applied sciences

MDPI

Review

MALDI-TOF/MS Analysis of Extracellular Vesicles Released by Cancer Cells

Carlo Zambonin

Dipartimento di Chimica, Università degli Studi di Bari "Aldo Moro", Via E. Orabona 4, 70124 Bari, Italy; carlo.zambonin@uniba.it

Abstract: The direct shedding of extracellular vesicles (EVs) from the plasma membrane is a recognized fundamental method for the intercellular transfer of properties in both physiological and pathological conditions. EVs are classified according to origin, biogenesis, size, content, surface markers, and/or functional properties, and contain various bioactive molecules depending on the physiological state and the type of the cells of origin including lipids, nucleic acids, and proteins. The presence of tumor-derived EVs in body fluids such as blood, ascites, urine, and saliva, together with the important role played in the tumor microenvironment where they intervene at different levels from oncogenesis to metastasis, make EVs a priority target for cancer studies. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) can play a leading role in the analysis and characterization of EVs and their load due to its intrinsic advantages such as high throughput, low sample consumption, speed, the cost-effectiveness of the analysis, and the ease of use. This work reviews the main MALDI-TOF applications for the analysis and characterization of extracellular vesicles in the tumor field.

Keywords: MALDI-TOF MS; cancer; extracellular vesicles; exosomes; diagnosis

Citation: Zambonin, C. MALDI-TOF/MS Analysis of Extracellular Vesicles Released by Cancer Cells. *Appl. Sci.* **2022**, *12*, 6149. https://doi.org/10.3390/app12126149

Academic Editor: Marco G. Alves

Received: 15 April 2022
Accepted: 15 June 2022
Published: 16 June 2022

1. Introduction

The sharing of information between cells in multicellular organisms occurs through cell-to-cell signaling, a series of indispensable functions carried out by gap junctions and synapses between physically close cells [1,2], or by systemic signals based on alternative pathways such as receptor–ligand interactions. Another emerging mechanism of intercellular communication is represented by the direct shedding from the plasma membrane of extracellular vesicles (EVs) containing a variety of bioactive molecules depending on the physiological state and the type of the cells of origin, which are mainly lipids [3], nucleic acids [4], and proteins [5]. EVs constitute a heterogeneous group of phospholipidic bilayer-encased membranous nanovesicles, whose structure provides stability to the transported material and a consequent greater ability to travel long distances when compared to free molecules. They circulate in biofluids such as blood, ascites, urine, and saliva, and are transported to close and distant recipient cells allowing the intercellular exchange of their content and exerting variable biological effects.

According to their origin, biogenesis, size, content, surface markers, and/or functional properties, EVs are classified [6] as apoptotic bodies and microvesicles, both originating from the plasma membrane and characterized by diameter sizes that range between 500–2000 and 100–500 nm, respectively, and exosomes, which have an endosomal origin and a size between 30 and 150 nm. However, since the nomenclature can be quite confusing, the International Society of Extracellular Vesicles (ISEV) has proposed the generic term EVs for the vesicles released from the cell [7].

Cancers are heterogeneous diseases affecting any part of the body and constituting a leading global cause of death. Oncogenesis depends on the combined accumulation and functional cooperation between genetic and epigenetic changes that alter the control

systems between cell proliferation and death [8], leading to the transformation of healthy cells into malignant ones. During the process, cancer cells must actively communicate with neighboring cells and the tumor microenvironment [9,10], where EVs are considered key components in cell-to-cell mediation and intervene at different levels from oncogenesis to metastasis. Therefore, their potential use for cancer therapy and vaccines or drug delivery systems is being seriously considered [11,12]. Furthermore, the abundant presence of tumor-derived EVs in biological fluids, together with the variability of their internal composition related to the physiological state, makes them interesting clinical targets to be used as biomarkers for the diagnosis and prognosis of diseases, carrying tumor-specific molecular signatures [13,14].

However, the characterization of extracellular vesicles and their load is not an easy task due to a series of issues, such as the standardization of isolation procedures, obtaining good quality extracts, long analysis time, high costs, the need for technical training, and many others [15], that make their use difficult in clinical practice. It has therefore become clear that reliable methods for a rapid and cost-effective analysis of EVs are highly advisable.

A leading role in the field of complex samples analysis is certainly played by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), a technique that has clearly demonstrated its potential in providing information from complex biological samples for diagnostic purposes in a relatively simple way [16], particularly when it comes to cancer [17]. In fact, MALDI is characterized by high throughput, low sample consumption, speed, and cost-effectiveness of the analysis, as well as ease of use even by unskilled personnel, allowing the fast generation of spectra and/or profiles from the matrix under study. Specifically, the potential, disadvantages, and possible implementation strategies of MALDI analysis of exosomes have recently been thoroughly discussed [18].

The purpose of the present work is to comprehensively review the main existing literature relating to MALDI-TOF applications to the analysis and characterization of extracellular vesicles in the tumor field.

2. Applications

2.1. Mesothelioma

Malignant mesothelioma [19] is a rare and aggressive malignancy with a poor prognosis, whose development is related to asbestos fiber exposure and genetic predisposition. The gold standard for its diagnosis is invasive pleural biopsy, and since no recommended screening tests are available for this cancer, the identification of novel biomarkers for its detection is vital. In what was the first application of MALDI-TOF as a tool for the analysis of EVs secreted by tumor cells, Hegmans et al. [20] performed the characterization of the protein composition of exosomes released by malignant mesothelioma cell lines. Exosome preparations were subjected to one-dimensional electrophoresis (1-DE) and excised protein spots were then trypsin-digested and characterized using MALDI-TOF. The identified proteins were involved in antigen presentation, signal transduction, migration, and adhesion. Some years later the same authors reported a procedure [21] for the isolation of exosomes from cell lines in vitro followed by 1-DE coupled to MALDI analysis to characterize their protein composition. It was highlighted that this technology can provide useful information on the biological functions of exosomes, as demonstrated in the case of the exosomes derived from mesothelioma that may promote tumor growth.

