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Review

Extracellular Vesicles and Artificial Intelligence: Unique Weapons against Breast Cancer

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Abstract: Breast cancer (BC) caused 685,000 deaths globally in 2020, earning the title of the most common type of tumor among females. With a multifactorial genesis, BC is influenced by several factors such as age, genetic and epigenetic predisposition, and an individual's exposome, and its classification is based on morphological/histological, invasiveness, and molecular futures. Extracellular vesicles (EVs) are cell-derived lipid-bilayer-delimited nanoparticles, which are distinguishable by size, genesis, and the markers expressed in exosomes (40 to 150 nm), microvesicles (40 to 10,000 nm), and apoptotic bodies (100–5000 nm). Produced in physiological and pathological cellular contexts, EVs are shuttles of biological material and are implicated in cell-to-cell communications, thus attracting significant interest in diagnostic and drug delivery research. We report and discuss the latest evidence regarding the important role of EVs in BC, deepening their implication in tumorigenesis and metastatic mechanisms. On the other hand, the use of BC-derived EVs as prognostic biomarkers and therapeutic approaches is undergoing investigation. Hence, EVs have become new weapons in precision medicine; however, only with the support of advanced algorithms such as artificial intelligence (AI) can we develop a wide range of information. Looking ahead, it is possible to see the application of AI in the prognosis and diagnosis of different pathologies.

Keywords: hormonal receptors; extracellular vesicles; machine learning; prevention; precision medicine; drug resistance; nanovectors; drug delivery; artificial intelligence

Citation: Serretiello, E.; Smimmo, A.; Ballini, A.; Parmeggiani, D.; Agresti, M.; Bassi, P.; Moccia, G.; Sciarra, A.; De Angelis, A.; Della Monica, P.; et al. Extracellular Vesicles and Artificial Intelligence: Unique Weapons against Breast Cancer. *Appl. Sci.* **2024**, *14*, 1639. https://doi.org/10.3390/app14041639

Academic Editors: Roger Narayan and Stefan H. Bossmann

Received: 20 December 2023
Revised: 31 January 2024
Accepted: 15 February 2024
Published: 18 February 2024

1. Introduction

1.1. Breast Cancer Epidemiology

The World Health Organization (WHO) reports noncommunicable diseases (NCDs), such as cardiovascular diseases and cancer, as leading causes of premature death (2019) [1]. In particular, breast cancer (BC) was reported to be one of the twenty global principal causes of death in a 2019 report [1,2]. BC is the most frequently diagnosed cancer worldwide, followed by lung cancer and colorectal cancer, with different percentages stratified by age group (40% in young people vs. 22% in older people) [2]. On 12 July 2023, the World Health Organization (WHO) asserted that by the end of 2020, breast cancer had been diagnosed in 7.8 million women over the past five years, causing 685,000 deaths globally in 2020 [3,4]. Male individuals can also have breast cancer, although with a very low incidence percentage (approximately 0.5–1% of breast cancers) [4]. Although global statistics on BC have not been published, different countries have reported their individual statistics. In Italy, the most

recent report (2022) estimated 55,700 new diagnoses of BC in women, an incidence of deaths of 12,500 in 2021, and a net survival rate of 88% 5 years after diagnosis [5]. The American Cancer Society's estimates for 2023 are that 297,790 new cases of invasive breast cancer will be diagnosed in women and 43,700 deaths will occur [6]; moreover, the COVID-19 pandemic might have caused a delay in cancer diagnosis and management [7,8]. BC occurs in women at any age after puberty and can occur in the absence of or in correspondence with different risk factors such as age, hormonal, reproductive, and metabolic factors, an unhealthy lifestyle, previous radiotherapy administration or breast dysplasia or neoplasm, and a family history of cancer, as it is heredity. In this context, it is important to remember that one of the Sustainable Development Goals (SDGs) of the 2023 Agenda is to reduce premature mortality from noncommunicable diseases (NCDs) by one-third with respect to the 2015 levels [9]. Therefore, reducing risk factors and improving early diagnosis and therapy strategies will be necessary to reach this goal. In this context, artificial intelligence (AI) could be a promising contributor [10].

1.2. Breast Cancer Classification

BC can be classified using morphological/histological features based on the cells giving rise to the tumor; lobule cells (lobular carcinoma) or milk ducts (ductal carcinoma) are the most frequent. Their ability to invade other tissues to metastasize classifies them as "non-invasive" or "invasive". The in situ (noninvasive) forms of ductal and lobular carcinomas are abbreviated as DCIS (ductal carcinoma in situ) and LCIS (lobular carcinoma in situ), respectively. Ductal and lobular carcinomas can also reach the metastatic stage, such as tubular, papillary, mucinous, or cribriform carcinomas (less common forms). Using the TNM classification, the parameter "T" evaluates the size of the tumor on a scale of 1 to 4 (T1–T4), "N" refers to the involvement of lymph nodes adjacent to the tumor, and "M" indicates the presence of metastases. Moreover, the molecular characterization of invasive breast cancer involves the presence and dosage of specific hormonal cellular receptors (HRs), such as estrogen-positive (ER+), progesterone-positive (PR+), and human epidermal growth factor receptor2-positive (HER2+) receptors, and a triple-negative tumor is characterized by the absence of all the abovementioned receptors. HR overexpression induces a high replicative power in the tumor, with consequent aggressiveness, and is therefore useful for predicting its growth over time. Hormone-responsive tumors represent approximately 70%, HER2-positive tumors represent about 20%, and triple-negative breast cancer cases comprise 15% [11]. BC classifications and their relative prevalence percentages and invasiveness/colonization processes are reported in Figure 1.

The evaluation of biological parameters, the cancer prognosis, the psycho-physical condition of the patient, and previous treatments received in a neoadjuvant/adjuvant context affect the choice of systemic treatment by a clinician. BC patients' treatment is based on a surgical approach, preceded/followed by neoadjuvant/adjuvant treatment such as hormone therapy and targeted chemotherapy drugs, according to the type of tumor. Immunotherapies try to stimulate the lost immune system activity using vaccines, immune checkpoint inhibitors [11], and the modification of immune cells and microbiota, as occurs in endometrial diseases [12]. With awareness in the media of the topic and the regional distribution of screening campaigns as a preventive approach, accompanied by diagnostic–therapeutic implementation and the availability of new anticancer drugs, the overall survival of critical metastatic patients has significantly increased. There are many steps forward for the characterization and identification of BC and a therapeutic approach to its treatment, but there are still many points that need to be clarified. A classical approach investigated the alterations in canonical molecular pathways and the identification of new biomarkers such as miRNA involved in cancer progression, metastasis, and drug chemoresistance [13]. The identification of new pathways through the characterization of alternative forms of cell–cell communication that play a role in carcinogenesis and tumor aggressiveness is needed. In the context of microparticles, the involvement of extracellular vesicles (EVs) in many different types of tumors has already been investigated. The next

section briefly introduces and describes EVs, the underlying reasons for their importance, and the success achieved in recent years.

