REVIEW ARTICLE

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Circulating Extracellular Vesicles in Human Disease

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An illustrated glossary and a video overview of extracellular vesicles are available at NEJM.org

It is well known that cells release fluid-filled sacs (vesicles) to the extracellular environment during cell death, or apoptosis, but it has been increasingly recognized that healthy cells may also release vesicles in the process of normal functions. Vesicles that are released by healthy cells have a wide variety of names (e.g., ectosomes, microparticles, microvesicles, exosomes, and oncosomes), with the term "extracellular vesicles" typically used as a generic reference to secreted vesicles.[1] Extracellular vesicles are found in circulation and contain cell-derived biomolecules (e.g., RNA, protein, and metabolites).

Extracellular vesicles are implicated in trafficking of molecules between cells and as such have an effect on physiologic function and serve as biomarkers for disease (see video). Nevertheless, important limitations — including practical difficulties in assaying low concentrations of extracellular vesicles in circulation, identifying their tissue of origin, and specifying which molecular cargo is most relevant — have restrained enthusiasm for research into the role of extracellular vesicles in vivo. The goal of this article is to provide a brief introduction to extracellular vesicles, with a specific focus on translational and clinical studies to highlight emerging evidence that suggests a potential role in human disease. Given the explosion of work in this field, it is difficult to cover the breadth of diseases in which extracellular vesicles may be functionally relevant. As such, the reader is referred to the expanding literature in this field for more details.[2,3]

What Is an Extracellular Vesicle?

Extracellular vesicles are membrane-bound organelles that are extruded from tissues containing different types of molecular cargo (e.g., RNA, protein, and metabolites). The classic dichotomy of extracellular vesicles has relied on size and biogenesis. For example, exosomes are defined as having a diameter of less than 150 nm, whereas ectosomes or microparticles (microvesicles) are defined as having a diameter up to 1000 nm. Regarding the formation of extracellular vesicles, exosomes are described as arising from multivesicular bodies and a wide range of cells. Although the extant literature relies on the size-based definition, recent expert consensus has recognized that understanding the biogenesis and differences in the molecular contents of different-sized extracellular vesicles may be relevant.[4] Currently, the mechanisms of extracellular-vesicle biogenesis and intracellular signaling pathways that compel their release are areas of ongoing study that are outside the scope of this review. We note that many studies focus on exosomes specifically; throughout this review, we refer to "extracellular vesicles" to specify the breadth of circulating extracellular vesicles, with the caveat that more investigations into their physical and biochemical properties are warranted.

