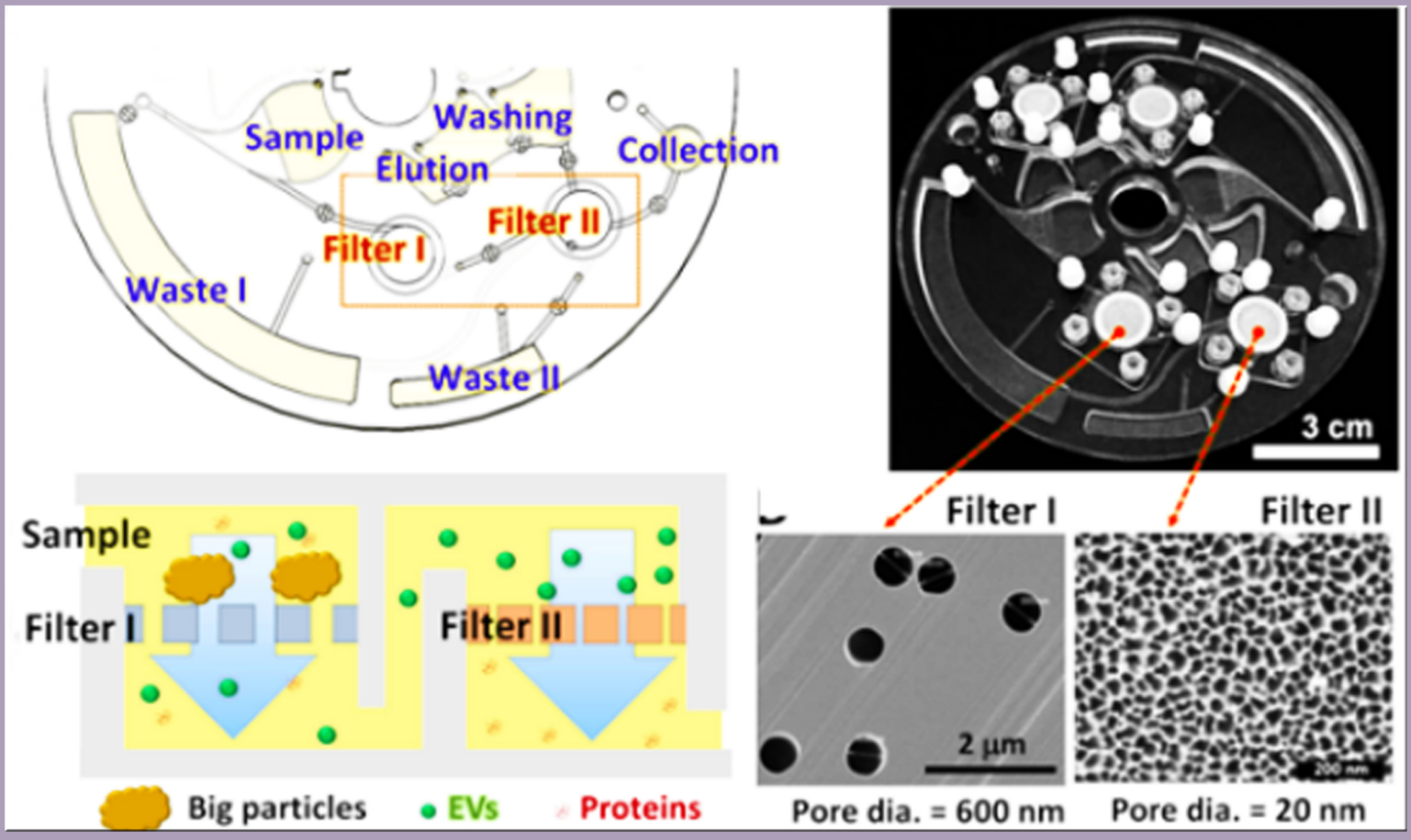
Volume 2 I Issue 1 I March 2021

Extracellular Vesicles and Circulating Nucleic Acids

evcna

Exodisc centrifugation/nano-filtration enrichment of EVs



EDITORIAL BOARD

GENERAL INFORMATION

About the Journal

Extracellular Vesicles and Circulating Nucleic Acids (*EVCNA*), is an international, peer-reviewed, open access journal. *Extracellular Vesicles and Circulating Nucleic Acids* provides an online platform for the sharing of research data, new methodology, reviews and commentaries in the areas of extracellular vesicles and circulating nucleic acids including DNA, RNA, and miRNA and their therapeutic use. The journal is committed to the rapid publication of original findings that increase our understanding of the molecular and cell biology, biogenesis, and origin of extracellular vesicles and circulating nucleic acids; and their use as biomarkers for the diagnosis, prognostication and surveillance of disease states, and in therapeutics. Manuscripts with clinical relevance are especially encouraged to promote the translation from basic science to clinical applications. The criteria for acceptance are scientific excellence and originality. All works involving the use of animals and human subjects must have been approved by institutional review committees and adhere to accepted international ethical standards.

Information for Authors

Manuscripts should be prepared in accordance with Author Instructions.
Please check www.evcnajournal.com/pages/view/author_instructions for details.
All manuscripts should be submitted online at https://oaemesas.com/login?JournalId=evcna.

Copyright

Permissions

Disclaimer

Publisher

OAE Publishing Inc.
245 E Main Street st112, Alhambra, CA 91801, USA
Website: www.oaepublish.com

Contacts

E-mail: editorialoffice@evcnajournal.com
Website: www.evcnajournal.com

Extracellular Vesicles and Circulating Nucleic Acids

CONTENTS

Volume 2 / Issue 1 / March 2021

Loh. *Extracell Vesicles Circ Nucleic Acids* 2021;2:1-2
DOI: 10.20517/evcna.2021.09

Extracellular Vesicles and Circulating Nucleic Acids

Editorial

Open Access

Advances in EV isolation technology and function

Y. Peng Loh

American Biochemist and Molecular Biologist, Bethesda, MD 20817, USA.

Correspondence to: Dr. Y. Peng Loh, American Biochemist and Molecular Biologist, Bethesda, MD 20817, USA.
E-mail: oaepub2019@yahoo.com

How to cite this article: Loh YP. Advances in EV isolation technology and function . *Extracell Vesicles Circ Nucleic Acids* 2021;2:1-2. https://dx.doi.org/10.20517/evcna.2021.09

Received: 30 Mar 2021 **Accepted:** 30 Mar 2021 **Available online:** 30 Mar 2021

Academic Editor: Yoke Peng Loh **Copy Editor:** Xi-Jun Chen **Production Editor:** Xi-Jun Chen

I would like to introduce to you our second issue of *EVCNA*. In this issue we have assembled 5 articles which represent the breadth of our journal. They include 2 reviews and 1 research article covering new technologies in extracellular vesicle (EV) isolation, production and characterization. Dr. Choi's[1] group reviews new platforms in the production of therapeutic exosomes from human cell lines. A review from Dr. Soper's[2] team discusses the pros and cons of traditional and non-traditional, including microfluidics and resistive pulse sensing technologies for extracellular vesicle isolation and detection. A paper from Dr. Baccarelli's[3] lab presents new procedures for the isolation and characterization of extracellular vesicles from saliva of children with asthma, which will facilitate the use of saliva exosomes as biomarkers in the future. Additionally, in this issue, Dr. Holliday's[4] lab has contributed an interesting review focusing on direct communication between extracellular vesicles in osteoblasts/osteocytes with osteoclasts in bone remodeling, offering a thought-provoking prospect in the field. Finally, a review from Dr. Loh's[5] group discusses the function of exosomes in various neurological disorders and brain cancer, highlighting many potential biomarkers, especially miRNAs, in serum - derived exosomes associated with these diseases that can be used as non-invasive diagnostic tools, since exosomes cross the blood brain and conversional biopsy is not possible. This issue also contains a report by Dr. Ikezu[6] on exciting papers presented at the Alzheimer's/Parkinson's Disease (ADPD) 2021 conference, which included the use of blood and CSF-derived exosomes in liquid biopsy to diagnose brain atrophy and cognitive dysfunction, and identification of specific EV cargoes from patients associated with various neurological deficits. Readers will find the articles in this issue especially useful in updating the current knowledge of EV research in many directions from understanding the pathophysiological roles of exosomes to new isolation technologies and potential applications of EVs in therapies.

DECLARATIONS

Author's contribution

Preparing the manuscript draft: Loh YP

Availability of data and materials

Not applicable.

Financial support and sponsorship

The author has not declared a specific grant for this editorial from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Kim J, Song Y, Park CH, Choi C. Platform technologies and human cell lines for the production of therapeutic exosomes. *Extracell Vesicles Circ Nucleic Acids* 2021;2:3-17. DOI
2. Zhao Z, Wijerathne H, Godwin AK, Soper SA. Isolation and analysis methods of extracellular vesicles (EVs). *Extracell Vesicles Circ Nucleic Acids* 2021;2:79-102. DOI
3. Comfort N, Bloomquist TR, Shephard AP, Petty CR, Cunningham A, Hauptman M, Phipatanakul W, Baccarelli A. Isolation and characterization of extracellular vesicles in saliva of children with asthma. *Extracell Vesicles Circ Nucleic Acids* 2021;2:28-47. DOI
4. Holliday LS, Patel SS, Rody WJ Jr. RANKL and RANK in extracellular vesicles: surprising new players in bone remodeling. *Extracell Vesicles Circ Nucleic Acids* 2021;2:18-27. DOI
5. Xiao L, Hareendran S, Loh YP. Function of exosomes in neurological disorders and brain tumors. *Extracell Vesicles Circ Nucleic Acids* 2021;2:54-78. DOI
6. Ikezu T. Current Trends in Extracellular Vesicle Research on Neuroscience from ADPD2021 Meeting. *Extracell Vesicles Circ Nucleic Acids* 2021;2:48-53. DOI

Kim *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:3-17
DOI: 10.20517/evcna.2020.01

Extracellular Vesicles and Circulating Nucleic Acids

Review

Open Access

Platform technologies and human cell lines for the production of therapeutic exosomes

Jiyoon Kim[1,#], Yonghee Song[1,#], Cheol Hyoung Park[1], Chulhee Choi[1,2]

[1]ILIAS Biologics Inc., Daejeon 34014, South Korea.
[2]Department of Bio and Brain Engineering, KAIST, Daejeon 34141, South Korea.
[#]Authors contributed equally.