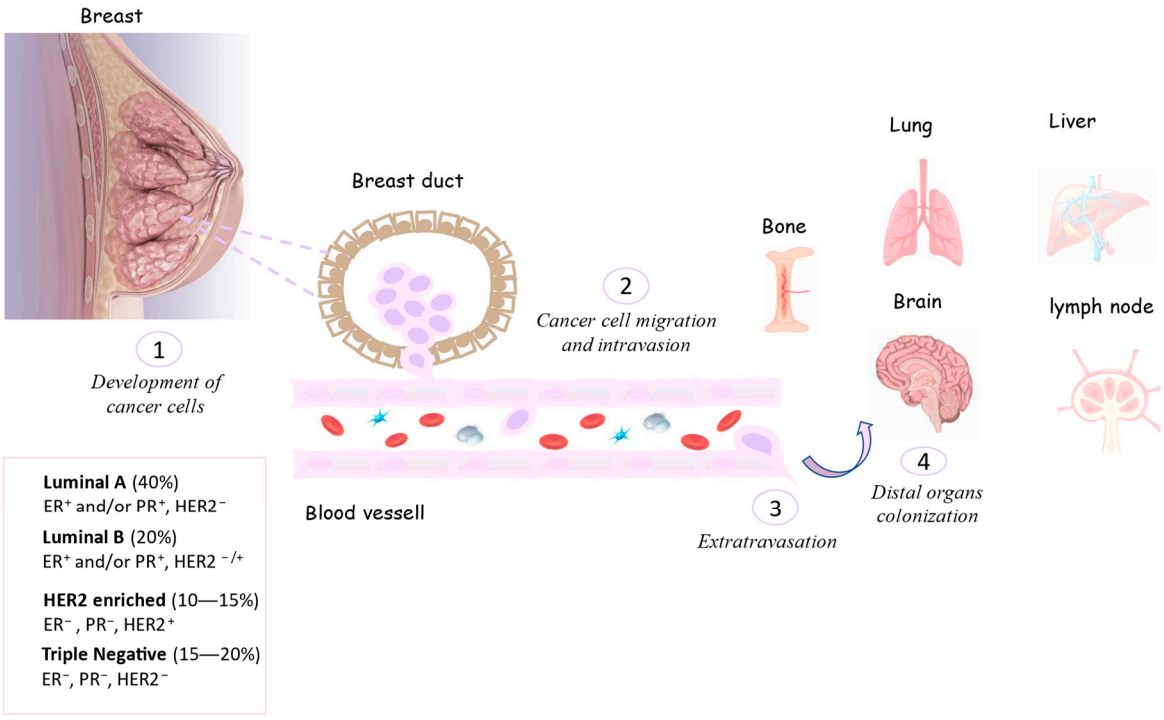

Figure 1. Process of the invasiveness and colonization of breast tumor cells in other organs.

1.3. Extracellular Vesicles (EVs)

EVs are very small lipid bilayer particles of a cellular origin, measuring in the order of nanometers to micrometers; they lack replicative power and express receptors that give them specificity and are produced by all three domains of Bacteria, Archaea, and Eukarya. Based on their size, the specific biomarkers expressed, and biogenesis mechanisms, they are divided into exosomes (40 to 150 nm), microvesicles (40 to 10,000 nm), and apoptotic bodies (100–5000 nm). According to the current classification, exosomes are subdivided into small and large (Exo-S, measuring 40–80 nm, and Exo-L, measuring 80–150 nm) exosomes, microvesicles are subdivided into ARMMs (arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles measuring 40–100 nm), which are distinguished from classical large microvesicles (~150–1000 nm) and large oncosomes (1–10 µm), and apoptotic EVs are subdivided into apoptotic bodies (1–5 µm) and apoptotic vesicles (~100–1000 nm) [14].

EVs' cargo consists of several cell-derived components such as lipids, proteins, nuclei acids, and viral-derived particles (in case of infection), and their bilayer composition protects EVs' cargo from aspecific cytotoxicity and consequent degradation. Figure 2 shows a typical exosome structure and main cargo component.

EVs are secreted by all cell types, both in physiological and pathological conditions, and are released by several biological fluids, such as blood, liquor, saliva, urine, milk, and others [15–17]. In the last decade, their characterization and functional studies have been implemented thanks to the use of highly technological and well-performing tools, reevaluating their importance. EVs have been identified as an alternative cell-to-cell communication strategy due to their cargo release to the receiving cell. Therefore, their contribution to homeostasis processes, tumoral context, and viral infections is well reported in the literature [18]. Thanks to their small size, ability to cross the blood–brain barrier, low toxicity, and nonimmunogenic phenotype, EVs can be engineered and directed to a specific target by the expression of particular surface structures (anchoring proteins and transmembrane), becoming valid candidates for drug delivery for precision medicine approaches.

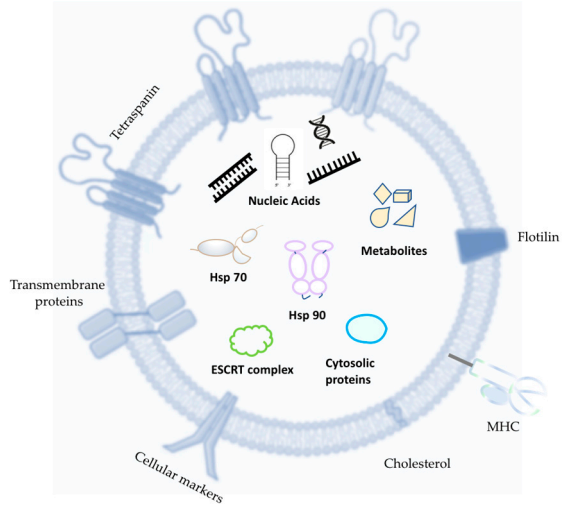

Figure 2. Generic EV futures and cargo. The most common EV tetraspanins are Motility-Related Protein-1 (CD9), human leucocyte surface antigen (CD53), Cluster of Differentiation 36 (CD36), Cluster of Differentiation 81 (CD81), and Cluster of Differentiation 82 (CD82). Typical cytosolic proteins are tumor susceptibility gene 101 (TSG 101), ALG-2-interacting protein X (ALIX), annexins (ANXAS), C-X-C chemokine receptor type 4 (CXCR4), and epithelial cell adhesion molecule (EPCAM). Abbreviations: Major Histocompatibility Complex (MCH); heat shock protein 70 (Hsp70); heat shock protein 90 (Hsp90); Endosomal Sorting Complexes Required for Transport (ESCRT).

