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PERSPECTIVE

Evidence-Based Clinical Use of Nanoscale Extracellular Vesicles in Nanomedicine

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DOI: 10.1021/acsnano.5b08015
ACS Nano XXXX, XXX, XXX−XXX

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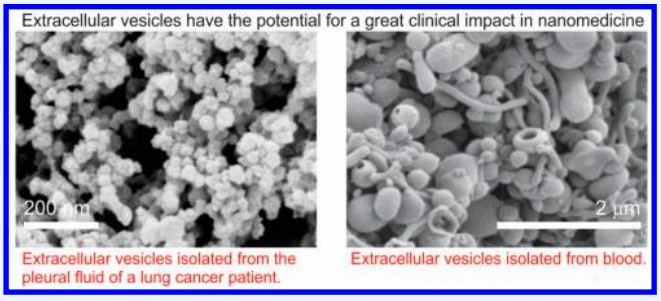
ABSTRACT: Recent research has demonstrated that all body fluids assessed contain substantial amounts of vesicles that range in size from 30 to 1000 nm and that are surrounded by phospholipid membranes containing different membrane microdomains such as lipid rafts and caveolae. The most prominent representatives of these so-called extracellular vesicles (EVs) are nanosized exosomes (70−150 nm), which are derivatives of the endosomal system, and microvesicles (100−1000 nm), which are produced by outward budding of the plasma membrane. Nanosized EVs are released by almost all cell types and mediate targeted intercellular communication under physiological and pathophysiological conditions. Containing cell-type-specific signatures, EVs have been proposed as biomarkers in a variety of diseases. Furthermore, according to their physical functions, EVs of selected cell types have been used as therapeutic agents in immune therapy, vaccination trials, regenerative medicine, and drug delivery. Undoubtedly, the rapidly emerging field of basic and applied EV research will significantly influence the biomedicinal landscape in the future. In this Perspective, we, a network of European scientists from clinical, academic, and industry settings collaborating through the H2020 *European Cooperation in Science and Technology* (COST) program *European Network on Microvesicles and Exosomes in Health and Disease* (ME-HAD), demonstrate the high potential of nanosized EVs for both diagnostic and therapeutic (*i.e.*, theranostic) areas of nanomedicine.



Strategic platforms for nanomedicine seek to exploit the improved (and often novel) physical, chemical, and biological properties of nanomaterials. However, these documents specify that there is an urgent need for biomimetism, namely, the process of simulating what occurs in nature.[1−3]

Extracellular vesicles (EVs), such as exosomes and small microvesicles, are nanovesicles, naturally released from cells in both normal or diseased states. Reflecting their cells of origin, these EVs are assembled by specific sets of molecules including proteins, lipids, metabolites, and nucleic acids. According to their molecular signature, they are able to interact specifically with selected target cells at local or distant sites, within or between organs.[4] Considered to be a vectorized signaling system, they seem to bind to specific membrane microdomains on their target cells; among others, these membrane microdomains contain transmembrane receptors, integrins, and cell-adhesion molecules. To transmit their information, they either fuse with the plasma membrane or get incorporated by endocytotic processes (Figure 1). Thus, in addition to direct cell−cell contact and soluble factors (*e.g.*, cytokines, chemokines, and hormones), EV-mediated signaling provides a third complex and targeted mode of intercellular communication.[5] According to their features, EVs are ideal candidates to serve as biomarkers, nanosized drug-delivery vehicles, and mediators for a variety of therapeutics in oncology, immune therapy, and regenerative medicine.[4,6] Thus, EVs have the potential for great clinical impact in nanomedicine. The dual potential of EVs as diagnostic tools and as therapeutic agents supports their use in "theranostics". This area of nanomedicine focuses on multidisciplinary research to set up new systems for various

DOI: 10.1021/acsnano.5b08015
ACS Nano XXXX, XXX, XXX−XXX

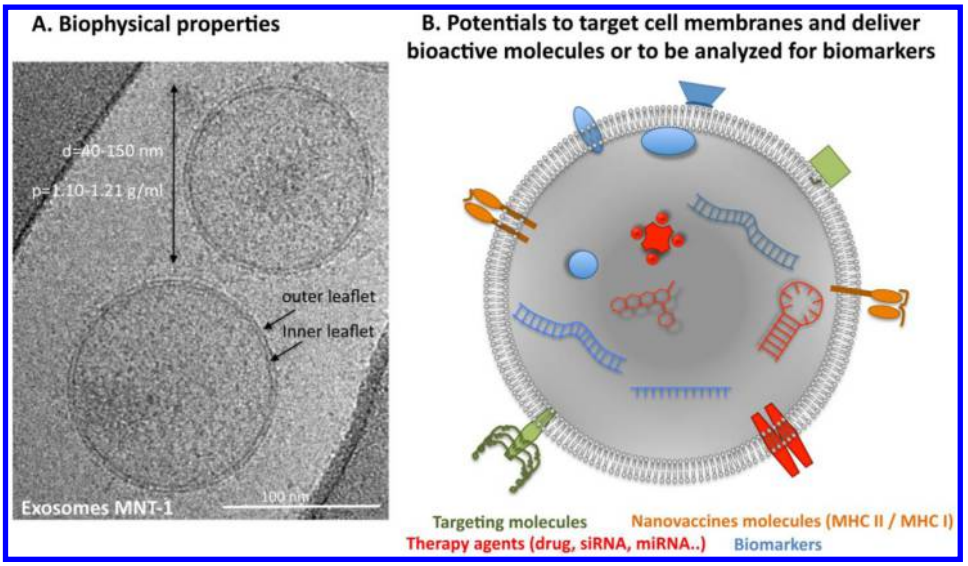


Figure 1. Exosomes, a natural source of nanoparticles to target cell membranes and deliver bioactive molecules or to be analyzed for biomarkers. (A) Extracellular vesicles are 50−300 nm vesicles surrounded by a lipid bilayer. Such physical characteristics are uniquely observed by cryo-electron microscopy (exemplified by a picture of exosomes derived from a human melanocytic cell line observed by cryo-EM. Credit: G. van Niel and A. Di Cicco. (B) Schematic representation of extracellular vesicles and the potential bioactive molecules and biomarkers that can be associated. Families of molecules of interest are classified by color codes as detailed in the text beneath. Credit: G. van Niel.

