Universidade de Lisboa

Faculdade de Farmácia

Extracellular vesicles in cancer: therapeutic implications

Luísa Cardoso Ferreira Pereira Bastos

Monografia orientada pelo Doutor André Daniel Lopes Simão, Investigador Científico e coorientada pelo Professor Doutor Rui Eduardo Mota Castro, Professor Auxiliar.

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Abstract

Extracellular vesicles encompass several types of very diverse bioparticles composed by a lipid bilayer, without a functional nucleus, which contain information in the form of proteins, lipids, and nucleic acids, such as DNA, RNA, or microRNA.

Extracellular vesicles are an important way of communication between cells. They can mediate intercellular communication, with a very wide range of functions in both physiological and pathological conditions. It is already recognized that extracellular vesicles can promote coagulation and bone regeneration, allow reticulocyte maturation, modulate immune response, and mediate neurodegeneration, cardiovascular diseases, and host permissiveness during infections.

These vesicles seem to present a relevant role in cancer progression, through oncogenic signalling, tumour microenvironment modulation and immunomodulation, which might influence tumour growth, invasiveness, angiogenesis, and metastasis. A better understanding of their origin, cargo, and functions in cancer, may be useful to develop new strategies for inhibiting oncologic development and the disease progression. Further research is needed in this area since several of extracellular vesicles' roles are not yet fully studied.

Considering the recent advances in the field of research of extracellular vesicles in cancer, it seems evident that they will play an increasingly important role in cancer diagnosis and prognosis through non-invasive diagnostic methods, such as liquid biopsy. Extracellular vesicles could be soon considered relevant biomarkers for cancer diagnosis and prognosis, as well as to therapeutic monitoring.

Extracellular vesicles are also promising research targets as drug delivery agents. It is even hypothesized that extracellular vesicles could work actively against cancer. Extracellular vesicles-based therapies have just started being developed and researched, there is still a long way to go. We will see in the future how far they can take us in the fight against cancer.

Keywords: Biomarkers; Cancer; EV-based therapeutics; Extracellular vesicles; Multidrug resistance.

Resumo

O termo vesículas extracelulares engloba diversos tipos de biopartículas muito diversas, compostas por uma bicamada lipídica, sem núcleo funcional e que contêm informação na forma de proteínas, lípidos e ácidos nucleicos, como DNA, RNA ou microRNAs.

As vesículas extracelulares são uma importante forma de comunicação entre as células. Podem mediar a comunicação intercelular em condições fisiológicas e patológicas, tendo uma ampla gama de funções. É já reconhecido que as vesículas extracelulares podem promover a coagulação e a regeneração óssea, permitir a maturação de reticulócitos, modular a resposta imune, e também, mediar a neurodegeneração, doenças cardiovasculares e a permissividade do hospedeiro durante infeções.

As vesículas extracelulares parecem apresentar um papel relevante na progressão do cancro, através da sinalização oncogénica, da modulação do microambiente tumoral e da imunomodulação, que podem influenciar o crescimento tumoral, a capacidade de invasão, a angiogénese e a metastização. Descobrir mais sobre a sua origem, conteúdo e funções no cancro poderá ser útil no futuro para o desenvolvimento de novas estratégias de inibição do desenvolvimento oncológico e de progressão da doença. É necessária mais investigação nesta área, uma vez que várias das funções destas vesiculas ainda estão totalmente estudadas.

Com os avanços recentes na investigação da área das vesículas extracelulares e do seu papel no cancro, parece evidente que elas terão um papel cada vez mais importante no diagnóstico e prognóstico do cancro, através de novos métodos, como a biópsia líquida. As vesículas extracelulares poderão em breve ser consideradas como biomarcadores relevantes para o diagnóstico e prognóstico do cancro, assim como para a monitorização da resposta terapêutica. São alvos de pesquisa promissores como agentes de distribuição de fármacos, sendo ainda hipoteticamente possível que as vesículas extracelulares também possam ter um papel ativo contra o cancro. As terapêuticas baseadas em vesiculas extracelulares apenas começaram agora a ser desenvolvidas e investigadas, ainda existe um longo caminho por percorrer. Veremos no futuro até onde nos podem levar na luta contra o cancro.

Palavras-chave: Biomarcadores; Cancro; Multirresistência; Terapêuticas baseadas em vesículas extracelulares; Vesículas extracelulares.

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Neste momento, recordo-me da noite em que soube que tinha ingressado no Mestrado Integrado em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade de Lisboa, e da imensa alegria e motivação que a minha mãe, pai, e namorado me transmitiram. O apoio deles ao longo de todo este percurso foi fundamental.

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Por último, gostaria de dedicar esta monografia a uma pessoa muito especial e sábia, que me acompanha desde pequena, à minha Avó Evinha.

Abbreviations

ABs: Apoptotic bodies

cfDNA: Cell-free DNA

cfRNA: Cell-free RNA

CTCs: Circulating tumour cells

ctDNA: Circulating-tumour DNA

DCs: Dendritic cells

dsDNA: Double-stranded DNA

EMT: Epithelial to mesenchymal transition

ESCRT: Endosomal Sorting Complex Required for Transport

EVs: Extracellular vesicles

EXOs: Exosomes

HSPs: Heat-shock proteins

ILVs: Intraluminal vesicles

MDR: Multidrug resistance

MHC-1: Major Histocompatibility Complex class I

MHC-2: Major Histocompatibility Complex class II

mRNA: Messenger RNA

miRNA: Micro RNA

MSCs: Mesenchymal stem cells

mtDNA: Mitochondrial DNA

ncRNA: Non-coding RNA

MVs: Microvesicles

MVBs: Multivesicular bodies

PS: Phosphatidylserine

P-gp: P-glycoprotein

ssDNA: Single-stranded DNA

SEVs: Stem-derived extracellular vesicles

STR: Short tandem repeat

TEVs: Tumour-derived extracellular vesicles

TF: Tissue factor

TLR: Toll-like receptors

TME: Tumour microenvironment

TSGs: Tumour suppressive genes

Index

Index of Figures

1 Introduction

Extracellular Vesicles (EVs) have important roles on biological processes in our organism, both in healthiness and sickness. One of these functions is the intercellular communication, when cells that are not able to communicate through direct cell-to-cell contact or secreted molecules transfer (1). Initially, the importance of EVs in the process of intercellular communication was not totally evident, since cell debris elimination mechanisms were more likely.

When EVs were observed nearly 50 years ago they were described as "platelet dust" (2) and associated with coagulation. In the 1980s, they were shown to have a role in the process of reticulocyte maturation into erythrocytes, and quality control of protein production, based on several studies done with reticulocytes (3–5). Later, in 1996, the function of EVs in antigen presentation was demonstrated (6), which opened doors for a different line of investigation, based on the roles of EVs for intercellular communication (7).

In the last two decades, research in this area has evolved significantly and has helped us to describe previously unknown functions. Now we understand that EVs are implicated in pathological processes, such as cancer and neurodegenerative diseases (1). Particularly, in cancer EVs are being propose as tools for its diagnosis and treatment. In fact, aiming to improve cancer prevention, detection, and treatment, researchers are working intensively, despite the complexity of this topic.

Data suggests that cancer cells can produce and release more EVs than healthy cells (8). Therefore, with this type of deregulation, some specific types of EVs are frequently present in higher quantities in biological fluids of cancer patients. It is estimated that the blood of a cancer patient may have twice as many exosomes (EXOs) compared to a healthy individual without this diagnosis (9). It is possible that there is a correlation between EVs amount and carcinogenic activity, but further research is still need. Researchers are focused on EVs and their pathological functions in cancer, looking in EVs potential to become useful tools for cancer diagnosis, prognosis, and treatment, the so called "theranostics" (10).

There are many types of EVs considered in literature. They are divided and stratified mostly regarding their biogenesis, origin, and physical characteristics, in majority by size. The scientific community is currently looking for new methods and protocols of isolation, purification, and characterization for EVs, in order to better identify all existing EVs subtypes (11–13).

In 2014, the International Society for Extracellular Vesicles (ISEV) provided the first harmonized guidelines to research in EVs area (11). In 2018, more information was included, and some instructions were updated or clarified (14). In fact, instructions from ISEV should now be used to standardize procedures, results, and conclusions.

For EVs studies, it is extremely important to register and thoroughly describe the culture medium composition, harvesting conditions and preparation details in *in vitro* studies and also, fully characterize the biological fluids or tissues under investigation and their respective storage conditions in *in vivo* and human studies (14).

Nowadays, it is still difficult to obtain pure EVs and samples free from non-vesicular components to carry out the necessary functional studies, due to the complexity of the extracellular milieu.

On that account, ISEV defined some minimal requirements for claiming the presence of EVs in isolates. The harvest of biological fluids which allegedly incorporate EVs should be gentle, restricting cell disruption, to avoid samples with large quantities of impurities. ISEV has also suggested that the characterization of EVs should include at least three different protein markers, and that the unexpected proteins should also be evaluated. ISEV also validated analytic methods such as Western Blot, flow cytometry and mass spectrometry for EVs isolates. In terms of single-vesicle analysis, electron microscopy and atomic force microscopy, can be considered for imaging purposes and nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing, for EVs' size analysis. It also highlighted the relevance of systematic negative controls, such as "mock" EVs, cultured without the cells of interest. Separation techniques should also be confirmed by quantitative PCR or other biochemical detection methods (11).