2.2. Breast Cancer

Breast cancer is the leading cause of cancer-related death in women worldwide [22]. It has six molecular subtypes that differ in terms of clinical relevance, therapeutic approaches, responses to treatment, and prognosis. Breast cancer has a relatively high (80–92%) five-year survival rate that strongly decreases (<25%) in the case of metastasis, making its early diagnosis extremely important. The most used approach for prevention is mammography, characterized by some known limitations such as high rates of false negatives and false positives. Therefore, the discovery of novel approaches for its screening and

follow-up is highly required. Extracellular vesicles' characterization by MALDI-TOF has been useful for the identification of biomarkers for breast cancer. A proteomic study was conducted on exosome-like vesicles isolated by ultracentrifugation from MDA-MB-231 breast cancer cell cultures, proteins were then separated by 2-DE, and eventually analyzed by MALDI-TOF [23] searching for possible specific biomarkers. Among the 179 identified proteins, 32 isoforms were upregulated in the vesicles in comparison to whole cell lysates, suggesting that the observed distinctive proteome could represent the signature of breast cancer vesicles that play key roles in cancer progression. MALDI-TOF and 2-DE and were also used [24] to compare the proteins secreted by two different breast cancer cell lines (BT474 and SKBR3) with those released by normal human mammary epithelial cells (184A1) and with the proteomes of the corresponding whole cell lysates, to understand how cell-to-cell communication becomes altered in the presence of cancer, hypothesizing that the tumor secretome could reflect the editing process associated with oncogenesis. Similar secretome profiles were found for BT474 and SKBR3 cells, whereas 184A1 cells exhibited different patterns. The results implied that all the investigated cell lines released exosomes and that those secreted by tumor cells presented higher amounts of proteins involved in antigen-processing and presentation and glycolytic metabolism, pathways associated with the evasion of tumor immunosurveillance and the deregulating of cellular energetics, two of the emerging cancer signatures. In another study, plasma concentrations of exosomes were found to be higher in breast cancer patients than in healthy women [25]. Electrophoresis separation showed exosomal proteins between 10 and 250 kDa and differences between healthy and cancer subjects in the expression level and number of proteins that were planned to be identified by MALDI TOF in view of their potential utility as breast cancer markers.

Tutanov et al. isolated total blood exosomes (plasma exosomes and those associated with blood cells) from healthy donors and breast cancer patients by ultrafiltration followed by ultracentrifugation in two different works, focused on the search for new protein tumor markers for breast cancer [26] and the assessment of the role of blood exosomes in tumor dissemination [27], respectively. In the first work, the maps of the proteome contained in the vesicles were obtained by 2D-E and compared between the two sets of samples, permitting the individuation of remarkable differences between normal and pathological subjects. A MALDI-TOF analysis allowed the identification of 99 proteins in the blood exosomes of both the investigated groups and, according to the exosome database Exocarta, some of them were observed for the first time. Interestingly, proteins associated with breast cancer were specifically detected in the total blood exosomes of cancer patients, even if further experiments were required to validate their role as biomarkers for the disease. In the second study, a MALDI analysis allowed the identification of 111 and 146 proteins in the blood exosomes (48 for the first time) of the healthy and pathological samples, respectively. Using a database of the differently expressed proteins in human cancer, it was found that 64% of the proteins of blood cell-associated exosomes from cancer patients were of tumor origin. These and other findings highlighted the role of plasma and blood cell-associated exosomes in the dissemination of the tumor process. Very recently, a sequential size-exclusion chromatography approach [28] was successfully used by Zheng et al. for the fast (less than 2 h) and efficient isolation of exosome fractions from human plasma samples collected from breast cancer (79), pancreatic cancer (57), and healthy (84) individuals. The vesicle membrane was then destructed to release their content and to proceed to the obtainment of MALDI fingerprints. A multi-classifier artificial neural network model was eventually adopted using extracted MS feature peaks as inputs, successfully allowing for the discrimination of the different types of samples.

2.3. Colorectal Cancer

Colorectal cancer is a global health issue with high incidence and mortality [29]. The American Joint Committee on Cancer classifies patients into five stages (from 0 to IV) based on the presence of tumors (T), the number of lymph node metastases (N), and the presence

of distant metastases (M). Although early-diagnosed and localized tumors can be easily removed (five-year survival rate > 90%), advanced-stage tumors have poor survival rates and only half of the patients survive within five years of diagnosis. Currently, a colorectal cancer diagnosis is achieved by coupling colonoscopy and histopathological examinations. However, due to their invasive nature, many patients are reluctant to undergo these tests, which are also hindered by several issues. Therefore, alternative detection methods for colorectal cancer are clearly needed.

The extracellular proteome of colorectal cancer cells was the target of an immunoscreening approach [30] for the identification of tumor biomarkers. The secretome of five colorectal cancer cell lines was separated by 2D-E, immobilized on polyvinylidene difluoride membranes, and used for serological screening with serum from 21 colorectal cancer patients compared to 24 healthy controls. Autoantigen candidates were defined based on immunoreactivities and the corresponding proteins isolated, which were identified by MALDI-MS and/or by nano-HPLC/ESI-MS/MS and confirmed by Duplex Western blotting. Glod4, a poorly characterized glyoxalase domain-containing protein, and a C-terminal fragment of agrin, a prominent large heparan sulfate proteoglycan resident in basement membranes, were proposed as potential markers for colorectal cancer. EVs released from different colon cancer cell populations, CCL-228 as the primary tumor, the lymph node metastasis CCL-227, and subclones resistant to different doses of the chemotherapy agent 5-fluorouracil, were successfully differentiated by MALDI protein profiling [31]. EVs were collected from cell culture supernatant by ultracentrifugation, the proteins were solvent-extracted and eventually analyzed using different MALDI-TOF-MS instruments. Hierarchical clustering, principal component analysis, and partial least-squares data analysis allowed to obtain protein patterns able to discriminate EVs secreted from different cell lines, making the approach a promising noninvasive tool for colon cancer diagnosis and therapy monitoring.

2.4. Melanoma

Melanoma is a frequent, aggressive, and deadly skin cancer, primarily affecting young and middle-aged people, and is mainly related to UV-radiation exposure, the number of nevi, and genetic susceptibility [32]. Its incidence and mortality have constantly increased especially in the Caucasian population. Nevertheless, immunotherapies and targeted therapies have markedly improved survival rates compared with the use of chemotherapy. The MALDI analysis of exosomes derived from melanoma cells can provide great help in deciphering the complex mechanisms underlying its onset.