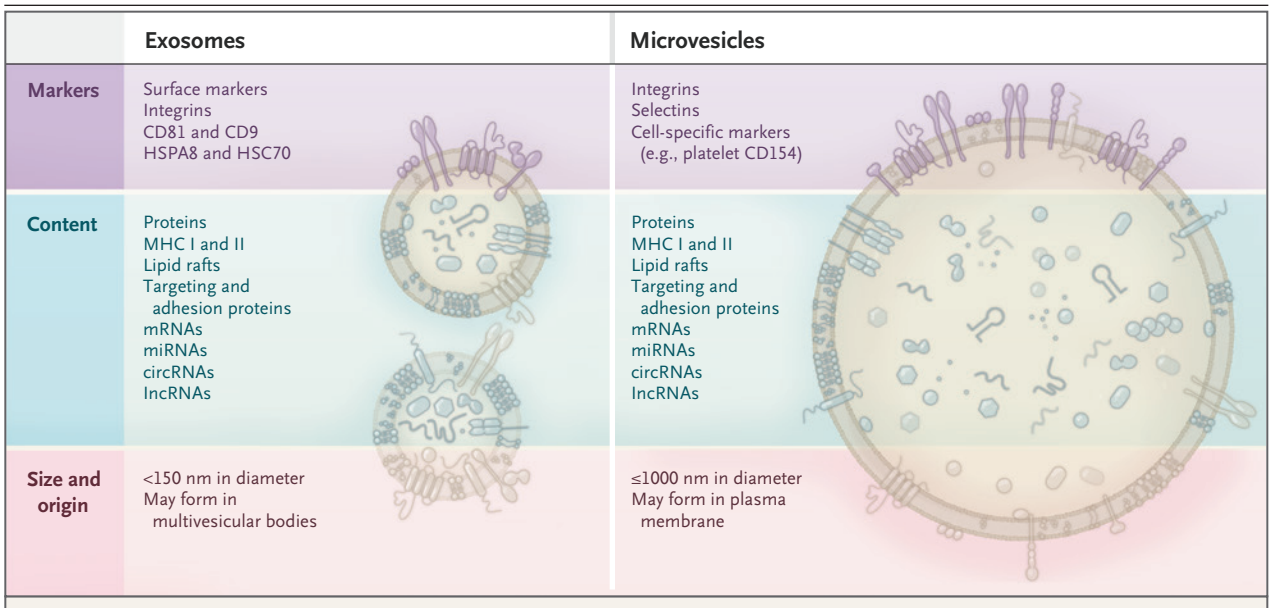

Figure 1. Size and Contents of Extracellular Vesicles.

Classic descriptions of extracellular vesicles have relied on size, with exosomes defined as having a diameter of less than 150 nm and larger vesicles (microvesicles, including ectosomes, microparticles, and oncosomes) measuring up to 1000 nm in diameter. Microvesicles may contain endocytic markers that distinguish them from other organelles with internal membranes, such as autophagic bodies or multilamellar lysosomes, although overlap in these markers is known to occur. Exosomes may contain protein markers associated with the endosomal pathway that is specific to their mode of formation. Extracellular vesicles that are formed by shedding from the plasma membrane may contain markers such as integrins and P-selectin. Extracellular vesicles may contain a variety of proteins, lipids, and nucleic acids that may be specific for, and thereby reflect, the cell of origin. They may also contain a wide variety of small noncoding RNAs, such as microRNAs, piwi-interacting RNA, and small nucleolar RNAs. However, there is substantial overlap in content among extracellular vesicles of various sizes and origins. MHC denotes major histocompatibility complex, and piwi P-element–induced wimpy testis; the RNA designations are circular (circ), long noncoding (lnc), messenger (m), and micro (mi).

BIOMARKERS OF PHYSIOLOGIC FUNCTION AND DISEASE

In the normal circulation, a large number of extracellular vesicles may arise from platelets or megakaryocytes,[1] although most cells are thought to release extracellular vesicles. Accordingly, extracellular vesicles are present in many different biofluids in addition to blood (e.g., breast milk, saliva, urine, and cerebrospinal fluid). In terms of contents, extracellular vesicles contain proteins, metabolites (including lipids), and nucleic acids (e.g., RNA) that may reflect cellular origin and function (Fig. 1).

The functional relevance of the release of extracellular vesicles into the circulation (and the cellular mechanisms leading to release and metabolism) continue to be investigated. Extracellular vesicles may function in intercellular communication through the transfer of proteins and RNA, with a relevant effect on systemic processes such as immune function[5] and inflammation,[6] as well as a host of disease- and organ-specific processes. In light of their importance in intercellular signaling and cell-to-cell communication, there is increasing interest in their potential role as noninvasive biomarkers for disease detection and prognosis. However, several key limitations have blunted enthusiasm for their widespread adoption into the clinical realm, as outlined below. With these caveats, we focus next on a selection of diseases (by no means exhaustive) in which extracellular vesicles have been suggested as functional biomarkers (Fig. 2).

CANCER

The role of extracellular vesicles and their contents as potential contributors to oncogenesis, metastatic disease, and resistance to chemotherapy is a rapidly expanding area of research in cancer biology. Extracellular vesicles can fun-