2 Materials and methods

For the development of this monography, the information was collected from research and review articles from PubMed, websites and publications from relevant authorities (WHO, NIH and Direção-Geral da Saúde) and from the book *The Cell: a Molecular approach* by Geoffrey M. Cooper. The research was performed on the databases PubMed, Google Scholar and on the specialized website of the Journal of Extracellular Vesicles, in English, and mostly without any restriction (publication date and authors). The keywords used on these databases were: extracellular vesicles, exosomes, microvesicles, resistance, cancer, metastasis, angiogenesis, oncogenesis, therapeutic, therapy, among others. The keywords were used alone and sometimes strategically combined between them, e.g., exosomes cancer.

From all the research done a total of 277 articles, websites and books were consulted, leading to 197 references whose content was considered very relevant, or even essential, for the development of this work.

3 Extracellular vesicles

EVs are a large group of heterogeneous single-membrane vesicles which are able to transfer information from one cell to another. EVs are produced by almost every cell in the human organism. They do not have a functional nucleus, and therefore, the EVs are uncapable of replicating themselves.

Research has shown that EVs present systemic properties (8), enabling the flow of information between nearby and distant cells. They can circulate in biological fluids, such as blood, saliva, urine, bile, breast milk, semen, amniotic fluid, ascites fluid and even cerebrospinal fluid (1).

The laboratory process necessary for studying these vesicles is complex. There are several confounding factors, both in terms of qualification and quantification, such as sample contamination with viral-like particles, proteins, lipids (8) or a high number of dead cells. Strategies were defined to minimize these risks, including culture-controlled mediums, determination of the percentage of dead cells present in the sample or full evaluation of the donor cell identity under investigation, using for example short tandem repeat (STR) analysis (14).

3.1 Types of extracellular vesicles

At first, Trans *et al*. (15) classified EVs based on their size using the differential centrifugation method. There were two well-defined groups of EVs: small vesicles (approximately 40 nm in diameter) and large vesicles (diameter greater than 500 nm). A nomenclature that is still used today, “exosomes” (for small vesicles) and “microvesicles” (for large vesicles), emerged over time. But these terms have adapted with the evolution of knowledge in the area, relying now on the biogenesis of the vesicles rather than on the size of EVs.

Regarding the biogenesis, EVs can be divided in three main groups: exosomes (EXOs), from 30 to 200 nm of diameter, microvesicles (MVs), 50-1000 nm, and apoptotic bodies (ABs), 1000-3000 nm. The biogenesis of EVs is complex, involving many cellular and metabolic pathways, such as the Endosomal Sorting Complex Required for Transport (ESCRT) machinery (16). The process can also be directly influenced by the external cell environment, leading to heterogenicity and diversity. EVs’ biogenesis is also very variable depending on the donor cell type and its homeostasis needs (17).

EXOs are the smallest EVs and derive from the endosomal system. The biogenesis and release of EXOs starts with a specific type of endosomes called multivesicular bodies (MVBs). Inside MVBs, intraluminal vesicles (ILVs) are formed through budding of the endosomal membrane itself. MVBs can follow two different pathways: lysosome fusion and consequent content degradation or releasing the ILVs to the extracellular environment, a process called exocytosis (18). After leaving the cell and passing into the extracellular space, these types of vesicles are then called EXOs (19).

EXOs are enriched in some relevant endosomal-specific proteins (like SNAREs and Flotilin-1 and -2), important adhesion proteins named tetraspanins (CD9, CD63, CD81 and CD82), in antigen presentation proteins, namely Major Histocompatibility Complex Class I and II (MHC-1 and MHC-2), and in surface proteins like chaperones Hcs70, Hsp90, Hsp99 and PKM2 (20). Inside EXOs, remain some proteins associated with their own biogenesis and cargo sorting, such as those related to the ESCRT machinery, like ALIX and TSG101 (21). Even though these markers are not totally exosome-specific, they are sometimes still used to differentiate at a general level EXOs from MVs.

Unlike EXOs, MVs are directly shed by the plasma membrane (hence the term "shedding-vesicles" some investigators use when referring to MVs) and they reach the extracellular space thanks to this budding and shedding mechanism. Their biogenesis process is far less clear and seems to be more complex to explain than the formation of MVBs. In the MVs synthesis process, there is an increase of some specific membrane proteins and lipid rafts (cholesterol-rich microdomains) (22). Some specific ligands, such as Ca^{2+}, serine proteases, thrombin, inflammatory cytokines, and growth factors, can act as a stimulus for the formation and release of MVs (23).

In general, MVs present a high level of conjugated proteins, such as glycoproteins and phosphoproteins (24) and are enriched in proteins from the endoplasmic reticulum, proteasome, and mitochondria. Beyond proteins, EXOs and MVs also differ in terms of lipid composition, where glycolipids and free fatty acids are predominant in EXOs, while ceramides and sphingomyelins characterize MVs (25).

ABs are generally the biggest type of EVs. ABs are released by membrane blebbing in cells undergoing a specific process: apoptosis, or programmed cell death. They have a very specific function, the elimination of cell waste, and for this reason, ABs are less relevant than EXOs and MVs to the cancer development.

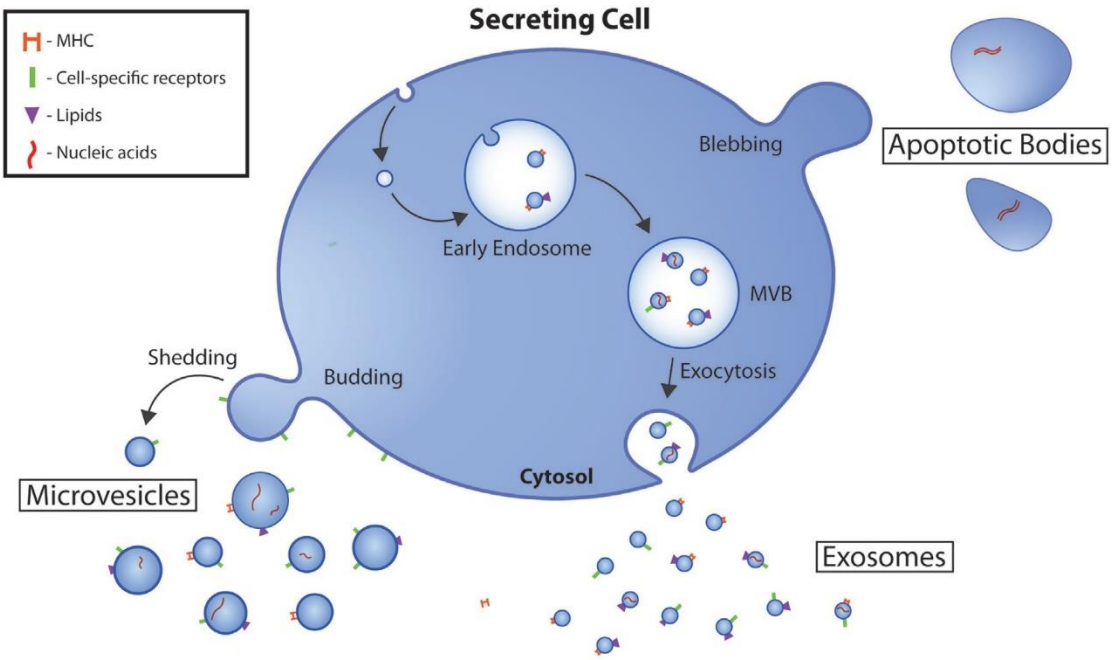

Figure 1. Biogenesis and release of extracellular vesicles.

Adapted from Gustafson *et al.* 2017 (26)

3.2 Extracellular vesicles cargo and uptake

Collecting and analysing the EVs content is an important step to understand their functions. Part of the cargo is considered functional, but some other compounds carried by them do not have known functions at this time. Due to the complexity of the EVs' content standards, databases such as ExoCarta (27) and Vesiclepedia (28) were created and are available online.

Information can be carried in these vesicles in the form of proteins, lipids, and nucleic acids. These bioactive components may induce behavioural changes, both physiologic and pathologic, in cells that uptake them. The content of the donor cell itself and the conditions under which the biogenesis takes place influence the cargo of EVs. Studies have shown that stress conditions can also affect EVs' cargo composition and sorting (29–31).

Proteins are the most abundant and studied cargo component of EVs. In EVs there are cytoskeletal, cytosolic, heat-shock proteins (HSPs), plasma membrane proteins, enzymes, proteins that allow signal transduction (like EGFR), and proteins related to transport functions, important for directing EVs to their respective targets (32). Among EVs most common proteins, it would be important to highlight some of the protein markers already referred in the previous

subtopic, such as tetraspanins (CD9 and CD81), MHC-1, MHC-II, TSG101 and the ESCRT-3 binding protein ALIX.

EVs also contain lipids and, even though they are mostly associated to their structural functions, they can also participate in intercellular communication regulating physiological and pathological cellular processes (33), such as fertilization (34), inflammation (35,36) and cancer angiogenesis (37). Cholesterol, phosphatidylserine (PS), and glycosphingolipids are commonly found in EVs, as well as other bioactive lipids such as leukotrienes, prostaglandins, and sphingomyelin (32).

