delivery of small molecules like doxorubicin, proteins and nucleic acids can also be delivered by EVs. These vesicles avoid phagocytosis or degradation by macrophages and protect proteins and nucleic acids from proteases and nucleases respectively. For example, Ohno *et al.* (2012) showed that EVs can efficiently deliver miRNA to epidermal growth factor receptor (EGFR)-expressing breast cancer cells. Active targeting was achieved by coupling the GE11 peptide, which binds specifically to EGFR, to the surface of the EVs.[88] However, some issues regarding the feasibility of loading EVs with nucleic acids of interest are described. In a pre-formation loading approach, cells are transfected with high concentrations of small RNAs. Subsequently, EVs are secreted in the extracellular fluid and are collected. This loading approach is difficult to control because of the fact that the mechanisms for RNA sorting in EVs is not yet fully understood.[89] Two methods for post-formation loading of EVs were proposed, but each has their limitations. Employing a commercial fusogenic lipofection reagent requires a large number of purification steps and the original vesicle composition might be drastically altered. The second method, electroporation, was shown to be less efficient than first postulated.[90] In an attempt to discover a new post-formation loading method, siRNAs were attached to the surface of isolated EVs by a cholesterol anchor. Nonetheless, it was not possible to functionally deliver the associated RNAs.[89]

The fact that EVs have a lot of advantages compared to other drug delivery systems, like limited (or no) undesired immunogenicity, a greater stability in the blood circulation, efficient delivery of the cargo into the cytosol and less off-target effects, makes them an important subject of interest. Nonetheless, some obstacles still exist. For example, the current isolation methods are expensive and yield low quantities of EVs. Also, EVs may contain pathogen-derived antigens and cytokines that activate pro-inflammatory pathways. Finally, EVs have diverse effects on health and disease that are not thoroughly understood and this may lead to adverse effects when used in the clinic.[91] One of the approaches to overcome these problems is the production of artificial EVs.[92] These EV mimetic delivery systems can deliver anti-tumour drugs in a more controlled way and are scalable for clinical settings. Summarised, EVs seem to be an interesting tool for advanced drug delivery into tumour cells, although a lot of research still needs to be conducted.

EVS AND THEIR LIMITATIONS

ISOLATION OF EVS

A problem in the use of EVs as a tool in the diagnosis and treatment of cancer is the fact that there is no consensus on the isolation method that should be used. Van Deun *et al.* compared two precipitation methods (ExoQuick and Total Exosome Isolation) with differential ultracentrifugation and OptiPrep density gradient centrifugation.[5] They concluded that OptiPrep density gradient centrifugation outperforms the other methods in terms of purity while EVs isolated with the precipitation methods contain lots of contaminating proteins. The precipitation methods are much less time consuming compared to differential ultracentrifugation and OptiPrep density gradient centrifugation and are therefore frequently used in EV research. The results from experiments using these precipitation methods however are disputable since protein complexes are co-isolated with EVs. Unfortunately differential ultracentrifugation and OptiPrep density gradient centrifugation are much more time consuming and cannot be performed by unexperienced researchers, hampering the implementation of these techniques in a diagnostic lab. We can conclude that technological improvements are necessary so that the use of EVs in the clinic would become more accessible. An alternative to this approach could be the direct analysis of EVs in the biofluid. In this approach EVs could be used without the need of purification for detection of a biomarker or for quantification. More research regarding the possibility of this approach needs to be conducted.