nanobiomedical applications, ranging from the medical use of nanoplatform-based diagnostic agents, to therapeutic agents, to possible future applications of diagnosis and therapy.[7] Theranostics includes the early detection of diseases, the monitoring of therapeutic responses, and the targeted delivery of therapeutic agents. Theranostics at the nanoscale encompasses nanoprobes, nanocarriers, and nanodiagnostics. However, the most important task of a theranostic strategy concerns theranostic nanoformulations, which deal with the development of new agents based on a "whole-in-one approach", which should have maximal application in the field of personalized medicine. Extracellular vesicles appear to be ideal nanovectors for theranostics, with maximal potential for targeting the disease site with only minimal side effects. If successful, the proof-of-concept in the use of EVs as autologous or allogeneic nanovectors for both diagnosis and therapy of major diseases will enable widespread preclinical and clinical applications.

NANOSIZED EXTRACELLULAR VESICLES AS DISEASE BIOMARKERS

In this section, we present data supporting the future of nanosized EVs as potentially the most reliable biomarkers in medicine. The majority of the available clinical data have been obtained from studies of cancer patients. However, based on the more limited data emerging from studies of other pathologies, the ensemble of the data supports EVs found in bodily fluids as a source of biomarkers for all human diseases evaluated thus far. The current "equipment" of disease biomarkers represents an unmet clinical need, and so far, many approaches have searched for single molecules as biomarkers. As an example, prostate-specific antigen (PSA) is a prominent molecule that is used as a prostate cancer (PCa) marker. Plasma PSA determination is now used worldwide in PCa screening, and it rapidly replaced digital rectal examination for early detection of cancer.[8,9] Plasma PSA is controversial as a PCa biomarker, however,[10−12] due to the likelihood of false positives, including benign prostatic hyperplasia (BPH).[11] Since PSA testing fails to discriminate between BPH and tumors, the use of this analysis causes overdiagnosis and overtreatment with consequent patients suffering side effects.[11,13−15] Prostate-specific antigen values above 4.0 ng per milliliter are considered abnormal; however, cutoff levels can change with age, race, and individual physiological condition,[11,13,14] with no significant progress in the last decades.[16] As multimolecular aggregates, EVs offer the unique opportunity to use a combination of different markers specifically expressed on tumor-derived EVs. In fact, serum PSA has been detected on plasma and urine- derived EVs in a large clinical study.[17,18]

> EVs have the potential for great clinical impact in nanomedicine.

Tumors. Tumor-derived EVs are proposed to contain a tumor-specific molecular signature, qualifying them as potential biomarkers in tumor diagnostics.[19] Such EVs can be harvested from biofluids such as blood and, for some cancer types, urine. In addition to PSA, clinical studies on other EV-associated cancer biomarkers have already been described and are summarized in Table 1. For example, a retrospective study on EV-associated biomarkers in stages III and IV melanoma patients showed increased levels of plasmatic caveolin-1 and CD63-positive EVs.[20] Researchers found that EV-associated caveolin-1 displayed a sensitivity of 69% and specificity of 96.3%, whereas a conventional cancer biomarker used in the follow up of melanoma patients, such as lactate dehydrogenase (LDH) serum levels, was altered in only 12.5% of patients.[20] More recently, a study in patients with pancreatic cancer found that glypican-1 (GPC1)-positive EVs were detectable in the serum of patients with pancreatic cancer with high levels of specificity and sensitivity and could distinguish healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage pancreatic cancer.[21] Moreover, breast cancer patients also presented high levels of GPC1 on EVs, suggesting that an increase of certain EV subtypes might represent a hallmark

DOI: 10.1021/acsnano.5b08015
ACS Nano XXXX, XXX, XXX−XXX

Table 1. Clinical Data Showing the Role of Nanosized Extracellular Vesicles as Tumor Biomarkers