1.3.1. EV Biogenesis

Exosomes are generated via the endo-lysosomal pathway by a process dependent on or independent from the endosomal sorting complex required for transport (ESCRT), through the intraluminal formation of multivesicular bodies and their fusion with the cell membrane, with consequent exocytosis, and they have a morphology typically defined as "cup-shaped" [19]. The chaperone Cdc37 assists protein kinase folding during exosome biogenesis and secretion, whereas autophagy-activating kinase ULK1, membrane-bound kinase SRC, and lipid kinase VPS34 intervene in exosome formation and release [20]. Microvesicles are generated by the extrusion of the cell membrane (budding/shedding), as well as apoptotic bodies when in an apoptotic cell. Apoptotic bodies can also derive from the endoplasmic reticulum, in which case they will not contain DNA. Although MVs originate as an extrusion of the plasma membrane, their arrangement in the bilayer of some lipids such as phosphatidylserine, aminophospholipids, and ethanolamine varies with respect to the structure of the mother membrane, resulting in their homogeneous (and not asymmetrical) distribution in the helix of the membrane [17].

EVs differ from each other in size, biogenesis process, density, and the presence/abundance of specific markers. However, their characterization becomes difficult due to the overlap of the size range of MVs and exosomes. The International Society for Extracellular Vesicles (ISEV) first published the Minimal Information for Studies of Extracellular Vesicles (MISEV) in 2014, updated in 2018, underlining the importance of defining the EV subcategories. Based on the latest evidence, the ISEV defines "extracellular vesicle (EV) as particles naturally released by cells, delimited by a lipid bilayer without the ability to replicate" [21].

1.3.2. EVs–Recipient Cell Interaction

The particular compositions of EV surface markers of different natures and structures (protein, lipid, etc.) are closely related to their original cell, and this makes EVs capable of specific interactions with recipient cells. In the presence of surface receptors and/or internalization, with the release of their cargo contents, EVs can influence the recipient cell's physiological or pathological condition. Exosomes can interact with target cells through different mechanisms: interactions between proteins exposed by exosomes and the receptors of the target cell, activating them; modifications to exosomal membrane proteins

whose fragments can act as ligands for cell surface receptors; and the fusion of exosomes with the target cell, resulting in the release of their contents.

Transmembrane proteins or adhesion molecules (CAM), integrins (ITG), tetraspanins (CD9, CD63, CD81) [22], growth factor receptors, heterotrimeric G proteins (GNA), and phosphatidyl-serine-binding MFGE8/lactadherin are able to mediate the interaction between the recipient cell and the extracellular vesicle, resulting in cargo release. Furthermore, cytosolic binding membrane receptor proteins, such as membrane-binding proteins (TSG101, annexins, Rabs), proteins responsible for signal transduction or scaffold proteins (syntenin), and extracellular proteins binding specifically or nonspecifically to membranes, such as fibronectin, acetylcholinesterase, collagen, and soluble secreted proteins (metalloproteinases, cytokines, growth factors), are implicated in EV uptake [23–26]. The mentioned EV uptake strategies are reported in Figure 3, such as EVs' important role as diagnostic and prognostic biomarkers and their implication in therapeutic approaches and clinical applications (Figure 3).

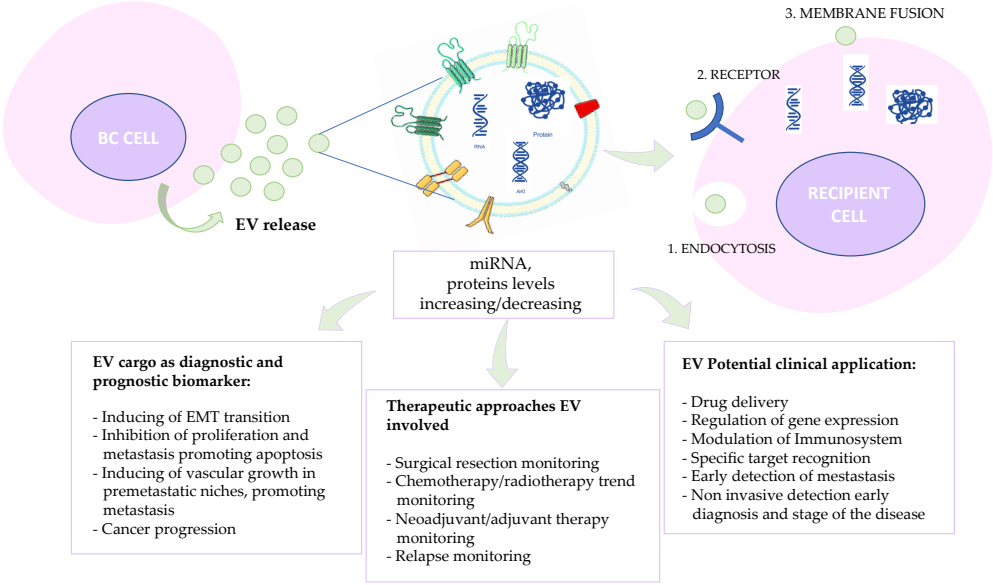

Figure 3. Schematic representation of alternative exosome-mediated cell–cell communication via different modes: (1) endocytosis-mediated communication; (2) direct surface-bound receptor-mediated communication; and (3) exosome fusion with the recipient cell membrane. EVs feature as diagnostic and prognostic biomarkers, and their implications for therapeutic approaches and clinical applications are also reported.