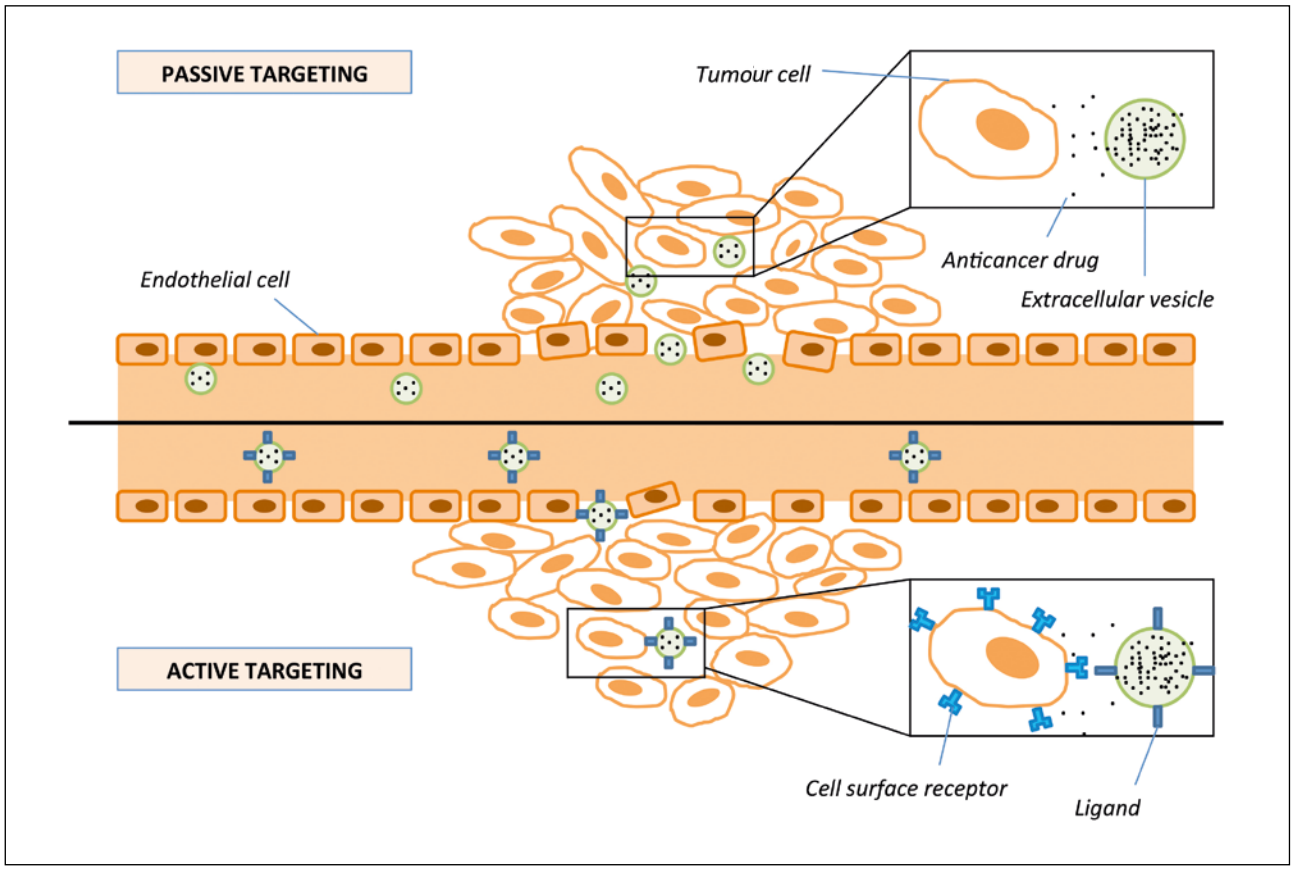

FIGURE 4. EVs can be used as a drug delivery system and can be targeted to tumour tissue in a passive and active way. Passive targeting is achieved by a prolonged circulation of the drug and a selective 'leaking' of EVs in the typical low-quality tumour blood vessels. Active targeting makes use of specific biological processes, such as ligand-receptor recognition, to make EVs accumulate in the tumour tissue.

DETECTION OF EVS

Also the detection of EVs encounters some problems. As EVs are very small, many of them fall under the resolution limit of optical microscopes. For that reason only electron microscopy offers a resolution small enough to visualise EVs. Other methods can be used to investigate the isolated EVs, for example Nanoparticle Tracking Analysis (NTA). The particles in suspension are brought into a chamber with a known volume. A laser beam is passed through the chamber and each particle scatters light, which is detected with a camera. The movement of the particles, due to the Brownian motion, is followed and correlated to the size. Finally, the number of particles, but also the size distribution of the particles in the sample is calculated. Next to the use of Nanoparticle Tracking Analysis, also other techniques are available to detect EVs (e.g. resistive pulse sensor).[93]

Besides the small size of the EVs, the concentration of EVs in the biofluid obtained from the patient leads to some limitations. To obtain pure EVs many purification steps are needed. Every purification step leads to a reduction in EV recovery. Lower yields of protein, RNA, etc., are obtained which causes the need for optimisation of downstream omics approaches for biomarker discovery. Besides using EVs to find a biomarker, the amount of EVs circulating in the blood stream (measured by e.g. high resolution flow cytometry) could offer interesting information as the concentration of EVs is increased in cancer patients. Due to the loss of EVs during the different steps of the isolation protocol a reference standard should be included to account for variation.

To date it was not possible to distinguish exosomes, which have a specific intracellular origin, from microvesicles, that bud directly from the plasma membrane. It would be interesting to solve this problem in the future as exosomes and microvesicles each can

KEY MESSAGES FOR CLINICAL PRACTICE

1. **EVs provide a unique source of biological information about a possible tumour in the body and for that reason they can serve as a powerful biomarker in cancer diagnosis and prognosis.**
2. **Besides the use of EVs in cancer diagnosis, also different strategies are available to use them in therapy e.g. as a drug delivery system.**
3. **EVs can be obtained in a non-invasive way by liquid biopsy sampling.**
4. **Although EVs seem very promising, a lot of work still needs to be done before they can reach clinical practice.**

have different opportunities in cancer research.

CONCLUSION

Although EVs have only been intensively studied during the last decade, it has become clear that they hold great promise for the diagnosis and the treatment of cancer. To further warrant the future implementation of EVs in the clinic, technological advances and insights in EV biogenesis and functions are needed. The concentration of EVs is increased in cancer patients, but the current methods for EV isolation tend to be time-consuming especially since EVs need to be separated from RNA-protein complexes, protein aggregates and abundant blood proteins. Technological advances together with reference standards are needed to allow for the clinical implementation of EVs for diagnosis, prognosis and therapy monitoring. In addition, a broader understanding of the biogenesis and functions of these small membrane vesicles will pave the way towards clinical trials involving EV-based therapeutic strategies.

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