Cancer cells are known to use EVs for several functions including throwing out harmful molecules (for instance, differentiation-inducing proteins such as histone H1.0, or antitumor drugs) and bringing molecules such as mRNA, microRNA, and proteins to other cells that can be functional in that new environment. Schiera et al. reported evidence that EVs released from A375 melanoma cells contain both a modified form of H1.0 histone and the corresponding mRNA [33]. Moreover, the specific search for RNA binding proteins, which have a recognized role in oncogenesis, led the authors to demonstrate the presence of the transcription factor MYEF2 (a DNA-binding repressor of the gene encoding the myelin basic protein) by MALDI-TOF analysis coupled to an affinity chromatography approach. In a further study, Zhu et al. [34] rapidly obtained MALDI fingerprints of intact exosomes shed by cell lines derived from different melanoma stages (SBCI2, WM115, and WM239), permitting their classification through mathematical analysis. In addition, it was proven that the approach allowed the tracking of specific proteins through their detection in both cells and exosomes. Well-known biomarkers for Melanoma were also individuated in the exosome mass spectral fingerprints. The method may represent a valuable option for the early diagnosis and follow-up of the disease via liquid biopsies and could be extended to other cancer types. The MALDI profiling of intact melanoma cells and related exosomes was also recently carried out by Lobasso et al. [35]. Melanoma cell lines (LCP and SK-Mel28) with a different metastatic tendency toward the bone were targeted and compared to obtain

the relevant lipid profiles and detect potential distinctive lipid markers for each line. The study provided useful information on the lipid content of melanoma, which could be exploited to clarify the role played by exosomes in cancer and to develop new therapies. Specifically, saturated fatty acids with shorter chains were observed more in LCP cells than in SK-Mel28; sphingomyelin, lysophosphatidylcholine, and phosphatidic acid levels were higher in exosome membranes than in parental cells, and bis(monoacylglycero)phosphate was proposed as a specific lipid marker of exosomes.

2.5. Lung Cancer

Lung cancer is the most frequent cancer worldwide and the major cause of death among all types [36]. It presents different histological subtypes, namely the most common non-small-cell lung cancer (NSCLC), which is divided into squamous cell cancer and adenocarcinoma, and the less diffused small-cell lung cancer (SCLC). NSCLC has a five-year overall survival rate of less than 20% [37], deeply influenced by the stage at the time of diagnosis. It has a silent onset, so that often most patients ignore the initial symptoms and refuse to undergo expensive and/or invasive conventional diagnostic tests, making the discovery of novel screening methods vital.

The phospholipid profiles of EVs shed by non-small-cell lung cancer cell lines were successfully obtained by Jung et al. [38], with the aim of finding marker peaks able to predict resistance to the signal transduction inhibitor gefitinib. Phospholipids were extracted using the Bligh–Dyer method from EVs released by cell lines resistant (PC9R) and responsive (PC9) to the drug, respectively, and subsequently analyzed by MALDI-TOF. The results indicated that the phospholipidomes of the vesicles of the two sets of samples were clearly distinguishable, indicating that EV phospholipids could be used as predictive biomarkers of gefitinib resistance. Lung cancer has also been the subject of study in a recent paper focused on the search for new protein tumor markers in serum EVs. Choi et al. [39] used 2D-E followed by MALDI-TOF analysis to characterize the proteome of EVs previously isolated from the serum samples of healthy and cancer individuals, respectively, via precipitation with polyethylene glycol and immunoaffinity separation. Among several differentially expressed proteins, seven were expressed at high levels in the EVs of tumor patients, as ascertained by Western blot analysis. Furthermore, the protein CD5L was suggested as a biomarker for the early noninvasive detection of the disease targeting the circulating vesicles because its serum expression correlated with that found in cancer tissues.

As with any analytical procedure, the data derived from MALDI-based proteomic studies are strictly dependent on pre-analytical variables [40,41], which can profoundly influence the quality of the entire analysis. Thus, in a proteomic study based on MALDI-TOF combined with differential ultracentrifugation for the identification of novel biomarkers in EVs released by a human lung carcinoma cell line (A549), Yu et al. [42] explored and optimized specific experimental parameters, finding that cell culture conditions, matrix selection, and EV storage were key factors for the obtainment of good results. The optimized conditions allowed the authors to identify two potential markers having a recognized role in lung carcinogenesis, S100A10 (S100 calcium-binding protein A10) and the ribosomal protein RPS27A.

2.6. Genitourinary Cancers

Genitourinary cancers are a heterogeneous group of cancers, in which the most common subtypes are bladder, prostate, and kidney cancers. Prostate and bladder cancers are among the most frequent worldwide. The use of the serum prostate-specific antigen measurement, the current screening procedure for prostate cancer detection and surveillance, has resulted in a significant reduction in prostate cancer mortality even if the test is characterized by a high false-positive rate [43] and patients with increased PSA values still need to confirm the presence of cancer by undergoing a prostate biopsy. The diagnostic techniques for bladder cancer, such as urine cytology, cystoscopy, and biopsy, have difficulties in distinguishing low-grade cancerous cells from healthy cells [44]. Therefore,

new diagnostic strategies for the early diagnosis of these pathologies are crucial for proper management, improving the efficacy of treatments and survival rates of patients.

Prostasomes are membranous microvesicles secreted by the epithelial cells of the prostate gland to deliver important information to sperm cells in semen, both in normal and in prostate cancer cells. A proteomic study on prostasomes from vertebral metastases of prostate cancer was conducted by Goran Ronquist et al. [45] using 2D-E coupled to MALDI-TOF. The presence among the proteins identified in the cancer-derived prostasomes of annexins (A1, A3, and A5) and dimethylarginine dimethylaminohydrolase 1, angiogenic factors that can favor tumor vascularization, supported the idea that prostasomes are involved in the interaction between tumor cells and their environment. Three complementary approaches based on MALDI, normal-phase liquid chromatography, and triple quadrupole MS, respectively, were used in a further application to obtain *N*-linked glycan profiles of expressed prostatic secretions and exosomes arising from normal individuals and high- and low-grade prostate cancer patients [46], finding changes in glycosylation that appeared to reflect the clinical status of prostate cancer.

Exosomes were extracted from HT1376 bladder cancer cell lines using sucrose cushion ultracentrifugation and the analysis of the relevant proteome was performed by LC-MALDI-TOF/TOF [47]. High-quality identification was achieved for 353 exosomal proteins (72 for the first time), finding strong correlations between the observed proteome and carcinoma of the bladder and other sites using the ExoCarta and Gene Ontology databases. Preliminary data relevant to the possible presence of candidate markers in urinary exosomes from bladder cancer patients were also reported. In another work, urinary exosomes were purified by ultracentrifugation from the urine of patients affected by urothelial carcinoma, the predominant histologic type of bladder cancer, and normal subjects prior to the MALDI-TOF analysis, with the aim of detecting new reliable biomarkers for the pathology [48]. Using optimized isolation and storage protocols, specific m/z ions (3367, 3441, 3483, and 10,884) were individuated as exosome markers, whereas the m/z ions 5593 and 5947, which were identified as fragmented peptides of alpha-1-antitrypsin and histone H2B1K, respectively, were proposed as biomarkers for urothelial carcinoma. The discovery of these markers, also verified by immunohistochemical analysis, could represent a valid aid for the rapid diagnosis and prediction of the risks for relapse and progression. The potential of exosomes as a source of cancer biomarkers was also explored by Zou et al. in a study focusing on characterizing their glycoconjugate content for future uses of compounds identified as biomarkers for genitourinary tract diseases [49]. Vesicles were isolated from normal urine samples using multistep differential centrifugation. Then, MALDI and LC/MS-MS analyses were independently performed on the extracts obtained through different pre-treatment steps, permitting the detection and identification of paucimannosidic, high-mannose, and complex-type glycans.