Nucleic acids are also an important part of EVs' cargo. Almost all nucleic acids have already been identified in EVs. DNA has not had a very prominent role, but it is found in ABs (especially oncogenic DNA) and in other EVs in the form mitochondrial DNA (mtDNA), single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA) (38–40). In fact, the presence of DNA in EVs has already been recognized to be important for clinical practice in the future, helping to detect (41) and genetically characterize the cancer (42). In contrast to DNA, the presence of RNA has been more approached. Inside EVs exists both intact and fragmented messenger RNA (mRNA), ribosomal RNAs, fragments of transfer RNA, piwi-interacting RNA, vault RNA (43,44), Y RNA (45) and non-coding RNAs (ncRNA) (46), in which it is important to highlight the presence of microRNA (miRNA) (47), whose regulatory function is linked to carcinogenesis (48).

To perform its functions, EVs must establish communication with their respective target cells. They are able to do it by three different types of mechanisms: Fusion of EXOs or MVs membrane with the target cell membrane, followed by cargo transfer (49); ligand-receptor binding and intracellular signalling activation (50,51); and receptor-mediated endocytosis, pinocytosis (52) and phagocytosis (53).

The mechanism of uptake is highly dependent on the available resources, mainly proteins, glycoproteins and lipids found on the external leaflets of both the EVs and the recipient cells. There are several endocytic pathways involved in the uptake of EVs, with clathrin-dependent endocytosis, caveolin-mediated uptake, macropinocytosis, phagocytosis, and lipid-raft mediated endocytosis being the most prevalent endocytic routes (54).

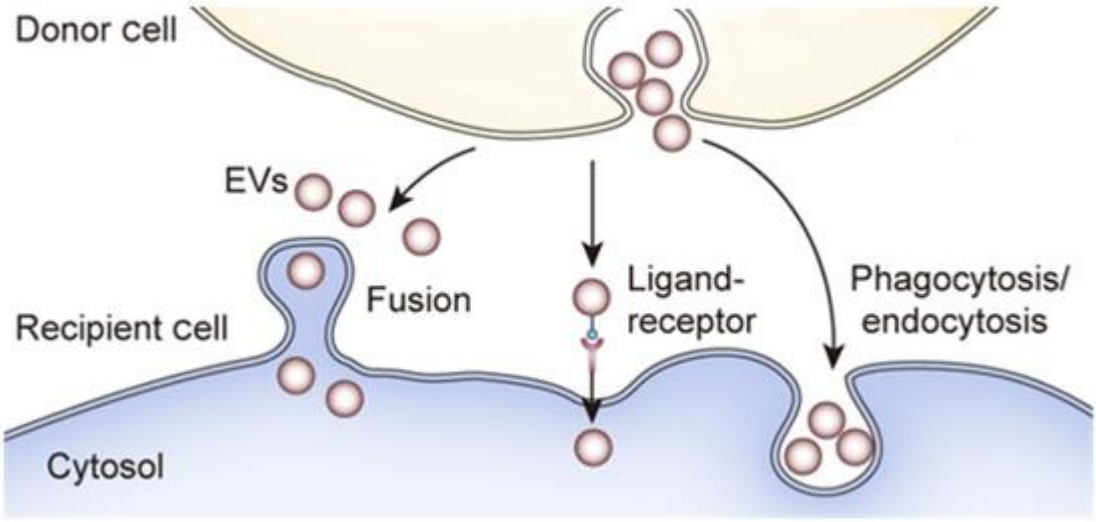

Figure 2. Extracellular vesicle interaction with recipient cells.

Adapted from Gao *et al.* 2021 (55)

3.3 Functions of extracellular vesicles

In the last two decades, EVs have been an important subject of study due to their wide capacity to intervene in numerous functions. EVs can transmit both signals and substances between different cells and to remodel the extracellular matrix in context of physiologic development, tissue homeostasis, immunitary response or in pathological processes such as cancer progression (56).

The functions of EVs in the immunitary response are relevant for understanding its role in cancer progression. Immunitary and cancer cells use EVs to modulate the immune response promoting either immune activation or immune suppression. Since it became known that B lymphocyte-derived EVs take part in antigen presentation (6), it became important to understand how EVs related to the immune cells. The EVs produced by B lymphocytes carried MHC-II and were able to induce antigen-specific MHC class II-restricted T cell response. Recently, NK-derived EVs have been linked to cytotoxic functions (57) and Treg-derived EVs to immune tolerance (58), important hallmarks for understanding the broad spectrum of EVs interactions within the immune system cells and processes. Early on, studies showed that immune-derived EVs could participate in proinflammatory mechanisms produced by macrophages, dendritic cells (DCs) and mast cells (59). Macrophages- and DCs- released EVs showed to promote inflammation through the leukotriene biosynthesis and granulocyte recruitment (35). In fact, the mast cell-derived EVs were able to transport activable

phospholipases and prostaglandins (36), which are substances implicated in the arachidonic acid cascade, allowing not only inflammation development, but also platelet aggregation, via other type of eicosanoids produced in that cascade: thromboxanes.

The role of EVs in coagulation is also related to cancer. In fact, procoagulant functions of EVs were long ago identified as being amplified in some cancer-derived EVs (60). Most EVs with coagulant properties contain Tissue Factor (TF) and PS. The PS presence on membrane of EVs creates a favourable environment for the functioning of the coagulation cascade, through the activation of several Ca^{2+}-dependent coagulation factors (61). TF is a glycoprotein present in the membrane of blood cells and EVs produced by those blood cells (platelets, monocytes, and neutrophils), that induces coagulation through the extrinsic pathway, which is related to vascular injury (32). TF allows conversion of factors VII/VIIa and favours thrombin production contributing to clot formation. Hypercoagulation has been associated to increased amounts of TF. In fact, thrombotic events are frequent in cancer patients, due at least in part to the high quantities of circulating EVs which include TF (62).

Several studies related the EVs' modulation importance in the field of immunitary response, coagulation, and cancer. Nevertheless, EVs are also being studied in other areas, such as cardiovascular diseases (63–66), bone regeneration (67,68), diabetes (69,70), neurodegeneration and neuroregeneration (71), senescence (72,73) and also COVID-19 (74).

4 Extracellular vesicles in cancer

Cancer is a standard term used to define more than one hundred types of malignant diseases. Other synonyms commonly used are malignant neoplasm or malignant tumour. The word cancer was idealized by Hippocrates (460-370 BC), the "Father of Medicine", using the Greek term *karkinos* (crab) to describe the tumours. Later, a Roman physician, Celsus (28-50 BC) translated the term into Latin, *cancer*, and Galen (AD 130-200), a Greek physician, used the Greek word *oncos* (swelling) referring to tumours. All these three terminologies are still up-to-date, and they are used frequently (75).

In all types of cancer, there is a heterogeneous mass of cells which divide in an abnormal and uncontrolled way (tumour), showing ability to invade adjacent tissues. These malignant cells can spread to different organs and continue to develop in a new place, just as they were doing in their original site, a process called metastasis (76). However, it is important to mention that not all tumours are malignant. Tumours which do not present ability to tissue invasion and metastasis are called benign.

Cancer has been widely studied in recent years, as one of the major health problems in the world. Cancer is a leading cause of death worldwide, after cardiovascular diseases, being estimated that cancer may have caused nearly 10 million deaths in 2020 (77), a number that is expected to continue to increase, with life expectancy increases (78).

In normal conditions, the immune system could detect a pre-cancerous cell and eliminate it using the apoptosis process. But sometimes the process fails, and the pre-cancerous lesion evolve to a malignant neoplasm. The natural cellular repair mechanisms are less effective in elderly people and once the development of cancer has started, it is difficult to reverse it (77).

The current knowledge has allowed us to better synthetize and understand cancer development and progression, with the important biological characteristics and capacities that cancer cells present, such as genetic instability and alteration, deregulated energetics, triggering inflammation, maintained proliferative signalling, evading growth inhibitors, resisting cell death, achieving replicative immortality, inducing angiogenesis and, activating invasion and metastasis (79).

Cancer cells have three important immune system evasion allies: Tumour-derived extracellular vesicles (TEVs), enzymes (such as Treg-expressing ectonucleotidases) and Toll-like receptors

(TLR) (80). These mechanisms prevent the immune system from detecting and attacking these malignant cells and can favour the disease progression.

TEVs, namely EXOs and oncosomes which are cancer-derived MVs have important roles in evasion mechanisms, namely, oncogenic signalling, angiogenesis, and metastasis (39,80–83). Beyond these different functions in cancer progression, TEVs also have direct effects on the cancer mass, which will be addressed later, such as therapeutic resistance transfer (84,85).

4.1 Roles of extracellular vesicles in cancer

Oncobiology is a complex area, with many cellular and extracellular intervening entities. It is important to emphasize that not only EVs affect the development and progression of the disease. Oncobiology and molecular medicine evolved a lot in the past few years, and cancer cell molecular signatures became more important. The erratic behaviour of cancer cells is related to some genetic factors present in their genome: mutations, amplifications, deletions, or translocations. These genome alterations in proto-oncogenes and tumour suppressor genes (TSGs) will allow the uncontrolled cell growth and proliferation of the pre-cancerous cell, resulting in cancer (86).

Nowadays, it is possible to sequence these genes (87) and additionally, investigate the deregulated pathways and their molecular signatures. Sequencing RNAs and the analysis of oncoproteins is possible through different and modern methods; like massive hybridization, microarrays, and even single-cell methods, like cytogenomic-based integrated analysis (88). Recently, high-throughput technology was applied to molecular characterization of independent circulating tumour cells (CTCs) and EVs, becoming them in very interesting targets for cancer molecular signature analysis (89).