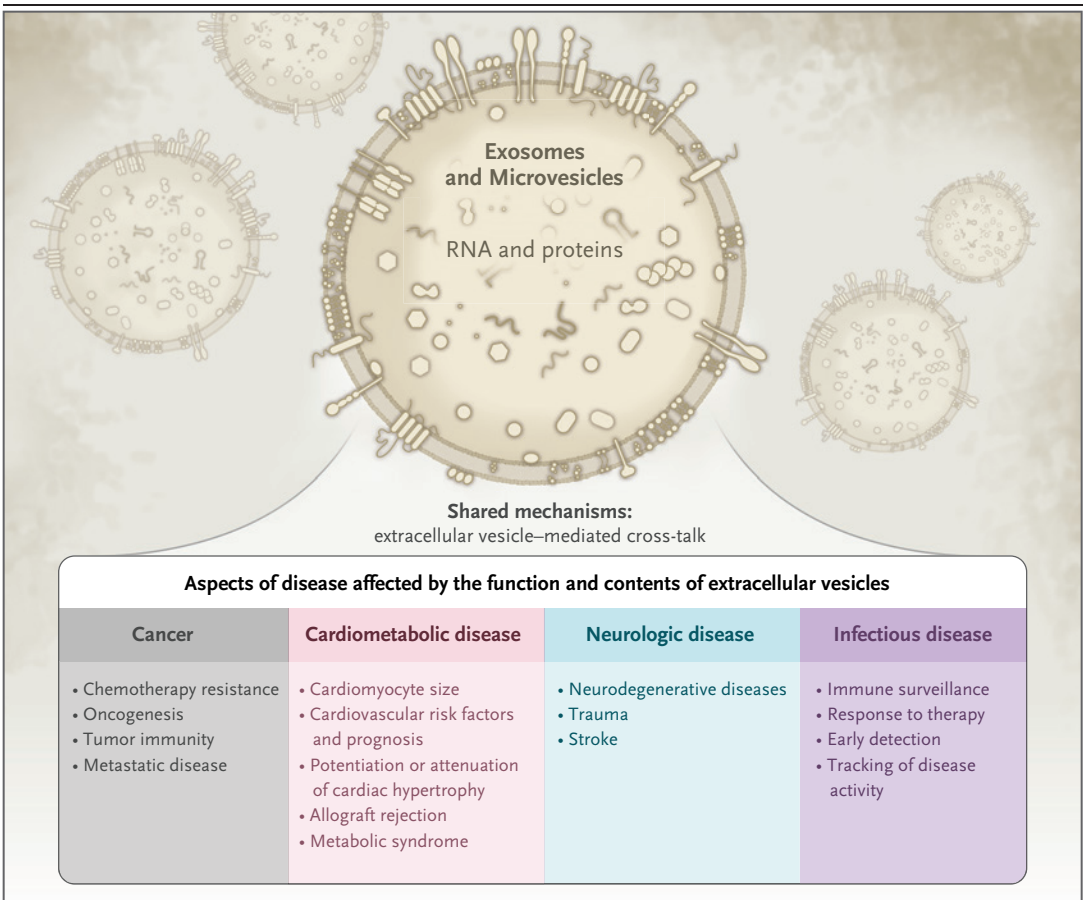

Figure 2. Possible Role of Extracellular Vesicles in the Diagnosis and Prognosis of Various Diseases.

Extracellular vesicles contain partially overlapping contents and shared mechanisms for communication and function. Investigations into a broad variety of diseases — oncologic, cardiometabolic, neurologic, and infectious — support a functional and potentially therapeutic role for extracellular vesicles. The results of these studies suggest that extracellular vesicles may be useful as biomarkers of disease progression or therapeutic response.

nel chemotherapeutic agents out of a cancer cell through bulk transport within vesicles or active efflux mechanisms[7,8] and may also express molecules that divert biologic agents away from malignant cells (e.g., human epidermal growth factor receptor 2 [HER2] in breast cancer[9]). In ovarian cancer, interactions between stromal tissue and cancer cells that are mediated by extracellular vesicles may transfer microRNA 21, which can potentiate resistance to chemotherapy.[10] Extracellular vesicles may also be involved in metastasis by harboring molecules that are involved in the epithelial–mesenchymal transition[11] or preparing target tissues for metastasis.[12] Furthermore, studies of breast cancer–derived exosomes suggest that they contain proteins required for microRNA-mediated gene silencing and may transform nonmalignant cells.[13] Finally, on the basis of the premise that extracellular vesicles bearing antigens derived from cancer cells may originate from the parent cancer cells, investigators have isolated extracellular vesicles from the plasma of patients with acute myeloid leukemia to evaluate whether these vesicles may alter the expression of molecules important in immune-cell function.[14] These findings implicate extracellular vesicles in different steps of carcinogenesis and therapeutic responses and suggest that they may play a functional role in specific aspects of cancer.