cancer biomarker		indication	biofluid	clinical study size	ref
PSA	prostate cancer	screening/early diagnosis	urine	controls N = 10; disease N = 24	17
PSA	prostate cancer	screening/early diagnosis	plasma	control N = 2; disease N = 5	18
EGFRvIII	glioblastoma	early diagnosis	serum	disease N = 30	137
(phospho)Met	melanoma	early diagnosis/ prognosis	plasma	Controls N = 7; stage III N = 24; stage IV N = 14	23
caveolin-1	melanoma	early diagnosis	plasma	controls N = 58; disease N = 90	20
survivin	prostate cancer	early diagnosis	olasma	HD N = 8; BPH N = 20; disease N = 39	25
CD24	breast cancer	early diagnosis	serum	HD N = 14, disease N = 18	138
EGRF	lung cancer	diagnosis/ personalized medicine	serum	HD N = 9; disease N = 9	139
miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	ovarian cancer	early diagnosis/ prognosis	serum	HD N = 10; stage I N = 10; stage II N = 10; stage III N = 20; stage IV N = 10	140
RNU6-1, miR-320, and miR-574-3p	glioblastoma	early diagnosis	serum	controls N = 50; disease N = 50	141
TMPRSS2:ERG2 and PCA3 mRNAs	prostate cancer	early diagnosis	urine	blinded prospective study N = 30	142
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	colorectal cancer	early diagnosis	serum	controls N = 22; disease N = 88	142
miR-21, miR1225-5p	gastric cancer	prognosis	peritoneal lavage fluid	disease N = 24	28
methylated LINE1 and SOX17 DNA	gastric cancer	diagnosis	gastric juice	HD N = 10; disease N = 20	143
CCR6 and HER-2/neu	gastric cancer	prognosis	plasma	HD N = 10; disease N = 37	144
miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p	lung cancer	early diagnosis	plasma	HD N = 10; benign disease N = 10; malignant disease N = 10	145
TGFB1 and MAGE3/6	ovarian cancer	prognosis/therapy monitoring	plasma	HD N = 10; benign disease N = 10; malignant disease N = 22	146
TYRP2, HSP70, HSC70, VLA-4	melanoma	prognosis	plasma	HD N = 9; stage I N = 2; stage III N = 7; stage IV N = 18	23
miR-21	human esophageal cell carcinoma	prognosis	serum	HD N = 41; disease N = 51	147
KRAS	pancreatic cancer	personalized medicine	serum	HD N = 2; disease N = 2	148
BRAFV600E, EGFR	lung cancer, melanoma	personalized medicine	plasma	*in vivo* model N = 8	96
Glypican-1	pancreatic cancer	early diagnosis	serum	HD N = 100; disease N = 190	21
Glypican-1	breast cancer	early diagnosis	serum	HD N = 100; disease N = 32	21
Hsp60	colon cancer	early diagnosis/ follow up	plasma	controls N = 40; disease N = 57	Cappello
MMP-9, DKP4, EMMPRIN, PODXL	renal cell carcinoma	biomarker discovery	urine	controls N = 23; RCC N = 29	149
EDIL-3/Del1	bladder cancer	diagnostic/ prognostic?	urine	controls N = 12; patients N = 12	150
Presence: LASS2, GALNT1 *Absence: ARHGEF39 and FOXO3*	bladder cancer	diagnosis	urine	controls N = 11; patients N = 8	151
TACSTD2	bladder cancer	diagnosis	urine	controls N = 29; patients N = 37	152
ITGA3 and ITGB1	metastatic prostate cancer	detection	urine	patients with BPH (N = 5), PCa (N = 5), and metastatic PCa (N = 3)	153
miR-34a	prostate cancer	response to treatment	urine	controls N = 36; patients N > 100 (different disease stage)	154
TM256, ADIRF, LAMTOR1 and others.	prostate cancer	diagnosis/follow up	urine	controls N = 15; prostate cancer N = 16	155
AGR2 splice variants	prostate cancer	screening/early diagnosis	urine	BPH N = 15; prostate cancer N = 24	156

of malignant cancers in general. In fact, EV concentration could also be used as an indicator of clinical status. For example, when the effect of treatment with imatinib due to a gastrointestinal stromal tumor was monitored, researchers found that the concentration of EVs before the treatment was increased with respect to the control.[22] Elevated levels of EV-expressing TYRP-2, VLA- 4, HSP70, and HSP90 have been detected in the plasma of melanoma patients.[23] Both HSP70 and HSP90 belong to the family of heat shock proteins (HSPs), which may emerge as a novel class of EV-associated cancer biomarkers.[19] Remarkably, EV-associated levels of HSP60 were dramatically decreased in colon cancer patients after surgical removal of the tumor.[24] As previously mentioned, EVs may also shuttle well-known tumor markers such as PSA. The EV-associated biomarker survivin has also been identified as a promising surrogate biomarker for early diagnosis of PCa.[25] Furthermore, in PCa patients, the EV concentration, as measured by nanoparticle tracking analysis (NTA), is higher than that in the plasma of healthy controls.[26] Interesting results were obtained by comparing *N*-glycan profiles of EVs from indolent and aggressive prostate cancer to those from noncancerous profiles.[27] Other series of clinical data of paramount importance are summarized in Table 1.

DOI: 10.1021/acsnano.5b08015
ACS Nano XXXX, XXX, XXX−XXX

Interestingly, in addition to plasma and serum biofluids, other biofluids may represent valuable sources of EV biomarkers. Peritoneal lavage and gastric juice, for example, may represent promising, noninvasive, and informative sources for gastric cancer diagnosis and/or follow up.[28]

Bronchoalveolar lavage (BALF) is an excellent bioresource for studying lung disorders, including cancer. Bronchoalveolar lavage contains EVs with the morphology, density range, and cargo with different size and vesicular forms compared to that of lung surfactant aggregates. In humans, EVs recovered from BALF of healthy individuals were shown to contain major histocompatibility complex (MHC) molecules that may regulate the local immune defense.[29] In sarcoidosis, however, the quantity of EVs is increased and they present a relatively greater quantity of MHC class I and class II molecules, as well as other bioactive molecules, such as neuregulin-1. Furthermore, they can activate autologous cells to produce inflammatory cytokines.[30] In asthma, BALF EVs exhibit particular microRNA (miRNA) profiles[31] and carry the biosynthetic machinery for leukotriene biosynthesis. Different miRNA contents were found in BALF from non-small-cell lung cancer compared to that from plasma.[32]