1.4. EV Characterization

The most common EV isolation methods include ultracentrifugation, density gradient centrifugation, the size exclusion chromatography approach, ultrafiltration, and immunoprecipitation assays [27]. Once isolated, EV subpopulations are characterized using quantitative and qualitative criteria. EV morphology can be detected via transmission electron microscopy (TEM) and scanning electron microscope (SEM). To determine the vesicle size and concentration, single-particle interferometric reflectance (SP-IRIS) and nanoparticle tracking analyses (NTAs) are used, which are methods that are able to detect the particles' intrinsic Brownian movement [28]. The number of particles can also be established via standard flow cytometry to evaluate large EVs and high-sensitivity flow cytometry (hsFCM) for small EVs [29], while resistive pulse sensing (RPS) is based on the pore size and adapted for a wide range of sizes [30]. Cryogenic electron microscopy (cryo-EM) and other technologies [31] are also used. EV characterization occurs via the identification of specific antigen–antibody recognition such as the use of an ELISA, Western blotting, specific antibodies for membrane proteins (tetraspanins) and cytosolic proteins (ALIX), peculiar markers of EV subgroups and the exclusion of other co-isolated materials,

and the multiple-reaction-monitoring (MRM) approach [32]. The super-resolution microscopy approach, direct stochastic optical reconstruction microscopy (dSTORM) [33,34], is able to ascertain how and where EVs are clustered and localized; while with advanced fluorescence microscopy, it is possible to detect single EVs in real time, observe EVs during their interactions and uptake within living cells, and visualize their post-internalization movements in a time lapse. To better investigate this specific subject, the authors refer readers to the MISEV 2023 guidelines [35].

1.5. EVs in Cancer

As explained in the preceding section, EVs carry a significant amount of information and can also be used for drug delivery. These two hallmarks make them an important weapon against cancer. Their implications in cancer are very intricate and are still being characterized. Certainly, their role as cellular information mediators makes them capable of modifying both local and distant tumoral microenvironments [36]. Tumor-derived EVs (TEVs) carry cell-derived molecules, such as coding or noncoding RNAs and DNA fragments, contributing to tumor progression and malignancy [37]. So, in both a paracrine and systemic manner, TEVs promote cancer progression, transferring aggressive phenotype information and conferring drug resistance [37–39]. They also interfere with the antitumor immune response and mediate intercellular communication among stromal cells and bone marrow. On the other hand, TEVs are able to promote the formation of pre-metastatic niches to encourage the outgrowth of incoming cancer cells distant from the primary tumor site [37,40,41]. Microvesicles derived from tumoral endothelial miR-9 cells were reported to promote not only MV-induced endothelial cell migration but also tumor angiogenesis [42–47]. Some authors revealed that oral squamous cell carcinoma cell-derived extracellular vesicles (OSCC_EVs) were enriched in microRNA (miR)-21-5p and that EVs were associated with enhanced resistance to cisplatin, an increased metastatic phenotype, stemness, and a poor outcome in OSCC patients [43]. By conducting liquid biopsies in a minimally invasive or noninvasive way, it is easy to observe circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), which are useful for monitoring patients and tumor evolution [45,46]. Similarly, EVs were found to be a source of prostate cancer biomarkers obtainable via liquid biopsies [47]. Another group correlated the pro-metastatic role of circulating EVs induced when undergoing neoadjuvant chemotherapy treatment with an enrichment in annexin A6 (ANXA6) in breast cancer patients [44].

2. EVs in Breast Cancer

An EV's cargo is transferred from a parent cell to a recipient cell, so it is subsequently involved in tumor initiation and progression, metastasis formation via the dissemination of the oncogene in target cells, and remodeling the extracellular matrix [48–52]. The involvement of TEVs in BC has been reported in the literature for many years. In 2013, exosomes derived from the triple-negative breast cancer (TNBC) Hs578Ts(i)8 cell line were investigated, and their increased invasiveness in the recipient cells utilized was noted; for example, TNBC patients' exosomes derived from sera were able to transfer phenotypic traits from the original cells to the receiving cells [53]. Certain authors have highlighted the role of exosomal miR-10b in tumoral invasiveness in the metastatic breast cancer MDA-MB-231 cell line [54]. Similar results were obtained for cancer-secreted miR-105, which regulated and induced cell migration via exosome-mediated transfer [55]. Tumor progression was investigated in response to hypoxic conditions; in particular, among the exosomal miRNAs, miR-16, let7a, miR-21, and miR-210 secreted by the MDA-MB-231 and T47D breast cancer cell lines, the latter was found to be significantly upregulated in hypoxic conditions and is thus a good target for a therapeutic approach [56]. Through the expression of peculiar exosomal integrins, exosomes can influence tumor cells' tropism to enable metastases (integrins $\alpha 6\beta 4$ and $\alpha 6\beta 1$ are associated with pulmonary metastases, while $\alpha v\beta 5$ is associated with liver metastases) [57]. Due to their own tendencies to be intrinsically biologically active and not only vectors of information, it has also been demonstrated that

some precursor microRNAs (pre-miRNAs) present in BC-derived TEVs were processed into mature miRNAs within TEVs in a cell-independent manner. On the other hand, BC-EVs derived from the cells and sera of patients induced nontumorigenic epithelial cells to cause cancer in a Dicer-dependent manner, promoting tumorigenesis [57]. As shown in the current literature, the role of mesenchymal stem cells (MSCs) is gaining significant interest, and the results are often controversial, sometimes showing pro-tumor activity while demonstrating the opposite role in other cases [58]. In 2014, Ono et al. demonstrated that the coculture of a bone marrow metastatic human breast cancer cell line (BM2) with human donor-derived bone marrow mesenchymal stem cells (BM-MSCs) induced the acquisition of dormant phenotypes in the BM2 cells, causing unresponsiveness to traditional chemotherapy approaches. The same effect was achieved by culturing the cells with exosomes isolated from BM-MSC cultures. This evidence, together with the overexpression of miR-23b in BM-MSC-derived exosomes, suggests the role of exosomes in promoting BC cell dormancy in the metastatic niche [59]. Researchers demonstrated the anti-angiogenic effect of MSC-derived exosomes enriched in miR-16, which target vascular endothelial growth factor (VEGF) with a consequent inhibition of angiogenesis [60]. Some reported higher levels of circulating exosomal miR-373 in triple-negative carcinomas than in luminal carcinomas, such as in estrogen-negative and progesterone-negative tumors, suggesting a correlation of this exosomal mi-RNA with the aggressiveness of breast carcinomas [61]. Other authors demonstrated that BC-EVs could carry miR-182-5p, which is highly expressed in breast cancer tissues and cells, and were able to downregulate CKLF-like MARVEL transmembrane domain-containing 7 (CMTM7) expression and activate the EGFR/AKT signaling pathway, with a consequent aggravation of breast cancer and the promotion of breast cancer angiogenesis through EVs-miR-182-5p [62,63]. In 2022, Qi et al. promoted the involvement of the pluripotent factor Lin28B-high, which is particularly upregulated in triple-negative BC, and low-let-7s exosomes (sourced from Lin28B-induced breast cancer stem cells) to generate lung metastases in breast cancer patients via exosomes, supporting cancer progression [60]. Great progress has been made in recent years to characterize new pathways and identify new biomarkers in tumors using machine learning approaches. A very interesting example is a study using a machine learning approach that identified COL11A1 as a hub gene that is highly expressed in breast cancer samples and associated with a poor prognosis [64].