Studies enrolling human participants have started to illuminate a role for extracellular vesicles in diagnosis, prognosis, and therapy in cancer. In ovarian cancer, the quantity of circulating

extracellular vesicles that presumably originated from tumor tissue (on the basis of a cell-surface marker) was proportional to the cancer stage and was greater than the quantity in healthy controls.[15] In addition, extracellular vesicles and their cargo have been explored as part of a diagnostic or prognostic strategy for various cancers, including cancers of the hepatobiliary system, breast, lung, gastrointestinal tract, skin (melanoma), prostate, and nasopharynx.[16-26] Efforts have focused on the discovery of biomarkers in extracellular vesicles across multiple biofluids relevant to each cancer, including proteins in circulating blood for colorectal cancer,[27] urinary microRNAs for prostate cancer[28] and proteins for bladder cancer,[29] and microRNA profiles in cerebrospinal fluid for brain cancer.[30] Specific molecules in extracellular vesicles have also been linked to diagnosis and staging (e.g., microRNA 21 for esophageal cancer[31]).

In light of this potential to affect the pathophysiological processes in cancer development, extracellular vesicles are increasingly being investigated as part of a new mode of cancer treatment.[32] Specific ongoing efforts include the use of extracellular vesicles to mediate anticancer immunity[33] and to serve as vectors for small-molecule delivery.[34] Further basic and clinical investigations of the specificity and off-target effects of extracellular vesicles are needed before such uses can be adopted in clinical practice.

CARDIOMETABOLIC DISEASE

The function of extracellular vesicles in cardiovascular and metabolic diseases shares features with its role in cancer, with emerging evidence of cross-talk between different cell types in the heart that is mediated by extracellular vesicles (Fig. 2). For example, angiotensin II elicits the release of extracellular vesicles from cardiac fibroblasts, which can potentiate cardiac hypertrophy through altering of gene expression in cardiomyocytes.[35] In addition, microRNA 155 that is contained within macrophage-derived extracellular vesicles decreases fibroblast proliferation and increases inflammation in mice,[36] which suggests that extracellular vesicle–mediated cross-talk between noncardiomyocyte-cell types may affect cardiac structure. Indeed, translational studies have borne out the concept that circulating extracellular vesicles obtained from patients with dilated cardiomyopathy can transfer a pathologic molecular phenotype to cardiomyocytes in culture,[37] similar to what has been observed in cancer.

In humans, the number of circulating extracellular vesicles can be increased in certain forms of cardiovascular disease (e.g., heart failure[38]). Most ongoing large cohort studies have focused on metabolites, proteins, and transcriptional analyses from whole plasma, and the isolation of extracellular vesicles to quantify these biomarkers is a newer field. The concentration of circulating exosomes in plasma is proportional to circulating levels of cardiac troponin and increases at 24 to 48 hours after coronary-artery bypass surgery.[39] The quantity of circulating microparticles has been associated with risk factors for cardiovascular disease[40] and with long-term cardiac prognosis (for endothelial-derived microparticles[41]). Protein studies of circulating extracellular vesicles involving recipients of cardiac transplants have suggested that the presence of a handful of proteins (some of which are involved in immunologic pathways) can identify patients with acute allograft rejection.[42] Furthermore, in a case–control study involving patients with and without heart failure after myocardial infarction, several microRNAs that are prognostic for heart failure and left ventricular remodeling were enriched in circulating extracellular vesicles.[43]

The interrogation of protein expression within extracellular vesicles has also uncovered several proteins that are related to acute coronary disease, including an immunoglobulin receptor, cystatin C, and complement.[44] In mice with myocardial infarction, the injection of extracellular vesicles derived from cardiac progenitor cells into the peri-infarct zone limited remodeling, similar to the result after the injection of progenitor cells. The benefits of extracellular vesicles may arise from effects on cardiomyocyte survival and myocardial fibrosis.[45,46]

