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Review

Molecular Profile Study of Extracellular Vesicles for the Identification of Useful Small "Hit" in Cancer Diagnosis

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Abstract: Tumor-secreted extracellular vesicles (EVs) are the main mediators of cell-cell communication, permitting cells to exchange proteins, lipids, and metabolites in varying physiological and pathological conditions. They contain signature tumor-derived molecules that reflect the intracellular status of their cell of origin. Recent studies have shown that tumor cell-derived EVs can aid in cancer metastasis through the modulation of the tumor microenvironment, suppression of the immune system, pre-metastatic niche formation, and subsequent metastasis. EVs can easily be isolated from a variety of biological fluids, and their content makes them useful biomarkers for the diagnosis, prognosis, monitorization of cancer progression, and response to treatment. This review aims to explore the biomarkers of cancer cell-derived EVs obtained from liquid biopsies, in order to understand cancer progression and metastatic evolution for early diagnosis and precision therapy.

Keywords: extracellular vesicles; liquid biopsy; biomarkers; tumor progression; metastasis

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1. Introduction

Carcinogenesis is a process in which unlimited/uncontrolled cell division occurs, leading to the formation of malignant tumors. Compared to normal cells, cancer cells acquire malignant properties through genetic mutations and other aberrations that give them adaptive and proliferative advantages. This malignant transformation is a multistage process involving the gradual accumulation of abnormalities necessary for cell tumor progression [1,2]. Sequential selection of variant subpopulations within the neoplastic clone are responsible for the progression of the neoplasm, and the development of intraneoplastic diversity is emerging as an important feature. In fact, heterogeneity allows cells to develop characteristics that allow them to proliferate, evade apoptosis, undergo angiogenesis, alter metabolism, and form metastases [3]. Findings suggest that cancer progression is related to altered protein expression and changes in metabolic pathways, which gives cancer cells the ability to survive and expand in a hostile microenvironment. Cancer cells use a variety of mechanisms through which they develop an extraordinary ability to adapt and expand in the microenvironment, including the release and uptake of extracellular vesicles (EVs).

EVs constitute a heterogeneous vesicle population secreted by virtually all cell types. They have been found in many body fluids, including blood [4], urine [5], saliva [6], bronchoalveolar lavage [7], and cerebrospinal fluid [8]. They are enriched with proteins, lipids, metabolites, DNA fragments, miRNA fragments, and non-coding RNAs [9]. According

to their size and biogenesis, EVs are classified into the following groups: exosomes, microvesicles, and apoptotic bodies. Exosomes (30–120 nm in diameter) are initially generated inside of multivesicular bodies (MVBs), existing as intraluminal vesicles (ILVs). When these MVBs fuse with the cell membrane, vesicles are released into the extracellular space, where they become known as "exosomes" [10]. In contrast to exosomes, microvesicles are formed by a characteristic "outward blebbing" of the plasma membrane, producing a class of heterogeneous vesicles of larger size (100 nm–1 µm in diameter). The formation of microvesicles appears to occur selectively in lipid-rich membrane microdomains (e.g., in lipid rafts) [11]. These vesicles are often given other names, including ectosomes, shedding vesicles, or oncosomes (in the case of cancer cells) [12]. Apoptotic bodies (1–5 µm in diameter) are released during the last steps of apoptosis through formation of membrane protrusions, such as microtubule spikes, apoptopodia, and beaded-apoptopodia. Although these classifications have been widely used in the literature, it is advised to use this nomenclature sparingly. This is due to the fact that most techniques currently used only allow the separation of small EVs from large EVs [12]. Consequently, the International Society for Extracellular Vesicles (ISEV) recommends using terms for EV subtypes, such as size, density, instead of using terms such as "exosomes" or "microvesicles", unless there is certainty of their biogenesis [13].

Liquid biopsies are a rich source of EVs and are typically obtained using minimally invasive medical procedures that allow sampling from healthy controls, and therefore allows the detection of minimal residual disease (MRD) or recurrence and tracking of tumor evolution. It has been demonstrated that cancer cell-associated EVs carry the signatures of proteins, lipids, metabolites, and RNA of the tumor cell of origin [9]. Additionally, cancer-derived EVs are important players in cancer progression, being able to facilitate intercellular communication with cells near and far away (e.g., stromal cells), promoting the formation of pre-metastatic niches. EVs are involved in different stages of tumorigenesis and metastasis by increasing angiogenesis and remodeling the extracellular matrix. This activity allows cells to escape from immune system recognition and induces resistance to various cancer therapies [14,15]. For this reason, molecular profiling of EVs can help us understand the behavior of the cancer, in order to obtain an early diagnosis and develop targeted therapies.

In this review, we aim to cover the fields' most recent findings in proteomic, lipidomic, and metabolomic studies in EVs. We will highlight the best practices for identification of tumor biomarkers by cancer cell-derived EVs, placing an emphasis on the hallmarks of cancer, promotion of invasion, and metastasis

2. EVs Performing Multiple Functions in Cancer Formation

EVs have been found to play an important role in various physiological and pathological conditions. Factors, such as hypoxia, inflammation, and extracellular acidification in the tumor microenvironment (TME) may contribute to the increased secretion rate of EVs [16]. In fact, it has been reported that malignant cells secrete an increased number of EVs with an altered composition to those from non-malignant cells of the same type [17]. However, diagnostic and prognostic evaluations using the total abundance of EVs may be subject to confounding effects from non-malignant cells responsible for circulating EVs in some disease conditions. Even so, studies have demonstrated that EVs are enriched with a highly heterogeneous pool of biological cargo, i.e., proteins, lipids, RNA, and metabolites that modulate target cells [9]. As previously mentioned, these tumor-derived EVs are released into circulation in order to carry out functions that support tumorigenesis, such as stimulating angiogenesis and promoting metastatic diffusion [18,19].

2.1. EVs Promote Angiogenesis, Invasion, and Metastasis

EVs perform important functions throughout several stages of invasion and metastatic colonization, including angiogenesis, invasion, epithelial mesenchymal transition (EMT), migration, and pre-metastatic niche formation which are described below.

2.2. Angiogenesis

Angiogenesis is the formation of new blood vessels from an already existing vascular network. Several steps characterize angiogenic sprouting, such as enzymatic degradation of the vessel basement membrane, endothelial cell proliferation, migration, germination, branching, and formation of new vessels [19]. Angiogenesis is regulated by a precise balance between stimulatory and inhibitory signals, which can become altered in cancer pathologies [20]. The potential of EVs to induce angiogenesis is thought to be dependent on their uptake by recipient cells. For example, EVs released from glioblastomas contain a host of pro-angiogenic signals, such as proteoglycans glypican-1 and syndecan-4. These signals increase revascularization through proliferation and formation of endothelial cells and tubules [21].

EVs released by tumor cells are important mediators of the angiogenic cascade, regardless of their uptake. Tumor angiogenesis is mediated by a repertoire of membrane-bound proteins that could confer selective advantages for tumor growth and metastasis. EVs secreted by cancer cells in the brain and neck contain the ephrin type B receptor 2 (EPHB2), which is associated with tumor angiogenesis by activating the STAT3 signaling pathway via engagement of ephrin-B2 on the surface of endothelial cells [22]. Similarly, ovarian cancer cell-derived EVs contain the soluble form of E-cadherin on their surface, making them capable of stimulating tumor angiogenesis by forming a heterodimer with VE-cadherin on the surface of endothelial cells [23]. In addition, EVs secreted by tumor cells show various cytokines on their vesicular surface, such as IL-8 and VEGF, which can stimulate the growth, migration and/or formation of endothelial tubes [24]. Studies on the role of EVs in angiogenesis show that they are excellent tools for understanding the mechanisms underlying endothelial cell migration, alteration of vessel phenotype, and germination of solid tumors.

2.3. EVs in Promoting Metastasis Initiation and Progression

The metastatic process involves invasion and proliferation of tumor cells into nearby and distant tissues via the circulatory and lymphatic systems. Metastatic cells establish a microenvironment which favors angiogenic and proliferative processes, promoting cancer metastasis. EVs from initial tumor cells promote EMT by targeting EMT-related factors, such as transforming growth factor beta (TGFβ), hypoxia-inducible factor 1 alpha (HIF-1α), thus initiating metastasis. For example, EVs released by prostate cancer cells are enriched with lysosomal hyaluronidase, which promotes the mobility of stromal cells, aiding in metastasis [25]. Moreover, EVs secreted by highly metastatic cells can increase cell ability to migrate and invade low-metastatic cells by promoting an EMT process via MAPK/ERK signaling [26]. The hypoxia that characterizes the TME also influences tumor cells to release EVs enriched in matrix metalloproteinase-13 (MMP-13), with a consequent increase in vimentin and decrease in E-cadherin in normoxic cells, thus improving the metastases that occur via EMT [27].