Extracellular vesicles have also been isolated from nasal lavage fluid and can be used for studying upper airway diseases.[33] Urinary EVs have also gained much attention as a source of biomarkers, as urine can be collected noninvasively in large amounts, and the isolated EVs are as stable as those from other biofluids. Urine contains highly heterogeneous populations of EVs that are released by the epithelial cells of the genitourinary system,[34,35] and the molecular profiles of urinary EVs seem to directly reflect the pathophysiological state of this system. Therefore, EV-based diagnosis could represent an alternative to current diagnostics, which, for many diseases of the genito-urinary system (kidney, bladder, prostate), rely on poorly predictive, relatively inaccurate biomarkers and/or on biopsy, which is associated with patient morbidity. Recently described isolation, purification,[36] and analytical strategies for urinary EVs facilitate their in-depth molecular characterization in research settings[37,38] and also in hospital settings.[39] During pathogenesis, the released EVs are subjected to disease-specific alterations that can be detected by in-depth proteomic, transcriptomic miRNA analyses or by metabolomics studies[35] to reveal the disease-specific markers that may be validated in preclinical and clinical diagnostic platforms. Notably, studies of the molecular composition of urinary EVs have not been restricted to cancer. Extracellular vesicles may also provide a reliable source of molecules to help understand the metabolic and physiologic state of the urinary tract, providing suitable biomarkers for diseases such as kidney injury, glomerulonephritis, lupus nephritis, diabetic nephropathy, thin basement membrane nephropathy, polycystic kidney disease, and/or fibrosis.[35]

Neurodegenerative Diseases. Extracellular vesicles have been implicated in various neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's, and amyotrophic lateral sclerosis. Central nervous system resident neural and non-neural cells all release EVs that can be detected in biological fluids, thus constituting a potentially beneficial source of information. In recent years, several groups have investigated EVs in blood and cerebrospinal fluid (CSF) during neurological diseases.[40] In several cases, EV analysis is progressing to the clinic despite numerous technological limitations. Among stroke victims, several studies have reported that endothelium and platelets under stress conditions release EVs, whose increase in plasma is proportional to ischemic brain volume.[41] In neurodegenerative disorders, the release of neurotoxic protein aggregates in association with EVs has been reported,[42] and further investigations have explored the roles of EVs in the pathogenesis of these diseases.[43] In fact, an interesting feature of neurodegenerative diseases is that they are characterized by the deposition of certain misfolded proteins into amyloid/amyloid-like aggregates in distinct regions of the brains. The misfolded versions of the proteins are suggested to be the primary culprits in the pathogenesis of AD, for instance. Amyloid proteins are, in fact, released in association with EVs, fully in agreement with the intracellular pathways of amyloid-associated proteins. Both immunoelectron microscopy and density gradient separation of EVs demonstrate that they contain Aβ peptides, suggesting that cells released some of the Aβ peptides in association with EVs, which can enable further deposition of peptides into amyloid plaques or even facilitate long-range transport. Evidence that EVs can participate in the formation of amyloid plaques came from the observation that EVs contain many pro-amyloidogenic lipids such as cholesterol, gangliosides, and sphingolipids, further supporting the hypothesis that they may participate in amyloid formation. While many of the underlying studies indicate detrimental roles of EVs in promoting amyloids, there is some controversy in this regard, as EVs have also been proposed to have a protective role by aiding in the clearance of amyloids.[44,45] Extracellular vesicles detected in the CSF are also suggested to be a potential source of biomarkers for patients with dementia.[46] Similarly, in patients affected by neuroinflammatory diseases such as multiple sclerosis, CSF EVs have been proposed as biomarkers for microglia activation, with the possibility of revealing the activation type (*i.e.*, protective or detrimental), along with disease progression.[47] Finally, seminal work has shown that glioblastoma EVs can be detected in plasma and reflect the corresponding brain tumor volume and its response to treatment, which is an extraordinary potential advancement over invasive brain biopsies or repeated imaging of the brain.[48] These studies suggest that further investigations into the use of EVs as biomarkers are highly warranted for a series of neurological diseases.

Infectious Diseases. The definition of the role of EVs in the context of infection is still developing, as viruses, bacteria, fungi, protozoa, and helminths all secrete forms of EVs, and even prions have been detected in EVs.[49,50] Clinically important pathogens like HIV-1 and hepatitis C and A viruses use EVs either to alter the host cell or to transport themselves to host cells. Infected cells can, in turn, release EVs that contain pathogen-associated molecular patterns (PAMPs) to stimulate the immune response.[51] On the contrary, infectious agents can use EVs to spread infection, facilitating movement of infectious materials, and to evade the host immune system response.[52] The *Leishmania infantum* parasite cultivation strategy used to accumulate exogenous antigens dramatically influences the composition of the recovered exoproteome, where an enrichment of proteins that are known to be essential for infection, such as GP63 or EF1, was observed.[53] The first *in vivo* demonstration of EV secretion by a pathogen was reported in sand flies infected with *Leishmania major*.[54] In this study, parasite EVs were coegested with the parasite during the insect's bite, influencing the host's infectious process and exacerbating the disease symptoms. Thus, EVs have been proposed as relevant candidates to add to the repertoire of virulence factors associated with vector-transmitted infections.[54] Thus, there is great potential for EVs as future biomarkers for infectious diseases of different etiologies, including viral, bacterial, and parasitic diseases.[4]

DOI: 10.1021/acsnano.5b08015
ACS Nano XXXX, XXX, XXX−XXX

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Autoimmune and Other Diseases. Extracellular vesicles seem to play key roles in autoimmune diseases. Behcet's disease (BD) is a complex multiorgan chronic inflammatory condition of unknown etiology wherein the genetic background and environmental factors are thought to be important contributors to disease pathogenesis.[55] In BD patients, plasmapheresis has been shown to induce rapid short-term remission, suggesting that an unidentified plasma-associated factor could be a trigger of flare-ups.[56] These patients were found to have elevated EV numbers in their plasma, and the majority of those EVs were derived from platelets. It has been proposed that a plasma EV number-based stratification of BD could more precisely identify inactive and active disease states and so could aid in its pharmacological management.