Oncogenes are genes capable of inducing transformations in the cells, leading to cancer development. They are activated from proto-oncogenes, that suffered specific point mutations (90). The investigation in this area started in 1970 with retroviral oncogenes (91,92) from the Rous Sarcoma Virus (RSV) and Avian Sarcoma Virus (ASV). Soon in 1976, the origin of the oncogenes was clarified, indicating that there were normally proto-oncogenes in the genome of these viruses (93). Finally, in 1981, a study in human cancer cells showed that the activation of oncogenes was also able to induce cellular transformation in human and animal models (94).

Among oncogenes and proto-oncogenes implicated in cancer development and progression, it is important to mention *RAS* (*KRAS*, *NRAS* and *HRAS*), *MYC*, *RAF*, *EGFR*, *ERBB2*, *MET*, *BRAF*, *BCL*, *ALK*, *ABL*, *ROS1* and *PIK3CA* (88,90,95,96).

Oncogenes can encode a specific group of proteins, called oncoproteins, that modulate some important cancer signalling pathways able to induce altered or unregulated cell proliferation processes. Oncoproteins are usually mutated or abnormally expressed proteins, such as growth factors, growth factor receptors (mostly tyrosine-kinase receptors), and transcription factors (90).

As oncogenes, TSGs will allow and promote cancer development and growth. But oncogenes must be triggered and, oppositely, TSGs must be inactivated or lost to allow cancer cell proliferation. TSGs contain and prevent the pre-cancerous or early cancer cells from establishing and developing, and when silenced, they cannot perform their normal function correctly (97). Research in this area started in 1969, when Harris *et al.* (98) showed that when cancer and normal cells were combined, deriving into a hybrid cell line, the malignant features of tumour cells were suppressed. The molecular functioning of this suppression process was only proposed later, through the study of retinoblastoma with the identification of the *RB1* gene (99). After *RB1*, *P53* was the second TSG identified, and it continues to be one of the most studied genes in this category. Others followed, like *BRCA1*, *BRCA2*, *INK4*, *PTEN*, *APC*, *MADR2*, *CDK2*, *CDKN2A*, *CDKN1A*, *NF1*, *NF2*, *CASP8*, *PIK3R1* and others. Similarly to oncogenes, TSG usually encode proteins but, in this case, these are regulatory or repairing proteins, that inhibit cancer development such as TP53 (97,100).

In 2007, Valadi *et al.* (101) suggested a new mechanism of intercellular communication, the delivery of RNA (miRNA and mRNA) by transfer through EVs. Three years later, it was confirmed by various studies that these nucleic acids carried by EVs were delivered to target cells and were in fact functional (102–104). It was from this point onwards that research into EVs began to increase exponentially, and the cancer related EVs' research also began to intensify.

The oncomirs (intracellular clusters of cancer miRNAs) were recently described (105), opening new doors for understanding the miRNAs' roles in cancer. MiRNAs are small, endogenous, ncRNAs from 19 to 25 nucleotides, capable of regulating gene expression after DNA transcription. This would mean that miRNAs can interfere with RNAs, by silencing them, and

thereupon inhibit their functions within cells. Thus, protein synthesis can also be detained or diminished. Today, there are nearly two thousand human miRNAs already identified (106).

Considering the role of miRNAs in cancer, investigations took a big step in 2002, when two miRNAs (miR15 and miR16) were found to be down-regulated in leukaemia (107) and three years later, He *et al.* also found a specific group of miRNAs from a primary transcript named chr13ORF25 (miR-17, miR-18, miR-19a, miR-20, miR-19b, and miR-92) that were overexpressed in B cell lymphoma. They concluded that this miRNA cluster was implicated in cancer development (105). MiRNAs-contained EVs are not just being investigated for cancer molecular characterization, but also for its early detection and treatment (108–110).

The molecular characterization of cancer is increasingly more important for the understanding of cancer development and progression. This knowledge has already allowed the construction of therapeutic guidelines in order to obtain better therapeutic results. It has also proved to be of importance for TEVs analysis, since these can be very varied and can affect many steps of cancer progression, from cancer growth to metastasis, through multiple pathways.

4.1.1 Extracellular vesicles in tumour growth and invasiveness

EVs can facilitate the process of tumour growth and intensify the level of invasiveness of the malignancy. Recent findings have suggested that stress conditions such as shear stress (111), hypoxia (112), acidosis (113), thermal and oxidative stress (114) can induce an increased release of EVs. Depending on cargo they carry, they can directly modulate both cancer and immune cells behaviour or indirectly, the tumour microenvironment (TME).

In fact, mechanisms of EVs-mediated cancer progression can facilitate the path to metastasis and may have therapeutic implications like anticancer drug resistance transfer (83,84).

Many factors can influence the cell selection and tumour-stroma/stroma-tumour crosstalk, affecting the migration and invasiveness capacity of the cancer cells (115–117). Cancer cells present a high diversity and plasticity, sometimes even with stem-like phenotypes, becoming them very difficult to treat.

For tumour growth, the available space, oxygen levels and other resources may function as tumour cell selection factors, allowing the most capable cancer cells continue their development (118). Due to their high level of proliferation, one of the first selection challenges is the limited space in which the cancer can develop. TEVs can help generate space with extracellular matrix

remodelling. In fact, cancer growth-related TEVs can contain soluble proteins, like cytokines, growth factors or proteolytic enzymes, such as metalloproteinases (22), that promote extracellular matrix degradation and remodelling (11), with relevant roles in subsequent tumour growth.

Hypoxia is also a frequent condition through which cancer cells have to undergo, being usually more frequent in the central region of the tumour, the cancer core, due to limitations in oxygen diffusion towards that area. Ultimately, the core can turn necrotic and the adjacent cells around it will start suffering hypoxia too. Cancer cells developed mechanisms to combat hypoxia, such as the formation of new blood vessels (angiogenesis) and several other metabolic, molecular, and genetic changes. The TME will also adjust and facilitate the cancer growth and spread (112). Recently, several studies showed that a hypoxic environment during cancer increase EVs' biogenesis and release (119), favouring the intercellular communication between cancer cells (120–122).

Temperature can work as a selective factor for cancer cell proliferation as well. Last year, Otsuka *et al.* (123) pioneered an investigation on the release of temperature-dependent EVs in breast cancer, discovering that cancer growth and EVs release were correlated and associated with poor prognosis. The undergoing mechanism was led by the *LDLR* gene, which triggered these EVs secretion.

EVs also play an important role in the TME and local immunomodulation. The TME is fundamentally constituted by cancer cells, adjacent endothelial, stromal, and mesenchymal cells, cancer associated fibroblasts and immune cells, such as DC, B-lymphocytes, T-lymphocytes, NK cells and macrophages (115,116). The immune cells within the TME are also important for helping to contain the tumour in terms of size and invasiveness. The intercellular communication in TME can be mediated by EVs (84,124,125), as a method to evade immune control mechanisms and promote the spread through the lymphatic system (126), by interacting with the immune cells (127).

Furthermore, EVs are also implicated in a novel area with great potential. In 2015, Haga *et al.* (128) studied the EVs-mediated interactions between tumour and mesenchymal stem cells (MSCs) in human cholangiocarcinoma, which is a type of cancer highly connected and communicated with its adjacent cells and tissues. It was shown that, when TEVs from this cancer contact with the MSCs, migratory capability risen and expression of bioactive mediators (alpha-smooth muscle actin, IL-6, CXCL-1 and CCL2) enhanced cancer cell proliferation and

fibroblastic differentiation, that may contribute to the close relationship between tumour and stroma in this type of cancer. Similarly, in other types of cancer such as breast adenocarcinoma (129), ovary adenocarcinoma (130) and gastric cancer (131), the EVs-related fibroblast differentiation has also been identified.

Different oncogenic signalling pathways that use EVs as intermediaries have been addressed. These pathways can be more general (like WNT and TGF-B pathway) or more cancer related, such as oncogenic proliferation or metastasis signalling cascades, like immune checkpoint PD-1 or *VEGF* signalling (132).

As already referred, TF is present in tumour cells and TEVs, and it also contributes to cancer progression processes, such as tumour growth, angiogenesis, and metastasis. Nowadays, the knowledge on EVs roles in tumour growth and invasiveness is still limited, but intervening on TEVs is certainly a great opportunity for minimizing cancer growth and invasiveness.

4.1.2 Extracellular vesicles in angiogenesis

The formation of new blood vessels is important for cancer cell proliferation and progression. When tumours reach a size superior to 1 mm of diameter, it is very likely that the cells situated in the core will start having less available resources and can possibly turn hypoxic and necrotic (112). Angiogenesis provides the solution for these challenges, and tumours depend on it (133,134). EVs may mediate cancer-related angiogenesis through endothelial cells activation, by the presence of substances that promote angiogenesis (like TF, sphingomyelin, αvβ6 integrin and VGEF) or mediating the intercellular communication between aggressive cancer stem cells and stroma.