Interestingly, Hoshino et al. demonstrated that cancer-derived EVs express a series of integrins that regulate adhesion to specific cancer cells and the extracellular matrix (ECM) molecules expressed in some organs [28]. They revealed that EVs containing ITGα6β4 and ITGα6β1 are associated with lung metastases through their ability to bind to lung-resident fibroblasts and epithelial cells. Similarly, integrin-expressing EVs containing αvβ5 are linked to liver metastases because of their ability to bind to Kupffer cells [28]. Macrophage migration inhibitory factor in EVs from pancreatic cancer can alter growth factor β production in Kupffer cells and increase fibronectin secretion of hepatic stellate cells [28]. Gastric tumor cell-derived EVs mediate the formation of a liver-like microenvironment through EGFR-mediated hepatocyte growth factor upregulation, ameliorating metastasis [29]. Many studies have also reported brain-derived secretory proteins, which can alter the brain microenvironment to promote the colonization of cancer cells, leading to brain metastasis [29]. In this respect, proteins present within EVs derived from the brain can increase the adhesive and invasive capacity of non-brain metastatic cells [30].

These results indicate that cancer-derived EVs released into the body have the potential to remodel cancerous and non-cancerous cells, leading to pre-metastatic niche formation and metastatic organotropism.

2.4. EVs in Immunomodulation

The metastatic cascade may be favored by the ability of EVs to mediate immunosuppression through the transport of inflammatory factors [31]. Importantly, tumor-derived EVs can be transmitting inflammatory factors to recipient cells to regulate their behaviors. For instance, the transfer of tumor-derived EVs into human THP-1 monocytic cells led to the production and secretion of various pro-inflammatory cytokines and tumor necrosis factor (TNF) -α, via TLR2 and TLR4 binding [32]. Moreover, EVs from breast cancer expressing Annexin A2 mediate an increase in the secretion of IL-6 and TNF-α by M1 macrophage activation [33].

Tumor-derived EVs can precondition an environment for future metastasis using other mechanisms as well. For example, EVs derived from gastric cancer express transforming growth factor (TGF)-β1, which is associated with lymph node metastasis through the induction of regulatory T cell (Treg) formation [34]. Contrarily, it has been observed that TGF-β1 in EVs derived from breast cancer cells can suppress T cell proliferation [35]. In addition, tumor-derived EVs appear to be capable of inhibiting the activation of T lymphocytes and induce apoptosis through the expression of the ligand FasL, a member of the tumor necrosis factor (TNF) family, which can promote the apoptosis of lymphocytes [36].

Cancer-derived EVs can also enhance immune evasion of cancer cells through the suppression of T cell activity and natural killer (NK) cells. For example, it has been identified that tumor-derived EVs can block IL-2-mediated activation of NK cells and their cytotoxic activity [37].

Conversely, EVs derived from immune cells can be considered attractive tools for fighting against cancer. For example, NK cell-derived EVs can induce the immune response by compromising the spread of solid tumors [38]. However, the role of EVs released by these cells is still poorly understood.

2.5. EVs in Reprogramming Energy Metabolism

The metabolic adaptations made possible by cancer cells allow them to adapt to conditions of nutritional deprivation and/or stress. TEM is heterogeneous in cancer cells, fibroblasts, endothelial cells, mesenchymal stem cells, and extracellular matrix, participating in the metabolic reprogramming crucial for cancer progression [39].

EVs released from cancer cells contribute to the transformation of normal fibroblasts into distinct functional subtypes. Cancer-associated fibroblasts (CAFs), a major component in TME, promote tumor growth and progression through various pathways, e.g., by the secretion of inflammatory factors [40]. On this note, early- and late-stage colorectal cancer (CRC) cell-derived EVs both can activate normal quiescent fibroblasts in phenotypically and functionally distinct subsets of CAFs, which could facilitate tumor progression [40]. Additionally, triple negative breast cancer (TNBC) cells overexpressing ITGB4 are capable of remodeling fibroblast metabolism and promoting glycolysis in CAFs [41].

EVs released from cancer-associated endothelial cells (CAECs) might also have an important influence on tumor progression. Analyses suggest that a decrease in levels of EC-derived EVs after chemotherapy in patients with metastatic breast cancer is associated with disease-free survival [42]. The intercellular communication between MSC and cancer cells is not well understood, and neither are the metabolic changes due to this interaction. However, TME could promote the development of cancer-associated mesenchymal stem cells (CA-MSCs) with the ability to differentiate into CAFs and influence cancer cells by secreting various metabolites by EVs. In fact, the metabolic crosstalk between tumor cells and CAFs profoundly affects TME remodeling and drives tumor growth [43]. In addition, glioma cell-derived EVs induced tumor-like phenotypes in MSCs by activating

glycolysis [44] and EVs from prostate cancer promote the formation of a pre-metastatic niche through the transfer of PKM2 into MSCs [45] (Figure 1).

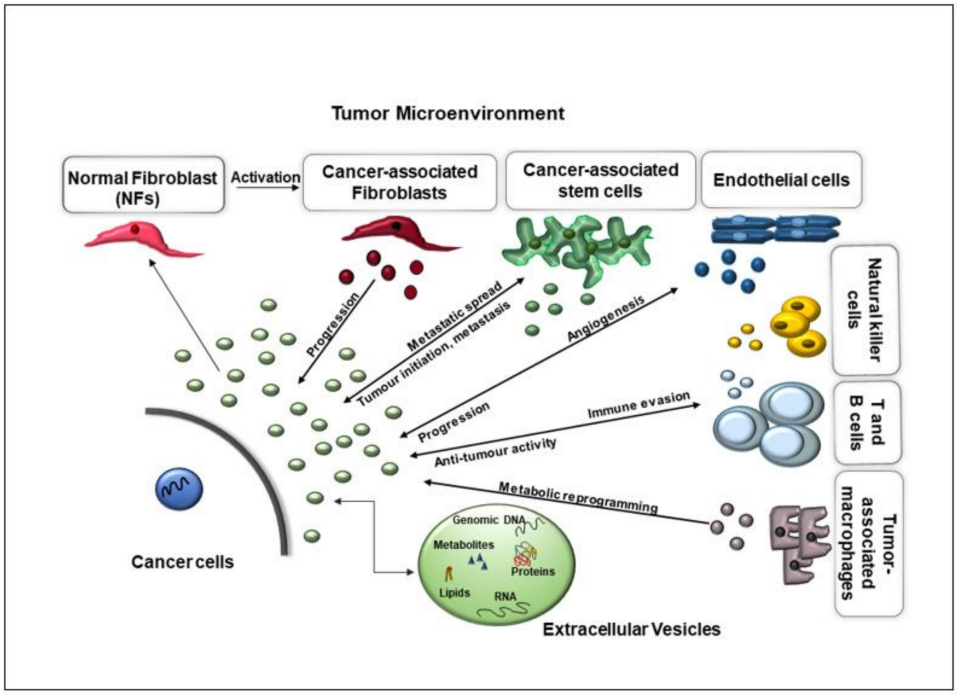

Figure 1. Schematic illustration of EV-mediated interactions between cancer cells and their TME. In cancer, EVs are involved in the process of transporting bioactive molecules between stromal and transformed cells, including endothelial, mesenchymal, immune, and CAF cells. Several subtypes of immune cells can also be found in the TME, including dendritic cells (DCs), B and T lymphocytes, and macrophages. EVs released within TME contribute to TME heterogeneity by creating a suitable environment for tumor growth, progression, and metastasis. EVs derived from immune cells which exhibit anti-cancer activity are an exception. Modified from [46].

3. EV Isolation from Different Body Fluids in Cancer

EVs can be isolated from many biofluids, including serum, plasma, urine, saliva, ascitic fluid, bile, cerebrospinal fluid (CSF), and pathological effusions from tumors [47]. The pre-analytical phase represents an important step in the analysis of EVs in terms of anatomical position, accessibility, and specific biophysical and chemical characteristics. For example, biofluids such as urine or blood are easily accessible via non-invasive/minimally invasive methods, making them ideal for longitudinal disease monitoring, and are commonly used samples for diagnostic purposes. Isolation of EVs from CSF is probably preferable over other biofluids for the study of neurological diseases. However, acquiring it is invasive and requires highly qualified professionals, creating potentially serious complications. Below is a description of some of the most commonly used biofluids.