Similar to cardiovascular disease, cardiometabolic diseases (e.g., obesity) are also characterized by an increased number of circulating microparticles.[47] Recent studies in mouse models have shown that extracellular vesicles from adipose tissue can modulate hepatic gene expression in a manner that is dependent on noncoding RNAs,[48] which suggests a potential pathogenic role for extracellular vesicles acting at a distance

in metabolic diseases. Much of the clinical data in this area remains correlative but suggests important links among diabetes, obesity, and cardiovascular disease. For example, shifts in the microRNA cargo in extracellular vesicles in patients with diabetes (e.g., reduced amounts of microRNA 126 and 26a in endothelial cell–derived microparticles) may be associated with cardiovascular disease,[49] which potentially links the two conditions. In turn, therapies directed against dysglycemia may alter circulating extracellular vesicles. In a small study[50] involving patients who had undergone bariatric surgery, the postsurgical shift in insulin resistance was accompanied by changes in microRNAs in extracellular vesicles that are implicated in insulin signaling. Although a great deal of work remains to translate early animal-based findings to humans, these preliminary findings suggest that extracellular vesicles could serve as functional biomarkers of cardiovascular and cardiometabolic diseases.

NEUROLOGIC DISEASE

With the emergence of similar themes in cancer, cardiovascular disease, and neurologic disease, researchers have intense interest in exploring the potential role of extracellular vesicles in neurodegeneration, trauma, and stroke. In models of traumatic brain injury, the presence of increased amounts of microRNA 124 in extracellular vesicles from microglia has been associated with decreased inflammation and improved regrowth after injury.[51] Similarly, in models of stroke, microRNA 133b contained within extracellular vesicles from stromal cells may be involved in improvements in neural structure.[52] The notion that extracellular vesicles may be involved in transferring phenotypes between diseased and healthy tissues, as has been observed in patients with cancer and cardiovascular disease, may also apply to neurocognitive diseases (e.g., Lewy body dementia[53]).

Emerging studies suggest a role for extracellular-vesicle cargo in neurocognitive diseases. Phosphorylated tau protein that is associated with extracellular vesicles in cerebrospinal fluid appears early in Alzheimer's disease,[54] and secretion of extracellular vesicles containing tau may be mechanistically important in Alzheimer's disease.[55] Altered expression of key proteins that are directly involved in synaptic physiologic function can be found in extracellular vesicles from plasma in direct proportion to cognitive dysfunction in adults with Alzheimer's disease,[56] and select extracellular-vesicle protein contents in circulating blood can indicate a high risk of Alzheimer's disease several years before clinical diagnosis.[57]

Next-generation RNA sequencing and polymerase-chain-reaction assay of extracellular vesicles from serum have identified a panel of 16 microRNAs that are dysregulated in Alzheimer's disease.[58] In addition, specific microRNA contents in extracellular vesicles from cerebrospinal fluid may be distinct in different neurocognitive diseases (e.g., Parkinson's and Alzheimer's disease[59]), findings that may be applicable to diagnosis if they are verified in large studies. In one study, circulating levels of extracellular vesicles containing tau protein were higher in players in the National Football League than in healthy controls and were related to poorer neurocognitive performance in football players,[60] findings that are consistent with an emerging recognition of chronic traumatic encephalopathy as a related disease entity. These results are especially intriguing in light of the emergence of point-of-care detection of circulating neuronal-cell–derived exosomes that are present after concussive injury in mice.[61] Similar to approaches in cancer, extracellular vesicle–based therapies that target neurodegenerative disease are rapidly emerging — for example, engineering of extracellular vesicles containing small interfering RNAs that alter the expression of an enzyme involved in the generation of beta-amyloid deposits.[62]

INFECTIOUS DISEASE

Viruses can harness the cellular machinery of extracellular vesicles for multiple purposes, including increasing infectivity and evading the immune system.[63] Extracellular vesicles that are derived from hepatoma cells infected with hepatitis C in vitro contain genetic information and proteins that promote infection in the absence of active interaction between viruses and target cells. Moreover, this extracellular vesicle–mediated infection may elude antibody-mediated immune clearance.[64]