On the other hand, several studies reported EV proteomic signature profiling, which established a strong application of EV cargo in predicting clinical outcomes and monitoring disease progression and evolution. Using label-free quantitative phospho-proteomics, 144 phospho-proteins were identified in plasma EVs, the levels of which were found to be higher in BC-positive patients compared to healthy controls, showing the utility of EVs in cancer monitoring and screening [65]. Several proteins correlated with the tumor context were reported as EV cargo and found to be useful for tumor/health discrimination and tumor stage identification. For example, EGFR, epithelial cell adhesion molecule (EpCAM), and HER2 on circulating EVs were investigated as candidates to distinguish malignant BC patients from healthy controls; they were found to have sensitivities of 97.37% and 92.31% in detecting early stage I BC [66]. Moreover, a liquid biopsy was used to identify CD63/EpCAM/mucin 1-triple-positive circulating EVs in BC-positive patients, discriminating between patients and healthy samples with a sensitivity of 91% [67].

2.1. EVs' Involvement in BC Drug Resistance

Therapeutic approaches in BC treatment are strongly related to the tumor classification, which provides information about the intrinsic future of drug resistance. The positive hormone receptors (HRs) in BC, such as the estrogen receptor (ER+) and the progesterone receptor (PR+), are generally sensitive to selective estrogen receptor modulators (SERMs), such as tamoxifen, and selective estrogen receptor downregulators (SERDs), such as fulvestrant [68]. Trastuzumab and lapatinib were found to be efficient in human epidermal growth factor receptor 2 (HER2)-positive BC treatment [69,70]. For both HR+ and HER2+

BC, chemotherapy and radiotherapy are suggested as neoadjuvant/adjuvant treatments to support a surgical approach. The cyclin-dependent kinase 4/6 inhibitor is now used in HR+/HER2-BC treatment and was recently proposed for TNBC treatment [71]. TNBCs cause low/absent expression levels of all HRs and present poor sensitivity to treatment, earning a reputation as a very aggressive and difficult-to-treat type of tumor. Regarding the intrinsic resistance phenomena, due to tumor heterogeneity, genetic and epigenetic mutations/futures, and the absence of expression of the drug target, acquired resistance is accompanied by a progressive loss of sensitivity to treatment over time, possibly due to alternative pathway expression or mutations that prevent drug efficiency. Alterations could occur in both cancer cells and/or at the expense of the tumor microenvironment (TME) [72]. The immunosuppressive microenvironment that can occur due to the presence of tumor-associated neutrophils (TANs), regulatory T cells (Tregs), and tumor-associated macrophages (TAMs), such as the secretion of immune suppression molecules, for instance, programmed cell death ligand 1 (PD-L1), leads to the acquisition of a treatment-resistant phenotype in BC. EVs are involved in therapy resistance; through their cargo, they influence the TME, although in a loop, TME cells communicate with BC cells via EVs. First, BC-derived EVs containing upregulated miR-146a are able to induce the transition of fibroblasts into cancer-associated fibroblasts (CAFs), a fundamental event in the formation of the TME [73]. CAFs promote more aggressive disease via several mechanisms, such as CAF-derived EVs enriched in miR-92 that induce increased PD-L1 expression in BC, inhibiting T cell performance with a consequent resistance to immunotherapies through immune suppression mechanisms [74].

MSCs are able to differentiate into adipocytes, chondrocytes, and osteoblasts, exhibiting plastic adherence, and they can be defined according to the presence/absence of the following biomarkers: CD73+, CD90+, CD105+ cells, CD14−, CD34−, CD45−, and human leukocyte antigen–DR isotype (HLA-DR) [75]. In physiological conditions, MSCs act as immunomodulators, but in BC-promoting tumor progression [76], EVs collected from MSCs promote increases in migration and proliferation in ER + BC via the wnt/β-catenin pathway, which is recognized to be implicated in BC drug resistance [77]. The controversial tumor-suppressor role of EV-derived MSCs in BC requires further research because they are reportedly able to suppress angiogenesis via the downregulation of VEGF, partially through miR-16 [60], and through the modulation of mTOR/HIF-1alpha/VEGF signaling suppression through miR-100 [78].

The involvement of RNA/proteins in EV cargo in the polarization of M2-type anti-inflammatory/immunosuppressive macrophages (alternatively activated) is well characterized and connected to cancer progression, lymph node metastasis, and responses to chemotherapy. An M2 immunosuppressive phenotype can be induced via an EV-mediated interplay between macrophages, cancer cells, and TME cells, which is associated with a poor prognostic outcome and lymph node metastasis [79–84]. Both M1-type pro-inflammatory (classically activated) and M2 macrophages were identified in the TME. In particular, the anticancer progression role of EVs was underlined in 2021 through the identification of miR-33-containing BC-derived EVs, which were able to induce M1 polarization in mouse macrophages [85], and the evidence that M1 macrophage-derived EVs cause BC sensitivity to chemotherapeutics [86]. Moreover, miRNA-205 is upregulated in tamoxifen-resistant MCF-7/TAMR-1 (M/T) cells and, also, in M/T cell-derived exosomes (M/T-Exo). Exosomal miRNA-205 could confer tamoxifen chemoresistance and tumorigenesis progress in recipient tumor cells by targeting E2F Transcription Factor 1 (E2F1) in human BC cells (BCCs) [87]. On the other hand, cancer stem cells (CSCs) are able to self-renew and differentiate into diverse cells, resulting in being resistant to chemotherapy; thus, they are a good therapeutic target. CD44+/CD24− surface markers were found to be minimum biomarkers for breast CSCs (BCSCs), and Her2- and CD44-positive EVs have been associated with tumor recurrence and metastasis [25].