3.1. Serum and Plasma

A crucial step in the development of biomarkers is to determine which blood sample is optimal for the study. A large limitation of serum collection is that platelets can become easily activated and release EVs, which alter the fluid composition. In addition, several variables play a significant role in the analysis of EV composition, such as patient status, syringe use, and anticoagulant use. For example, citrate and ethylenediamine tetraacetic acid (EDTA) are the most commonly used anticoagulants in preparation, in order to prevent platelet activation and release of platelet EVs. Depending on the choice of the downstream EVs analysis, all these variables must necessarily be taken into account for the standardization of the sample collection protocol [13,48]. Another important aspect is

the lack of quality control (QC) for plasma samples to be used in clinical trials. Recently, researchers have agreed that human sample quality must be standardized prior to any molecular analysis [49].

3.2. Urine

Unlike serum, urine can be collected in large volumes with little or no patient discomfort. It contains a considerable number of EVs, proteins, metabolites, and cells derived from the urogenital tract and filtration of glomerular plasma. For this reason, it is ideal for the analysis of physical and pathological conditions of an individual [50]. Urine is a unique biofluid characterized by varying pH ranges, osmolarity, composition and concentration of dispersed solutes, even within the same individual [51]. Urinary EVs (uEVs) released predominantly by the kidneys, urinary tract epithelium, and male reproductive tract, constitute a heterogeneous population [52]. In addition, uEVs can be strongly influenced by factors, such as medication, exercise, food intake, and the presence of urinary pigments [53]. Consequently, careful standardization of urine collection and development of protocols compatible with downstream analyses/reproducibility of data are recommended.

3.3. Saliva

Human saliva is easy to collect, inexpensive, and can be performed in a non-invasive manner. It is also enriched in salivary extracellular vesicles (SEV) [54]. A similarity between the salivary and plasma proteome has been previously described in literature, therefore SEV should be derived partly from the salivary glands, and partly from circulation [55]. Saliva has advantages over blood as it does not coagulate, and its composition does not undergo changes brought about by the release of EVs from platelets. However, interference of high-abundance amylases and proline-rich proteins may affect the isolation and purification of SEVs, as well as the identification of low abundance proteins present inside the SEVs. Therefore, removal of amylase and other proteins from saliva prior to extraction of SEVs would be a necessary pre-analytic step for accurate downstream analysis (e.g., proteomic analysis) [56]. In addition, the composition and concentration of analytes in saliva are influenced by varying collection conditions and processing methods. Non-contaminated saliva can be collected via a buccal swab or by spitting into a test tube (which are subject to variation depending on tongue and/or cheek movements). Saliva production can be stimulated through use of chewing gum, for example, producing a volume three times greater than the volume of unstimulated saliva [57]. Whole saliva usually contains cellular debris and bacterial cells. Taking all of these variabilities into account, it is necessary to develop a standardized protocol to minimize variability when studying the composition of saliva for the identification of biomarkers.

3.4. CSF

CSF is a transparent fluid that provides a unique insight into neurological disease. CSF is the result of the ultrafiltration of blood plasma by ependymal cells in the choroid plexuses of the cerebral ventricles, containing few cells and almost devoid of proteins [58]. CSF biopsies are particularly important as they allow the collection of brain tissue from living individuals, however, sampling is more invasive than blood or urine sampling [59]. In this case, it is necessary also to pay attention to the pre-analytical procedures, since errors in the collection, storage, and exchange of biofluids are the most represented laboratory errors [59]. Cerebrospinal fluid can be collected from external ventricular drainage (i.e., during shunt or extraventricular placement) or by lumbar puncture (spinal tap). A clearly defined and consistent protocol for lumbar CSF collection ensures identical processing and the ability to compare results across institutions. Lumbar CSF typically does not contain blood, however, there are many differences between centrifuged and non-centrifuged components, and short-term temporal collections are difficult to obtain [60].

The choice of a specific isolation technique is not only dependent on the type of sample but also on the type of downstream analyses used for '-omics' characterization (e.g., proteomics).

4. Studies of the Molecular Profiling of EVs as Potential Biomarkers

EVs carry different classes of molecules, including proteins, lipids, nucleic acids, and other metabolites [9], and their content is affected by different environmental factors [61]. This, along with their ability to act as powerful cell-cell communication mediators, has contributed to their emergence as biomarkers of numerous diseases, including cancer [62]. However, mainly due to its small size, the purification of EVs is still a challenge and different isolation methods are used to obtain EVs efficiently and with high purity [63].

The techniques widely used for isolating EVs include differential ultracentrifugation (dUC), size exclusion chromatography (SEC), density gradient flotation, immunoprecipitation, and polymer-based precipitation, as well as commercially available kits [64]. dUC is the most extensively used method for isolating EVs, consisting of sequential centrifugations with increasing speed and time, to separate particles depending on their size and density [65]. This method can obtain a high yield of EVs, but it is time-expensive and requires specialized equipment [66]. Furthermore, it could damage the integrity of these vesicles, affecting their profiles of RNA, protein, and other metabolites which are a potential source of biomarkers [67]. Hence, other techniques, such as SEC and density gradient centrifugation are gaining relevance to isolate EVs [66].

On the other hand, it has been reported that the choice of a method for EVs isolation could impact down-stream analysis of their cargo, either due to the isolation of different EV populations, or the co-isolation of contaminating proteins and other possible matrix contaminants [68,69]. In addition, various challenges come into play when isolating EVs from different biological sources, such as possible co-purification of chylomicrons and lipoprotein particles with EVs in serum and plasma [70], or the presence of Tamm–Horsfall protein in urine [71], which can interfere in posterior biomarker analysis. Thus, a methodological standardization of EV isolation is still needed, in order to assure the reproducibility of the subsequent analyses, especially for clinical settings [72]. A better understanding of the protein, lipid, and metabolic composition of EVs and the extent to which EVs composition reflects the source cell composition provides a solid basis for further development of diagnostics and therapeutics [9].

In this review, we summarize the recent advances in bioactive EVs contents focusing on proteins, lipids, and metabolites that could play a significant role as diagnostic markers.

4.1. Proteome Profiling Analysis of EVs in Multiple Cancers

Proteins are an important class of molecules that are transported by EVs, and some of them are integral constituents of EV structures. Analysis of EV protein composition is crucial to understanding the mechanisms of their biogenesis and function under physiological and pathological conditions. It is well-known that EVs are highly enriched in membrane proteins, such as tetraspanins, including CD9, CD63, CD81, CD82, CD151, and Tspan8 [73,74]; proteins involved in EVs biogenesis as well as ESCRT-related proteins, such as ALIX, TSG101 (the stereotypical biomarkers for EVs characterization), and syntenin [75]. Other typical EV proteins include cell adhesion-related proteins (integrins, LFA-1, and ICAM -1) [76], and those participating in cytoskeletal construction (actin and tubulin) and vesicle trafficking (e.g., Rab family proteins). MHC class I and II complexes, involved in antigen presentation, have been also reported [77], as well as heat shock proteins, which facilitate protein folding and balance of proteostasis (i.e., Hsp60, Hsp70, and Hsp90) [78].

In addition to self-proteins, EV content reflects the physiological state of the cell from which they originated. Since EVs are normally isolated in small amounts, highly sensitive analyses are needed. In recent years, technology have been widely used to allow the massive identification and relative quantification of proteins present in EVs from different biological samples [79,80]. Moreover, many public databases have been created to share

data among the scientific community, such as EVpedia, ExoCarta, and Vesiclepedia [81–83]. Current proteomic technology based on mass spectrometry (MS) is the basis for the study and discovery of specific non-invasive biomarkers in the oncology field and beyond. Its high sensitivity makes it capable of identifying low abundance proteins over wide dynamic ranges, including post-translational changes. Additionally, ELISA and Western blotting can be used to identify potential biomarkers. Although many analytical methods have been standardized, unique challenges are associated with different applications of proteomics. On this note, we have considered some studies on cancer-derived-EVs to assess the recent research progress in the field, and at the same time highlight the potential biomarkers reported.

In the literature there are many examples of potential biomarkers found in EVs derived from breast cancer (BC) from both cell lines and biological fluid. An early study on serum-derived EVs of BC patients revealed the presence of CD24, with low epithelial cell adhesion molecules (EpCAM) expression, providing evidence that EpCAM could be cleaved from EVs via serum metalloproteinases [84] (Table 1). CD24 is a glycosylphosphatidylinositol-anchored membrane protein considered to be a negative cancer stem cell marker, specifically in BC [85]. EpCAM is an epithelial cell marker which modulates biological processes, such as cell proliferation, migration, and invasion.