2.7. Brain Tumors

Brain tumors can be categorized into several kinds. Among them, the most diffused and lethal primary brain cancers are the heterogeneous group of gliomas, which can be classified according to their histological features, molecular specifications, location, differentiation patterns, and anaplasia features [50]. Through tissues arising from biopsy or tumor resection, the World Health Organization discriminates between low- (stage I and II with a relatively good prognosis) and high-grade (stage III and IV, or glioblastoma) tumors. Noninvasive approaches for the diagnosis of glioma tumors are highly needed, and EVs could play a key role in their diagnosis, prognosis, and follow-up, as demonstrated by several existing applications [50]. Useful information was also obtained by MALDI analysis of exosomes.

Costa et al. selected three different cell lines, human embryonic kidney 293, human H4 glioma, and mouse glioma Tu-2449, for a MALDI-based study dedicated to the characterization of N-glycans from EV glycoproteins [51]. Specifically, EVs secreted by the cells were isolated using ultracentrifugation and compared with total cellular membranes. Peptide N-

glycosidase F was used to release N-Glycans, which were labeled with 2-aminobenzamide and analyzed by MALDI after an LC separation step. In all the cell lines, complex N-glycans (high amounts) were observed only in EVs, whereas high-mannose glycans were present in both EVs (small amounts) and cellular membranes (high amounts). In addition, some glycans specific to human and mouse cell lines have also been identified. The approach could represent a valuable help for the individuation of new glioma biomarkers and could further aid the understanding of the functions of glycans in EVs. Glioblastoma, the highest-grade form of glioma as well as one of the most aggressive types among all cancers, exhibits a high cell proliferation rate and infiltrating capacity of the surrounding tissues, which are related to the presence of cancer stem cells, small populations of tumor cells similar to stem cells that are characterized by stem cell marker expression, high self-renewal, and resistance to radiation/chemotherapy agents. In a study developed by Di giuseppe et al. [52], EVs released in vitro by cancer stem-like cells obtained from two patients with primary glioblastoma were isolated by sequential centrifugal ultrafiltration and divided into two subtypes by electron microscopy and Western blot analysis. Then, 2-DE followed by MALDI-TOF analysis permitted the identification of specific proteins with different functions for each EV subpopulation, which could represent novel biomarkers or potential drug targets. However, a greater number of samples need to be analyzed to draw definitive conclusions. The major role played by EVs in pathological conditions was also understood by Shtam and co-workers, who described methods for the production, isolation, and characterization of the protein content of human cancer cell-derived exosomes in vitro [53]. Specifically, MALDI-TOF coupled to two-dimensional electrophoresis (2-DE) was exploited to obtain more detailed information on the proteome of exosomes secreted by brain tumor cells.

2.8. Osteosarcoma

Osteosarcoma is the most common primary bone tumor among children and teenagers [54] and the third most common cancer in adolescence. Even though the combination of surgery and chemotherapy is improving the survival rate, many still suffer from metastases with the lung as the most common site. Since highly invasive biopsies are used for osteosarcoma diagnosis, new approaches for early diagnosis are needed for successful treatments and a good prognosis.

As far as MALDI-exosome applications are concerned, Han and co-workers recently conducted a proteomic analysis of plasma exosomes from osteosarcoma patients in two distinct works devoted to rapid detection and metastasis evaluation, respectively. In both the applications, the vesicles were isolated from the plasma samples using ultracentrifugation and subsequently analyzed by MALDI-TOF. In one study [55], data arising from surface-enhanced Raman scattering and MALDI were synergically used to obtain high precision discrimination between osteosarcoma patients and healthy subjects. In the other work [56], MALDI and multivariate statistical analyses allowed for the differentiation between plasma exosomes from osteosarcoma patients with lung metastasis, osteosarcoma patients without lung metastasis, and healthy individuals. Furthermore, seven proteins were proposed as potential biomarkers of osteosarcoma lung metastasis using machine learning methods and LC-MS/MS analysis. Both methods demonstrate great potential for the clinical diagnosis of osteosarcoma.

2.9. Liver Cancer

Hepatocellular carcinoma is the most common type of liver cancer and among the most lethal malignancy worldwide [57]. It shows a poor prognosis and frequent recurrence, regardless of the extensive advances in treatment approaches such as surgery, liver transplant, radiation, ablation, embolization, targeted therapy, and immunotherapy. It is then crucial to understand the mechanisms at the base of hepatocellular carcinoma occurrence, development, and progression, as well as to individuate new biomarkers for its early diagnosis. To reach this aim, exosomes represent an ideal analytical target [57], playing

significant roles in tumor occurrence, development, metastasis, immune regulation, and drug resistance.

An epithelial-to-mesenchymal transition is a crucial event that enables epithelial cancer cells to differentiate and acquire a mesenchymal phenotype [58]. Losing their cell–cell adhesions and becoming more motile, these differentiated cells can migrate to distant anatomical sites leading to the development of metastatic growth. It has also been observed that many cancers can metastasize even if the differentiation process is not complete. The mesenchymal transition is regulated by specific zinc fingers (Snail, Slug ZEB1, ZEB2) and helix-loop-helix (E47, Twist) transcription factors, through many events which have not yet been fully deciphered. For instance, Snail and Slug repress the expression of the epithelial marker E-cadherin silencing the CDH1 gene promoter through the methylation of its DNA sequences. Karaosmanoglu et al. [59] compared complete and partial epithelial-to-mesenchymal transition in different Slug over-expressing hepatocellular carcinoma-derived cell lines (HepG2 and Huh7). MALDI-TOF/TOF and ELISA were used to identify exosomal proteins, looking for the presence of chemo-resistance and markers for the transition. The results indicated that Slug over-expression induced both complete (with downregulation of E-cadherin and upregulation of ZEB2) and partial transitions (with upregulation of E-cadherin and downregulation of vimentin and ZEB2), together with chemo-resistance through the expression of CD133, the downregulation of ABCB1, and the upregulation ABCG2. Furthermore, fibronectin 1, collagen type II alpha 1, and fibrinogen gamma chain were suggested as biomarkers for chemo-resistance and partial epithelial-to-mesenchymal transition.