In fact, TEVs effects on TME endothelial cells are currently being researched. In HeLa cells, a cancer cell line widely used in cancer research, produce TEVs capable of interacting with TME endothelial cells, and research has shown that the vascular integrity and permeability can be altered by these HeLa-derived TEVs interact with TME endothelial cells, changing their vascular integrity and permeability, through a mechanism which is miRNA independent, but unknown (135).

Several angiogenesis promoters have been found in TEVs. Among them, the activity of sphingomyelin, a sphingolipid found in cellular membranes and on EVs, mostly located in the outer leaflet of the lipid bilayers, has been recently described (37). Moreover, Krishn *et al.*

showed that prostate cancer cell-derived EVs containing αvβ6 integrin, similarly to sphingomyelin, can also enhance neovascularization, through activation of TME endothelial cells. The αvβ6 integrin promoted endothelial cell motility and the initiation of tubular endothelial formation, stimulating tumour related angiogenesis (81). In addition, it would be important to mention TF once more. TF has been found to activate the PAR2 signalling pathway, leading to an increased expression of proangiogenic factors (62).

Angiogenesis is considered a therapeutic target for anticancer therapies. In 2019, Zeng *et al.* (136) investigated how EVs can negatively affect anti-angiogenic drugs. They mimicked miR-9 mediated angiogenesis in endothelial cells (*in vitro* and *in vivo*) and administrated vandetanib (a VEGF, EGFR, and tyrosine kinases inhibitor), showing that miR-9 mediated angiogenesis was abolished, but EVs release was enhanced. The EVs produced by the resistant cells were enriched in VEGF, leading again to new vessel formation and, therefore, possible therapeutic failure.

Finally, the relevance of EVs regarding angiogenesis has also been studied in a severe cancer, glioblastoma multiforme, which harbours an important subpopulation of cancer stem-like cells in its TME. These cells actively contribute to the aggressiveness of this type of cancer since they are capable of enhancing the angiogenesis very effectively in the brain microvascular endothelium, through the release of VEGF-A-enriched TEVs (137).

4.1.3 Extracellular vesicles in metastasis

Metastasis is the dynamic and complex process that allows malignant neoplasms to spread from the primary tumour to other different distant tissues and organs and originate new cancerous formations. It is intimately connected to advanced cancer stages and cancer lethality (77). This process is highly dependent on different tumour-stroma ways of communication, including EVs.

Several routes of dormant cancer cells transport (138) are implicated in metastasis, being the most relevant among them the lymphatic spread (the most prevalent route) and blood (which originates hematogenous metastases). For example, TF contained in EVs can be responsible for overlaying cells with fibrin and allowing the hematogenous metastasis (62).

Different cytokines, growth factors, and EVs are key elements for the metastasis process. The angiogenic and oncogenic signalling mediated by EVs may also create an optimal environment

for the cancer migration (139). For instance, EVs can be crucial mediators of *P53* physiologic tumour suppressive action, or can also be used by mutant *P53*, a frequent mutation that occurs during cancer development associated to lower survival rates, to promote oncogenic signalling and reprograming tumour-stroma communication leading to metastasis (82).

Other studies suggest that EVs can also be related to the existent tissue tropism during metastasis, through mediation of protein receptors on TEVs outer leaflets, like integrins (140).

Recently, it was also demonstrated that the donor cell origin, primary or secondary tumour, has a direct impact on the EVs characteristics (141). In fact, TEVs produced by aggressive metastatic cells are able to instruct other cancer cells, to increase their invasive and vasculogenic properties, inducing more metastatic behaviour. Peinado *et al.* (83) showed an underlying mechanism in which melanoma-derived TEVs conveying the *MET* oncogene were able to induce permanent changes in the bone marrow progenitor cells.

Several cancers, like neuroblastoma, promote metastasis to bone marrow, which is associated to poor outcome. High levels of TEVs containing miR-375 were found in patients whose neuroblastoma was already metastasized to bone marrow, suggesting an important role of TEVs, as well as miR-375 in the metastatic progression (142).

Another relevant recent finding is that metastatic TEVs can also intervene in epithelial cell transformation and epithelial to mesenchymal transition (EMT), by creating a more suitable TME for cell migration. Last year, Ono *et al*. (143) concluded that TEVs from oral cancer cells changed the TME by inducing tumour-associated macrophages polarization to an M2 phenotype, a transition that contributes to cancer progression.

4.2 Extracellular vesicles as cancer biomarkers

In the last decade, EVs have emerged as potential clinical biomarkers for cancer diagnosis and prognosis, due to their differential production by cancer cells throughout the evolution of the disease and their release in biological fluids. However, their usefulness is still limited in the clinical practice (10), considering that understanding EVs and TEVs origin, roles, and function is a lengthy process. Research is evolving fast and some EVs with interesting roles in cancer were already identified as potential biomarkers.

Cancer biomarkers are biomolecules produced by healthy cells in response to cancer or by cancer cells themselves, that easy to obtain with minimum discomfort or risk to the patient and are found in high quantities or being modulated during cancer. They may facilitate the diagnosis, but also prognosis, therapeutic prediction and monitoring of different types of cancer.

In cancer, early detection is fundamental since the treatment is easier when the original lesion is localized in a single organ. It is more likely to survive the disease when screenings tests are performed and when a prompt diagnosis is reached (77). The most common methods for cancer diagnosis encompass tissue biopsy, endoscopic techniques, CT-scan, MRI, Nuclear scan, PET-scan and other imaging techniques (144).

The search of EVs and other circulating tumour-derived material in biological fluids, also called "liquid biopsies" are less invasive than some these traditional methods for cancer diagnosis, mainly the tissue biopsy, and consequently, a preferable method for both diagnosis and monitoring. Additionally, they could be extremely helpful to detect for those cancers which do not have an easy access, like brain tumours (25,145).

Liquid biopsies are already considered valuable methods for detection of cancer related mutations (like KRAS and P53) in cell-free DNA (cfDNA) even before cancer development (146), allowing healthcare professionals to select high-risk patients for cancer screenings and contributing to early detection (147,148). It was also verified that this method could be used for cancer monitoring and management (149), using the search of CTCs (150), circulating-tumour DNA (ctDNA), cfDNA (151), cell-free RNA (cfRNA) (152), miRNAs (153) and EVs (148,154–158). Importantly, liquid biopsies, in addition to evaluate disease progression, are able to determine the possibility of cancer recurrence after treatment, being a versatile method, with a great potential to decrease cancer-associated lethality rates.

4.2.1 Extracellular vesicles in cancer diagnosis

As already discussed, early detection is fundamental to have greater chances of cancer survival. An early diagnosis can be achieved by being aware of the cancer symptoms and seeking medical attention as soon as they are detected (77). However, this can be extremely complicated in some types of cancer which present late or non-specific symptoms, such as pancreatic cancer (109).

EVs are versatile, and easy to collect and sometimes cancer-, stage- and patient-specific, which could be an important tool in establishment of personalized medicine (159).

The EVs' miRNAs content has been considered a great candidate for diagnosis purposes. For instance, some miRNAs (like miR-16-5p, miR-23a-3p, miR-23b-3p, miR-27a-3p, miR-27b-3p, miR-30b-5p, miR-30c-5p and miR-222-3p) were found in elevated levels in blood plasma of colorectal cancer patients. Their plasma concentration correlates with staging and treatment response, and were significantly reduced after tumour resection surgery (110).

In addition, TEVs from metastatic melanoma, ovarian, and breast cancer, were recently shown to be enriched in two mitochondrial membrane proteins, MT-CO2 and COX6C, which are considerably elevated in this specific cancer stage (metastasis) and less expressed in early stages (160), serving as two good candidates for staging the disease.

In fact, EVs enriched in other protein cancer markers are being studied to help cancer early diagnosis. In 2013, Bijnsdorp *et al.* (161) found TEVs enriched in some specific integrins, ITGA3 and ITGB1, in urine of metastatic prostate cancer patients. These vesicles were able to establish contact with non-cancerous cells, promote metastasis, and can be detected in urine, therefore presenting potential to accurate and early identification of metastatic prostate cancer through a non-invasive analysis technique.

The study of urinary TEVs in some types of urologic cancer has been emerged due to their easier and less-invasive way to harvest, compared with blood plasma. For that, several new feasible EVs isolation protocols in urine have been developed and proposed.

4.2.2 Extracellular vesicles in cancer prognosis and monitoring

Cancer prognosis is a multifactorial prevision of how the disease will progress in a particular patient. Several factors must be considered. Historically, the type of cancer and stage at the time of diagnosis were important variables (162). Nowadays, molecular signatures, genetic characterization and response to therapy are becoming more relevant. EVs are already being studied for prognosis purposes, mostly for the evaluation of therapeutic responsiveness and effectiveness.

In 2014, TEVs produced by glioma and lung cancer cells were used to evaluate the phosphorylation of protein kinases rates in the cancer cells presenting *EGFR* mutation. EGFR variant III increased phosphorylation of EGFR, AKT and ERK. Interestingly, the levels of AKT protein in the collected EVs decreased significantly in erlotinib treated patients (163).

Similarly, EVs also present potential for the management and prediction of cancer immunotherapy response. Cancer cells are able to use the immune checkpoint ligand PD-L1 for evading immune surveillance. The pharmacological inhibition of this T cell immune surveillance mechanism, PD-1 blockade (Nobel Prize in Physiology in 2018) or PD-L1 blockade, was ground-breaking for cancer treatment. Monoclonal antibodies such as nivolumab, pembrolizumab (anti-PD-1) or atezolizumab and avelumab (anti-PD-L1) became very relevant options for cancer treatment. However, nearly half of patients did not respond to these type of treatment (164).