Human epidermal growth factor receptor-2 (HER2) positive breast cancer represents 15–20% of all breast cancers. The primary mechanism of HER2 activation in breast cancer is its gene amplification, which causes its overexpression, ultimately activating several signaling pathways [86]. It has been revealed that HER2 is overexpressed in EVs derived from plasma of BC patients [87] (Table 1).

Table 1. Summary of the potential protein biomarkers identified in EVs from different cancer types.

Cancer Type	Biomarker	Sample	Authors
Thyroid Cancer	TLN1, ITGB2, SRC and CAPNS1	serum derived-EVs	[4]
Breast Cancer	CD24 and EpCAM	serum derived-EVs	[84]
Breast Cancer	HER2	plasma derived-EVs	[87]
Breast Cancer	GPC-1, ADAM10, GLUT-1 and desintegrin	*In vitro*: MDA-MB-231 and MCF-10A cell lines	[88]
Breast Cancer	Del-1, 14-3-3 epsilon protein, β-actin, annexin A1 / 5, heat shock protein 71, and galectin-binding protein 3	*In vitro*: cell line MDA-MB-231	[89]
Pancreatic Cancer	GPC-1	serum derived-EVs	[90]
Colorectal Cancer	GPC-1	plasma derived-EVs	[91]
Colorectal Cancer	CK19, TAG72, and CA125	plasma derived-EVs, CRC cells, tumor interstitial fluid	[92]
Ovarian Cancer	CA125 and HE4	serum derived-EVs	[93,94]
Colon Cancer	CD147	*In vitro*: HCT15 and HCT116	[95]
Colon Cancer	TSPAN1	*In vitro*: HCT-116 and HT-29 CC cell lines	[96]
Colon Cancer	annexin	plasma derived-EVs	[97]
Hepatic Cancer	AMPN, VNN1, pIgR, FCN1 and NEP	serum derived-EVs	[98]
Hepatic Cancer	FIBG, A1AG1 and S100A8	serum derived-EVs	[98]
Cholangiocarcinoma	FCN2, ITIH4, FIBG; MUC1, EGFR, EpCAM, and others.	serum derived-EVs; EGI1, TFK1 cell lines and non-tumor SV40-immortalized human cholangiocytes	[98]
Cholangiocarcinoma	EpCAM, ASGPR1, annexin V and taMPs	serum derived-EVs	[99]
Cholangiocarcinoma	fetuin-A and HSP90B	*In vitro*: M213 and M213D5 cell lines	[100]
Hepatic Cancer	CAP1	*In vitro*: MHCC97-H and MHCC97-L cell lines	[101]
Gastric Cancer	PSMA3 and PSMA6	serum derived-EVs	[102]

Table 1. *Cont.*

Cancer Type	Biomarker	Sample	Authors
Prostate Cancer	FABP5, Granulin, AMBP, CHMP4A, and CHMP4C	urine derived-EVs	[103]
Bladder Cancer	MUC1, CEA, EPS8L2 and moesin	urine derived-EVs	[104]
Lung Cancer	MUC1	plasma derived-EVs	[105]
Lung Cancer	NY-ESO-1, EGFR, PLAP and EpCam	plasma derived-EVs	[106]
Lung Cancer	LBP	serum derived-EVs	[107]
Lung Cancer	BPIFA1, CRNN, MUC5B, and IQGAP1	saliva derived-Evs	[108]
Lung Cancer	LRG1	urine derived-EVs	[109]
Glioblastoma	annexin A2, vimentin, tenascin-C and others	*In vitro*: A172, Glia-Tr, Glia-L, Glia-R, and Glia-Sh cell lines	[110]
Osophageal squamous cell carcinoma	GPC1	*In vitro*: HEEpiC, Het-1A, TE-1, TE-5, TE-6, TE-8, TE-9, TE-10, TE-11, TE-14 and TE-15 and LK-2 cell lines	[111]
Nasopharyngeal carcinoma	ICAM-1, CD44v5 and TSP-1	*In vitro*: C666-1, NP69 and NP460 cell lines	[112]

Another study revealed that glypican-1 (GPC-1), disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), and glucose transporter 1 (GLUT-1) were upregulated in triple negative metastatic cancer cell line MDA-MB-231 (MDA) compared to the control immortalized epithelial breast tissue cell line MCF-10A (MCF) [88] (Table 1). GPC-1 is a cell surface proteoglycan protein involved in the control of cellular growth and differentiation by activation of mitogenic signaling by heparin-binding growth factors. It has been observed to be highly expressed in tissues from BC compared to healthy patients [88]. ADAM10, a transmembrane protease protein, is very abundant in high-grade tumors, and these levels correlated with negative outcomes for the basal subtypes of BC patients [113]. Lastly, GLUT-1 is a solute carrier protein that facilitates the transport of glucose across the plasma membranes. Its expression is correlated with high-grade BC cancer and increased proliferative activity, and its absence significantly increased disease-free survival in BC patients [114].

On the other hand, proteomic analyses from cell culture EVs have also provided other potential biomarker candidates in BC, including 14-3-3 epsilon protein, β-actin, annexin A1/5, heat shock protein 71, and galectin-binding protein 3 [89] (Table 1). In addition, Del-1 has been reported as an early-stage BC EVs biomarker [115]. Del-1 was first identified as an extracellular matrix protein having 3 N-terminal epidermal growth factor-like domains and the discoidin I-like or factor V C domains, C1 and C2 [116]. The striking decrease in Del-1 concentrations in plasma after surgery suggests that this protein could be a useful surveillance biomarker to assess the response of breast cancer patients to cancer therapies [115].

Proteomics have also contributed to identifying potential biomarkers in other types of cancer, such as GPC-1, through showing increased serum EVs of pancreatic cancer (PC) patients [90] (Table 1), as well as in plasma from colorectal cancer (CRC) patients [91] (Table 1). All the studies dealing with the presence of GPC-1 in EVs confirm its potential role as a clinical cancer biomarker. In addition, CK19, TAG72, and CA125 proteins were significantly enriched in EVs derived from CRC cells, tumor interstitial fluid, and patients' plasma [92] (Table 1). CA125 is an antigenic membrane protein of unknown function, however, the release of soluble proteolytic fragments of CA125 into the extracellular space appear to be associated with the conversion from benign to cancer cells [117]. In addition, CA125 together with HE4 are candidate biomarkers for ovarian cancer. So far, the serum EV CA125 and HE4 levels can significantly identify ovarian cancer at the early stage from healthy subjects, benign ovarian disease patients, and other gastrointestinal cancer patients [93,94].

In regard to CRC, protein profiling studies of individual EVs led to the identification of CD147-positive EVs with high predictive value [95] (Table 1). CD147 is a glycoprotein

released by tumor cells in a soluble form, or by EVs involved in progression, invasion, and metastasis, suggesting it as a relevant tumor biomarker for cancer diagnosis [118].

Despite the advances in colon cancer (CC) diagnosis, clinical outcomes and survival rate remain poor. Studies on EVs derived from HCT-116 and HT-29 CC cells and plasma from CC patients showed high levels of tetraspanin 1 (TSPAN1) [96] (Table 1). TSPAN 1 is a member of the tetraspanin family, which may be involved in cancer progression (e.g., proliferation, cell migration, and motility) [96].

Moreover, annexins were increased in EVs derived from plasma of CC patients compared to those from healthy controls (HCs), showing a sensitivity of 75.7% [97] (Table 1). Annexin 2A (ANXA2) is a calcium-binding cytoskeletal protein expressed on the surface of endothelial cells, macrophages, mononuclear cells, and various types of cancer cells [119]. Elevated ANXA2 expression correlates with cell migration and invasion [120].

Furthermore, high-performance analysis has identified differential proteomic profiles in EVs from the serum of intrahepatic carcinoma (iCCA), hepatocellular carcinoma (HCC), and primary sclerosing cholangitis (PSC) patients versus control donors. Reports demonstrated that aminopeptidase N (AMPN, also known as CD13), pantetheinase (VNN1), and polymeric immunoglobulin receptor (pIgR) showed good diagnostic capacity of CCA [98]. AMPN, ficolin-1 (FCN1), and neprilysin (NEP) showed good correlation with PSC compared to the healthy control group [98] (Table 1). VNN1 pantetheinase is a protein anchored to glycosylphosphatidylinositol (GPI) on the cell membrane, which participates in the synthetic pathway of pantothenic acid (vitamin B5), acting as a key regulator of tissue tolerance to stress in various diseases [121]. pIgR seems to be involved in the promotion of cell transformation and proliferation, providing new insights into the role of immunoglobulin receptors [122].