EV-released biomolecules can change the metabolism/physiology of cells, conferring information capable of inducing a drug-resistant phenotype, such as the miR-181 family

involved in drug resistance and metastasis progression in BC [88]. As shown in mouse models, CD63(+) cancer-associated fibroblasts (CAFs) confer tamoxifen resistance, and the sequestration of exosomal miR-22 induces overcoming tamoxifen resistance [89]. On the other hand, in an MCF7 xenograft mammary fat pad mouse model, Sansone et al. demonstrated the emergence from quiescence of metabolically dormant cells during hormonal treatment thanks to the transfer of exosome-derived mtDNA from CAFs to BC [90]. Previous evidence shows a controversial role for EVs, which, due to their content, are able to promote cancer progression and a drug resistance phenomenon or the inhibition of cancer progression [91–100]. Based on the evidence that EVs' cargo is fundamental to cellular functions and also influences their responsiveness to drugs, the idea of engineering these nanovesicles into drug vehicles or molecules capable of directing cells towards a phenotype of sensitivity to the chosen treatment is currently a great challenge in precision medicine [101–113].

2.2. Exosomes as a Weapon in Precision Medicine

The field of EVs is highly speculative because the characterization of EVs' contents, such as proteins and miRNAs, and their expression levels can be used as candidate markers for early detection, relapse, and metastasis prediction, such as susceptibility to therapeutic treatment in BC patients. Highly stratified by type and status, with a highly heterogeneous nature, BC is treated with typical approaches such as surgery and chemo/radio/hormone therapy but not personalized medicine [114–126]. The enrichment of protocols useful for obtaining and characterizing EVs through the arrival of cutting-edge technologies and the ease of isolating EVs from many biological matrices (blood, urine, milk, lymphatic exudate, etc.) in a noninvasive manner make them good candidates for biomarkers alone or in association with other emerging markers for different cancers [127–135]. The information obtained via the EVs' fluid characterization can be useful and applied in BC diagnosis, monitoring after surgical resection, and during neoadjuvant/adjuvant therapy. The research led to the identification of several cancer-related markers (CD24, CD29, CD44, and CD146) and platelet markers (CD41b, CD42a, and CD62p) on EVs of potential interest as biomarkers; however, further characterizations are needed [66]. Many studies promote some markers EVs, in particular circulating EVs, as tools for several tumor diagnoses and prognostic biomarkers [120–133].

EVs' low immune response and tolerability in the body, ease of internalization with the release of carried macromolecules, and ability to cross the blood–brain barrier due to their small size make them a good vehicle for drug delivery in precision medicine [136,137]. Although there are excellent prospects for the use of EVs, there still appear to be limitations, in part due to the half-life of the nanoparticles. In 2019, Saunderson et al. highlighted that the length of time for the clearance of exosomes from the blood of wild-type and $CD169^{-/-}$ mice was ~2 min, but a longer-lived reservoir of exosomes was present after 120 min in the spleen, although the majority of exosomes had been cleared from circulation [138]. The topical application of exosomes (buccal mucosa or ocular surface) shows a shorter exosome half-life caused by the rapid fluid turnover (saliva or tears) and the exposition of external elements. Due to the rapid clearance rate of circulating exogenous exosomes and consequently poor exosome enrichment in the target tissue, several studies in the literature promote strategies to increase the exosomes' half-life in circulation. However, in vivo exosome half-life and clearance are closely associated with administrative methods, the availability of recipient cells for EV internalization, the size of the EVs, and the cell-type origin [139]. It is known that the exposition of CD47 proteins upon an exosome's surface represents "don't eat me" signaling, evading the monocytes' phagocytosis with an increase in circulating exosomes [140]. The latest evidence suggests a hydrogel, an absorbable biological scaffold, as a vehicle able to achieve the stability and biological activity of exosomes [141]. In particular, hyaluronic-acid-based hydrogels have been used for a long time as an exosome delivery strategy in bone regeneration applications [142].

In particular, in Table 1, the main miRNAs and EV-derived biomarkers implicated in the diagnosis, prognosis, and drug sensitivity of BC and variations in their relative expression levels are shown.

Table 1. miRNA and biomarkers derived from EVs implicated in BC diagnosis (a), prognosis (b), and chemoresistance (c).

Diagnosis

Biomarkers				miRNA			
Markers	**Expression**	**Source**	**Ref.**	**miRNA**	**Expression**	**Source**	**Ref.**
EDIL3	↑	Cells	[91]	miR-122-5p	↑	Plasma	[103]
FN	↑	Cells/plasma	[92]	Let-7b-5p	↓	Plasma	[103]
FAK	↑	Plasma	[93]	miR-101 & miR-372	↑	Serum	[104]
MEK1	↑	Plasma	[93]	miR-188-5p	↓	Serum	[105]
CD47	↓	serum	[94]	miR-1246	↑	Plasma	[106]
GPC-1	↑	Cells	[95]	miR-21	↑	Plasma	[106,107]
GLUT-1	↑	Cells	[95]	miR-7641	↑	Cells/plasma	[108]
ADAM10	↑	Cells	[95]	miR-9	↑	Cells	[109]
EpCAM	↑	Cells/plasma	[96]	miR-155	↑	Cells	[110,111]
HER2	↑	Cells/plasma	[96–98]	miR-105	↑	Cells/Mouse	[112]
RALGAPA2, PKG1 & TJP2	↑	Plasma	[99]	miR-373	↑	Cells/Serum	[113]
LY6G6F, VWF, BSG, C1QA & ANGPT1/Ang1	↑	Plasma	[100]	miR-223-3p	↑	Plasma	[114]
Glycoprotein 130	↑	Cells	[101]				
CD147	↑	Serum	[102]				

(a)

Prognosis

Biomarkers				miRNA			
Markers	**Expression**	**Source**	**Ref.**	**miRNA**	**Expression**	**Source**	**Ref.**
Annexin A2	↑	Cells/serum	[115]	miR-21	↑	Plasma	[106,107]
NGF	↑	Serum	[116]	miR- 338-3p	↑	Serum	[121]
IGFRβ	↑	Plasma	[92]	miR-124-3p	↑	Serum	[121]
CD82	↑	Serum	[117]	miR-340-5p	↑	Serum	[121]
Del-1	↑	Plasma	[118]	miR-29b-3p, miR-20b-5p, miR-17-5p, miR-130a-3p, miR-18a-5p, miR-195-5p, miR-486-5p & miR-93-5p	↓	Serum	[121]
Survivin	↑	Serum	[119]	miR-16 & miR30b	↑	Plasma	[122]
MMP-1/CD63	↑	Urine	[120]	miR-93	↑	Plasma	[122]
				miR-373 & miR-24–2-5p	↑	Plasma	[123]
				miR-548b-5p, miR-655-3P & miR-376b-5p	↓	Plasma	[123]
				miR-421, miR-128–1 & miR- 128–2	↑	Serum	[124]