3. Conclusions

The extracellular vesicles released by cells have long been considered a way to discard unwanted products, by functioning as cellular waste disposers. Currently, they are recognized as key factors in intercellular crosstalk in both healthy and pathological tissues including cancer, a condition in which EVs are released in greater quantities and are involved in several processes, including angiogenesis, proliferation, metastasis, immune evasion, and drug resistance. The central functions performed by EVs in the progression of cancer make them a natural study target for inferring crucial information on different aspects of tumors. Over the years, MALDI-TOF mass spectrometry has been increasingly employed for the characterization of EVs and their internal cargo released by different types of cancer cells, demonstrating its potential in a wide range of applications. Most of the publications, summarized in Table 1, have been devoted to proteomic studies on topics such as the identification of new biomarkers for early diagnosis, metastasis, follow-up, drug resistance prediction, tumor differentiation, epithelial-to-mesenchymal transition, and many others. Of course, a full characterization of EVs is not possible using a single analytical technique, since they carry the components of different classes associated with different biological functions and diseases. In fact, some reported applications have been performed using combined LC-MS and MALDI approaches in an attempt to obtain a more complete picture of the system under investigation.

Table 1. Objectives and main findings of the MALDI-TOF applications for the analysis of tumor derived EVs.

Authors	Cancer Type	Objective	Main Findings	Ref
Hegmans et al.	Mesothelioma	Proteomic analysis of exosomes	Identification of several proteins	[20]
Palazzolo et al.	Breast	Proteomic analysis of exosome-like vesicles	179 proteins identified, 32 isoforms upregulated in the vesicles in comparison to whole cell lysates	[23]

Table 1. *Cont.*

Authors	Cancer Type	Objective	Main Findings	Ref
Klinke et al.	Breast	Proteomic analysis of secretome to detect alterations in cell-to-cell communication	Different secretome profiles observed between cancer and normal cells	[24]
Tamkovich et al.	Breast	Proteomic characterization of blood exosomes	Detection of several proteins with different expression between healthy and cancer patients	[25]
Tutanov et al.	Breast	Search for protein biomarkers in total blood exosomes	Proteins associated with breast cancer detected only in total blood exosomes of cancer patients	[26]
Tutanov et al.	Breast and pancreatic	Investigation on the role of blood exosomes in tumor dissemination	64% of the proteins of tumor origin identified in cancer patients	[27]
Zheng et al.	Breast	Isolation and fingerprinting of plasma exosomes	Differentiation of different cancer types through a multi-classifier artificial neural network model using MS peaks as inputs	[28]
Klein-Scory et al.	Colorectal	Immunoscreening of the secretome for the identification of tumor biomarkers	Two potential protein markers proposed, Glod4 and a C-terminal fragment of agrin	[30]
Stubiger et al.	Colorectal	Protein profiling of EVs to monitor chemoresistance	Discriminatory protein patterns as the result of increased chemoresistance of parent cells	[31]
Schiera et al.	Melanoma	Investigation on the presence in EVs of H1.0 linker histone variant	Evidence of the presence of modified H1.0 histone, the corresponding mRNA, and the transcription factor MYEF2	[33]
Zhu et al.	Melanoma	Protein fingerprinting of bloodstream-circulating whole exosomes	Classification of different lines from tumor stage level through mathematical analysis. Detection of biomarkers	[34]
Lobasso et al.	Melanoma	Lipid profiling of intact cells and related exosomes	Information on the lipid content of melanoma. Bis(monoacylglycero)phosphate proposed as a specific lipid marker of exosomes	[35]
Jung et al.	Lung	Phospholipid profiling of EVs searching for markers for gefitinib resistance prediction	Changes in EVs phospholipidomic profiles directly related to gefitinib resistance	[38]
Choi et al.	Lung	Search for new tumor markers in EVs from serum and secreted by cell lines	7 proteins expressed at high levels in tumor EVs. CD5L suggested as biomarker for the disease in serum EVs	[39]
Yu et al.	Lung	Optimization of experimental protocols for EVs protein profiling	Medium components and ultracentrifugation procedures indicated as key factors. Detection of S100A10 and RPS27A proteins	[42]
Goran Ronquist et al.	Prostate	Proteomic characterization of metastasis-derived prostasomes	Identification of angiogenic factors dimethylarginine dimethylaminohydrolase 1 and annexins A1, A3, A5	[45]

Table 1. *Cont.*

Authors	Cancer Type	Objective	Main Findings	Ref
Nyalwidhe et al.	Prostate	*N*-linked glycan profiling of expressed prostatic secretions and exosomes	Changes in glycosylation appeared to reflect the clinical status of prostate cancer	[46]
Welton et al.	Bladder	Proteomic analysis of exosomes	353 exosomic proteins (72 for the first time) identified. Strong correlations between the observed proteome and cancer	[47]
Lin et al.	Urothelial	Proteomic profiling of urinary exosomes	Identification of alpha-1-antitrypsin and histone H2B1K as diagnostic and prognostic markers	[48]
Costa et al.	Glioma	Characterization of N-glycans from EVs glycoproteins and comparison with total cellular membranes	Different profiles observed in EVs and in cellular membranes	[51]
Di Giuseppe et al.	Glioblastoma	Proteomic characterization of two subtypes of EVs shed by cancer stem-like cells	Identification of specific proteins with different functions for each subpopulation	[52]
Shtam et al.	Brain	Development of methods for exosomes analysis	Description of the protein composition of exosomes	[53]
Han et al.	Osteosarcoma	Plasma exosome profiling for rapid detection of cancer	Discrimination between cancer patients and healthy subjects	[55]
Han et al.	Osteosarcoma	Profiling of plasma exosomes to evaluate metastasis	Differentiation between patients with metastasis, patients without metastasis, and healthy individuals. 7 proteins proposed as possible metastasis biomarkers	[56]
Karaosmanoglu et al.	Hepatocellular	Search for biomarkers for partial epithelial-to-mesenchymal transition	Identification of fibronectin 1, collagen type II alpha 1 and fibrinogen gamma chain as markers for chemo-resistance and partial epithelial-to-mesenchymal transition	[59]