Since PSC is a major risk factor of CCA development, serum EV proteins were compared between CCA and PSC, and a selection of 10 overexpressed proteins in CCA were identified, including fibrinogen gamma chain (FIBG), alpha-1-acid glycoprotein 1 (A1AG1), and S100A8 proteins [98]. A1AG1 is a protein mainly expressed during the acute phase of the inflammatory process, that can play an important role in the tumor microenvironment, affecting immune modulation, drug resistance, and cancer progression [123], whereas S100A8 protein (calgranulin A) belongs to the S100 multigenic family of calcium-modulated proteins with roles in inflammation [124].

Discrimination between early-stage CCA (I-II) and PSC is possible through detection of ficolin-2 (FCN-2) proteins, which are involved in cancer immunity, suppression of EMT, and metastasis of HCC [125]. On this note, comparison of early-stage CCA (I-II) with PSC showed that ficolin-2, inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), and FIBG were most abundant in early-stage CCA. Further, MUC1, EGFR, EpCAM, among others, were abundant in EVs released from CCA human cell lines compared to normal human cholangiocytes (NHC) [98] (Table 1). Other enriched proteins after tumor resection found in serum EVs were EpCAM, asialoglycoprotein 1 receptor (ASGPR1), annexin V, and tumor-associated microparticles (taMPs), showing sensitivity/specificity scores and positive/negative predictive values (>78%), indicating their potential in diagnosis [99] (Table 1). Hepatic ASGPR1 is a transmembrane molecule specifically expressed on the sinusoidal and basolateral hepatocellular membranes, and not in other human tissue [126]. Furthermore, taMPs have recently emerged as novel vehicles for a horizontal crosstalk between different cells, especially in the setting of inflammatory conditions [127].

Differential exosomal phosphoproteome analysis of invasive M213 and M213D5 CCA cells has shown its potential as a biomarker in cancer. Reduced exosomal fetuin-A phosphorylation and high HSP90B phosphorylation has been detected in tissues from CCA patients with a low TNM stage, compared to those with a single high-stage TNM [100] (Table 1).

The analysis of EVs obtained from high- and low-grade metastatic HCC cell lines discriminated CCA from other tumors, highlighting adenylate cyclase associated protein 1 (CAP1) as enriched in metastatic tumor cells when compared to non-tumor/primary

cell lines [101] (Table 1). CAP1 is an actin monomer binding protein involved in the reorganization of the actin filament essential for cell migration. Its overexpression in HCC is closely related to tumor metastases [128], although its role needs to be further investigated.

EVs secreted in gastric cancer (GC) are also involved in tumorigenesis. A recent study suggests that proteasome subunits PSMA3 and PSMA6 levels in patients with metastatic GC (stage III/IV) were significantly higher in serum EVs than those in healthy controls and patients with early-stage GC [102] (Table 1). PSMAs are major components of the 20S proteasome core complex with potential diagnostic utility [129]. In prostate cancer (PC), there is a need to identify new markers, due to the fact that prostate specific antigen (PSA), both in free form and in EVs derived from plasma of PC lacks specificity and sensitivity [130], and therefore could lead to overdiagnosis [131]. In the search for new biomarkers, it has been shown that levels of fatty acid binding protein 5 (FABP5) in urine-derived EVs were higher in pathological groups, as well as the levels of granulin, AMBP, CHMP4A, and others [103] (Table 1). FABP5 belongs to the family of intracellular lipid-binding proteins, and is responsible for uptake and transport of fatty acid [132]. In PC cell lines, FABP5 regulates energy metabolism via ERRα activation, suggesting that a new FABP5-ERRα signaling axis plays an important role in the regulation of AMPK activity, which is a cellular energy state sensor that directs metabolic adaptation to support cell proliferation and survival [133]. Granulins, also known as granulin-epithelial precursors, are a growth factor which regulates inflammation and tumorigenesis. They can promote migration, invasion, and proliferation in PCa [134]. CHMP4A is a subunit of the charged multivesicular body, ESCRT-III complex, which is involved in multivesicular body (MVBs) formation and sorting of endosomal cargo proteins into MVBs [135].

Although urine is an excellent source of protein biomarkers for bladder cancers, there is a high degree of variability in these samples. In this regard, the enrichment of urine EVs could reduce the variability between samples, allowing us to identify functionally relevant proteins. For instance, 1222 total proteins were detected with high confidence in EVs derived from bladder cancer, validating some of them by Western blotting, such as mucin-1 (MUC1), carcinoembryonic antigen (CEA), epidermal substrate of growth factor receptor kinase 8-protein 2 (EPS8L2), and moesin, as proteins directly associated with cancer [104] (Table 1). MUC1s are membrane glycoproteins that play important roles in cell physiology, such as mediating anti-adhesive properties between cells and the extracellular matrix (ECM). Its deregulated expression is associated with cancer progression [136]. CEA, also known as CD66, is expected to have potential value in the early diagnosis of invasive urinary bladder cancer [137]. Eps8L2 belongs to the family of epidermal growth factor receptor kinase substrate 8 (EPS8)-related proteins, which are involved in actin remodeling in response to EGF [138]. Moesin is known to be associated with an aggressive phenotype in several malignant tumors, showing predictive ability for early detection of bladder urothelial carcinoma (BUC) invasion [139]. EGFR is a transmembrane receptor whose function is to regulate both cell proliferation and apoptosis via signal transduction pathways [140], which are highly related to lung cancer [141].

A large number of biomarkers is currently being investigated in lung cancer. Consequently, it has been shown that EVs released by non-small-cell lung cancers (NSCLC) patients' plasma are also particularly enriched in MUC1 [105] (Table 1). Furthermore, EVs isolated from lung cancer cells, lung biopsies, and plasma are enriched in EGFR, making them the most powerful prognostic biomarker [106] (Table 1). Sandfeld-Paulsen and co-workers also reported an enrichment in NY-ESO-1, phospholipase A-2-activating protein (PLAP), and EpCam proteins. NY-ESO-1, also known as cancer-testis antigen (CTAs), is regularly limited in its expression to germ and placental cells, but is re-expressed in tumor cells. Similarly, NY-ESO-1 expression was found in NSCLC and has been associated with a higher risk of relapse, poorer response to treatment, and shorter survival [142]. PLAP is a member of the WD-repeat protein, G-protein-transducin superfamily, which mediates eicosanoid generation and participates in inflammatory responses [143]. On the other hand, EpCAM is a transmembrane glycoprotein that affects intercellular adhesion, and is

overexpressed in various human epithelial carcinomas. It is involved in many important functions relevant to tumor progression, including cell proliferation [17]. Other works have shown that serum EVs can help distinguish patients with metastatic NSCLC from non-metastatic NSCLC by monitoring lipopolysaccharide-binding protein (LBP) levels [107] (Table 1). BPIFA1, CRNN, MUC5B, and IQGAP1 were also found highly abundant in salivary exosomes of lung cancer patients [108] (Table 1). BPIFA1 is a protein specifically expressed in the upper airways and nasopharyngeal regions. It was identified as a potential marker for the micro-metastasis of non-small cell lung cancer (NSCLC) [144]. MUC5B is a gel-forming mucin secreted from airway epithelial cells in the lung, associated with longer survival in primary EGFR mutant NSCLC [145], and IQGAP is a scaffold protein that may promote the regulation of cancer cell migration and metastasis [146]. Furthermore, proteomic analyses of urinary exosomes of NSCLC patients highlighted exosomal leucine-rich-alpha2-glycoprotein 1 (LRG1) as a candidate biomarker for non-invasive diagnosis, playing a role in epithelial-mesenchymal transition (EMT) and angiogenesis [109] (Table 1).

Analyses of the EV proteome have also provided potential new biomarkers for glioblastoma cancer (GBC). Naryzhny and colleagues (2020) provided a list of potential biomarkers by secretome profiling through LC-MS/MS, which included annexin A2, vimentin, and tenascin-C, among others [110] (Table 1). Vimentin is an intermediate filament protein that plays a central role in GBC progression [147], and tenascin-C is a non-filamentous protein that mediates cell-cell and cell-matrix interactions, which affects negatively proliferation and invasion in GBC [148].