EVs have recently been recognized as research targets for PD-1 and PD-L1 blockade response monitoring. In 2018, Chen *et al.* (165) reported that TEVs from melanoma cells contained PD-L1 and that it was possible to correlate the PD-L1-enriched TEVs levels with the therapeutic efficacy of pembrolizumab, since non-responder animal models had significantly higher levels of vesicular PD-L1. New methods for cancer prognosis and disease monitoring based on EVs and immune checkpoint PD-1 and PD-L1 are currently being developed and researched. In 2020, Hu *et al.* (166) developed a new non-invasive, sensitive, fast, and isothermal assay for vesicular PD-L1 detection, which can predict therapeutic response and select the more appropriate candidates for immune checkpoint inhibitor therapies.

HSPs, including HSP70, have also been strongly associated to cancer prognosis. TEVs which carry HSP70 are usually present during cancer metastasis. Through a recent prospective clinical pilot research, it was shown that HSP70-enriched TEVs are sensitive diagnostic and cancer stage-related biomarkers. In these patients, the HSP70 in TEVs was inversely correlated with therapeutic response and related to clinical outcome (167).

The current knowledge in EVs and cancer indicates that the amount of TEVs in biological fluids during cancer can be related to some clinical outcomes. In 2017, Menck *et al.* (168) demonstrated that TEVs could be useful biomarkers for predicting clinical outcomes. TEVs from different types of cancer (head and neck, lung, breast, colorectal, among others) were evaluated through flow cytometry, in comparison to a healthy control group. In all types of cancer, a poor overall survival was associated to the extracellular matrix metalloproteinase inducer (EMMPRIN) presence in those blood TEVs. Similarly, EVs could also hamper the cancer treatment process, through transferring of therapeutic resistance between different cancer cell phenotypes (84).

Cancer treatment is currently based on resection surgery, radiotherapy and/or anti-cancer therapies, such as chemotherapy, immunotherapy and hormonal therapy. More recently, innovative targeted, gene and stem-cell therapies are being developed (169–171). Even though these therapies are recent, and are not still implemented in clinical practice, it is possible that EVs will become a part of the innovative cancer treatments in the future (172–175).

4.3 Therapeutic resistance transfer via extracellular vesicles

Multidrug resistance (MDR) represents a major issue to cancer treatment (176). Patients whose cancer does not respond to a potent drug combination usually have a worse prognosis, and higher death rates. In these cases, anti-cancer therapies are not able to eradicate from the organism all the cancer cells within a tumour, and treatment may represent a positive pressure stimulus for select the more aggressive and resistant cancer cells (177).

Some studies have linked EVs to drug resistance (178). TEVs could contain proteins and nucleic acids which induce therapeutic resistance, such as the efflux pump P-glycoprotein (P-gp) (179). Ca^{2+} signalling is one of the pathways that coordinate vesiculation of cancer cells. This mechanism has been implicated in the transfer of MDR phenotypes between different cancer cells. Recently, Taylor *et al.* (23) demonstrated that abnormal mobilization of Ca^{2+} and calpain activation induce EV biogenesis and release, contributing to the calcium channel TRPC5 transfer from resistant to sensitive cancer cells.

Other studies have shown that EVs-mediated resistance transfer can be mediated through numerous and different mechanisms; like EMT induction, promotion of anti-apoptotic pathways, drug efflux or sequestration, immunomodulation and also, via stem-derived EVs (SEVs) (180).

Recently, studies showed that TEVs from triple-negative breast cancer (an aggressive form of breast cancer) induced resistance to docetaxel and doxorubicin in other sensitive cancer cells (181); transferred selectively P-gp to sensitive cells (179); and, mediated the transfer of resistance to trastuzumab, a monoclonal antibody used for HER2 inhibition (182).

The effects of MDR transfer through EVs are a major concern in cancers with a high fatality rate. In 2018, it was shown that TEVs from glioblastoma can induce drug resistance to an alkylating agent used for chemotherapy, temozolomide (183).

Research about the role of EVs in cancer drug resistance is becoming more frequently addressed and more relevant, but it is being mostly done *in-vitro*, and sometimes the TME is obliviated (85). Further research is needed, and cancer organoids hold a lot of potential to be extremely helpful for these investigations (184,185).

4.4 Extracellular vesicles as novel therapeutic agents

EVs can stimulate or inhibit cancer progression, depending on the cell that releases the vesicles and the TME conditions (125). Among their physiological functions, EVs can also promote cell death. NK cell-derived EVs were recently found to induce apoptosis in cancer cells (186). These EVs contained specific compounds, such as cytotoxic proteins and activated caspases, that could induce both caspase-dependent and -independent signalling pathways, culminating in apoptosis. ELISA assays assessed that these EVs were enriched in perforin, granzyme A, granzyme B, granulosin and FasL, showing that more than one cell mechanism was triggered simultaneously.

Limiting cancer progression by interfering with angiogenesis is also a very appropriated way to use EVs in our favour. In fact, last year it became known that mesenchymal stroma cells released EVs capable of inhibiting angiogenesis directly, through direct contact with the endothelium, and indirectly, through anti-angiogenic substances transfer, like TIMP-1, CD39 and CD73, which make endothelial migration more difficult and promote extracellular matrix remodelling (187).

In terms of strategies for novel cancer therapy development, the key is the EVs' versatility. It has been presumed that they could possibly work as drug delivery agents, to be targeted, blocked, or modified in order to work as anti-cancer therapeutics themselves. In fact, the use of EVs as drug delivery systems has also been used to soften adverse drug reactions, such as cardiotoxicity associated to doxorubicin chemotherapy (188).

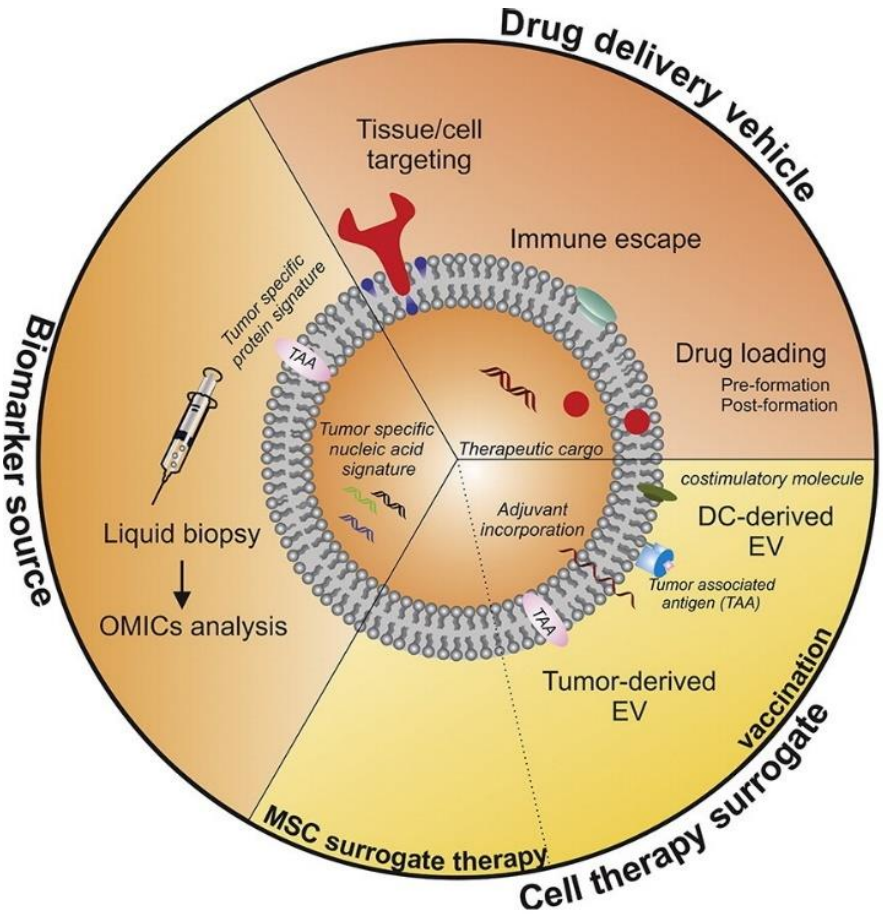

Figure 3. Possible future therapeutic uses of extracellular vesicles in cancer.
From Stremersch *et al.* 2016 (189)

EVs for therapeutic purposes usually derive from MSCs, which are considered appropriate for cell-based therapies (25). For instance, in 2016 Koojimans *et al.* (190) developed a new method to direct EVs to tumour cells effectively, where GPI-linked nanobodies in the surface of EVs showed to have a better uptake by EGFR-dependent cancer cells.

The distribution of other relevant contents, besides pharmacological or biological molecules, are also being investigated. Gene therapy using CRISPR/Cas9 is evolving very fast and presents a lot of potential to the cancer treatment. In addition to viral vectors, EVs could also be an advantageous option to direct these machineries to their targets. This year, it was suggested and confirmed by McAndrews *et al.* (191) that EVs could deliver functional CRISPR/Cas9 to target cells, allowing gene editing, including in the area of cancer.