Correspondence to: Prof. Chulhee Choi, ILIAS Biologics Inc., 40-20 Techno 6ro, Yuseong-gu, Daejeon 34014, South Korea. E-mail: cchoi@iliasbio.com

How to cite this article: Kim J, Song Y, Park CH, Choi C. Platform technologies and human cell lines for the production of therapeutic exosomes. *Extracell Vesicles Circ Nucleic Acids* 2021;2:3-17. https://dx.doi.org/10.20517/evcna.2020.01

Received: 20 Oct 2020 **First Decision:** 22 Dec 2020 **Revised:** 18 Jan 2021 **Accepted:** 27 Jan 2021 **Available online:** 30 Mar 2021

Academic Editor: Y. Peng Loh **Copy Editor:** Xi-Jun Chen **Production Editor:** Yue-Yue Zhang

Abstract

Exosomes are extracellular vesicles secreted by most cell types and represent various biological properties depending on their producing cells. They are also known to be important mediators of intercellular communication. Recent data suggest that exosomes can mediate the therapeutic effects of their parental cells; hence, they have been in the spotlight as novel therapeutics. To develop and manufacture effective therapeutic exosomes, customized strategies are needed to use appropriate technologies for exosome engineering and to select suitable production cell lines. In this review, we provide an overview of currently available exosome engineering platform technologies for loading active pharmaceutical ingredient cargo and the types of human cells/cell lines that are being used as exosome-producing cells, particularly focusing on their characteristics, advantages, and disadvantages.

Keywords: Therapeutic exosome, exosome engineering technologies, human cells, stem cells, HEK293, dendritic cells

INTRODUCTION

Extracellular vesicles (EVs) are nanometer-sized membrane-encapsulated vesicles which are secreted by most types of cells. They can mediate cell-to-cell communication through their bioactive cargo molecules, such as nucleotides, lipids, and proteins. The three major subtypes of EVs are exosomes, microvesicles, and

apoptotic bodies[1,2]. These EV subtypes can be distinguished based on their size and mode of biogenesis. Microvesicles and apoptotic bodies have a diameter of 0.05-1 m and 1-5 m, respectively, whereas exosomes are relatively smaller with a typical diameter of 30-200 nm[3]. These small secretory particles, "exosomes", are released from most eukaryotic and prokaryotic cells into the extracellular fluids, such as blood, plasma, urine, saliva, milk, semen, and tears. In recent years, of the three EV subtypes, exosomes have attracted attention as a therapeutic drug (e.g., NCT04173650) or vehicle for drug delivery based on several groundbreaking studies conducted on their therapeutic potential[4].

Exosomes have a different biogenesis pathway compared to that of other EVs. Exosome biogenesis is a multi-step process which starts with invagination of early endosomes from plasma membrane of the producer cells [Figure 1]. As previously reported, early endosomes mature into multivesicular bodies (MVBs) through the late endosomal stages. In the middle of MVB maturation, a second invagination called "membrane inward budding" of the exosome generates intraluminal vesicles (ILVs) inside the late endosomes. When ILVs bud inward of late endosomes, various cytoplasmic bioactive components, including proteins, lipids, and nucleic acids, are loaded into ILVs as payloads. In a few cases, early endosomes are produced directly by the endoplasmic reticulum or trans-Golgi, independent of membrane invagination[5,6]. MVBs are fused with lysosomes for degradation or with plasma membrane of the parent cells to release multi-components, including ILVs, into the extracellular fluids. The released materials are referred to as exosomes[7-9].

Much evidence exists for exosome biogenesis; however, understanding of their cellular uptake is in its infancy. To track the functionality, tropism to tissue/organ, and *in vivo* stability of exosomes, their cellular uptake mechanisms should be proposed. The internalization of exosomes into the cells involves distinct mechanisms, such as endocytosis[10-12], phagocytosis[11], micropinocytosis[13], and direct fusion[14] [Figure 1]. However, it is still unclear how the recipient cells respond differently for each exosome uptake mechanism. After being taken up by the recipient cells, exosomes enter the early endosomes and undergo one of the three fates: disintegrate to release internal components, fuse with lysosomes for degradation, or are released back into the extracellular fluid[9].

Many studies are ongoing to utilize the properties and biogenesis of exosomes in developing therapeutics for various intractable diseases. Specifically, various engineering technologies have been developed to load therapeutic biomolecules into exosomes for efficient delivery. To improve the efficacy and productivity of therapeutic exosomes, it is very important to identify the optimal exosome-producing cells, and various cells are being examined at academic institutions and pharmaceutical companies. In this review, we discuss current platform technologies for developing therapeutic exosomes and the exosome-producing human cells/cell lines.

PLATFORM TECHNOLOGIES FOR THERAPEUTIC EXOSOMES

Exosomes have various biological characteristics, such as biocompatibility, high penetrability, and biodegradability[15]. Although a lot of supportive evidence for exosome therapeutics is still needed, the unique biological properties of exosomes make it possible to expect therapeutic potential in the clinic. Exosomes are composed of various surface and intracellular molecules, reflecting the type and condition of their parental cells. They are found in all kinds of body fluids, making them suitable candidates for liquid biopsy. Exosomes not only can be used as diagnostic markers, but also can act as drug delivery vehicles or exert therapeutic effect itself, because of their characteristics to elicit minimal host immune responses[16,17] and ability to cross the biological barriers in the brain or placenta[18-20]. As reported, repeatedly injecting engineered exosomes specifically delivered RNAi to the mammalian host brain, while inducing minimal

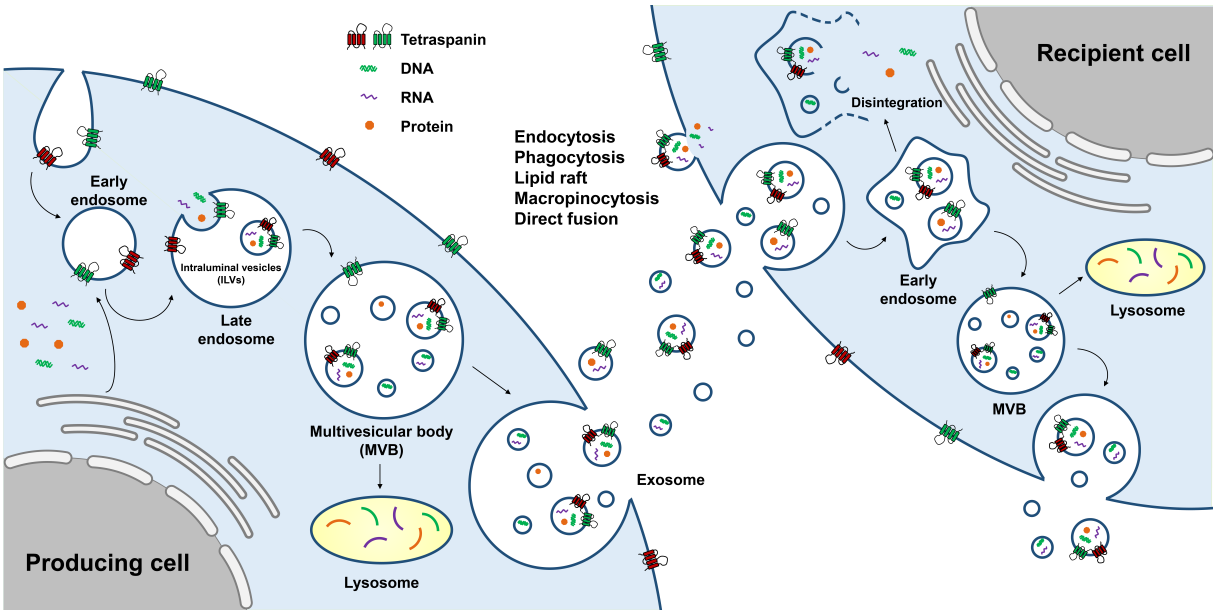


Figure 1. Schema describing biogenesis of exosomes. Biogenesis of exosomes starts with the first invagination of plasma membrane of the producing cells, followed by the formation of early endosomes. Then, the cytosolic components (nucleic acids and proteins) are loaded into ILVs of the late endosomes through the second invagination. After the maturation of MVBs, some components undergo degradation by fusion with lysosomes, while others are secreted into the extracellular fluids as "exosome". Exosomes enter the recipient cells via various mechanisms, such as endocytosis, phagocytosis, lipid raft, macropinocytosis, and direct fusion. After being taken up by the recipient cells, exosomes fuse with early endosomes and release their cargos into the intracellular area by disintegration. Other parts of the exosomes either are released back to the extracellular fluid by fusion with transmembrane or get degraded by lysosomes. ILVs: intraluminal vesicles; MVBs: multivesicular bodies.

immune response[19]. Because of the advantages of using exosomes as a drug delivery vehicle, the research for pharmaceutical exosomes has advanced significantly in recent years[21]. There are three different approaches to using exosomes as therapeutics [Figure 2].