Lastly, proteomic approaches have improved the knowledge of less known tumors thanks to the specificity of the proteins found in EVs. In this context, EVs isolated from serum of thyroid cancer lymph node metastases (LNM) patients had high amounts of talin-1 (TLN1), integrin beta-2 (ITGB2), SRC, and CAPNS1, compared with thyroid cancer without LNM [4]. TLN1 is a cytoskeletal protein involved in regulating the activity of cell adhesion proteins by coupling them to F-actin [149], it is also involved in adhesion, proliferation, survival, and tumor progression [150]. ITGB2 belongs to a family of cell surface receptors that play a key role in cell adhesion, migration, proliferation, and survival by forming physical interactions between the cell and the extracellular matrix [151]. SRC kinase activity and protein levels are elevated in several cancers, and are correlated with malignant progression [4]. Similarly, CAPNS1 belongs to a family of 15 calcium-dependent intracellular thiol proteases, whose aberrant expression or activity is involved in several diseases including cancer. Specifically, high calpain-1 levels were associated with papillary thyroid cancer, but its role in regulation of the proliferation and migration needs further investigation [4].

In esophageal squamous cell carcinoma (ESCC), GPC1 was identified as a novel biomarker [111] (Table 1). Additionally, EVs isolated either from NPC C666-1 cells or immortalized nasopharyngeal epithelial cells (NP69 and NP460) had large amounts of pro-angiogenic proteins, including intercellular adhesion molecule-1 (ICAM-1), and a variant isoform of CD44 (CD44v5), while the angio-suppressive protein thrombospondin-1 (TSP-1) was present at low levels in NPC C666-1 EVs [112] (Table 1). ICAM-1 is a member of the immunoglobulin superfamily, and CD44v5 a transmembrane glycoprotein involved in many biological activities, such as cell migration, tumor invasion, and metastasis [151].

4.2. Lipidome Profiling Analysis of EVs in Multiple Cancers

EVs represent an untapped source for the discovery of clinically relevant lipid biomarkers, which can be used in pre-clinical detection, as well as for following disease progression. Knowing the lipid profile of EVs from various tumor cell types is an important aspect, although a better understanding of how cancer cells evaluate their lipid resources to meet the metabolic demands of high proliferation rates could enhance novel anticancer therapeutic strategies. There is growing evidence about the transfer of biologically active lipids and lipid metabolites as one of the mechanisms used by cancer cells to alter the energy pathways within the tumor microenvironment [152]. It is now known that EVs transfer

lipids and lipid-related proteins to influence target cell function [153,154], making the study of the EV lipidome essential for the identification of biomarkers for diagnostic purposes.

The lipidomic profiling of EVs isolated from cell cultures and biofluids includes differences in lipid composition. Based on published studies, EVs are mainly made up of membrane lipids, although small amounts of other lipids could be captured by the cytosol during ILV formation. Therefore, the lipid composition of EVs should therefore reflect the composition of a lipid bilayer. In fact, it has been shown that there is an asymmetrical distribution of lipid classes in the two leaflets of the plasma membrane, where sphingolipids and phosphatidylcholine (PC) are mainly present in the outer leaflet, while other lipid classes are mainly found in the inner leaflet [155]. Recent advances in lipid analysis by LC-MS/MS have identified lipid classes and lipid species that appear to be enriched in EVs. An important lipidomic profile study of EVs derived from single cell types was realized by Skotland and co-workers in ten exosome preparations, including cell-to-exosome enrichment factors in eight of them, showing an enrichment of 2–3 times in cholesterol (CHOL), sphingomyelin (SM), glycosphingolipids (GSLs), and phosphatidylserine (PS). Notably, the membranes of most EVs show lower phosphatidylcholine (PC) and phosphatidylinositol (PI) content than their cells of origin, but contain similar levels of phosphatidylethanolamine (PE) [156].

The synthesis of cholesterol, also known as the mevalonate pathway, is an important pathway of lipid biosynthesis, since cholesterol is a major component of membranes involved in controlling membrane fluidity and the formation of lipid rafts. Consequently, altered intracellular cholesterol levels can greatly modulate membrane architecture, promoting plasma membrane fluidity, and, therefore, contribute to cell migration and metastasis. Considering this, the active lowering of cholesterol in an advanced stage of the disease can have negative effects [157]. Conversely, the establishment of primary tumors is highly dependent on growth-stimulating signaling pathways, promoted by cholesterol concentrations on the membrane through the formation of lipid rafts. In fact, cholesterol-rich lipid rafts facilitate the accumulation of tyrosine kinase receptors, such as HER2. In this case, blocking cholesterol synthesis could inhibit the onset and proliferation of cancer in the early stages of the disease [158]. In order to use cholesterol metabolism as a therapeutic target in cancer, it is necessary first to understand why cancer cells depend on cholesterol and how this affects the progression of the disease.

Glycosphingolipids (GSLs) are a subtype of glycolipids which mediate cell-cell interactions and modulate signal transduction pathways. Aberrant expression of specific GSLs and related enzymes is strongly associated with tumor formation and malignant transformation [159]. There are several studies in literature about the expression of various glycosphingolipids in specific tumors. For example, glycosphingolipids, such as GD3 and GD2, enhance the malignant properties of cancer cells, such as cell proliferation, cell invasion, and migration [160]. In contrast, mono-sialyl gangliosides, such as GM1 and GM2 often suppress malignant properties of cancerous cells [161]. The analysis of GSLs does remain challenging due to their amphiphilic nature and inherent complexity.

Sphingomyelin (SM) is also a key component of lipid rafts involved in the regulation of several signaling pathways [162]. It has been shown that low SM levels are associated with the tumorigenic transformation [163]. However, the role of SM in cancer is yet to be understood, since other studies have reported high levels of SM and different roles in cancer [164]. Considering the glycerophospholipids (GPLs) species, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) are abundant in mammalian cell membranes, there is growing evidence that these GPLs (particularly PC and PS) might be potential biomarkers of cancers. Evidence supports that the overexpression of PCs observed in cancer cells is mainly due to upregulation of choline kinase and activation of phospholipase C specific to phosphatidylcholine, the latter activated in the cycles of phosphatidylcholine induced by mitogens and oncogenes, with effects on signaling pathways, regulation of the cell cycle, and cell proliferation [165]. The phosphatidylinositol (PI) resides on the cytosolic surface of cell membranes, and differential phosphorylation generates distinct phosphoinositides, contributing to their signaling diversity, including

cell growth and proliferation. The phosphoinositides have a distinct localization in the cell through which they carry out their specific function localization (e.g., PI (4,5)P2 is enriched at the plasma membrane, while PI(3)P in early endosomes). Aberrant phosphoinositide signaling has been observed in cancers, suggesting its potential role as a biomarker [166].

A pioneering work quantified 22 classes of EV lipids derived from metastatic prostate cancer cell lines, such as PC-3 cells, finding enrichment in CHOL, SM, glycosphingolipids, and phosphatidylserine, indicating a particular lipid sorting in the exosome membrane compared with the source cells by MS analysis [167] (Table 2). In contrast to healthy cells, tumor cells expose the phosphatidylserine (PS) at the cell surface. An early event in apoptosis is the appearance of PS on the cell surface, which reduces the inflammatory response by alerting phagocytic cells to engulf the cell. Macrophages recognize PS on the surface of apoptotic cells while viable cancer cells with high external PS inhibit phagocytosis by displaying CD47 [168]. In addition, EVs derived from tumor cells might expose PS, suggesting that the source of PS is mostly derived from them, thus constituting a diagnostic biomarker for cancer [169].

Research done on six different prostate cell lines observed differences in the relative abundance of the classes of glycerophospholipids between cells and their EVs [170] (Table 2). Glycerolipids comprise all glycerol-containing lipids; i.e., mono-, di- and tri-substituted glycerols (MAG, DAG, and TAG, respectively), and are important constituents of the EV membrane [171]. On the other hand, the EV lipidomes released from three prostate cell lines, i.e., RWPE1 (non-tumorigenic), NB26 (tumorigenic), and PC-3 (metastatic), have been recently published by Brzozowski et al. [74]. These authors have shown a relative enrichment of lipid species, fatty acids, glycerolipids, and prenolic lipids in EVs from RWPE1, while sterol lipids, sphingolipids, and glycerophospholipids were more abundant in EVs from NB26 and PC-3 cells. They also found that the average CHOL content of EVs derived from PC cells was three times higher than EVs derived from RWPE-1 cells [74].

Table 2. Summary of the lipids and other metabolites identified in EVs with potential as biomarkers of different cancers.