Glycosylation changes of EVs are being considered as disease biomarkers. In addition, other types of molecules, such as glycans, have been shown to be EV-linked biomarkers of different diseases, including some inflammatory and autoimmune diseases. For example, urinary EVs from patients with classical galactosemia are characterized by complex-type N-linked glycosylation in contrast to healthy subjects whose EV glycosylation was mainly of high-mannose-type.[57] Surface glycosylation of urinary EVs was also analyzed in autosomal dominant polycystic kidney disease (ADPKD). Here, lectin microarray analysis revealed that 6 out of 43 different lectins have different binding intensity to EVs from individuals with ADPKD compared to EVs from healthy subjects.[58] All of these findings demonstrate the biomarker potential of EV glycans and the applicability of high-throughput techniques (such as lectin microarrays) in selecting lectins that can be used as the basis for establishing new diagnostic assays.

NANOSIZED EXTRACELLULAR VESICLES AS THERAPEUTIC AGENTS

Tumor and Infectious Disease Vaccination. As described above and previously reviewed,[4] EVs from different cell types exert a variety of different physiological functions. Initiated with the observation that B-cell-derived EVs carry functional MHC−peptide complexes on their surface and contain the potential to exert T cell stimulatory functions,[59] interest was raised in using EVs as immune modulatory agents. After it was shown that EVs derived from dendritic cells (DCs) pulsed with tumor antigens mediated antitumor responses,[60] limited numbers of preclinical and clinical trials investigated the role of DC-derived EVs as antitumor therapies. So far, two phase I clinical trials have been performed, one in France and one in the United States, to treat melanoma or small-cell lung carcinoma patients, respectively (Table 2).[61,62] The trials mainly demonstrated feasibility and safety; a small number of patients benefited from the treatment, resulting in the initiation of a clinical phase II trial in France to treat non-small-cell lung cancer patients.[63] Although the later therapy did not induce detectable effector T cell responses, a positive effect on natural killer (NK) cells was observed in some patients.[64] Following the same strategy, EVs from DCs pulsed with pathogens of infectious disease, such as fungi, bacteria, parasitic protozoa, and helminths, might be useful as agents in anti-infectious disease treatment. In fact, proof-of-principle trials have been performed with DC-EVs obtained from *Toxoplasma gondii*-pulsed DCs. Indeed, such EVs conferred protection against subsequent *Toxoplasma* infections in preclinical models.[65−67] Proof-of-principle vaccination trials have been also performed in preclinical animal models for malaria infection. Here, application of EVs from infected reticulocytes were found to protect mice from lethal *Plasmodium yoelii* infections,[68] thus reinforcing the use of EVs as a new therapeutic approach against parasitic diseases.

Table 2. Therapeutic Application of EVs in Human Clinical Trials and a Treatment Attempt

EV source	disease	EV modification	phase	official clinical study title	study size	ref
dendritic cells pulsed with antigenic peptides	melanoma		phase I		n = 15	61
dendritic cells pulsed with antigenic peptides	non-small lung cancer		phase I		n = 13	62
dendritic cells pulsed with antigenic peptides	non-small-cell lung cancer		phase II	phase II trial of a vaccination with tumor antigen-loaded dendritic cell-derived exosomes on patients with unresectable non-small-cell lung cancer responding to induction chemotherapy	n = 22	NCT01159288[64]
ascites	colorectal cancer		phase I		n = 40	74
MSCs	type I diabetes		phase I	phase I study of the effect of cell-free cord blood derived microvesicles on β-cell mass in type 1 diabetes mellitus (T1DM) patients	n = 20	NCT02138331
MSCs	GvHD		treatment attempt		n = 1	76
plant nanovesicles	colon cancer	curcumin loaded	phase I	phase I clinical trial investigating the ability of plant exosomes to deliver curcumin to normal and malignant colon tissue	n = 35	NCT01294072
tumor cells	malignant pleural effusion	chemotherapeutic drug loaded	phase II	phase II study of tumor cell-derived microparticles used as vectors of chemotherapeutic drugs to treat malignant ascites and pleural effusion	n = 22	NCT01854866

DOI: 10.1021/acsnano.5b08015
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In other settings, EVs directly released from pathogens or from pathogen-infected cells have been used to pulse DCs *in vitro* or for subsequent *in vivo* vaccination in a number of preclinical models.[6,69] In a similar context, outer membrane vesicles (OMVs), which are continuously produced by Gram-negative bacteria by vesiculation of the outer membrane,[70] have successfully been used as vaccines.[71] For example, an OMV-based vaccine named Bexsero has been generated by Novartis. It efficiently protects against *Neisseria meningitides* infections and is used as a vaccine against serogroup B meningococcal diseases in children.[72,73] Extracellular vesicles as vaccines have also been used in antitumor therapy. Specifically, in a phase I clinical trial performed in China, EVs from ascites fluid from colorectal cancer patients were used as a vaccine to trigger antitumor activities of DCs (Table 2). Feasibility and safety were demonstrated.[74] Preclinical and clinical EV-based vaccination trials for antitumor treatment or to fight infectious diseases indicate that this therapeutic concept is safe and feasible. The future will show how this can be translated as nanomedicinal approaches in clinics.