From a biomarker perspective, the morphologic features and quantity of extracellular vesicles appear to correspond to the activity of viral

infection. In patients who are infected with the human immunodeficiency virus type 1 (HIV-1), both the size and quantity of circulating extracellular vesicles are inversely proportional to the ratio of CD4 to CD8 T cells, with a smaller size and lower quantity associated with a higher CD4:CD8 ratio (indicating strong immune function). The control of HIV-1 infection is associated with near normalization of the morphologic features of extracellular vesicles.[65] Furthermore, treatment with antiretroviral therapy is associated with a reduced amount of microRNA 155 (a proinflammatory noncoding RNA) and of microRNA 223 in extracellular vesicles, which suggests that the contents of extracellular vesicles may provide a molecular signature of response to therapy.[65] The infectious potential of extracellular vesicles extends to prion disease,[66] in which extracellular vesicles bearing prion protein that has been converted into its pathogenic insoluble conformer (PrPSc) may transmit the disease.[67] Further studies of extracellular vesicles as biomarkers for therapy, early detection, or tracking of infection are warranted.

MAJOR CHALLENGES

Enthusiasm about basic, clinical, and translational studies of extracellular vesicles has spurred the formation of various international professional societies (e.g., the American Society for Exosomes and Microvesicles and the International Society for Extracellular Vesicles) to guide and standardize protocols for the study of extracellular vesicles and biomarker development.[4,68] However, despite such enthusiasm regarding extracellular vesicles as biomarkers of human disease, major technical and biologic roadblocks are only beginning to be addressed.

From the perspective of model-system investigations, a lack of clarity regarding how extracellular vesicles are formed and how to track their origin and destination in vivo represents an impediment to mechanistic studies. With respect to human translation, primary challenges in claiming extracellular vesicles as useful biomarkers of disease revolve around several major, interconnected themes. For example, there is no standardized method for isolating and characterizing extracellular vesicles from human biofluids. In addition, data are lacking on the influence of clinical factors (e.g., age, sex, and race) on the quantity and cargo of extracellular vesicles, independent of disease state. Moreover, the demands of blood-sample processing in the required volumes for the isolation of extracellular vesicles may limit the use of existing banked samples in human cohort studies.

Various methods have been advanced for isolating extracellular vesicles (Fig. 3). These approaches have included (but are not limited to) density gradient centrifugation, antibody affinity columns, and precipitation–ultracentrifugation techniques. Of these methods, ultracentrifugation appears to be the most commonly used technique worldwide.[69] Density gradient centrifugation combines serial centrifugation steps at different speeds with a density gradient to isolate extracellular vesicles, techniques that require substantial time, biofluid volume, and expertise and that therefore limit point-of-care clinical translation. Antibody purification techniques rely on surface proteins that are thought to be hallmarks of circulating extracellular vesicles (e.g., tetraspanins) and are therefore inherently limited by the nonuniform expression of these markers on all extracellular vesicles, along with the cost of using this technique in large patient cohorts. Isolation with proprietary polymer precipitation and centrifugation offers the promise of "off the shelf," rapid isolation with low input volumes and little additional expertise beyond standard techniques, which has led to its widespread adoption across many clinical and translational efforts at biomarker discovery. Nevertheless, this rapid technique may come at the price of increased contamination (e.g., with proteins).[70]

Furthermore, beyond the isolation of extracellular vesicles, there are various methods for the characterization of size and morphologic features (e.g., flow cytometry, atomic force microscopy, and particle-tracking technology). Given the requirement for robust, reproducible isolation of extracellular vesicles from small input quantities, it is clear that ongoing attempts at standardization will be critical to the development of biomarkers in this field.

Apart from such technical barriers, clinical factors relevant to general biomarker validation may have an effect on the distribution and contents of extracellular vesicles. For example, the amount of time between collection and isolation of extracellular vesicles may become relevant for plasma, given the tendency for platelets to re-