Cancer Type	Biomarker	Sample	Authors
Prostate Cancer	Glucoronate; increased creatinine, glucuronate, pantothenic acid, 4-pyridoxic acid in urina; lysine, kynurenine, threonine, tryptophan, cytidine in plasma	Urine and plasma-derived EVs	[5]
Prostate Cancer	Increased CHOL, sphingolipids and glycerophospholipids, decreased glycerolipids and prenolic lipids	*In vitro*: PC-3, RWPE1, and NB26 cell lines	[74]
Prostate Cancer	Increased glycerophospholipids and sphingolipids	*In vitro*: PC-3, DU145, VCaP, and RWPE1 cell lines	[170]
Colorectal Cancer	Increased glycerophospholipids, SM, CHOL, and PS	*In vitro*: LIM1215 cell line	[172]
Breast Cancer	Increased levels of CHOL and SM, decreased levels of PC	*In vitro*: D3H2LN and D3H1 cell lines	[173]
Glioblastoma and hepatocellular carcinoma	Increased SM and ceramides in glioblastoma than hepatocellular carcinoma	*In vitro*: Huh7 and U87 cell lines	[174]
Ovarian Cancer	Increased PS, PI, PE, and PG in HOSEPiC; Increased LPI, LPG, LPC, and LPS in SKOV-3	*In vitro*: SKOV-3 and HOSEPiC cells	[175]
Prostate Cancer	Increased PS and lactosylceramide	Urine-derived EVs	[176]
Prostate Cancer	DHEAS; acyl carnitines, citrate, and kynurenine	Urine-derived EVs: PCa and BPH patients	[177]
Endometrial adenocarcinoma	Cyclic alcohols, steroids, prenols, and amino acid conjugates	PC-1 cell line; plasma-derived EVs	[178]
Pancreatic Cancer	Alanylhistidine, 6-dimethylaminopurine, leucylproline, and methionine sulfoxide, others	Serum-derived EVs	[179]
Glioblastoma	Enrichment in glycerol, tryptophan, carnitine, and GSSG	*In vitro*: U118, LN-18, and A172 cell lines; normal human astrocytes	[180]

Additionally, differences in the relative fraction of glycerophospholipids, sphingolipids, glycerolipids, and sterol lipids were identified in LIM1215 colorectal cancer cells, and in their secreted EVs, respectively. Besides, SM, CHOL, and PS were more common in EVs derived from the colorectal cancer cell line LIM1215 than in parental cells. These authors identified a decrease in PC/PE ratios in EVs relative to LIM1215 parent cells [172] (Table 1). Moreover, a lipid composition study of EVs and cells of their origin and between

EVs derived from high and low metastasis triple negative breast cancer (TNBC) cell lines, D3H2LN and D3H1, showed an increase in the levels of CHOL, SM, and a decrease in the levels of PC in their EVs. In addition, EVs derived from D3H2LN were enriched in unsaturated diacylglycerols (DGs) compared with EVs from D3H1 [173] (Table 2).

Moreover, studies performed on glioblastoma, hepatocellular carcinoma, and human bone marrow-derived mesenchymal stem cells (MSCs) have shown high abundance of SM and ceramides in EVs released by glioblastoma cells (U87), while opposite results were reported for EVs secreted by hepatocellular carcinoma cells (Huh7). PS was only slightly enriched in EVs released by all three cell lines, while the PC and PI content were higher in cells than in EVs [174] (Table 2).

On another note, several lipid species present in EVs released from ovarian cancer cells (SKOV-3) differ when compared to those from ovarian surface epithelial cells (HOSEPiC). In particular, EVs from HOSEPiC cells were more abundant in PS, PI, PE, and phosphatidylglycerol (PG), while EVs secreted from SKOV-3 cells presented higher content in lysophosphatidylinositol (LPI), lysophosphatidylserine (LPS), lysophosphatidylinositol (LPG), lysophosphatidylcholine (LPC) [175] (Table 2). Lysophospholipids (LPLs) consist of lyso-glycerophospholipids and lysosphingolipids, which are implicated in important functional roles, e.g., through intracellular G protein-coupled receptor (GPCR)-mediated signaling. Although a comprehensive understanding of LPL levels and their distribution patterns is lacking, several studies have revealed that LPLs are associated with the development, progression, and metastasis of cancers, as in ovarian cancer [181].

On this note, analysis of the urine exosome lipid repertoire in patients with renal carcinoma suggested that lysophospholipids represent the most present lipid class than in healthy control cells [182]. Prostate cancer patients' urine exosomes (PCa) revealed up to nine differentially expressed lipid species, including lactosylceramide, with the highest patient/control ratio [176] (Table 2). To date, only a few studies have investigated the lipid composition of EVs in biofluids, due to difficulties in lipid isolation from these vesicles. There are increasing updates on the EVs' lipid composition, functionality, and potential use as biomarkers.

4.3. Metabolome Profiling Analysis of EVs in Multiple Cancers

The discovery of metabolic biomarkers in EVs is an important goal for diagnosing clinical relapses. A distinctive feature of cancer cells is their ability to perform metabolic reprogramming necessary for their high energy requirements [183]. To meet the metabolic needs associated with proliferation, a cancer cell must increase the import of nutrients from the environment. Classically, cancer metabolism has focused on carbon metabolism, including glycolysis and the tricarboxylic acid cycle (TCA cycle). Much is known about the role of glucose as a source of energy for cancer growth; however, amino acids are also important molecules in supporting cancer development. Glutamine is a non-essential amino acid abundant in circulation, which plays an important role in addition to its function as a constituent of proteins. It provides its two nitrogen atoms to synthesize hexosamines, nucleotides, and other amino acids; guides the uptake of essential amino acids; and is also a substrate for TCA, particularly under conditions of carbon diversion to the glycolytic pathways. The level of glutamine in tumor tissues *in vivo* was found to be significantly lower than in healthy surrounding tissues or plasma. Cancer cells accumulate oncogenic alterations that convey a significant degree of independence to make up for this lacking of glutamine. For instance, *c-Myc* hyperactivation results in altered levels of downstream transcriptional targets involved in glutamine uptake and metabolism. The lack of glutamine can induce apoptosis in a Myc-dependent manner [184]. On the other hand, glutamine, glycine, and aspartate serve as carbon and nitrogen donors for purine biosynthesis [185], whereas glycine, serine, and methionine provide one carbon unit through the methionine-folate cycle for nitrogenous bases [186].

Metabolites are small molecular analytes present inside the cell, which can provide real-time information on the biochemical events that occur at the time of sample collection,

and, therefore, are indicative of the physiological state of the patient [187]. As a result, important clinical information on disease progression can be obtained by monitoring metabolic changes in the patient's bio-fluids, such as blood, urine, saliva, and others, as well as EVs derived from cell lines. Interestingly, during the formation of EVs, small metabolites can be packed inside the vesicle or they can be produced as a result of the activity of metabolic enzymes within the EVs [188].

It should be noted that there is a definitive crossover between EV metabolomics and lipidomics, as the size of the biologically relevant lipids make them classified as metabolites. Because their circulating levels are very low and difficult to detect, metabolites can only be distinguished through use of highly sensitive identification techniques, such as mass spectrometry (LC-MS/MS) and magnetic resonance spectroscopy.

Analyses of urine-derived EVs (uEVs) have highlighted specific metabolites, including creatinine, glucuronate, pantothenic acid, 4-pyridoxic acid, and others. Metabolites specific for plasma-derived EVs (pEVs) include lysine, kynurenine, threonine, tryptophan, cytidine. Metabolites were found to differ in abundance between uEVs and pEVs. Interestingly, the authors observed that EVs released in pre-prostatectomy present low levels of adenosine, glucuronate, isobutyryl-L-carnitine, and D-ribose 5-phosphate, compared to EVs secreted post-surgery, as well as in control and untreated samples. Specifically, they found greater differences in glucuronate between treated and untreated cancer groups when compared to a control group [5]. Another study observed a statistically significant difference in many of the key metabolites in PCa patients, including acyl carnitines, citrate, and kynurenine among benign prostate hyperplasia (BPH) samples. Importantly, they found significantly elevated levels of the steroid hormone dehydroepiandrosterone sulphate (DHEAS) in uEVs from PCa patients compared to BHP patients. There were also a few molecules differentially expressed between two subgroups of PCa patients (stages 2 and 3), as acylcarnitine [189].