Naïve exosome

The first is to use naive exosomes that are isolated from various cell types, such as mesenchymal stem cells (MSC), embryonic stem cells, and immune cells, directly for disease treatment as is[22,23]. Although naïve exosomes are regarded as promising therapeutic agents, their exact pharmacological mechanisms are poorly understood. Hence, there is an imperative need to better understand the complexity of naturally secreted exosomes and to identify the components with therapeutic activities.

Direct cargo-loading

There are attempts to develop therapeutic exosomes by loading active pharmaceutical ingredients into exosomes using direct or indirect loading. The direct loading methods deliver therapeutic molecules to inside of the exosomes through various incubation methods, such as simple incubation or physical, chemical, or electrical stimulation. As an example of simple incubation, when EL-4-derived exosomes and curcumin were co-cultured at 22 °C, the exosome-encapsulated curcumin exhibited enhanced anti-inflammatory effects on sepsis mouse model compared to curcumin alone[24]. Similarly, when chemotherapeutic agent paclitaxel (PTX) was incubated with exosomes by shaking at 37 °C, it was incorporated into exosomes, and these engineered exosomes showed at least 50-fold enhanced cytotoxic activity compared to PTX alone[25]. Furthermore, electroporation and sonication are generally used for loading nucleotides[19]. For instance, exosomes loaded with RNAi and small-RNAs in the lumen reduced the effects of oncogenes in tumor cells[26,27]. Other cargo loading methods include extrusion, freeze-thaw, and

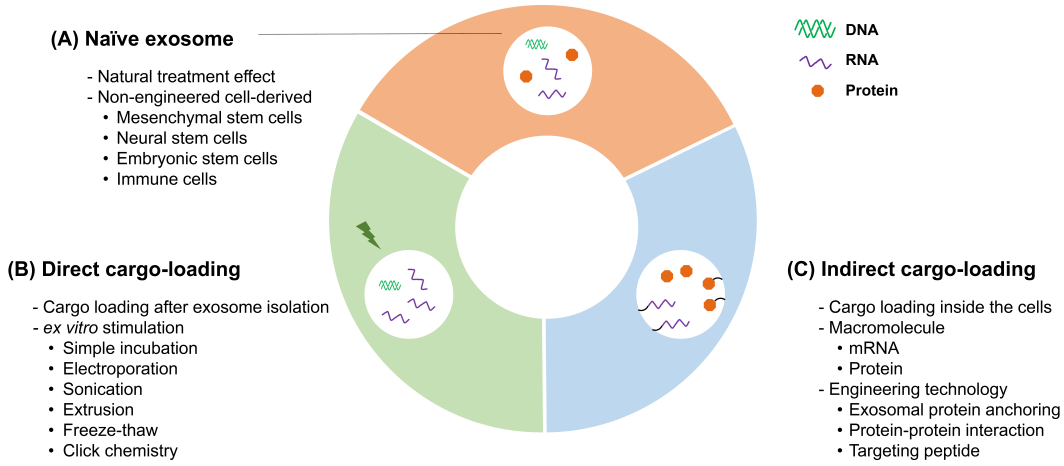


Figure 2. Platform technologies for therapeutic exosomes can be categorized into three subtypes. (A) Naïve exosomes are isolated from different types of cultured cells including stem cells and immune cells. The major limitation of this method is the control over type and amount of cargo molecules. (B) The direct cargo-loading method uses ex vitro stimulations after exosome isolation. Limitations with this method include exosome stability, productivity, and the size of the cargo molecules. (C) In the indirect cargo-loading method, selected and specific macromolecules can be loaded into exosomes by engineering producer cells. Currently, this technique is not only used for loading therapeutic macromolecules but also for targeting specific tissues and organs.

click chemistry[28,29]. Direct cargo-loading allows a more tailored therapeutic effect of exosomes by loading the desired molecule compared to naïve exosomes. However, the major impediment of these techniques is their low stability[30-32] and unsuitability for high molecular-weight cargos, such as mRNAs and proteins.

Indirect cargo-loading

Several pioneering groups have reported a new generation of techniques with indirect cargo loading. In recent years, diverse loading methods have been introduced without disrupting the structure and function of large size RNA molecules[33,34]. Fundamentally, these techniques use RNA binding proteins, which are known to interact with specific sequences of RNA molecules. In 2018, Kojima *et al.*[33] introduced a set of devices known as EXOsomal transfer into cells (EXOtic) and demonstrated its applicability using the interaction between C/D box RNA structure and L7Ae ribosomal protein. In detail, C/D box RNA was subsequently conjugated after therapeutic mRNA synthesis, and L7Ae was fused with CD63, which is naturally localized on the exosomal membrane. With this, mRNA was successfully loaded into exosomes[33]. In the same year, Wang *et al.*[34] developed a method to load mRNA into exosomes based on the interaction between trans-activating response (TAR) sequence and trans-activator of transcription (Tat) protein. They conjugated mRNA with TAR element and membrane protein ARMMs with Tat.

In addition to large size nucleotide loading, protein loading methods have been developed. The direct fusion of exosomal transmembrane proteins including tetraspanins (CD9, CD63, and CD81)[1] with cargo proteins has the potential to load specific cargo proteins into the exosomes. However, the functional area of the cargo proteins from direct fusion is limited only to the close proximity of the recipient cell's membrane. A platform that addresses this limitation is the "exosomes for protein loading via optically reversible protein-protein interaction" (EXPLOR®), which delivers cargo proteins into the cytosol in a free form[35]. Cargo protein and tetraspanin, especially CD9, interact with each other through the fusion with cryptochrome 2

(CRY2) and the truncated domain of calcium- and integrin-binding protein 1 (CIBN), respectively. CRY2 and CIBN bind with each other when exposed to specific wavelengths of blue light, and they dissociate in the absence of blue light. This selective interaction allows cargo proteins to be freely localized in the recipient cells. In addition, methods for targeting the therapeutic molecules to specific tissues have been devised.

Similar to cargo loading, membrane proteins are used as an anchor of targeting peptides. For example, Tian *et al.*[36] fused the exosomal membrane protein Lamp2b with the αv integrin-specific RGD (R, arginine; G, glycine; D, aspartic acid) peptide, which specifically targeted αv integrin expressing tumor cells and reduced the tumor progression. Most of the cargo loading approaches introduced above require not only engineering exosome-producing cells but also ways to increase exosome production. Therefore, it is important to select cells that are amenable for transfection and large-scale culture processes.

EXOSOME PRODUCING HUMAN CELL LINES SUITABLE FOR THERAPEUTIC APPLICATIONS AND SCALE-UP PRODUCTION

While exosomes have conserved proteins such as tetraspanins (CD81, CD63, and CD9), Tsg101, and Alix, which are responsible for their common biological activities, they also have specific components depending on their producer cell types, suggesting that exosomes can reflect the intrinsic properties of the producer cells. For example, miR-146a, which contributes to the protection against myocardial infarction, is highly enriched in the exosomes released from cardiosphere-derived cells (CDCs)[37]. Similarly, a high concentration of anti-inflammatory miR-223 is found in exosomes released from human peripheral blood mononuclear cells (PBMCs) and animal bone marrow-derived mesenchymal stem cells (MSCs)[38-40]. As the therapeutic effects of exosomes can be modulated not only by the type of parental cells but also by the environmental factors such as culture materials and conditions, the characteristics of cells should be carefully considered to select the suitable exosome-producing cell lines for basic research as well as for clinical applications. Scalability, consistency, and controllable manufacturing methods for culture will need to be established to produce clinical-grade exosomes. In the following section, we compare the genetic modification, productivity, scalability, and safety of exosome producing human cell lines and summarize research trends of several human cells currently in use for production [Table 1].

Stem cells: mesenchymal stem cells and neural stem cells

MSCs are multipotent stem cells that can be isolated from various human tissues including bone marrow, adipose tissue, amniotic fluid, umbilical cord blood, and others. They can be differentiated into multiple lineages such as adipocytes, chondrocytes, and osteocytes. These cells exhibit therapeutic effects and have been broadly used in clinical trials[82,83]. Especially, MSCs have been in the spotlight as source cells for regenerative medicine for various diseases including myocardial ischemia / reperfusion injury[84], graft-versus-host-disease (GvHD)[85], Alzheimer's disease[86], and skin injury[87]. Recently, it has been reported that MSC-derived exosomes have similar therapeutic effects to those of MSCs, suggesting that MSC-derived exosomes can also be used as therapeutic agents[88]. Similarly, the therapeutic effects of neural stem cells (NSCs) such as regeneration, plasticity, neurogenesis, and attenuated neuroinflammation in neurodegenerative disease have been reported to be mediated by NSC-derived exosomes which are enriched with specific miRNAs[89].