(b)

Table 1. *Cont.*

Chemioresistance							
Biomarkers				miRNA			
Markers	**Expression**	**Source**	**Ref.**	**miRNA**	**Expression**	**Source**	**Ref.**
PERP, ITB1, GNAS2 & GNA13	↓	Cells/plasma	[125]	miR-100, miR-222, miR-30a & miR-17	↑	Cells	[126]
GSTP1	↑	Cells/serum	[126]	miR-221/222	↑	Cells	[127]
UCHL-1	↑	Cells/serum	[127]	let-7a, let-7b, let-7c, miR-103a, miR-16, miR-23a, miR-23b, miR-27a & miR-30a	↑	Cells	[135]
TrpC5	↑	Cells/serum	[127]	miR-130a, miR-20b, miR-25, miR-425, miR-455-3p, miR-4725-5p, miR-551, miR-92	↓	Cells	[136]
BCRP, HER2	↑	Plasma	[126]	miR-9-5p, miR-195-5p & miR-203a-3p	↑	Cells	[135]
Annexin 6	↑	Cells	[125]	miR-378a-3p, miR-378d	↑	Cells	[136]
ATPases, annexins, tubulins, integrins & Rabs	↑	Cells	[127]	miR-155	↑	Cells	[137]
P-gp, CD44, galectin-3 & glycogenin-1	↑	Cells	[129]				
TP53	↑	Cells/plasma	[128–134]				

(c)

Thus, EVs are identified as biomarkers, support cancer immunotherapies, and can act as a drug delivery mode for therapeutic agents. In fact, EVs can be genetically engineered to express specific monoclonal antibodies on their surfaces that are able to exert a host immune system response or the delivery of specific drugs for BC treatment (doxorubicin and reverse multidrug resistance) [143]. Some authors proposed HELA-Exos, a α-lactalbumin (α-LA)-engineered BC-derived exosome, to form an in situ dendritic cell (DC) vaccine to boost antitumor activity by promoting the activation of conventional DCs (cDC1s) in situ and tumor-reactive CD8+ T cell responses [144]. In Table 2, we report an actual clinical trial on BC exosomes, which was updated on 19 December 2023 [145].

Table 2. Clinical trial: BC exosomes available at clinicaltrials.gov (last accessed on 19 December 2023).

NCT Number	Study Title	Study Status	Study Type
NCT05955521	Exosome as the Prognostic and Predictive Biomarker in EBC Patients	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT01344109	A Pilot Study of Tumor-Derived Exosomes as Diagnostic and Prognostic Markers in Breast Cancer Patients Receiving Neoadjuvant Chemotherapy	WITHDRAWN	OBSERVATIONAL
NCT05286684	Feasibility of Exosome Analysis in Cerebrospinal Fluid During the Diagnostic Workup of Metastatic Meningitis (Exo-LCR)	RECRUITING	INTERVENTIONAL
NCT04258735	Genetic Characteristics of Metastatic Breast Cancer Patients	RECRUITING	INTERVENTIONAL

Table 2. *Cont.*

NCT Number	Study Title	Study Status	Study Type
NCT04530890	Interest of Circulating Tumor DNA in Digestive and Gynecologic/Breast Cancer	RECRUITING	INTERVENTIONAL
NCT04653740	Omic Technologies to Track Resistance to Palbociclib in Metastatic Breast Cancer	UNKNOWN	INTERVENTIONAL
NCT04288141	A Study to Measure the Expression of the HER2-HER3 Dimer in Tumour and Blood (Exosomes) Samples From Patients With HER2 Positive Breast Cancer Receiving HER2 Targeted Therapies	RECRUITING	OBSERVATIONAL
NCT03974204	Analyses of Exosomes in the Cerebrospinal Fluid for Breast Cancer Patients With Suspicion of Leptomeningeal Metastasis.	WITHDRAWN	INTERVENTIONAL
NCT04781062	Development of a Horizontal Data Integration Classifier for Noninvasive Early Diagnosis of Breast Cancer	ACTIVE_NOT_RECRUITING	INTERVENTIONAL
NCT02977468	Effects of MK-3475 (Pembrolizumab) on the Breast Tumor Microenvironment in Triple Negative Breast Cancer	RECRUITING	INTERVENTIONAL
NCT02662621	Pilot Study With the Aim to Quantify a Stress Protein in the Blood and in the Urine for the Monitoring and Early Diagnosis of Malignant Solid Tumors	COMPLETED	INTERVENTIONAL
NCT02892734	Ipilimumab and Nivolumab in Treating Patients With Recurrent Stage IV HER2 Negative Inflammatory Breast Cancer	TERMINATED	INTERVENTIONAL
NCT04298398	Impact of Group Psychological Interventions on Extracellular Vesicles in People Who Had Cancer	UNKNOWN	INTERVENTIONAL

3. An Artificial Intelligence Approach to Precision Medicine

Artificial intelligence (AI) is crucial in applying the precision medicine approach since it is able to elaborate on a significant amount of any kind of information. To better understand the reasons for the bond between AI and EVs and as a reason to keep investigating this kind of approach, we briefly report the definition of AI and its importance at present, focusing on health and breast cancer. AI, defined as a set of sciences, theories, and techniques, including mathematical logic, statistics, probabilities, computational neurobiology, and computer science, was created during the 1940s and experienced a new boom in 2010, mainly due to improvements in computing science and access to massive quantities of data [146]. AI tries to imitate human intelligence; therefore, it is necessary to develop a machine that has the basic abilities of vision, hearing, touch, induction, reasoning, knowledge acquisition, and the ability to think logically. A machine with these skills should be able to make optimal decisions to improve human life [147]. AI has a variety of applications in our daily lives, from smart watches that use AI to recognize motor activity to online shopping sites that suggest products based on previous purchasers' records, but artificially intelligent systems have also made a great contribution to healthcare for patients' diagnosis, prognosis, and care [148,149]. In particular, in recent decades, AI's application for cancer diagnosis has increased quickly [150]. However, this kind of algorithm should be used carefully. In fact, AI helps in cancer detection, but only if it is combined with clinicians' expertise [151]. In this sense, there seems to be a more interesting use of AI in the field,

where the large number of parameters makes finding a correlation with cancer difficult. In this context, using living databases containing all kinds of patients' data, from miRNAs to symptoms, extremely complex models can be customized for therapy selection, dose calculation, surveillance modality and scheduling, and so on [152].