A proof-of-concept study to detect EVs metabolite biomarkers from plasma of endometrial adenocarcinoma (EAC) in patients versus control subjects revealed a clear separation of metabolites. Furthermore, EVs characterized by TGF-β-treated human pancreatic cell line (PANC 1) showed marked differences compared to the control group [177] (Table 2). A metabolomic study of EVs derived from patient blood before and after chemotherapy also detected the presence of different compounds, i.e., 6-dimethylaminopurine, leucyl proline, alanyl-histidine, and methionine sulfoxide [178] (Table 2). EVs of GBM subtypes were shown to contain significantly distinguishable metabolic content from astrocytoma cells. Furthermore, a significant difference was found in the metabolic profile between GBM-derived EVs and parental cells; with enrichment in glycerol, tryptophan, carnitine, and oxidized glutathione (GSSG) [179] (Table 2).

Currently, few metabolome-oriented studies have addressed EVs under tumorigenic conditions. These studies reflect the metabolic plasticity of cancer cells and their tendency to escape dependence on canonical pathways through metabolic reprogramming. Clearly, new and innovative combinatorial analysis strategies are needed to cover the entire spectrum of the metabolome. Moreover, the method of cell culture can also impact the metabolite composition of EVs, and may need to be taken into consideration when comparing results from different studies [180] (Table 2).

4.4. miRNA Profiling Analysis of EVs in Multiple Cancers

Tumor-derived EV microRNAs (miRNAs) have received much attention as biomarker candidates for non-invasive diagnostics, given their role in tumor progression and metastasis. Analysis of serum exosomal miRNA expression profiles of CRC patients revealed that miR-19a and miR-92a were significantly upregulated compared to HCs [190] (Table 3). Moreover, let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a were detectable at significantly higher levels in colon cancer cell lines and serum samples from CRC patients [191] (Table 3). A recent report demonstrated that miR-1246 and other miRNA markers were highly enriched in EVs derived from pancreatic cancer patients [192] (Table 3). In addition, miR-1246, miR-1290, miR-375 [193], miR-141 [194], and others [195]

were detectable in EVs isolated from plasma and serum samples of patients with prostate cancer, respectively. Furthermore, other works highlighted miR-21-5p and miR-92a-3p as emerging diagnostic biomarkers for HCC [196] (Table 3). Analysis of plasma-derived EVs from lung cancer patients identified miR-320, miR-126 [197], as well as let-7f, miR-146, miR-203, miR-106a, and miR-20b [198].

It has been reported that EV miRNAs confer high accuracy in identifying BC; in particular, a decreased expression of miR-142-5p and miR-150-5p were significantly associated with more advanced tumor grades (grade III), while the decreased expression of miR-142-5p and miR-320a was associated with a larger tumor size (<20 mm) [199] (Table 3). Some significant miRNAs derived from EVs were also associated with the severity of BC, including miR-939 implicated in drug resistance, miR-338, and others involved in TME [200–202] (Table 3). miRNA profiling of EVs has also revealed an increased expression of miR- 200 and miR-18, while a decreased expression of miR-100 and miR-125b has been reported in different histotypes of ovarian carcinomas, as compared to normal ovarian tissue [203]. Furthermore, Let-7 miRNA family members represent a diagnostic potency marker to manage follicular nodules in the thyroid gland [204]. miR-423-5p, miR-484, miR-142-5p, and miR-17-5p were discovered to be dysregulated in GC or implicated in GC tumorigenesis/metastasis in EVs isolated from serum samples [205]. Lucero and coworkers (2020) identified eight candidate miRNAs that may mediate EV-associated angiogenesis in glioblastoma, including miR-148a and miR-9–5p [206]. Different studies involving miRNAs associated with tumor progression and diagnostics are summarized in Table 3.

Table 3. Summary of the miRNAs identified in EVs with potential as biomarkers of different cancers.

Cancer Type	Biomarker	Sample	Authors
Colorectal Cancer	Increased miR-19a and miR-92a	Serum-derived EVs	[190]
Colorectal Cancer	Increased let-7a, miR-1229, miR-1246, miR-150, miR-21, 223, and miR-23a	Serum-derived EVs	[191]
Pancreatic Cancer	Increased miR-1246, miR-4644, mir_3976, and miR-4306	Plasma-derived EVs	[192]
Prostate Cancer	Increased miR-1246, miR-1290 and miR-375	Serum-derived EVs	[193]
Prostate Cancer	Increased levels of miR-141	Serum-derived EVs	[194]
Prostate Cancer	Increased miR-21-5p and let-7a-5p	Plasma-derived EVs	[195]
Hepatocellular Carcinoma	Increased miR-21-5p, miR-92a-3p	Plasma-derived EVs	[196]
Lung cancer	Increased miR-320 and miR-126	Plasma-derived EVs	[197]
Breast Cancer	Decreased miR-142-5p and miR-150-5p	Plasma-derived EVs	[199]
Breast Cancer	miR-200a, miR-200b, miR-200c, miR-429, and miR-141	*In vitro*: 4T1, 4TO7, 67NR, and MCF10CA cell lines.	[200]
Breast Cancer	miR-338-3p, miR-340-5p, and miR124-3p	Serum-derived EVs	[201,202]
Ovarian Cancer	Decresead of miR-100 and miR-125b	*In vitro*: SKOV3, HO-8910 and U937 cell lines.	[203]
Thyroid Cancer	Let-7 miRNA family serum-derived EVs	Serum-derived EVs	[204]
Gastric Cancer	miR-423-5p, miR-484, miR-142-5p, and miR-17-5p serum-derived EVs	Serum-derived EVs	[205]
Glioblastoma	Increased miR-1246	*In vitro*: GBM8 neurospheres	[207]
Glioblastoma	Increased miR-301a	*In vitro*: U87MG and U251 cell lines	[208]
Glioblastoma	Increased miR-21	Serum-derived EVs	[209]

Several approaches are being used to create a miRNA profile to classify different cancer histotypes, nonetheless, there is a deregulated expression of specific miRNAs. Some of them have found them as regularly loaded in EVs and associated with cancer progression, which could be used as biomarkers and in therapy design. However, the development of new technology is required to advance in this aspect.

5. Future Challenges and Conclusions

Different reports have identified numerous potential EV-based biomarkers that can aid in cancer diagnosis, disease monitoring, and development of targeted therapy. Currently,

studies in proteomics, lipidomics, and metabolomics have advanced our knowledge of the properties of EVs. However, limitations in sample quality, EV isolation methods, cargo analysis, and interpretation of results may contribute to the failure of them as biomarkers in achieving clinical utility.

One of the primary challenges in characterizing cancer-specific EVs in biofluids is that samples also contain large amounts of EVs secreted by healthy cells, as well as other biomolecules (e.g., albumin, lipoproteins). Methods of isolation and analysis have limited sensitivity/specificity for detecting specific tumor-secreted EVs in biofluids because they are based on a small sample size in which EVs are diluted. Therefore, it is necessary to optimize isolation methods *in vitro* in order to distinguish specific EV subgroups to address for specific clinical scenarios. In fact, the success of EV molecular profiling heavily relies on the isolation and separation process. Consequently, developing efficient isolation methods and enriching cancer-derived EVs or specific EV subpopulations from human biofluids are urgent in order to further advance in this field.

In recent years, the field of microfluidics has allowed for the development of novel exosome purification methods, starting from small sample quantities [210]. Microfluidic platforms have been shown to sort exosomes with a high level of purity and sensitivity by reducing cost, volume of reagents consumed, and time invested in the procedure. However, microfluidics platforms have the disadvantage of their manufacturing complexity [211]. The first microfluidic platform used for the isolation of EVs relied on exosome immunoaffinity and unlabeled detection. It quantified the levels of EpCAM and CD24 proteins measured in relation to CD63 (+) exosome counts, with a diagnostic accuracy of 97% in ovarian cancer ascitic fluids. Another important aspect of microfluidic platforms is their potential use in the identification of specific subpopulations of EVs. Multiplex microfluidics have enabled the selective and specific capture of IV HER2 (+) in serum from breast cancer patients [212].

EVs hold great potential for disease diagnostics, which is why it is important to further know their content and cell-targeting mechanisms. Further knowledge and classification of the proteins, lipids, and metabolites of EVs will be useful in order to gain deeper insight about their role in transmitting information between cells, as well as our understanding of disease biogenesis and progression. Online databases have been created for the purpose of cataloguing EV content. There, highly accessible websites are used to compare sequences, and upload new ones [81–83].

Despite their promising utility as cancer biomarkers, the use of EVs in the clinical setting is still far from use in everyday practice, due to difficulties in isolation through standard analysis techniques.

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