The MSC- and NSC-derived exosomes are known to have therapeutic potentials on their own without going through any engineering. Therefore, stem cells such as MSCs and NSCs that possess natural intrinsic therapeutic effects could be a good source for producing naïve exosomes[89,90]. To produce MSC-derived naïve exosomes, either autologous or allogeneic cell sources can be used depending on their advantages and

Table 1. Advantages and disadvantages of cells/cell lines used for production of therapeutic exosomes in preclinical studies

Cells	Advantages	Disadvantages	Exosome type	Target diseases
MSCs	Immunomodulatory; low immunogenicity	Limited scalability (adherent); limited growth capacity	Naïve	Myocardial ischaemia / reperfusion injury[41-43] Traumatic brain injury[44,45] Alzheimer's disease[46,47] Lung injury[48] Graft-versus-host-disease (GvHD)[49] Cutaneous injury (wound)[50] Kidney injury[51]
			Engineered	Tumor[52,53] Stroke[54] Rheumatoid arthritis[55] Acute lung injury[56] Myocardial infarction[57]
NSCs	Immunomodulatory; low immunogenicity	Limited scalability (adherent)	Naïve	Spinal cord injury[58] (Ischemic) stroke[59] Alzheimer's disease[60] Hypoxia-reperfusion injury[61]
			Semi-engineered (culture media-modified)	Spinal cord injury[62]
HEK293	High scalability (possible to suspension); High growth capacity (immortalized cell line); Easy manipulation	Tumor metastatic potential	Engineered	Drug addiction[63] Tumor[64-67] Parkinson's disease[33] Sepsis[68] Heart disease[69] Alzheimer's disease[70]
DCs	Immunomodulatory; low immunogenicity	Limited scalability (adherent)	Engineered	Alzheimer's disease[19] Parkinson's disease[71] Cancer (vaccine)[72,73]
CDCs	Low immunogenicity	Limited scalability (adherent)	Naïve	Myocardial infarctio[37,74] Duchenne muscular dystrophy[75]
Amniotic cells	Low immunogenicity	Limited scalability (adherent)	Naïve	Ovarian dysfunction[76] Wound healing[77,78] Lung injury[79,80]
CAR-T	Low toxicity; low immunosuppression	Limited scalability	T cell-engineered	Tumor[81]

MSCs: Mesenchymal Stem Cells; NSCs: neural Stem Cells; HEK293: human embryonic kidney 293 cells; DCs: dendritic cells; CDCs: cardiosphere-derived cell; CAR-T: chimeric antigen receptor-T cells.

limitations. While autologous cells are non-immunogenic, they have limitations with scalability. In contrast, allogeneic cells have the opposite characteristics.

Productivity of exosomes

In terms of exosome productivity, MSCs produce a relatively large number of exosomes per cell[82,91]. However, for more efficient and scalable production of therapeutic exosomes, requirements such as use of chemically defined materials and serum-free suspension culture should be met. The current conventional *in vitro* monolayer culture method used for MSCs is a major limitation to scale up the production of therapeutic exosomes. Recently, a three-dimensional culture method was reported to increase the productivity of MSC-derived therapeutic exosomes[92], although the growth capacity of the cells was limited. Immortalization of MSCs by introducing oncogenes such as c-myc or human telomerase reverse transcriptase is being considered to provide MSCs with sustained growth capabilities[91,93]. Furthermore, the conditionally immortalized hNSC line, generated by c-MycER^{TAM} technology[94], fulfilled the needs of brain research field and clinical market by enabling large-scale cell production, and hNSCs are being studied in clinical trials for stroke and critical limb ischemia conditions[95]. This suggests that the exosomes produced by immortalized hNSC cells may also have therapeutic effect. However, the immortalization strategy

requires careful attention, as it poses potential safety issues such as tumorigenicity[96].

Safety

MSC-derived exosomes have advantages with regards to safety. MSCs are considered to be non-immunogenic with a lower risk of allogeneic immune rejection by the host[90], and they are currently undergoing extensive clinical trials. However, the pro-proliferative effects of MSC-derived exosomes on injured cells suggest the possibility of their involvement in cancer progression, even though the pro- or anti-cancer effect of MSC-derived exosomes is currently controversial[97-99]. Another safety concern with stem cells is the use of animal-derived serum for cell growth. If serums such as fetal bovine serum (FBS) are not completely removed before the final exosome production, there is a high possibility that these serum contaminants will be loaded into the exosomes during the culture. The retention of these serum contaminants in exosomes can be a problem from a regulatory standpoint in the production of therapeutic agents. Alternatively, using xeno-free culture media components or exosome-depleted FBS could be considered; however, this may affect the compositions or physiological properties of exosomes and result in stress-induced phenotype by loading reactive oxygen species and stress-related proteins[100].

HEK293 cells

HEK293 is a human embryonic kidney cell line and has been commonly used in research and bio-industrial field for more than 30 years[101]. HEK293 cells have various technological advantages required for the development of biopharmaceuticals such as rapid growth, simple culturing, and easy manipulation, owing to their high transfection efficiency. In addition, HEK293 has been widely used as a cellular model in research for producing exosomes as drug carriers due to its high exosome productivity and neutral phenotype[68,102].

Productivity of exosomes

For high scalability of biopharmaceutical productions including therapeutic exosomes, it is advantageous for the producing cell lines to be adapted to single-cell suspension culture at high density. HEK293F cell line, developed by Invitrogen[103], has been adapted to grow in serum-free suspension culture and is useful for the large-scale production of industrial bio-products. Several biopharmaceuticals produced using HEK293 cells have been recently approved by the US Food and Drug Association (FDA) or European Medicines Agency (EMA), suggesting that HEK293 cells can be utilized for clinical-grade biological production during the development of new therapeutics[104].

Engineering of exosomes

To produce engineered exosomes loaded with specific regulatory genes or proteins with enhanced targeting ability, efficient transfection protocols are needed. HEK293 cell line is extensively used in exosome production because of its ease of handling and transfection. For instance, HEK293T cells engineered with "EXOtic" devices were designed to load therapeutic mRNAs in exosomes to deliver cargoes to specific targets, and they have been used for the production of therapeutic research exosomes[33]. In addition, a HEK293T cell line engineered to stably express specific recombinant proteins using EXPLOR® technology allowed loading target proteins into exosomes[68].

Safety

Although HEK293 is not a cancer cell line, there may be concerns regarding the tumorigenic or toxic potential of the exosomes derived from these cells because the cell line is immortalized. However, omics research on HEK293T-derived exosomes indicated the enrichment of only a few disease- or cancer-related components and demonstrated their insignificant relationship with the physiological and pathological

processes, suggesting that HEK293T-derived exosomes are safe to be used as *in vivo* drug delivery vehicle[105]. Furthermore, treatment with varying doses of HEK293-derived exosomes neither had a significant effect on viability and function of other cells such as macrophages and HepG2 nor induced pro-inflammatory cytokine response[106,107]. In addition, administration of mice with HEK293-derived naïve and engineered exosomes for several weeks did not induce any significant toxicity[17]. These results demonstrate that HEK293-derived exosomes have low *in vitro* and *in vivo* toxicity and immunogenicity.

Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells that induce an antigen-specific T-cell immune response and mediate between innate and adaptive immunity. The unique characteristic of DC-derived exosomes (Dex) is their ability to present antigen and immune-related proteins (major histocompatibility complex I and II); hence, they can induce an anti-tumor immune response followed by tumor regression. Based on these attributes, Dex has been used for cancer vaccination and treatment in several preclinical and clinical studies[72,108,109]. Three phase I clinical trials and one phase II clinical trial with Dex have been completed or are ongoing[109]. In one of the phase I clinical trials, Dex was produced using autologous monocyte-derived DCs loaded with antigenic HLA-presented peptides of melanoma-associated antigen from a single leukapheresis for cancer vaccine generation[110].

Engineering and productivity of exosomes

Dex can be engineered by modifying the producer cells through transfection[111] or viral transduction[112]. For instance, to express neuron-specific rabies viral glycoprotein (RVG) peptides on the surface of Dex and target them to neurons and microglia in the brain, DCs were genetically modified by transfection with plasmid DNA encoding a fusion protein composed of an exosomal membrane protein, Lamp2b, and RVG peptides[19]. Furthermore, DCs have been modified by viral transduction to produce tumor antigen-presenting exosomes[73]. Since immature DCs have an inherent ability to take up proteins and peptides from surrounding fluids and tissues, special loading techniques are not required to load proteins and peptides into Dex[109]. Therefore, model tumor antigens such as ovalbumin (OVA) were co-incubated with immature DCs to obtain OVA-loaded exosomes[113]. However, immature DCs produce limited numbers of exosomes[19].

Safety

Dex have been proven to be safe in clinical trials[114]. Furthermore, exosomes produced from self-derived immature DCs are devoid of immunostimulatory surface markers such as CD40, CD86, and MHC-II, which reduces their immunogenicity[115]. Two of the phase I clinical trials demonstrated low toxicity of exosomes derived from tumor peptide-loaded DCs in metastatic melanoma or non-small cell lung cancer patients[116]. However, in a recent phase II clinical trial in non-small cell lung cancer (NSCLC), exosomes derived from DCs matured by interferon-gamma and loaded with MHC class I- and class II-restricted cancer antigens did not meet the primary endpoint of 50% non-progressors by post-chemotherapy[117]. Furthermore, mature DC-derived exosomes have been reported to increase endothelial inflammation and atherosclerosis[118]; hence, the use of Dex as a therapeutic agent requires in-depth research and additional controls.

Others: CDCs, amniotic cells, and CAR-T cells

CDCs improve angiogenesis and restore heart function after myocardial infarction by secreting exosomes[74]. The microRNA miR-146a is highly enriched in the CDC-derived exosomes and plays an important role in thickening up of the infarction walls[37]. Recent studies have reported that CDC-derived exosomes might be potentially useful in treating Duchenne muscular dystrophy by restoring skeletal muscle and heart functions[75].

CDCs were immortalized using simian virus 40 large and small T antigens. The use of imCDC$^{sh\text{-}test}$-derived exosomes as therapeutics is currently being investigated in preclinical research for cell-free therapies[119].