Certainly, the application of these mass spectrometry techniques to such complex bioanalytical problems presents some contraindications, especially with regard to the potential extension of strategies from basic science to patient care. Particularly, more effective, and less expensive isolation protocols for EVs should be developed, that can integrate with the analytical instrumentation in an automated way and be standardized. Furthermore, the task is made more complicated by inherent problems, such as the complexity of the biological matrices from which EVs must be extracted and the changeable and heterogeneous composition of the content of EVs with a large number of variables. On the other hand, even though EVs have often been analyzed using LC-MS, likely because chromatographic separation helps to reduce the complexity of these matrices, MALDI-TOF surely represents a more appropriate instrument for extending the methods to clinical practice due to its intrinsic advantages in terms of high-throughput, rapidity, cost-effectiveness, and ease of use. In fact, it is important to point out that more than half the articles reviewed in this work have been published in the last five years, evidence that represents a clear sign of how the MALDI-TOF approach is becoming increasingly central in the context of studying exosomes.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The author thanks Manuela Triggiani for her statistical support.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Raposo, G.; Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J. Cell Biol.* **2013**, *200*, 373–383. [CrossRef] [PubMed]
2. Jabeen, S.; Thirumalai, V. The interplay between electrical and chemical synaptogenesis. *J. Neurophysiol.* **2018**, *120*, 1914–1922. [CrossRef] [PubMed]
3. Dang, V.D.; Jella, K.K.; Ragheb, R.R.T.; Denslow, N.D.; Alli, A.A. Lipidomic and proteomic analysis of exosomes from mouse cortical collecting duct cells. *FASEB J.* **2017**, *31*, 5399–5408. [CrossRef] [PubMed]
4. Ridder, K.; Keller, S.; Dams, M.; Rupp, A.K.; Schlaudraff, J.; Del Turco, D.; Starmann, J.; Macas, J.; Karpova, D.; Devraj, K.; et al. Extracellular Vesicle-Mediated Transfer of Genetic Information between the Hematopoietic System and the Brain in Response to Inflammation. *PLoS Biol.* **2014**, *12*, e1001874. [CrossRef] [PubMed]
5. Doyle, L.M.; Wang, M.Z. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. *Cells* **2019**, *8*, 727. [CrossRef]
6. EL Andaloussi, S.; Mager, I.; Breakefield, X.; Wood, M. Extracellular vesicles: Biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* **2013**, *12*, 347–357. [CrossRef]
7. Théry, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K.; et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell Vesic.* **2018**, *7*, 1535750. [CrossRef]
8. Ilango, S.; Paital, B.; Jayachandran, P.; Padma, P.R.; Nirmaladevi, R. Epigenetic alterations in cancer. *Front. Biosci.–Landmark* **2020**, *25*, 1058–1109.
9. Milane, L.; Singh, A.; Mattheolabakis, G.; Suresh, M.; Amiji, M.M. Exosome mediated communication within the tumor microenvironment. *J. Control. Release* **2015**, *219*, 278–294. [CrossRef]
10. Han, L.; Lam, E.W.F.; Sun, Y. Extracellular vesicles in the tumor microenvironment: Old stories, but new tales. *Mol. Cancer* **2019**, *18*, 1–14. [CrossRef]
11. Xu, R.; Rai, A.; Chen, M.; Suwakulsiri, W.; Greening, D.W.; Simpson, R.J. Extracellular vesicles in cancer—implications for future improvements in cancer care. *Nat. Rev. Clin. Oncol.* **2018**, *15*, 617–638. [CrossRef] [PubMed]
12. Thakur, A.; Parra, D.C.; Motallebnejad, P.; Brocchi, M.; Chen, H.J. Exosomes: Small vesicles with big roles in cancer, vaccine development, and therapeutics. *Bioact. Mater.* **2022**, *10*, 281–294. [CrossRef] [PubMed]
13. Yokoi, A.; Ochiya, T. Exosomes and extracellular vesicles: Rethinking the essential values in cancer biology. *Semin. Cancer Biol.* **2021**, *74*, 79–91. [CrossRef] [PubMed]
14. Szwedowicz, U.; Lapinska, Z.; Gajewska-Naryniecka, A.; Choromanska, A. Exosomes and Other Extracellular Vesicles with High Therapeutic Potential: Their Applications in Oncology, Neurology, and Dermatology. *Molecules* **2022**, *27*, 1303. [CrossRef] [PubMed]
15. Li, P.; Kaslan, M.; Lee, S.H.; Yao, J.; Gao, Z. Progress in Exosome Isolation Techniques. *Theranostics* **2017**, *7*, 789–804. [CrossRef]
16. Cho, Y.T.; Su, H.; Huang, T.L.; Chen, H.C.; Wu, P.W.J.; Wu, C.; Wu, D.C.; Shiea, J. Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry for clinical diagnosis. *Clin. Chim. Acta* **2013**, *415*, 266–275. [CrossRef]
17. Zambonin, C.; Aresta, A. MALDI-TOF/MS Analysis of Non-Invasive Human Urine and Saliva Samples for the Identification of New Cancer Biomarkers. *Molecules* **2022**, *27*, 1925. [CrossRef]
18. Jalaludin, I.; Lubman, D.M.; Kim, J. MALDI-MS: A Powerful but Underutilized Mass Spectrometric Technique for Exosome Research. *Mass Spectrom. Lett.* **2021**, *12*, 93–105.
19. Lagniau, S.; Lamote, K.; van Meerbeeck, J.P.; Vermaelen, K.Y. Biomarkers for early diagnosis of malignant mesothelioma: Do we need another moonshot? *Oncotarget* **2017**, *32*, 53751–53762. [CrossRef]
20. Hegmans, J.P.J.J.; Bard, M.P.L.; Hemmes, A.; Luider, T.M.; Kleijmeer, M.J.; Prins, J.B.; Zitvogel, L.; Burgers, S.A.; Hoogsteden, H.C.; Lambrecht, B.N. Proteomic Analysis of Exosomes Secreted by Human Mesothelioma Cells. *Am. J. Pathol.* **2004**, *164*, 1807–1815. [CrossRef]
21. Hegmans, J.P.J.J.; Gerber, P.J.; Lambrecht, B.N. Exosomes. *Methods Mol. Biol.* **2008**, *484*, 97–109. [PubMed]
22. Wu, H.-J.; Chu, P.-Y. Recent Discoveries of Macromolecule- and Cell-Based Biomarkers and Therapeutic Implications in Breast Cancer. *Int. J. Mol. Sci.* **2021**, *22*, 636. [CrossRef] [PubMed]