In addition, other EV-based therapies are currently being developed and studied. In 2019, Hong *et al.* (192) tried to activate DCs through EVs. The result was positive and the modified EVs PH20, enriched in hyaluronidase, were found to induce cancer cell degradation. Moreover, the enrichment of EVs from MSCs with TRAIL, a pro-apoptotic molecule that alone presents problems of bioavailability and distribution, selectively induced apoptosis in target cancer cells (193). Similarly, EVs could carry the oncolytic virus genome and protect it from the immune system until target, the cancer cells (172).

Researchers are working on several other options to use EVs for treatment and diagnosis purposes. EVs present a lot of potential. To correctly classify and systemize all the information obtained from EVs research, in terms of synthesis, artificial modifications, production, and quality, researchers must define strategies for their design and manufacture. The high standards of the pharmaceutical products require that researchers have taken this into account (194).

Until 03/09/2021, in the ClinicalTrials.gov platform (195) from the NIH: National Library of Medicine (https://clinicaltrials.gov/ct2/home), a database of clinical studies conducted around the World, it is possible to find a total of 84 clinical studies and clinical trials related to the keyword "extracellular vesicles" on the most varied areas, such as renal, infection, inflammatory, cardiovascular and neurodegenerative diseases. From these clinical studies, 19 are related to the keyword "cancer", where "neoplasm" and "tumor" were automatically added. EVs are found to be studied in melanoma, lung, breast, thyroid, colorectal, prostate, brain, gastric and oral cancer, as cancer biomarkers (196), and their potential for drug delivery and novel therapies has also already been identified. Therefore, research must continue. In the future, we will see if all their potential is confirmed through validated scientific evidence.

5 Conclusions

Extracellular vesicles can be extremely important in cancer progression. The processes of cancer growth, invasiveness, angiogenesis, and metastasis can be led by extracellular vesicles. Extracellular vesicles can also present anti-cancer properties, through tumour microenvironment immunomodulation, apoptosis induction, and anti-angiogenic properties. Their full profile of functions and underlying mechanisms is not yet completely known. It is necessary further research in this area, but the potential of extracellular vesicles has already been identified.

Nowadays, medical approach tends to move towards personalized medicine, looking forward to the administration of the most appropriate medicine for that specific patient and extracellular vesicles will probably help the scientific community to achieve this goal. Extracellular vesicles could be soon considered relevant biomarkers for cancer diagnosis, prognosis, and monitoring, as well as promising targets, potential drug delivery agents, and it is even hypothesized that extracellular vesicles could work actively against cancer. EV-based therapies could be extremely useful for cancer treatment in the future, but there is still a long way to go.

Therefore, extracellular vesicles constitute valuable research targets for cancer diagnosis, prognosis, and treatment.

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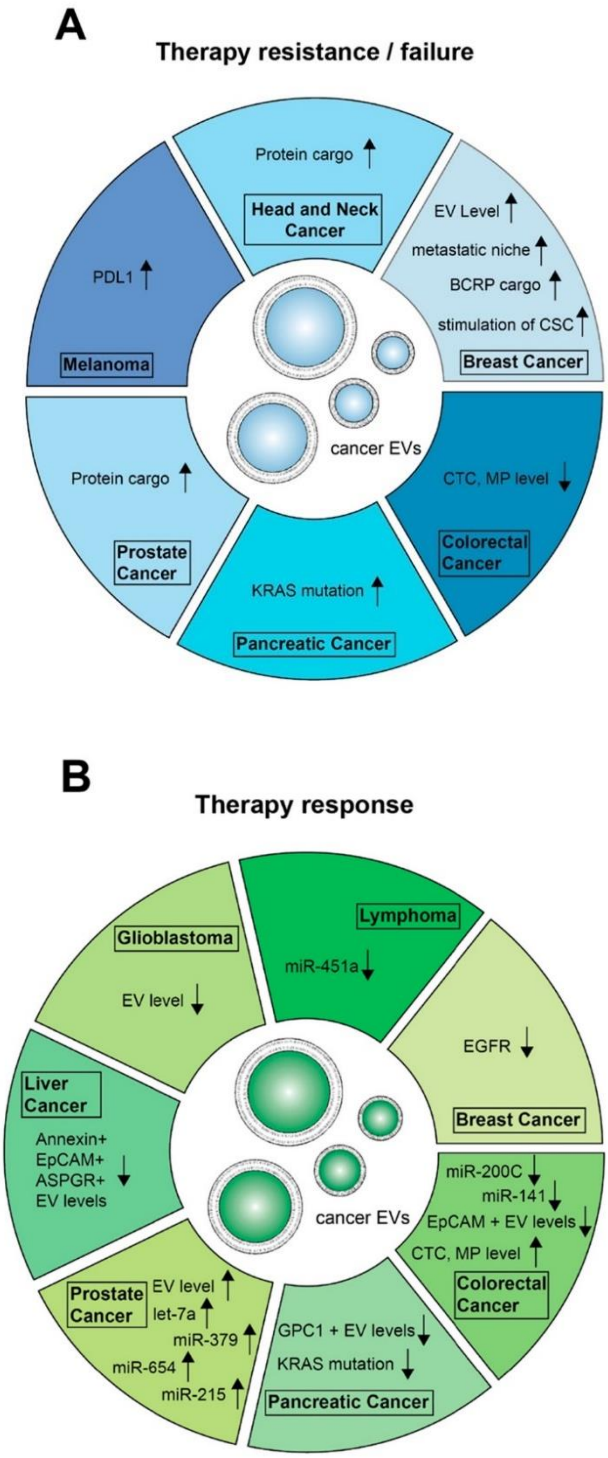
Appendix

A1. Extracellular vesicles in resistance and response to anti-cancer therapy



Adapted from Stevic I, Buescher G, Ricklefs FL. Monitoring Therapy Efficiency in Cancer through Extracellular Vesicles. Cells. 2020; 9(1):130 (197).

A2. Extracellular vesicles as potential cancer biomarkers

Table II. Molecules in EV from body fluids of patients with cancer as potential markers for personalized medicine

Caner type	Material	Body fluids	Biomarkers	Potential application
Prostate cancer	Protein	Plasma, serum	Survivin	Monitoring
	Protein	Plasma	PSA	Diagnosis/prognosis
	Protein	Urine	PSA	Diagnosis/monitoring
	Protein	Urine	β-catenin	Screening
	Protein	Urine	PCA-3, TMPRSS2:ERG	Diagnosis/monitoring
	miRNA	Plasma, serum and urine	miR-107, miR-141, miR-375, miR-574-3p	Diagnosis/prognosis
	miRNA	Serum	miR-141	Diagnosis/prognosis
	miRNA	Serum	15 miRNAs	Diagnosis/monitoring
	Vesicle	Serum	Platelet microparticle number	Prognosis
	Vesicle	Plasma	Microvesicle number	Diagnosis/prognosis
Ovarian cancer	Protein	Plasma	Claudin-4	Screening
	Protein	Plasma	TGF-beta1, MAGE3/6	Prediction/monitoring
	Protein	Serum	L1CAM, CD24, ADAM10, EMMPRIN	Diagnosis/prognosis
	Protein	Serum	IgG recognized exosome antigen	Diagnosis
	Protein	Ascites	CD24, EpCAM	Diagnosis
	Protein	Ascites	MMP2, MMP9, uPA	Diagnosis
	miRNA	Serum	12 miRNAs	Screening
	Vesicle	Serum	EpCAM(+)exosome number	Screening
Lung cancer	Protein	Serum	EGRF	Prognosis
	Protein	Urine	LRG1	Diagnosis
	Protein	Pleural effusion	SNX25, BTG1, PEDF, thrombospondin	Diagnosis
	miRNA	Serum	miR-486, miR-30d, miR-1, miR-499	Diagnosis/prognosis
	miRNA	Plasma	6 miRNAs	Diagnosis
	miRNA	Plasma	let-7f, miR-30e-3p, miR-223, miR-301	Diagnosis
	miRNA	Plasma	miR-205, miR-19a, miR-19b, miR-30b, miR-20a	Monitoring
	miRNA	Plasma	10 miRNAs	Screening
	miRNA	Plasma	12 miRNAs	Screening/prognosis
	Vesicle	Plasma	EpCAM(+)exosome number	Screening/prognosis
	Vesicle	Plasma	Microvesicle number	Prognosis
Glioblastoma	Protein	Plasma	EGFR, EGFRvIII, PDPN, IDH1R132H	Monitoring
	Protein	Serum	EGFRvIII	Prognosis
	miRNA	Serum	miR-21	Diagnosis/prognosis
	miRNA	Serum	RNU6-1, miR-320, miR-574-3p	Diagnosis
	miRNA	Cerebrospinal fluid	miR-21	Diagnosis
Breast cancer	miRNA	Blood, milk, ductal fluids	miR-16, miR-1246, miR-451, miR-720	Prognosis
	miRNA	Serum	miR-200a, miR-200c, miR-205	Diagnosis
	Protein		CD24, EpCAM	
	Protein	Serum	HER-2	Monitoring
	miRNA	Serum	miR-21	Prognosis
Pancreatic cancer	miRNA	Serum	miR-17-5p, miR-21	Diagnosis
	DNA	Serum	KRAS	Companion diagnosis/prognosis
	Protein	Plasma	EGFR	Diagnosis
	mRNA	Saliva	Apbb1ip, ASPN, Daf2, FoxP1, Bco31781, Gng2	Diagnosis
Colorectal cancer	Protein	Ascites	Claudin-3	Diagnosis
	miRNA	Serum	7 miRNAs	Diagnosis
	Vesicle	Plasma	Microvesicle number	Diagnosis/prognosis
Melanoma	Protein	Plasma	Caveolin-1	Diagnosis/prognosis
	Protein	Plasma	TYRP2, VLA-4, HSP70, HSP90‘	Prognosis