As explained in previous sections, prevention and screening are the first steps in reducing BC mortality. Currently, mammography is used as a method of early detection, and the introduction of mammography screening was proven to significantly reduce BC mortality [153], although it can lead to false negatives and false positives and thus unnecessary invasive exams [154]. Therefore, in recent years, liquid biopsy has become a good candidate to surpass this limitation. A liquid biopsy allows for noninvasive sample collection and enables an improvement in BC's early diagnosis, screening, prognosis, relapse, disease monitoring, and response to therapy. Many components of tumor cells, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free RNA, miRNA, tumor-educated platelets, and EVs, can be analyzed in a liquid biopsy [155,156]. In this scenario, miRNAs appear as liquid biopsy biomarkers for early and noninvasive BC detection and prognosis [157–160]. These biomarkers come from both free-circulating miRNAs or miRNAs within EVs [161–163]. Thanks to the implementation of high-throughput technologies, such as microarray and NGS, thousands of miRNAs in BC samples can be profiled. Therefore, discerning a causal correlation between miRNAs' expression and a coincidental one is impossible for clinicians or researchers. Here, AI becomes the key to this limitation. Moreover, AI can integrate patients' miRNA profiles with omics data to define a precise strategy for diagnosis and therapy [164,165]. As reported before, EVs' miRNAs carry more personalized information with respect to the circulating miRNA. Here resides the indissoluble bond between AI and EVs. EVs' importance was recognized only in recent decades; therefore, AI applications to EVs' miRNA are still limited. However, it will have future applications. Therefore, we propose an overview of AI's applications with regard to miRNAs in BC for diagnosis and therapy. Rehman et al., using ML algorithms integrated with feature-selection methods, demonstrated that with just three selected miRNAs, classifiers can still detect breast cancer in comparison to the use of many more miRNAs [166]. Other studies report interesting results from integrating the data of circulating miRNAs using multiple classification algorithms, an ensemble of decision trees, neural networks, and support vector machines [167,168]. In recent years, many models have been proposed to detect biomarkers in miRNAs, from a tree-based model to the most advanced algorithms such as deep learning (DL) or convolutional neural networks (CNNs), in different types of cancer, but their applications are still limited. On the other hand, machine learning algorithms are widely used in BC imaging diagnoses, for example, mammography [147,169–171]. AI can detect breast lumps (masses), mass segmentation, breast density, and the risk of breast cancer [172]. However, many scientists have identified challenging issues in these techniques [173]. miRNAs can also be useful for classifying different subtypes of BC; several studies in the literature have shown a particular pattern of expression of miRNAs for each subtype [174,175]. This is probably the most challenging field for clinicians and AI since miRNAs' expression could also be influenced by a patient's specific characteristics. Nowadays, few studies report the use of AI algorithms to detect miRNA as a subtype of biomarker [176]. Just as in BC early detection, the most common applications in BC subtypes' detection are related to clinicopathologic or imaging parameters. Other authors implemented a predictive model based on an advanced ML algorithm using clinicopathological variables. Using a powerful ML algorithm and a support vector machine (SMV), which is ideal for categorization tasks, with the Firefly algorithm, they achieved excellent results compared to other algorithms. They were able to identify TNBC and nonTNBC with high accuracy [177]. Furthermore, a radiomics-based model proposed showed promising results in BC subtype detection, with an accuracy of 0.902 [178]. Nevertheless, this field of AI still has many limitations and necessitates further study. Few studies report the application of AI in prognosis using an miRNA profile [178]. Despite the challenges presented, the exploration of AI in the analysis of miRNA for cancer holds

great promise as a research area. As AI technology continues to advance and larger miRNA expression datasets become available, it is poised to assume an increasingly significant role in the realms of cancer diagnosis, prognosis, and treatment. In the foreseeable future, improvements in AI algorithms and heightened collaboration among researchers, clinicians, and industry stakeholders are anticipated to further facilitate the seamless integration of AI into the routine management of BC. Finally, the amalgamation of miRNA analyses with other omics data has the potential to offer a more comprehensive understanding of BC biology, paving the way for the development of targeted therapeutic approaches in relation to other cancers.

4. Conclusions

Considered waste-disposal mechanisms in the past, nanometer-size lipid EVs derived from living or apoptotic cells provide essential biological functions in intercellular communication. Actually, EVs' proteomic and miRNome profiles have become a popular way to detect and characterize BC, a promising approach for cancer detection and monitoring. Moreover, EV cargo analyses are useful to understand drug resistance mechanisms in cancers and to characterize a particular future, which is useful for an early diagnosis. A multiomics analysis of tumor-derived EVs, which collects information obtained from exosome cargo and uses a machine learning approach, should be attempted to develop an panel of tumor types (phenotypes). Subsequently, the next step should be optimizing the extraction and characterization of exosomes obtained from biological fluids directly at a patient's bedside in order to rapidly obtain predictive factors about tumor type and/or sensitivity and drug therapy to better direct the clinical approach toward a personalized approach. To reach this gold standard, the integration of AI will be necessary. Improvements in AI algorithms and heightened collaboration among researchers, clinicians, and industry stakeholders will be fundamental for the routine management of BC. Moreover, EVs' features, such as their small dimensions, nontoxicity, organotropism target, and low level of immunogenicity, position them as nanocarriers to drive drug delivery.

Author Contributions: Resources, writing—original draft, and writing—review and editing, E.S., A.S. (Annafrancesca Smimmo) and A.B.; investigation and supervision, D.P. and M.D.D.; investigation, resources, and writing—original draft, M.A. and P.B.; drawing of figures, tables, bibliographic research, A.B., A.S. (Annafrancesca Smimmo) and E.S.; investigation, data curation, and software, G.M. and A.S. (Antonella Sciarra); resources, formal analysis, and software, A.D.A. and P.D.M.; investigation and data curation, M.M.M.; critical revision of the manuscript for important intellectual content and final approval, M.D.D. and A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Union—NextGenerationEU through the Italian Ministry of University and Research under PNRR—M4C2-I1.3 Project PE_00000019 "HEAL ITALIA HEALTH EXTENDED ALLIANCE FOR INNOVATIVE THERAPIES, ADVANCED LAB-RESEARCH, AND INTEGRATED APPROACHES OF PRECISION MEDICINE".

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

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