CDCs express MHC I but not MHC II; hence, they evade recognition by CD4 lymphocytes and have low immunogenicity. Xenogeneic CDC-derived exosomes are primarily safe as they induce low immunogenicity[74].

Human amniotic epithelial cells (hAECs), found mainly in the placenta, preserve the properties of embryonic stem cells such as anti-inflammatory and low immunogenicity, making them suitable for regenerative medicine. Recent studies have reported that hAEC-derived exosomes enhance lung repair after injury[79], expedite wound healing[77], and restore ovarian function[76]. Furthermore, there are no ethical issues in using amniotic cells and its exosomes. Owing to these advantages, amniotic cells can be considered as one of the suitable candidates for therapeutic exosome research.

Chimeric antigen receptor (CAR)-T cells are emerging as novel immunotherapy agents for a wide range of cancers, and numerous clinical trials have been conducted on CAR-T cells. Recently, two CAR-T cell therapies, Kymriah from Novartis and Yescarta from Kite Pharma, were approved by the US FDA for treatment of diffuse large B-cell lymphoma and B-cell acute lymphoblastic leukemia[120]. Similar to other exosomes, CAR-T cell-derived exosomes express CAR-T cell-specific factors, such as CAR and cytotoxic molecules on their surface, and have potential as excellent cancer-targeting and anti-cancer agents[81]. Unlike CAR-T cells, the exosomes derived from CAR-T cells do not cause toxicity. Moreover, they do not express programmed cell death protein 1 (PD-1) on their surface, so their anti-cancer effects do not weaken. This suggests that CAR-T exosomes could be used as anti-cancer immunotherapy agents in the future[81].

THERAPEUTIC APPROACHES OF EXOSOMES

The therapeutic effect of exosomes derived from different types of producer cells is promising, as shown by various studies on different diseases. For instance, the macrophage-derived exosomes loaded with anti-cancer therapeutic compound PTX were used to treat multi-drug resistant cancer. PTX-loaded exosomes demonstrated an increased anti-cancer effect in murine lung tumor models[25].

Small RNAs have been regarded as suitable regulators for modulating oncogene expression. However, the major drawback for their therapeutic use is the lack of an appropriate *in vivo* delivery system. Ohno *et al.*[66] systemically injected let-7a miRNA-loaded exosomes into epidermal growth factor receptor (EGFR) - positive breast cancer xenograft mouse models. The exosome membranes were modified to express GE11 peptide for targeting EGFR; the engineered exosomes efficiently delivered miRNA and suppressed tumor phenotype[66]. Mendt *et al.*[16] developed clinical grade siRNA-loaded MSC-derived exosomes called "iExosomes" which targeted KRAS (G12D) mutation in pancreatic cancer[27]. Additionally, iExosomes increased survival rate and reduced cancer phenotypes in various tumor models. Wang *et al.*[121] showed that miR-185-enriched MSC-derived exosomes inhibit oral leukoplakia by reducing proliferation and angiogenesis in oral potentially malignant disorders.

Severe and chronic infections lead to inflammatory-related syndromes. Sepsis is associated with high mortality rate and has no standard therapeutic options. Choi *et al.*[68] demonstrated that exosomes loaded with super-repressor IκB (srIκB), the constitutive active NF-κB signaling inhibition factor, using EXPLOR® technology successfully inhibited pro-inflammatory responses *in vitro* and *in vivo* and prolonged the survival of septic animals. Inflammation is also a high-risk factor of preterm birth (PTB)[122-124]. Over the last 30 years, diverse therapeutic methods have been developed; however, only a few drugs related to obstetrics

and gynecological diseases have reached clinical trials because of the difficulties in crossing the placental barrier[125]. By using cyclic recombinase-loaded exosomes, it was demonstrated that the engineered exosomes can pass through the placental barrier and efficiently deliver effective proteins to the fetus in the murine model[20]. These reports suggest that exosomes might be potent treatment agents for inflammation-induced PTB. Acute kidney injury is another immune-related disease with a high mortality rate and no definitive therapies[126]. Engineered exosomes delivering an anti-inflammatory modulator, interleukin-10, were shown to ameliorate kidney injury symptoms[127].

One of the important reasons for exosomes to be considered as an attractive drug delivery vehicle is their ability to cross bio-barriers consisting of multiple cell layers[128] as in PTB. Exosomes are rapidly emerging as a novel way to treat brain diseases as they can also cross the blood-brain barrier, one of the major hurdles for conventional drugs[129,130]. Alpha-synuclein (α-Syn) aggregation is the primary pathological phenotype of Parkinson`s disease. RVG-exosomes which specifically target the brain successfully delivered α-Syn-siRNAs to the brain of S129D α-Syn transgenic mice model[71]. Systemic administration of exosomes significantly reduced α-Syn protein aggregates in the brain of mice. In addition, catalase mRNA-loaded exosomes using EXOtic devices attenuated neurotoxicity and neuroinflammation in mouse brain.

CONCLUSION

Three technologies are being used for producing therapeutic exosomes: naïve exosomes, delivering drugs directly into exosomes, and indirect loading of drugs into exosomes through genetic modification of producer cells. Several studies in academia and industry are using these techniques for developing therapeutic exosomes, and various cell lines are being investigated for optimal production of therapeutic exosomes.

The exosomes derived from stem cells including MSCs and NSCs have shown intrinsic therapeutic efficacy. Therefore, it is important to know the exact mechanisms of how these effects are derived into the exosomes from their parent cells. In addition, further studies related to scalability, consistent quality, and compatibility of the cells with cGMP guidelines should be performed before their clinical application.

With its ease of manipulation, HEK293 cell line is a key platform to produce therapeutic cargo-loaded exosomes along with improving the production process. However, the intrinsic effects of naïve HEK293-derived exosomes are still unclear; hence, in-depth studies are required to characterize HEK293-derived exosomes.

It has been shown that the molecular compositions of exosomes are dependent not only on the cell type but also on the physiological status of the producing cells. However, currently, it is not easy to distinguish the subtypes of exosomes. Furthermore, the exact *in vivo* distribution and stability of exosomes remain unknown, and additional in-depth studies with highly sensitive analytic methods are needed for a more accurate understanding of the mechanisms of action and to obtain more information on pharmacologically active components of exosomes[102]. In addition, engineering technologies should be further improved to enhance the activities and targeting ability of exosomes. Likewise, the human cells/cell lines used for biopharmaceutical productions should be free from safety and toxicity concerns including tumorigenicity and immunogenicity.

DECLARATIONS

Authors' contributions

Wrote, discussed, and edited the manuscript: Kim J, Song Y

Reviewed and edited the manuscript: Park CH
Made substantial contributions to conception, reviewed, and final approved the manuscript: Choi C

Availability of data and materials
Not applicable.

Financial support and sponsorship
This work was supported by a grant (2016M3A9B6945931) from Ministry of Science and ICT, Republic of Korea.

Conflicts of interest
Choi C, the founder and shareholder of ILIAS Biologics Inc., is an inventor of a patent related to "EXPLOR® " technology filed by ILIAS Biologics Inc. (no. KR 10-1877010 and US 10,702,581).

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

REFERENCES
1. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002;2:569-79. DOI PubMed
2. Raposo G. Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373-83. DOI PubMed PMC
3. Tikhomirov R, Donnell BR, Catapano F, et al. Exosomes: from potential culprits to new therapeutic promise in the setting of cardiac fibrosis. *Cells* 2020;9:592. DOI PubMed PMC
4. Wiklander OPB, Brennan MÁ, Lötvall J, Breakefield XO, El Andaloussi S. Advances in therapeutic applications of extracellular vesicles. *Sci Transl Med* 2019;11:eaav8521. DOI PubMed PMC
5. Babst M. MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Curr Opin Cell Bio* ;23:452-7. DOI PubMed PMC
6. Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci* 2019;9:19. DOI PubMed PMC
7. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* 2009;10:513-25. DOI PubMed
8. Sinha S, Hoshino D, Hong NH, et al. Cortactin promotes exosome secretion by controlling branched actin dynamics. *J Cell Biol* 2016;214:197-213. DOI PubMed PMC
9. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
10. Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. *Nat Rev Mol Cell Biol* 2007;8:603-12. DOI PubMed
11. Feng D, Zhao WL, Ye YY, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 2010;11:675-87. DOI PubMed
12. Svensson KJ, Christianson HC, Wittrup A, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. *J Biol Chem* 2013;288:17713-24. DOI PubMed PMC
13. Tian T, Zhu YL, Zhou YY, et al. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem* 2014;289:22258-67. DOI PubMed PMC
14. Parolini I, Federici C, Raggi C, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;284:34211-22. DOI PubMed PMC
15. Delcayre A, Estelles A, Sperinde J, et al. Exosome display technology: applications to the development of new diagnostics and therapeutics. *Blood Cells Mol Dis* 2005;35:158-68. DOI PubMed
16. Mendt M, Kamerkar S, Sugimoto H, et al. Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight* 2018;3:e99263. DOI PubMed PMC
17. Zhu X, Badawi M, Pomeroy S, et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following

sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles* 2017;6:1324730. DOI PubMed PMC