Immune Suppressive and Regenerative Therapies. Patient cohorts with a variety of different degenerative and inflammatory diseases have been treated with somatic stem cells, especially with mesenchymal stem cells (MSC), either to promote regeneration or to suppress inflammation.[75] Contrary to the original assumption that stem cells integrate into affected tissue to exert their therapeutic function, they instead seem to act in a paracrine rather than in a cellular manner. The results of increasing numbers of studies in preclinical models and a single treatment attempt of a graft *versus* host disease patient suggest that EVs exert the stem cells' therapeutic effects.[6,76−78] Head-to-head comparisons of MSC and MSC-EV applications have been performed in animal models for acute kidney failure[79] and ischemic stroke.[80] Significant differences were undetected.

Thus, it is feasible that, in the future, stem-cell-derived EVs could be used instead of stem cells to treat various diseases. There are several challenges to be addressed before stem-cell-derived EVs can be approved for the treatment of certain diseases, but compared to therapies with stem cells, they provide a variety of advantages. In contrast to cells as non-self-renewing units, EVs lack any endogenous tumor-formation potential. Furthermore, they can be sterilized by filtration through 0.22 μm filters and can be handled, stored, and characterized more easily than cells. However, it has to be considered that any given EV samples may provide heterogeneous mixtures of different EV subentities, all containing different compositions. For biological activity, heterogeneity may be an important parameter, as EVs may concomitantly convey multiple signals that act synergistically for a defined activity. However, this heterogeneity provides a challenge to the standardization of EV preparations. Recent findings indicate that EVs released from stem/progenitor cells promote tissue regeneration by modulation of gene transcription and induction of epigenetic changes in recipient cells and by delivering growth factors,[81] but studies on the mode of action and identification of potentially healing molecules carried by EVs are a challenge for the field. Rapid translation of EV products for therapeutic use is also challenged by the lack of standard purification and characterization methods that can be used in clinical settings.[6] However, a number of research groups and companies are working on these challenges. It is highly likely that stem-cell-derived EVs as well as EVs from other cell types (*e.g.*, endothelial cell or regulatory T cells[82−87]) will advance to clinical applications within the next few years. Treatments of a range of diseases have been considered as potentially profiting from EV therapies, including autoimmune, chronic, and acute inflammatory diseases such as rheumatoid arthritis, inflammation of connective and vascular tissues, autoimmune inflammatory disease, intestinal chronic inflammatory diseases, Crohn's diseases and ulcerative colitis, type 1 diabetes, multiple sclerosis, cystic fibrosis, graft *versus* host disease, as well as diseases associated with acute tissue damage such as myocardial infarction, ischemic stroke, acute and chronic kidney failure, drug-induced liver injury, hypoxia-induced pulmonary hypertension, hind limb ischemia, and perinatal asphyxia.[6]

Further, within the context of EV research, parasites (including helminths) have been shown to produce EVs expressing immunomodulatory molecules.[50] Such EVs have been considered for the treatment of autoimmune disorders.[88] Indeed, recent studies have shown the usefulness of EVs from *Heligmosomoides polygyrus*, a parasitic roundworm, in a rodent model of allergy.[89]

Drug Delivery. From an applied perspective, synthetic lipoproteins have long been considered to be viable nanocarriers for targeted delivery of drugs[90−93] because numerous cancers overexpress light density lipoprotein receptor. The most widely exploited drug-delivery platform is based on liposomes or lipid-based nanoparticles (LNPs). These nanoformulations have been used effectively to encapsulate various macromolecular drugs including proteins, chemotherapeutics, imaging agents, and different species of therapeutic RNAs (*e.g.*, small interfering RNA, siRNA). Many of these bind to apolipoprotein E (ApoE) in blood and facilitate efficient delivery to the liver.[92] Despite being effective, the main limitations with current nanocarriers based on LNPs are potential toxicity/immunogenecity and limited ability to penetrate organs and tissues outside the reticuloendothelial system (RES). Hence, EVs have emerged as candidates for drug delivery. Several reports have indicated the high delivery potential of EVs, such as paclitaxel in autologous prostate cancer EVs,[93] in particular, in relation to endogenous protein and miRNA transfer.[94] Furthermore, they can contain gDNA.[95,96] Extracellular vesicles have also successfully been used to deliver exogenous drugs such as small molecules, miRNAs, and siRNAs.[97] Recently, it was demonstrated that even an exogenous protein (catalase) can be loaded into EVs and subsequently confer neuroprotection in models of Parkinson's disease.[98]

By engineering EVs to display targeting moieties, tissues beyond the RES are amenable to targeting even after systemic delivery.[99,100] Although EVs hold true potential as drug-delivery platforms, we note that the efficacy of loading of the lipophilic small drugs is good,[94] but in the case of siRNA, it is very low.[101] Similarly, in the case of endogenous miRNA transfer with EVs, caution has to be taken, as the majority of extracellular RNA is not associated with EVs.[102] Thus, strategies are needed that can increase exogenous drug loading or methods of manipulating producer cells that permit selective loading of proteins or RNA into EVs. Examples where loading of drugs (in addition to the self-assembly of lipophilic drugs) could be achieved include the use of extruded vesicles from cells as well as synthetic EVs.[103−105] However, it remains to be shown whether such systems are equally effective and safe as naturally secreted and purified EVs. In this context, it is interesting to note that exosomes released from melanocytes and melanoma cells were recently found to interact physically with ApoE-associated