23. Palazzolo, G.; Albanese, N.N.; Di Cara, G.; Gygax, D.; Vittorelli, M.L.; Pucci-Minafra, I. Proteomic Analysis of Exosome-like Vesicles Derived from Breast Cancer Cells. *Anticancer Res.* **2012**, *32*, 847–860. [PubMed]
24. Klinke, D.J., II; Kulkarni, Y.M.; Wu, Y.; Byrne-Hoffman, C. Inferring Alterations in Cell-to-Cell Communication in HER2þ Breast Cancer Using Secretome Profiling of Three Cell Models. *Biotechnol. Bioeng.* **2014**, *111*, 1853–1863. [CrossRef] [PubMed]
25. Tamkovich, S.N.; Somov, A.K.; Karpukhina, K.V.; Grigoreva, A.E.; Yunusova, N.V.; Stakheeva, M.N.; Voytsitskiy, V.E.; Kondakova, I.V.; Laktionov, P.P. Isolation and characterization of exosomes from blood of patients with mastopathy and breast cancer. *AIP Conf. Proc.* **2017**, *1882*, 020075.
26. Tutanov, O.S.; Bakakina, Y.S.; Proskura, K.V.; Grigoryeva, A.E.; Syakhovich, V.E.; Beliaev, S.A.; Ryabchikova, E.I.; Tsentalovich, Y.P.; Laktionov, P.P.; Tamkovich1, S.N. Search for Breast Cancer Proteomic Markers in Total Blood Exosomes. *Sib. J. Oncol.* **2020**, *19*, 49–61. [CrossRef]
27. Tutanov, O.S.; Proskura, K.V.; Grigoryeva, A.E.; Tsentalovich, Y.P.; Tamkovich, S.N. Identification of Tumor Dissemination Facilitating Proteins in Exosomes Associated with Blood Cells of Breast Cancer Patients. *Russ. J. Bioorg. Chem.* **2020**, *46*, 1018–1033. [CrossRef]
28. Zheng, H.; Zhao, J.; Wang, X.; Yan, S.; Chu, H.; Gao, M.; Zhang, X. Integrated Pipeline of Rapid Isolation and Analysis of Human Plasma Exosomes for Cancer Discrimination Based on Deep Learning of MALDI-TOF MS Fingerprints. *Anal. Chem.* **2022**, *94*, 1831–1839. [CrossRef]
29. Tieng, F.Y.F.; Abu, N.; Nasir, S.N.; Lee, L.-H.; Ab Mutalib, N.-S. Liquid Biopsy-Based Colorectal Cancer Screening via Surface Markers of Circulating Tumor Cells. *Diagnostics* **2021**, *11*, 2136. [CrossRef]
30. Klein-Scory, S.; Kübler, S.; Diehl, H.; Eilert-Micus, C.; Reinacher-Schick, A.; Stühler, K.; Warscheid, B.; Meyer, H.E.; Schmiegel, W.; Schwarte-Waldhoff, I. Immunoscreening of the extracellular proteome of colorectal cancer cells. *BMC Cancer* **2010**, *10*, 70. [CrossRef]
31. Stubiger, G.; Nairn, M.D.; Abban, T.K.; Openshaw, M.E.; Mancera, L.; Herzig, B.; Wuczkowski, M.; Senfter, D.; Mader, R.M. MALDI-MS Protein Profiling of Chemoresistance in Extracellular Vesicles of Cancer Cells. *Anal. Chem.* **2018**, *90*, 13178–13182. [CrossRef] [PubMed]
32. Teixido, C.; Castillo, P.; Martinez-Vila, C.; Arance, A.; Alos, L. Molecular Markers and Targets in Melanoma. *Cells* **2021**, *10*, 2320. [CrossRef] [PubMed]
33. Schiera, G.; Di Liegro, C.M.; Puleo, V.; Colletta, O.; Fricano, A.; Cancemi, P.; Di Cara, G.; Di Liegro, I. Extracellular vesicles shed by melanoma cells contain a modified form of H1.0 linker histone and H1.0 mRNA-binding proteins. *Internat. J. Oncol.* **2016**, *49*, 1807–1814. [CrossRef] [PubMed]
34. Zhu, Y.; Pick, H.; Gasilova, N.; Li, X.; Lin, T.E.; Laeubli, H.P.; Zippelius, A.; Ho, P.C.; Girault, H.H. MALDI Detection of Exosomes: A Potential Tool for Cancer Studies. *Chem* **2019**, *5*, 1318–1336. [CrossRef]
35. Lobasso, S.; Tanzarella, P.; Mannavola, F.; Tucci, M.; Silvestris, F.; Felici, C.; Ingrosso, C.; Corcelli, A.; Lopalco, P. A Lipidomic Approach to Identify Potential Biomarkers in Exosomes From Melanoma Cells with Different Metastatic Potential. *Front. Physiol.* **2021**, *12*, 748895. [CrossRef]
36. Dai, W.; Feng, J.; Hu, X.; Chen, Y.; Gu, Q.; Gong, W.; Feng, T.; Wu, J. SLC7A7 is a prognostic biomarker correlated with immune infiltrates in non-small cell lung cancer. *Cancer Cell Int.* **2021**, *21*, N106. [CrossRef]
37. Jia, Z.; Patra, A.; Kutty, V.K.; Venkatesan, T. Critical Review of Volatile Organic Compound Analysis in Breath and In Vitro Cell Culture for Detection of Lung Cancer. *Metabolites* **2019**, *9*, 52. [CrossRef]
38. Jung, J.H.; Lee, M.Y.; Choi, D.Y.; Lee, J.W.; You, S.; Lee, K.Y.; Kim, J.; Kim, K.P. Phospholipids of tumor extracellular vesicles stratify gefitinib-resistant nonsmall cell lung cancer cells from gefitinib-sensitive cells. *Proteomics* **2015**, *15*, 824–835. [CrossRef]
39. Choi, E.S.; Faruque, H.A.; Kim, J.H.; Kim, K.J.; Choi, J.E.; Kim, B.A.; Kim, B.; Kim, Y.J.; Woo, M.H.; Park, J.Y.; et al. CD5L as an Extracellular Vesicle-Derived Biomarker for Liquid Biopsy of Lung Cancer. *Diagnostics* **2021**, *11*, 620. [CrossRef]
40. Aresta, A.; Calvano, C.D.; Palmisano, F.; Zambonin, C.; Monaco, A.; Tommasi, S.; Pilato, B.; Paradiso, A. Impact of sample preparation in peptide/protein profiling in human serum by MALDI-TOF mass spectrometry. *J. Pharm. Biomed. Anal.* **2008**, *46*, 157–164. [CrossRef]
41. Calvano, C.D.; Aresta, A.; Iacovone, M.; De Benedetto, G.E.; Zambonin, C.; Battaglia, M.; Ditonno, P.; Rutigliano, M.; Bettocchi, C. Optimization of analytical and pre-analytical conditions for MALDI-TOF-MS human urine protein profiles. *J. Pharm. Biomed. Anal.* **2010**, *51*, 907–914. [CrossRef] [PubMed]
42. Yu, Z.; Zhao, C.; Hu, S.; Zhang, H.; Li, W.; Zhang, R.; Luo, Q.; Yang, H. MALDI-MS-based biomarker analysis of extracellular vesicles from human lung carcinoma cells. *RSC Adv.* **2021**, *11*, 25375. [CrossRef] [PubMed]
43. Harvey, P.; Basuita, A.; Endersby, D.; Curtis, B.; Iacovidou, A.; Walker, M. A systematic review of the diagnostic accuracy of prostate specific antigen. *BMC Urol.* **2009**, *9*, 14–22. [CrossRef] [PubMed]
44. Alberice, J.V.; Amaral, A.F.; Armitage, E.G.; Lorente, J.A.; Algaba, F.; Carrilho, E.; Marquez, M.; Garcia, A.; Malats, N.; Barbas, C. Searching for urine biomarkers of bladder cancer recurrence using a liquid chromatography-mass spectrometry and capillary electrophoresis-mass spectrometry metabolomics approach. *J. Chromatogr. A* **2013**, *13*, 163–170. [CrossRef]
45. Goran Ronquist, K.; Ronquist, G.; Larsson, A.; Carlsson, L. Proteomic Analysis of Prostate Cancer Metastasis-derived Prostasomes. *Anticancer Res.* **2010**, *30*, 285–290.