18. Zhuang X, Xiang X, Grizzle W, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* 2011;19:1769-79. DOI PubMed PMC
19. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJA. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;29:341-5. DOI PubMed
20. Sheller-Miller S, Choi K, Choi C, Menon R. Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy. *Am J Obstet Gynecol* 2019;221:502.e1-e12. DOI PubMed
21. Song Y, Kim Y, Ha S, et al. The emerging role of exosomes as novel therapeutics: Biology, technologies, clinical applications, and the next. *Am J Reprod Immunol* 2020:e13329. DOI PubMed PMC
22. Lai RC, Arslan F, Tan SS, et al. Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J Mol Cell Cardiol* 2010;48:1215-24. DOI PubMed
23. Zhu L, Kalimuthu S, Gangadaran P, et al. Exosomes derived from natural killer cells exert therapeutic effect in melanoma. *Theranostics* 2017;7:2732-45. DOI PubMed PMC
24. Sun D, Zhuang X, Xiang X, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther* 2010;18:1606-14. DOI PubMed PMC
25. Kim MS, Haney MJ, Zhao Y, et al. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine* 2016;12:655-64. DOI PubMed PMC
26. Lamichhane TN, Jeyaram A, Patel DB, et al. Oncogene knockdown via active loading of small RNAs into extracellular vesicles by sonication. *Cell Mol Bioeng* 2016;9:315-24. DOI PubMed PMC
27. Kamerkar S, LeBleu VS, Sugimoto H, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 2017;546:498-503. DOI PubMed PMC
28. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM. Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *J Control Release* 2015;205:35-44. DOI PubMed
29. Hood JL. Post isolation modification of exosomes for nanomedicine applications. *Nanomedicine (Lond)* 2016;11:1745-56. DOI PubMed PMC
30. Hood JL, Scott MJ, Wickline SA. Maximizing exosome colloidal stability following electroporation. *Anal Biochem* 2014;448:41-9. DOI PubMed PMC
31. Haney MJ, Klyachko NL, Zhao Y, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* 2015;207:18-30. DOI PubMed PMC
32. Luan X, Sansanaphongpricha K, Myers I, Chen H, Yuan H, Sun D. Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacol Sin* 2017;38:754-63. DOI PubMed PMC
33. Kojima R, Bojar D, Rizzi G, et al. Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment. *Nat Commun* 2018;9:1305. DOI PubMed PMC
34. Wang Q, Yu J, Kadungure T, Beyene J, Zhang H, Lu Q. ARMMs as a versatile platform for intracellular delivery of macromolecules. *Nat Commun* 2018;9:960. DOI PubMed PMC
35. Yim N, Ryu SW, Choi K, et al. Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. *Nat Commun* 2016;7:12277. DOI PubMed PMC
36. Tian Y, Li S, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 2014;35:2383-90. DOI PubMed
37. Ibrahim AG, Cheng K, Marbán E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Reports* 2014;2:606-19. DOI PubMed PMC
38. Ismail N, Wang Y, Dakhlallah D, et al. Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. *Blood* 2013;121:984-95. DOI PubMed PMC
39. Taibi F, Metzinger-Le Meuth V, Massy ZA, Metzinger L. miR-223: an inflammatory oncomiR enters the cardiovascular field. *Biochim Biophys Acta* 2014;1842:1001-9. DOI PubMed
40. Wang X, Gu H, Qin D, et al. Exosomal miR-223 contributes to mesenchymal stem cell-elicited cardioprotection in polymicrobial sepsis. *Sci Re* 2015;5:13721. DOI PubMed PMC
41. Xin H, Li Y, Cui Y, Yang JJ, Zhang ZG, Chopp M. Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013;33:1711-5. DOI PubMed PMC
42. Feng Y, Huang W, Wani M, Yu X, Ashraf M. Ischemic preconditioning potentiates the protective effect of stem cells through secretion of exosomes by targeting Mecp2 via miR-22. *PLoS One* 2014;9:e88685. DOI PubMed PMC
43. Liu L, Jin X, Hu CF, Li R, Zhou Z, Shen CX. Exosomes derived from mesenchymal stem cells rescue myocardial ischaemia/reperfusion injury by inducing cardiomyocyte autophagy via AMPK and Akt pathways. *Cell Physiol Biochem* 2017;43:52-68. DOI PubMed
44. Zhang Y, Chopp M, Meng Y, et al. Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. *J Neurosurg* 2015;122:856-67. DOI PubMed PMC
45. Kim DK, Nishida H, An SY, Shetty AK, Bartosh TJ, Prockop DJ. Chromatographically isolated CD63 + CD81 + extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. *Proc Natl Acad Sci U S A* 2016;113:170-5. DOI PubMed PMC
46. Wang SS, Jia J, Wang Z. Mesenchymal stem cell-derived extracellular vesicles suppresses iNOS expression and ameliorates neural

impairment in Alzheimer's disease mice. *J Alzheimers Dis* 2018;61:1005-13. DOI PubMed

47. Reza-Zaldivar EE, Hernandez-Sapiens MA, Gutierrez-Mercado YK, et al. Mesenchymal stem cell-derived exosomes promote neurogenesis and cognitive function recovery in a mouse model of Alzheimer's disease. *Neural Regen Res* 2019;14:1626-34. DOI PubMed PMC
48. Zhu YG, Feng XM, Abbott J, et al. Human mesenchymal stem cell microvesicles for treatment of Escherichia coli endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;32:116-25. DOI PubMed PMC
49. Lai P, Chen X, Guo L, et al. A potent immunomodulatory role of exosomes derived from mesenchymal stromal cells in preventing cGVHD. *J Hematol Oncol* 2018;11:135. DOI PubMed PMC
50. He X, Dong Z, Cao Y, et al. MSC-derived exosome promotes M2 polarization and enhances vutaneous wound healing. *Stem Cells Int* 2019;2019:7132708. DOI PubMed PMC
51. Nargesi A, Lerman LO, Eirin A. Mesenchymal stem cell-derived extracellular vesicles for kidney repair: current status and looming challenges. Stem. *Cell Res Ther* 2017;8:273. DOI PubMed PMC
52. Katakowski M, Buller B, Zheng X, et al. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett* 2013;335:201-4. DOI PubMed PMC
53. Melzer C, Rehn V, Yang Y, Bähre H, von der Ohe J, Hass R. Taxol-loaded MSC-derived exosomes provide a therapeutic vehicle to target metastatic breast cancer and other carcinoma cells. *Cancers (Basel)* 2019;11:798. DOI PubMed PMC
54. Xin H, Katakowski M, Wang F, et al. MicroRNA cluster miR-17-92 cluster in exosomes enhance neuroplasticity and functional recovery after stroke in rats. *Stroke* 2017;48:747-53. DOI PubMed PMC
55. Chen Z, Wang H, Xia Y, Yan F, Lu Y. Therapeutic potential of mesenchymal cell-derived miRNA-150-5p-expressing exosomes in rheumatoid arthritis mediated by the modulation of MMP14 and VEGF. *J Immunol* 2018;201:2472-82. DOI PubMed PMC
56. Yi X, Wei X, Lv H, et al. Exosomes derived from microRNA-30b-3p-overexpressing mesenchymal stem cells protect against lipopolysaccharide-induced acute lung injury by inhibiting SAA3. *Exp Cell Res* 2019;383:111454. DOI PubMed
57. Chen Y, Zhao Y, Chen W, et al. MicroRNA-133 overexpression promotes the therapeutic efficacy of mesenchymal stem cells on acute myocardial infarction. *Stem Cell Res Ther* 2017;8:268. DOI PubMed PMC
58. Rong Y, Liu W, Wang J, et al. Neural stem cell-derived small extracellular vesicles attenuate apoptosis and neuroinflammation after traumatic spinal cord injury by activating autophagy. *Cell Death Dis* 2019;10:340. DOI PubMed PMC
59. Webb RL, Kaiser EE, Jurgielewicz BJ, et al. Human neural stem cell extracellular vesicles improve recovery in a porcine model of ischemic stroke. *Stroke* 2018;49:1248-56. DOI PubMed PMC
60. Li B, Liu J, Gu G, Han X, Zhang Q, Zhang W. Impact of neural stem cell-derived extracellular vesicles on mitochondrial dysfunction, sirtuin 1 level, and synaptic deficits in Alzheimer's disease. *J Neurochem* 2020;154:502-18. DOI PubMed
61. Liu Q, Tan Y, Qu T, Zhang J, et al. Therapeutic mechanism of human neural stem cell-derived extracellular vesicles against hypoxia-reperfusion injury in vitro. *Life Sci* 2020;254:117772. DOI PubMed
62. Ma K, Xu H, Zhang J, et al. Insulin-like growth factor-1 enhances neuroprotective effects of neural stem cell exosomes after spinal cord injury via an miR-219a-2-3p/YY1 mechanism. *Aging (Albany NY)* 2019;11:12278-94. DOI PubMed PMC
63. Liu Y, Li D, Liu Z, et al. Targeted exosome-mediated delivery of opioid receptor Mu siRNA for the treatment of morphine relapse. *Sci Rep* 2015;5:17543. DOI PubMed PMC
64. Kosaka N, Iguchi H, Yoshioka Y, Hagiwara K, Takeshita F, Ochiya T. Competitive interactions of cancer cells and normal cells via secretory microRNAs. *J Biol Chem* 2012;287:1397-405. DOI PubMed PMC
65. Mizrak A, Bolukbasi MF, Ozdener GB, et al. Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth. *Mol Ther* 2013;21:101-8. DOI PubMed PMC
66. Ohno S, Takanashi M, Sudo K, et al. Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol Ther* 2013;21:185-91. DOI PubMed PMC
67. Faruqu FN, Xu L, Al-Jamal KT. Preparation of exosomes for siRNA delivery to cancer cells. *J Vis Exp* 2018. DOI PubMed PMC
68. Choi H, Kim Y, Mirzaaghasi A, et al. Exosome-based delivery of super-repressor IkappaBalpha relieves sepsis-associated organ damage and mortality. *Sci Adv* 2020;6:eaaz6980. DOI PubMed PMC
69. Kim H, Yun N, Mun D, et al. Cardiac-specific delivery by cardiac tissue-targeting peptide-expressing exosomes. *Biochem Biophys Res Commun* 2018;499:803-8. DOI PubMed
70. Jahangard Y, Monfared H, Moradi A, Zare M, Mirnajafi-Zadeh J, Mowla SJ. Therapeutic effects of transplanted exosomes containing miR-29b to a rat model of Alzheimer's disease. *Front Neurosci* 2020;14:564. DOI PubMed PMC
71. Cooper JM, Wiklander PB, Nordin JZ, et al. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov Disord* 2014;29:1476-85. DOI PubMed PMC
72. Hsu DH, Paz P, Villaflor G, et al. Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides. *J Immunothe* ;26:440-50. DOI PubMed
73. Lu Z, Zuo B, Jing R, et al. Dendritic cell-derived exosomes elicit tumor regression in autochthonous hepatocellular carcinoma mouse models. *J Hepatol* 2017;67:739-48. DOI PubMed
74. Gallet R, Dawkins J, Valle J, et al. Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic porcine myocardial infarction. *Eur Heart J* 2017;38:201-11. DOI PubMed PMC
75. Aminzadeh MA, Rogers RG, Fournier M, et al. Exosome-mediated benefits of cell therapy in mouse and human models of duchenne muscular dystrophy. *Stem Cell Reports* 2018;10:942-55. DOI PubMed PMC
76. Zhang Q, Sun J, Huang Y, et al. Human amniotic epithelial cell-derived exosomes restore ovarian function by transferring microRNAs against apoptosis. *Mol Ther Nucleic Acids* 2019;16:407-18. DOI PubMed PMC