DOI: 10.1021/acsnano.5b08015
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lipoparticles, maybe indicating that each of the different nanomessengers can be combined to make use of each of their advantages as a drug-delivery tool.[106]

Nanoparticle PEGylation (PEG is a coiled polymer of repeating ethylene ether units with dynamic conformations) is the current standard for stealth in nanoparticle drug delivery. However, potential immunological response and absence of active targeting prevent its widespread use.[107] PEGylated nanoparticles rely on the enhanced permeability and retention (EPR) effect for tumor targeting, which is absent if primary tumors or metastases are smaller than 100 mm^3.[108] Bioconjugation approaches of PEGylated nanoparticles with targeting ligands to self-organize into some useful conformation are ambiguous because of denaturation of proteins during the conjugation process and the overall difficulty of duplicating biological complexity on the nanoscale.[109] These disadvantages are largely absent when functionalizing PLGA (poly(lactic-*co*-glycolic acid)), gold, or silicon nanoparticles with cellular plasma membranes. This has already been successfully demonstrated with cancer cell membranes to induce an immune response (*i.e.*, as a vaccination)[110] and by leukocyte and erythrocyte membranes to enhance circulation times (*i.e.*, by avoiding immune uptake)[109,111] and increasing cancer cell specificity.[111] These hybrids possess the ease-of-use and flexibility of synthetic materials, as well as the functionality and complexity of natural materials. Thus, EV-sized, cell-membrane-camouflaged nanoparticles are a delivery strategy with the potential to improve the therapeutic efficacy of the treatment of a variety of diseases.

Extracellular Vesicles in Milk. According to epidemiological analysis, human milk is better than artificial infant formula in allowing appropriate metabolic programming and protecting the baby against conditions such as type 2 diabetes, obesity, and hypertension in later life. Purification of EVs from breast milk has been described.[112,113]

EV-sized, cell-membrane-camouflaged nanoparticles are a delivery strategy with the potential to improve the therapeutic efficacy of the treatment of a variety of diseases.

Breast milk is rich in many bioactive molecules all sent to the baby in different packaging (*e.g.*, exfoliated cells, microvesicles, fat globules). Finding and using natural sources of EVs loaded with bioactive miRNA from mammals will require extensive effort in purifying and characterizing EVs both from milk and from digestive fluids of the baby. The design of artificial nanoparticles for breast milk supplementation remains unresolved.

Other Therapeutic Implications. In discussing EVs' potential for therapy, a number of glycobiological aspects of EVs are worth mentioning. First, from a fundamental point of view, glycans (as other molecules) are specifically enriched or excluded from EVs. The fact that A/B blood group antigens are excluded from EVs compared to the plasma membrane is what enables EVs to be used therapeutically.[114] Second, from a technological point of view, specific targeting of EVs loaded with therapeutics may be accomplished by displaying peptides on their surfaces. An associated issue is proteolytical degradation of such peptides in circulation, but this can be prevented by introducing a glycosylation motif at specific positions, without influencing protein–target interactions.[115] Third, for applications, specific glyco-profiles of EVs related to several diseases were detected by lectins, and new adjuvant cancer therapy strategies employing lectins to remove circulating cancer-derived EVs selectively have been proposed.[116]

Extracellular Vesicles in Cosmetics. Recent studies have highlighted roles for EVs in the skin. Maintenance of skin pigmentation, which is required for skin color and for photoprotection against harmful UV radiation, is the consequence of tight intercellular communication between keratinocytes and melanocytes. In an academic–industrial collaboration between the Raposo group and Clarins Laboratories, it was shown that human primary keratinocytes secrete EVs that are targeted to melanocytes to modulate pigmentation. Extracellular vesicles are key actors in skin pigmentation, enhancing melanin synthesis by increasing the expression and activity of melanosomal proteins.[117] These effects are connected to particular miRNA compositions. Furthermore, the function of keratinocyte-derived EVs has been demonstrated to be photo-type-dependent and is modulated by UVB. This study not only uncovers an important physiological function for EVs in our understanding of how pigmentation is regulated by intercellular communication but also opens new avenues for technological development. For example, based on these findings, Clarins recently launched a new product that, likely by acting on the composition of EVs, inhibits overproduction of melanin ("Sérum Mission Perfection de Clarins").

PRECLINICAL DATA SUPPORT A GREAT FUTURE FOR NANOSIZED EXTRACELLULAR VESICLES IN NANOMEDICINE

Based on the clinical evidence (outlined above) showing that EVs may be exploited as either disease biomarkers or therapeutic tools, it is conceivable that EVs may represent key players in the future of nanomedicine and, in particular, in the field aimed at defining the most biomimetic approach in nanomedicine. The presence of EVs in the plasma of both healthy individuals and those with various diseases suggests that EVs may serve as vectors for transferring information to tissues and organs far from their places of production, that is, acting in a paracrine manner.

These actions indicate that EVs may well diffuse normal, abnormal, or aberrant messages to cells both close to their origins and at distances. This, in turn, suggests that EVs may play key roles as nanodevices belonging to integrated networks involved in multiple pathophysiologies. Our current understanding is that EVs are key regulators of normal functions of the body.[4]

It is conceivable that in the near future nanosized EVs may be helpful in the screening and diagnosis of viral diseases. In fact, we have evidence that EVs are natural delivery systems for a variety of viruses including EBV, HCV, HIV, coxsackie virus B1, and hepatitis A.[118−124] Moreover, prion proteins are shuttled by nanovesicles, although only preclinical data are available to date.[125−129] The data strongly suggest that EV-based tests will be included in new screening approaches for transmissible diseases, for example, in blood donors.