46. Nyalwidhe, J.O.; Betesh, L.R.; Powers, T.W.; Jones, E.E.; White, K.Y.; Burch, T.C.; Brooks, J.; Watson, M.T.; Lance, R.S.; Troyer, D.A.; et al. Increased bisecting N-acetylglucosamine and decreased branched chain glycans of N-linked glycoproteins in expressed prostatic secretions associated with prostate cancer progression. *Proteom. Clin. Appl.* **2013**, *7*, 677–689. [CrossRef]
47. Welton, J.L.; Khanna, S.; Giles, P.J.; Brennan, P.; Brewis, I.A.; Staffurth, J.; Mason, M.D.; Clayton, A. Proteomics Analysis of Bladder Cancer Exosomes. *Mol. Cell. Proteom.* **2010**, *9*, 1324–1338. [CrossRef]
48. Lin, S.Y.; Chang, C.H.; Wu, H.C.; Lin, C.C.; Chang, K.P.; Yang, C.R.; Huang, C.P.; Hsu, W.H.; Chang, C.T.; Chen, C.J. Proteome Profiling of Urinary Exosomes Identifies Alpha 1-Antitrypsin and H2B1K as Diagnostic and Prognostic Biomarkers for Urothelial Carcinoma. *Sci. Rep.* **2016**, *6*, 34446. [CrossRef]
49. Zou, G.; Benktander, J.D.; Gizaw, S.T.; Gaunitz, S.; Novotny, M.V. Comprehensive Analytical Approach toward Glycomic Characterization and Profiling in Urinary Exosomes. *Anal. Chem.* **2017**, *89*, 5364–5372. [CrossRef]
50. Sourani, A.; Saghaei, S.; Sabouri, M. A systematic review of extracellular vesicles as non-invasive biomarkers in glioma diagnosis, prognosis, and treatment response monitoring. *Mol. Biol. Rep.* **2021**, *48*, 6971–6985. [CrossRef]
51. Costa, J.; Gatermann, M.; Nimtz, M.; Kandzia, S.; Glatzel, M.; Conradt, H.S. N-Glycosylation of Extracellular Vesicles from HEK-293 and Glioma Cell Lines. *Anal. Chem.* **2018**, *90*, 7871–7879. [CrossRef]
52. Di Giuseppe, F.; Carluccio, M.; Zuccarini, M.; Giuliani, P.; Ricci-Vitiani, L.; Pallini, R.; De Sanctis, P.; Di Pietro, R.; Ciccarelli, R.; Angelucci, S. Proteomic Characterization of Two Extracellular Vesicle Subtypes Isolated from Human Glioblastoma Stem Cell Secretome by Sequential Centrifugal Ultrafiltration. *Biomedicines* **2021**, *9*, 146. [CrossRef] [PubMed]
53. Shtam, T.A.; Naiyzhny, S.N.; Landa, S.B.; Burdackov, V.S.; Artamonova, T.O.; Filatov, M.V. Isolation and proteomic analysis of exosomes secreted by human cancer cells in vitro. *Tsitologiya* **2012**, *54*, 430–438.
54. Behjati, S.; Tarpey, P.S.; Haase, K.; Ye, H.; Young, M.D.; Alexandrov, L.B.; Farndon, S.J.; Collord, G.; Wedge, D.C.; Martincorena, I.; et al. Recurrent mutation of IGF signalling genes and distinct patterns of genomic rearrangement in osteosarcoma. *Nat. Commun.* **2017**, *8*, 15936. [CrossRef] [PubMed]
55. Han, Z.; Yi, J.; Yang, Y.; Li, D.; Peng, C.; Long, S.; Peng, X.; Shen, Y.; Liu, B.; Qiao, L. SERS and MALDI-TOF MS based plasma exosome profiling for rapid detection of osteosarcoma. *Analyst* **2021**, *146*, 6496–6505. [CrossRef]
56. Han, Z.; Peng, C.; Yi, J.; Wang, Y.; Liu, Q.; Yang, Y.; Long, S.; Qiao, L.; Shen, Y. Matrix-assisted laser desorption ionization mass spectrometry profiling of plasma exosomes evaluates osteosarcoma metastasis. *iScience* **2021**, *24*, 102906. [CrossRef]
57. Li, S.; Chen, L. Exosomes in Pathogenesis, Diagnosis, and Treatment of Hepatocellular Carcinoma. *Front. Oncol.* **2022**, *12*, 793432. [CrossRef]
58. Tan, E.J.; Thuault, S.; Caja, L.; Carletti, T.; Heldin, C.H.; Moustakas, A. Regulation of Transcription Factor Twist Expression by the DNA Architectural Protein High Mobility Group A2 during Epithelial-to-Mesenchymal Transition. *Cell Biol.* **2012**, *287*, 7134–7145. [CrossRef]
59. Karaosmanoglu, O.; Banerjee, S.; Sivas, H. Identification of biomarkers associated with partial epithelial to mesenchymal transition in the secretome of slug over-expressing hepatocellular carcinoma cells. *Cell. Oncol.* **2018**, *41*, 439–453. [CrossRef]