77. Zhao B, Zhang Y, Han S, et al. Exosomes derived from human amniotic epithelial cells accelerate wound healing and inhibit scar formation. *J Mol Histol* 2017;48:121-32. DOI PubMed
78. Zhao B, Li X, Shi X, et al. Exosomal microRNAs derived from human amniotic epithelial cells accelerate wound healing by promoting the proliferation and migration of fibroblasts. *Stem Cells Int* 2018;2018:5420463. DOI PubMed PMC
79. Tan JL, Lau SN, Leaw B, et al. Amnion epithelial cell-derived exosomes restrict lung injury and enhance endogenous lung repair. *Stem Cells Transl Med* 2018;7:180-96. DOI PubMed PMC
80. Royce SG, Patel KP, Mao W, Zhu D, Lim R, Samuel CS. Serelaxin enhances the therapeutic effects of human amnion epithelial cell-derived exosomes in experimental models of lung disease. *Br J Pharmacol* 2019;176:2195-208. DOI PubMed PMC
81. Fu W, Lei C, Liu S, et al. CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity. *Nat Commun* 2019;10:4355. DOI PubMed PMC
82. Yeo RW, Lai RC, Zhang B, et al. Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013;65:336-41. DOI PubMed
83. Can A, Celikkan FT, Cinar O. Umbilical cord mesenchymal stromal cell transplantations: A systemic analysis of clinical trials. *Cytotherapy* 2017;19:1351-82. DOI PubMed
84. Lai RC, Arslan F, Lee MM, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;4:214-22. DOI PubMed
85. Elgaz S, Kuçi Z, Kuçi S, Bönig H, Bader P. Clinical use of mesenchymal stromal cells in the treatment of acute graft-versus-host disease. *Transfus Med Hemother* 2019;46:27-34. DOI PubMed PMC
86. Chakari-Khiavi F, Dolati S, Chakari-Khiavi A, et al. Prospects for the application of mesenchymal stem cells in Alzheimer's disease treatment. *Life Sci* 2019;231:116564. DOI PubMed
87. Hu P, Yang Q, Wang Q, et al. Mesenchymal stromal cells-exosomes: a promising cell-free therapeutic tool for wound healing and cutaneous regeneration. *Burns Trauma* 2019;7:38. DOI PubMed PMC
88. Katsuda T, Ochiya T. Molecular signatures of mesenchymal stem cell-derived extracellular vesicle-mediated tissue repair. *Stem Cell Res Ther* 2015;6:212. DOI PubMed PMC
89. Vogel A, Upadhya R, Shetty AK. Neural stem cell derived extracellular vesicles: Attributes and prospects for treating neurodegenerative disorders. *EBioMedicine* 2018;38:273-82. DOI PubMed PMC
90. Baek G, Choi H, Kim Y, Lee HC, Choi C. Mesenchymal stem cell-derived extracellular vesicles as therapeutics and as a drug delivery platform. *Stem Cells Transl Med* 2019;8:880-6. DOI PubMed PMC
91. Chen TS, Arslan F, Yin Y, et al. Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *J Transl Med* 2011;9:47. DOI PubMed PMC
92. Cha JM, Shin EK, Sung JH, et al. Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells. *Sci Rep* 2018;8:1171. DOI PubMed PMC
93. James S, Fox J, Afsari F, et al. Multiparameter analysis of human bone marrow stromal cells identifies distinct immunomodulatory and differentiation-competent subtypes. *Stem Cell Reports* 2015;4:1004-15. DOI PubMed PMC
94. Stevanato L, Corteling RL, Stroemer P, et al. c-MycERTAM transgene silencing in a genetically modified human neural stem cell line implanted into MCAo rodent brain. *BMC Neurosci* 2009;10:86. DOI PubMed PMC
95. Sinden JD, Hicks C, Stroemer P, Vishnubhatla I, Corteling R. Human neural stem cell therapy for chronic ischemic stroke: charting progress from laboratory to patients. *Stem Cells Dev* 2017;26:933-47. DOI PubMed PMC
96. Serakinci N, Guldberg P, Burns JS, et al. Adult human mesenchymal stem cell as a target for neoplastic transformation. *Oncogene* 2004;23:5095-8. DOI PubMed
97. Bruno S, Collino F, Deregibus MC, Grange C, Tetta C, Camussi G. Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. *Stem Cells Dev* 2013;22:758-71. DOI PubMed
98. Roccaro AM, Sacco A, Maiso P, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest* 2013;123:1542-55. DOI PubMed PMC
99. Zhang X, Tu H, Yang Y, Fang L, Wu Q, Li J. Mesenchymal stem cell-derived extracellular vesicles: roles in tumor growth, progression, and drug resistance. *Stem Cells Int* 2017;2017:1758139. DOI PubMed PMC
100. Li J, Lee Y, Johansson HJ, et al. Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles. *J Extracell Vesicles* 2015;4:26883. DOI PubMed PMC
101. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977;36:59-74. DOI PubMed
102. Ferguson SW, Nguyen J. Exosomes as therapeutics: The implications of molecular composition and exosomal heterogeneity. *J Control Release* 2016;228:179-90. DOI PubMed
103. Vink T, Oudshoorn-Dickmann M, Roza M, Reitsma JJ, de Jong RN. A simple, robust and highly efficient transient expression system for producing antibodies. *Methods* 2014;65:5-10. DOI PubMed
104. Dumont J, Euwart D, Mei B, Estes S, Kshirsagar R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit Rev Biotechnol* 2016;36:1110-22. DOI PubMed PMC
105. Li J, Chen X, Yi J, et al. Identification and characterization of 293T cell-derived exosomes by profiling the protein, mRNA and MicroRNA components. *PLoS One* 2016;11:e0163043. DOI PubMed PMC
106. Ferguson S, Kim S, Lee C, Deci M, Nguyen J. The phenotypic effects of exosomes secreted from distinct cellular sources: a comparative study based on miRNA composition. *AAPS* ;20:67. DOI PubMed PMC
107. Saleh AF, Lázaro-Ibáñez E, Forsgard MA, et al. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity.