Preclinical data also support the use of EVs as the most biomimetic nanovectors for a variety of molecules, including proteins, nucleic acids, and chemicals. Nanosized EV-encapsulated curcumin, delivered by the intranasal route, is efficient in preventing brain inflammation and is more effective than curcumin alone.[130] Moreover, EVs released by human tumor cells or human tumors treated with cisplatin contain cisplatin in its active/native form.[131] The future of the clinical use of EVs

DOI: 10.1021/acsnano.5b08015
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depends on a high level of networking between researchers involved in the field and a strategic approach on how to guide future research. A level of consensus was recently achieved by the International Society for Extracellular Vesicles (ISEV), although it has not yet been fully implemented in clinical studies.[6,132,133]

Funded by Europe's Horizon 2020 program, a consortium of academic, clinical, and industry partners with a common interest in EVs has been established. This cooperation in science and technology, entitled the European Network on Microvesicles and Exosomes in Health and Disease (ME-HaD), includes EV researchers from 27 European countries and allied groups from the United States and Australia. The aim of ME-HaD is to foster multidisciplinary approaches to research in this field, including the theranostic relevance of EVs, with the ultimate goal of exploiting EVs for clinical applications, which is achievable only through coordinated efforts and valorization. Guided, mentored, and trained by more experienced EV researchers within ME-HaD, this consortium currently includes membership of more than 250 early stage researchers, who will hopefully be the future leaders in the field of EV research and application.

THE FUTURE OF EXTRACELLULAR VESICLES IN NANOMEDICINE AND INDUSTRY INVESTMENT

The life science market is remarkably conservative, relative to the extremely dynamic EV market. For instance, ultracentrifugation is still the gold standard for EV isolation, used by ~60% of researchers in the field. The acceptance of novel commercial tools is slow. The pharma industry, however, is open to EV-based solutions in companion diagnostics and personalized medicine if they are reliable and specific for EVs. Thus, EV analysis will likely enable rapid *in vitro* diagnostic or laboratory-developed/exoteric tests for hospitals or centralized laboratories and will also be tools for quality control of production processes and surrogate markers for the development of novel therapies.

1. In order to surmount regulatory hurdles (which are diverse and rapidly evolving in the biggest markets, such as the United States, the European Union, and Asia) and both market and cultural insertion, extensive clinical validation and technology beta testing is needed. This calls for time, money, and collaborative research efforts including multiple stakeholders so as to produce definitive evidence that EV marker assays outperform and/or complement conventional diagnostics, thus leading to a broad acceptance from clinicians and patients.

2. The technological readiness level of EV analysis might not be sufficiently robust. Fabrication of novel materials and sophisticated devices (microfluidic chips or specific sensors) has produced some exciting proof-of-concept applications of advanced technologies. These have limited application in routine laboratory practice, however, due to cost or because they still are not guaranteed to work in "all hands", according to their inventors. On the other hand, we have convincing evidence of EV detection and analysis using cost-effective and familiar formats of assays that are compatible with off-the-shelf laboratory equipment such as plate readers or polymerase chain reaction (PCR) cyclers.[20,134]

Extensive developments in the field of EVs, in particular, the promising preliminary results from using EVs, therapeutically and as diagnostics markers, has resulted in a number of start-ups that have initiated commercialization of these achievements. Big and small pharmaceutical companies have already taken first steps in evaluating development, costs of the investments, and registration and commercialization strategies. Promising results and demands for new therapeutic EV development will, undoubtedly, stimulate pharmaceutical industry interest in the production of therapeutic EVs at larger scales.

The active participation of the pharmaceutical industry should support the development of the field of EVs. Large companies, with a high volume of starting material and the availability of analytical tools, will accelerate development of the detection and characterization of EVs by both the evaluation of commonly used techniques and the development of new techniques. In addition, the pharmaceutical industry's high demands for quality regulation will accelerate standardization of EV sample collection, isolation, and analysis methods, which are highly desirable outcomes.

CONCLUSIONS AND PROSPECTS

Nanosized EVs, which may both contain disease biomarkers and/or be the vectors of potential therapeutic molecules, thus represent the ideal theranostic approach. This new multidisciplinary field focuses on building nanosystems for future joint applications of diagnosis and therapy. The theranostic "all-in-one approach" has great potential in the field of personalized medicine, as it enables the detection and monitoring of a disease in individual patients, possibly in early clinical stages, as well as targeted drug delivery at the site of the disease. Here, we have included data dealing with clinical studies and provided evidence that EVs are currently used in clinical research as biomarkers of disease and as therapeutic tools. Thus, this Perspective emphasizes the evidence that natural nanosized EVs are critical to the future of nanomedicine.

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Notes

Views expressed in this Perspective are those of the authors and not necessarily the views of the ACS.
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the european COST action for supporting the European Network on Microvesicles and Exosomes in Health and Disease (ME-HaD, BM1202, www.cost.eu/COST_Actions/BMBS/Actions/BM1202) who funded parts of the publication of this work. Pictures of the graphical abstract were adapted from Šuštar *et al.*[135] and Mrvar-Brecko *et al.*[136]

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DOI: 10.1021/acsnano.5b08015
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