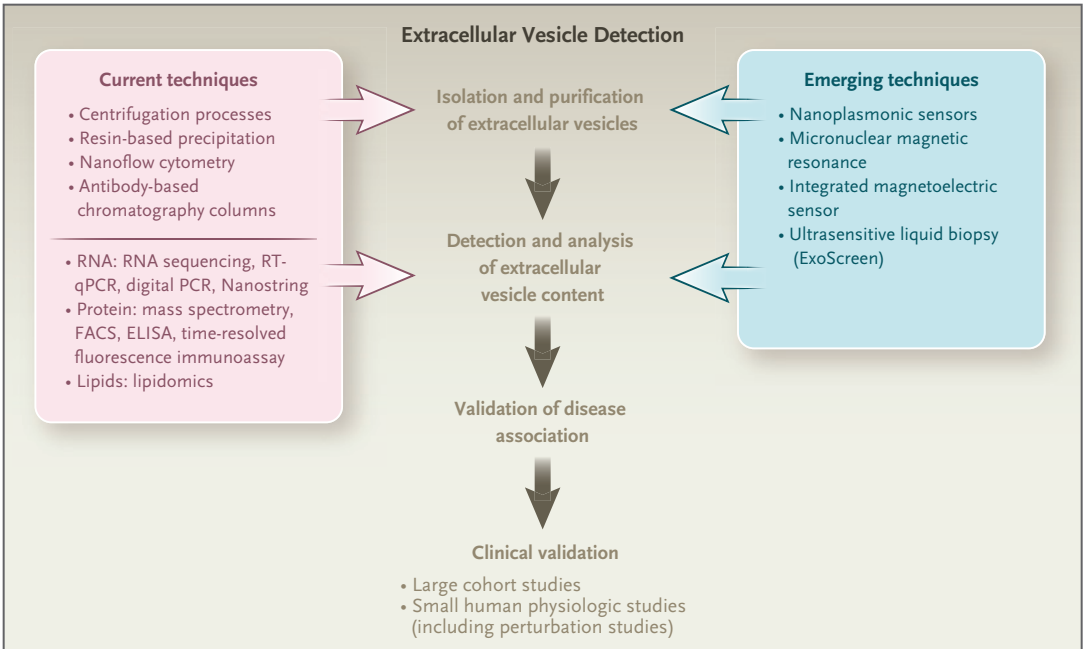

Figure 3. Current and Emerging Methods of Detection of Extracellular Vesicles.

Current methods of isolating extracellular vesicles (e.g., density gradient centrifugation) are both time and labor intensive. Emerging technologies that may overcome some of these challenges include those that enable the characterization of single extracellular vesicles and those that allow for ultrasensitive detection of associated proteins or RNAs without the need for isolating extracellular vesicles. Even after isolation, analysis of the contents of extracellular vesicles is not currently standardized, with different methods for analyzing morphologic features and contents (e.g., RNA sequencing and proteomics). ELISA denotes enzyme-linked immunosorbent assay, FACS fluorescence-activated cell sorting, and RT-qPCR quantitative reverse transcriptase–polymerase chain reaction.

lease extracellular vesicles.[68] As noted above, it remains unclear how age, sex, and race affect the contents of extracellular vesicles, and clarification of these associations is necessary in biomarker development and in understanding the role of extracellular vesicles in human pathophysiology. Furthermore, volumes that are required for mass spectrometric analyses of proteins or metabolites are generally smaller than those required for high-quality extracellular-vesicle isolation, which limits the ability to interrogate the long-term prognostic and diagnostic importance of the quantity and contents of extracellular vesicles in limited plasma volumes from extant cohort studies. Finally, identifying the tissues of origin for circulating extracellular vesicles may be especially important in understanding their relevance to disease, although selective reagents to isolate tissue-specific extracellular vesicles require development and validation.

INFLUENCE ON CLINICAL PRACTICE

Newly discovered biomarkers of human disease should reflect disease pathogenesis, change with intervention, and offer diagnostic or prognostic value beyond current measures. Although extracellular vesicles may be functional in disease, it remains to be seen whether such analyses will improve disease detection or guide therapy beyond current biomarkers and clinical metrics. For the above-mentioned reasons, key technical and practical factors currently limit the clinical use of extracellular vesicles, and further efforts to standardize practice are needed. Nevertheless, promising studies across several types of disease — oncologic, cardiometabolic, neurologic, and infectious — suggest a role for extracellular vesicles both in causing disease and in preserving homeostasis. Furthermore, using emerging technologies, we are beginning to recognize extracellular vesicles as potential drug-delivery

vectors in a whole new class of therapeutic agents.[33,62] With improvements in methods and translational efforts, the medical community may fully uncover the potential for extracellular vesicles to complement available diagnostic, prognostic, and therapeutic information in human disease.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

An audio interview with Dr. Patel is available at NEJM.org

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