Table II (*Continued*)

Caner type	Material	Body fluids	Biomarkers	Potential application
Gastric cancer	Protein	Serum	CCR6, HER-2/neu, EMMPRIN, MAGE-1, C-MET	Diagnosis/prognosis
	Vesicle	Plasma	Platelet microparticle number	Diagnosis
Bladder cancer	Protein	Serum	EPS812, mucin-4	Diagnosis
Mucinous adenocarcinoma	Protein	Plasma	Tissue factor, MUC1	Monitoring
Oesophageal squamous cell carcinoma	miRNA	Serum	miR-21	Prognosis
Cervical cancer	miRNA	Cervicovaginal lavage	miR-21, miR-146a	Diagnosis
Acute leukaemia	miRNA	Plasma	miR-92	Diagnosis
Hepatocellular carcinoma	Vesicle	Serum	Microvesicle number	Diagnosis

Adapted from Taixue An, Sihua Qin, Yong Xu, Yueting Tang, Yiyao Huang, Bo Situ, Jameel M. Inal & Lei Zheng (2015) Exosomes serve as tumour markers for personalized diagnostics owing to their important role in cancer metastasis, Journal of Extracellular Vesicles, 4:1 (159).

A3. Extracellular vesicles in clinical studies

ClinicalTrials.gov Search Results 09/05/2021

	Title	Status	Study Results	Conditions	Interventions	Characteristics	Sponsor/Collaborators
1	Impact of Group Psychological Interventions on Extracellular Vesicles in People Who Had Cancer	Not yet recruiting	No Results Available	•Cancer	•Behavioral: Mindfulness Based-Cognitive Therapy (MBCT) •Behavioral: Emotion Focused Therapy Group for Cancer Recovery (EFT-CR) •Other: Treatment as usual (no intervention)	Study Type: Interventional	•Instituto Portugues de Oncologia, Francisco Gentil, Porto •Polytechnic Institute of Porto •VTT Technical Research Centre, Finland •University of Oulu •Linnaeus University •European Commission
2	Contents of Circulating Extracellular Vesicles: Biomarkers in Colorectal Cancer Patients	Recruiting	No Results Available	•Colorectal Cancer	•Biological: analysis (protein, lipid, RNA ...) of circulating exosomes, size and number •Other: Gathering additional information about the patient's cancer •Diagnostic Test: Diagnostic test	Study Type: Observational	•Centre Hospitalier Universitaire Dijon
3	A Prospective Feasibility Study Evaluating Extracellular Vesicles Obtained by Liquid Biopsy for Neoadjuvant Treatment Response Assessment in Rectal Cancer	Not yet recruiting	No Results Available	•Rectal Cancer •Liquid Biopsy	•Procedure: Supplementary blood samples collection during the normal follow up of the patients	Study Type: Observational	•University Hospital, Bordeaux
4	The Modulatory Role of Internet MBCT on Extracellular Vesicles and Distress in Cancer Patients - Study Protocol	Not yet recruiting	No Results Available	•Cancer	•Behavioral: Mindfulness Based Cognitive Therapy (MBCT) •Other: Treatment as Usual	Study Type: Interventional	•Instituto Portugues de Oncologia, Francisco Gentil, Porto •European Commission •University of Coimbra •VTT Technical Research Centre, Finland •University of Oulu •Linnaeus University
5	Pimo Study: Extracellular Vesicle-based Liquid Biopsy to Detect Hypoxia in Tumours	Completed	No Results Available	•Cancer	•Other: Hypoxia marker	Study Type: Interventional	•Institute of Cancer Research, United Kingdom
6	Development of Liquid Biopsy Technologies for Noninvasive Cancer Diagnostics in Patients With Suspicious Thyroid Nodules or Thyroid Cancer	Recruiting	No Results Available	•Thyroid Gland Carcinoma •Thyroid Gland Nodule	•Procedure: Biospecimen Collection •Other: Electronic Health Record Review	Study Type: Observational	•Jonsson Comprehensive Cancer Center
7	New Strategies to Detect Cancers in Carriers of Mutations in RB1	Recruiting	No Results Available	•Retinoblastoma •Secondary Primary Malignancies After Retinoblastoma	•Other: blood draw	Study Type: Observational	•VU University Medical Center •University Hospital, Essen •Institut Curie

8	Validation of ClarityDX Prostate as a Reflex Test to Refine the Prediction of Clinically-significant Prostate Cancer	Recruiting	No Results Available	•Prostate Cancer	•Diagnostic Test: Blood test: ClarityDX Prostate	Study Type: Observational	•Nanostics •Alberta Prostate Cancer Research Initiative, APCaRI •DynaLIFE Medical Laboratories •Prostate Cancer Centre, Calgary •Northern Alberta Urology Centre •Alberta Cancer Foundation •Alberta Innovates Health Solutions •Motorcycle Ride for Dad •University Hospital Foundation - The Kaye Fund Competition
9	Olmutinib Trial in T790M (+) NSCLC Patients Detected by Liquid Biopsy Using BALF Extracellular Vesicular DNA	Completed	No Results Available	•Non Small Cell Lung Cancer	•Drug: Olmutinib	Study Type: Interventional	•Konkuk University Medical Center •Hanmi Pharmaceutical Company Limited
10	Multicenter Clinical Research for Early Diagnosis of Lung Cancer Using Blood Plasma Derived Exosome	Active, not recruiting	No Results Available	•Lung Cancer	•Diagnostic Test: Exosome sampling	Study Type: Observational	•Korea University Guro Hospital
11	Intestinal Microbiota in Prostate Cancer Patients as a Biomarker for Radiation-INduced Toxicity (IMPRINT)	Recruiting	No Results Available	•Prostate Cancer •Prostate Adenocarcinoma •Prostatic Neoplasms	•Other: Collection of human biofluids •Other: Patient reported outcome measures	Study Type: Interventional	•University Hospital, Ghent
12	Pilot Study With the Aim to Quantify a Stress Protein in the Blood and in the Urine for the Monitoring and Early Diagnosis of Malignant Solid Tumors	Completed	No Results Available	•Cancer	•Other: blood samples •Other: Urine samples	Study Type: Interventional	•Centre Georges Francois Leclerc
13	Prospectively Predict the Efficacy of Treatment of Gastrointestinal Tumors Based on Peripheral Multi-omics Liquid Biopsy	Recruiting	No Results Available	•Advanced Gastric Adenocarcinoma •Immunotherapy	•Device: EV-array	Study Type: Observational	•Shen Lin •Peking University
14	A Phase III Trial of Pertuzumab Retreatment in Previously Pertuzumab Treated Her2-Positive Advanced Breast Cancer	Active, not recruiting	No Results Available	•HER2-positive Locally Advanced or Metastatic Breast Cancer	•Drug: Trastuzumab •Drug: Pertuzumab •Drug: Docetaxel •Drug: Paclitaxel •Drug: Nab-paclitaxel •Drug: Vinorelbine •Drug: Eribulin •Drug: Capecitabine •Drug: Gemcitabine	Study Type: Interventional	•Japan Breast Cancer Research Group •Chugai Pharmaceutical

15	The Senstivity and Specificity of Using Salivary miRNAs in Detection of Malignant Transformation of Oral Lesions.	Completed	No Results Available	•Oral Premalignant Lesions	•Diagnostic Test: using salivary miRNA (412,512)	Study Type: Observational	•Cairo University
16	An Observational Study to Evaluate the Clinical Utility of the Oncomine Precision Assay Within the Exactis Network	Not yet recruiting	No Results Available	•NSCLC		Study Type: Observational	•Exactis Innovation
17	ncRNAs in Exosomes of Cholangiocarcinoma	Unknown status	No Results Available	•Cholangiocarcinoma •Benign Biliary Stricture		Study Type: Observational	•The Second Hospital of Nanjing Medical University
18	GMCI, Nivolumab, and Radiation Therapy in Treating Patients With Newly Diagnosed High-Grade Gliomas	Active, not recruiting	No Results Available	•Glioma, Malignant	•Biological: AdV-tk •Drug: Valacyclovir •Radiation: Radiation •Drug: Temozolomide •Biological: Nivolumab •Other: Laboratory Biomarker Analysis	Study Type: Interventional	•Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins •Candel Therapeutics, Inc. •National Cancer Institute (NCI) •Bristol-Myers Squibb
19	Radiation Therapy, Plasma Exchange, and Immunotherapy (Pembrolizumab or Nivolumab) for the Treatment of Melanoma	Recruiting	No Results Available	•Melanoma	•Biological: Nivolumab •Biological: Pembrolizumab •Radiation: Radiation Therapy •Biological: Therapeutic Exchange Plasma	Study Type: Interventional	•Mayo Clinic •National Cancer Institute (NCI)

Adapted from ClinicalTrials.gov, available from https://clinicaltrials.gov/ct2/results?cond=Cancer&term=extracellular+vesicles&cntry=&state=&city=&dist=, accessed on 05/09/2021 (196).