Nanoscale 2019;11:6990-7001. DOI PubMed

108. Chen S, Lv M, Fang S, Ye W, Gao Y, Xu Y. Poly(I:C) enhanced anti-cervical cancer immunities induced by dendritic cells-derived exosomes. *Int J Biol Macromol* 2018;113:1182-7. DOI PubMed
109. Markov O, Oshchepkova A, Mironova N. Immunotherapy based on dendritic cell-targeted/-derived extracellular vesicles-a novel strategy for enhancement of the anti-tumor immune response. *Front Pharmacol* 2019;10:1152. DOI PubMed PMC
110. Pitt JM, André F, Amigorena S, et al. Dendritic cell-derived exosomes for cancer therapy. *J Clin Inves* ;126:1224-32. DOI PubMed PMC
111. Gehrmann U, Hiltbrunner S, Georgoudaki AM, Karlsson MC, Näslund TI, Gabrielsson S. Synergistic induction of adaptive antitumor immunity by codelivery of antigen with alpha-galactosylceramide on exosomes. *Cancer Re* ;73:3865-76. DOI PubMed
112. Wang L, Xie Y, Ahmed KA, et al. Exosomal pMHC-I complex targets T cell-based vaccine to directly stimulate CTL responses leading to antitumor immunity in transgenic FVBneuN and HLA-A2/HER2 mice and eradicating trastuzumab-resistant tumor in athymic nude mice. *Breast Cancer Res Treat* 2013;140:273-84. DOI PubMed
113. Damo M, Wilson DS, Simeoni E, Hubbell JA. TLR-3 stimulation improves anti-tumor immunity elicited by dendritic cell exosome-based vaccines in a murine model of melanoma. *Sci Rep* 2015;5:17622. DOI PubMed PMC
114. Morse MA, Garst J, Osada T, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* 2005;3:9. DOI PubMed PMC
115. Tian Y, Jiang X, Chen X, Shao Z, Yang W. Doxorubicin-loaded magnetic silk fibroin nanoparticles for targeted therapy of multidrug-resistant cancer. *Adv Mater* 2014;26:7393-8. DOI PubMed
116. Escudier B, Dorval T, Chaput N, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. *J Transl Med* 2005;3:10. DOI PubMed PMC
117. Besse B, Charrier M, Lapierre V, et al. Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* 2016;5:e1071008. DOI PubMed PMC
118. Gao W, Liu H, Yuan J, et al. Exosomes derived from mature dendritic cells increase endothelial inflammation and atherosclerosis via membrane TNF-alpha mediated NF-kappaB pathway. *J Cell Mol Med* 2016;20:2318-27. DOI PubMed PMC
119. Ibrahim AGE, Li C, Rogers R, et al. Augmenting canonical Wnt signalling in therapeutically inert cells converts them into therapeutically potent exosome factories. *Nat Biomed Eng* 2019;3:695-705. DOI PubMed PMC
120. Fournier C, Martin F, Zitvogel L, Kroemer G, Galluzzi L, Apetoh L. Trial watch: adoptively transferred cells for anticancer immunotherapy. *Oncoimmunology* 2017;6:e1363139. DOI PubMed PMC
121. Wang L, Yin P, Wang J, et al. Delivery of mesenchymal stem cells-derived extracellular vesicles with enriched miR-185 inhibits progression of OPMD. *Artif Cells Nanomed Biotechnol* 2019;47:2481-91. DOI PubMed
122. Romero R, Espinoza J, Kusanovic JP, et al. The preterm parturition syndrome. *BJOG* 2006;113 Suppl 3:17-42. DOI PubMed PMC
123. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. *Science* 2014;345:760-5. DOI PubMed PMC
124. Xu Y, Romero R, Miller D, et al. Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. *Am J Reprod Immunol* 2018;79:e12820. DOI PubMed PMC
125. Beck S, Wojdyla D, Say L, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010;88:31-8. DOI PubMed PMC
126. Ronco C, Bellomo R, Kellum JA. Acute kidney injury. *Lancet* 2019;394:1949-64. DOI PubMed
127. Tang TT, Wang B, Wu M, et al. Extracellular vesicle-encapsulated IL-10 as novel nanotherapeutics against ischemic AKI. *Sci Adv* 2020;6:eaaz0748. DOI PubMed PMC
128. Ha D, Yang N, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. *Acta Pharm Sin B* 2016;6:287-96. DOI PubMed PMC
129. Perets N, Hertz S, London M, Offen D. Intranasal administration of exosomes derived from mesenchymal stem cells ameliorates autistic-like behaviors of BTBR mice. *Mol Autism* 2018;9:57. DOI PubMed PMC
130. Williams AM, Dennahy IS, Bhatti UF, et al. Mesenchymal stem cell-derived exosomes provide neuroprotection and improve long-term neurologic outcomes in a swine model of traumatic brain injury and hemorrhagic shock. *J Neurotrauma* 2019;36:54-60. DOI PubMed

Holliday *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:18-28
DOI: 10.20517/evcna.2020.02

Extracellular Vesicles and Circulating Nucleic Acids

Review

Open Access

RANKL and RANK in extracellular vesicles: surprising new players in bone remodeling

L. Shannon Holliday[1,3], Shivani S. Patel[1], Wellington J. Rody, Jr[2]

[1]Department of Orthodontics, University of Florida College of Dentistry, Gainesville, FL 32610, USA.
[2]Department of Orthodontics and Pediatric Dentistry, Stony Brook School of Dental Medicine, Stony Brook, NY 11794, USA.
[3]Department of Anatomy & Cell Biology, University of Florida College of Medicine, Gainesville, FL 32610, USA.

Correspondence to: Prof. L. Shannon Holliday, PhD, Department of Orthodontics, University of Florida College of Dentistry, 1600 SW Archer Road, Gainesville, FL 32610, USA. E-mail: sholliday@dental.ufl.edu

How to cite this article: Holliday LS, Patel SS, Rody WJ, Jr. RANKL and RANK in extracellular vesicles: surprising new players in bone remodeling. *Extracell Vesicles Circ Nucleic Acids* 2021;2:18-28. https://dx.doi.org/10.20517/evcna.2020.02

Received: 10 Nov 2020 **First Decision:** 5 Jan 2021 **Revised:** 11 Jan 2021 **Accepted:** 14 Jan 2021 **Available online:** 30 Mar 2021

Academic Editor: Y. Peng Loh **Copy Editor:** Yue-Yue Zhang **Production Editor:** Xi-Jun Chen

Abstract

Receptor activator of nuclear factor kappa B-ligand (RANKL), its receptor RANK, and osteoprotegerin which binds RANKL and acts as a soluble decoy receptor, are essential controllers of bone remodeling. They also play important roles in establishing immune tolerance and in the development of the lymphatic system and mammary glands. In bone, RANKL stimulates osteoclast formation by binding RANK on osteoclast precursors and osteoclasts. This is required for bone resorption. Recently, RANKL and RANK have been shown to be functional components of extracellular vesicles (EVs). Data linking RANKL and RANK in EVs to biological regulatory roles are reviewed, and crucial unanswered questions are examined. RANKL and RANK are transmembrane proteins and their presence in EVs allows them to act at a distance from their cell of origin. Because RANKL-bearing osteocytes and osteoblasts are often spatially distant from RANK-containing osteoclasts in vivo, this may be crucial for the stimulation of osteoclast formation and bone resorption. RANK in EVs from osteoclasts has the capacity to stimulate a RANKL reverse signaling pathway in osteoblasts that promotes bone formation. This serves to couple bone resorption with bone formation and has inspired novel bifunctional therapeutic agents. RANKL- and RANK- containing EVs in serum may serve as biomarkers for bone and immune pathologies. In summary, EVs containing RANKL and RANK have been identified as intercellular regulators in bone biology. They add complexity to the central signaling network responsible for maintaining bone. RANKL- and RANK-containing EVs are attractive as drug targets and as biomarkers.

Keywords: Cell signaling, osteoclast, osteoblast, osteocyte, exosome, microvesicle, coupling factor, apoptotic vesicle

INTRODUCTION

Receptor activator of nuclear factor kappa B-ligand (RANKL), its receptor RANK, and the RANKL-binding decoy receptor osteoprotegerin are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies[1]. In the late 1990s, these proteins were identified as essential for regulating the formation of osteoclasts and thus for bone remodeling[2,3]. Mice in which RANKL was knocked out had few or no osteoclasts, the hematopoietic cells specialized to resorb bone, and were severely osteopetrotic[4]. In addition, these mice had immune cell abnormalities and lacked certain lymph nodes. Female RANKL knockout mice died before maturity as a consequence of defective breast development[5]. Mice in which osteoprotegerin was knocked out were osteoporotic[2]. This led to the hypothesis that bone resorption was a function of the ratio of RANKL to osteoprotegerin.

These regulatory molecules were identified due to efforts to find the "osteoclast differentiation factor". Indirect evidence had suggested that there might be a specific regulatory molecule responsible for directing hematopoietic stem cells to differentiate into osteoclasts. Since bone diseases, in particular osteoporosis and metastatic bone cancer, are major clinical problems that are associated with excess bone resorption by osteoclasts, it was reasoned that if such a specific regulatory molecule existed, it would be an extremely attractive target for drug development[6,7]. Groups from Amgen and Snow Brand Milk Products concurrently identified RANKL (called "osteoprotegerin-ligand" by the Amgen group and "osteoclast differentiation factor" by the group from Snow Brand) as the osteoclast differentiation factor[2,3]. The nomenclature was later resolved to RANKL. Osteoprotegerin, which binds RANKL and blocks its interaction with RANK, and thus osteoclast formation, has not been developed directly into a therapeutic agent because it is promiscuous, binding other TNF superfamily members, and has a short half-life in circulation[6]. Amgen developed a humanized monoclonal antibody directed against RANKL (denosumab), which has become a major therapeutic for the treatment of osteoporosis (Prolia®) and bone cancer (Xgeva®) [7-9]. Although RANKL, RANK, and osteoprotegerin have roles in the immune system, the use of denosumab as a therapeutic has had relatively few off target effects.

Extracellular vesicles (EVs) include exosomes and microvesicle (also known as ectosomes)[10,11]. Exosomes are released from cells as cytosolic multivesicular bodies fuse with the plasma membrane. The multivesicular bodies are formed by inward budding of the bounding membrane of cytosolic vesicles. Microvesicles are formed by direct budding from the plasma membrane. Evidence suggests that there is considerable overlap in the composition and size range of exosomes and microvesicles. For this reason, the International Society for Extracellular Vesicles recommends using the term EV to describe these vesicles unless there is a clear evidence that the EVs are exosomes or microvesicles[12]. In this article we will use the term EV, though various nomenclatures were used in the original reports. A third type of EV is apoptotic vesicles that form during apoptosis, but these are larger and are usually separated from exosomes and microvesicles during standard isolations. During the past decade, many studies have supported the hypothesis that EVs serve as intercellular regulators[13].

The discovery of RANKL in EVs in 2015[14], and RANK in EVs in 2016[15], together with other recent advances in the understanding of bone biology, particularly the finding that most RANKL that stimulates bone resorption is from osteocytes[16-20], has opened up new lines of study [Figure 1]. Incorporation of these transmembrane proteins into EVs allows each to be, as needed, either a receptor or a ligand, serving as an

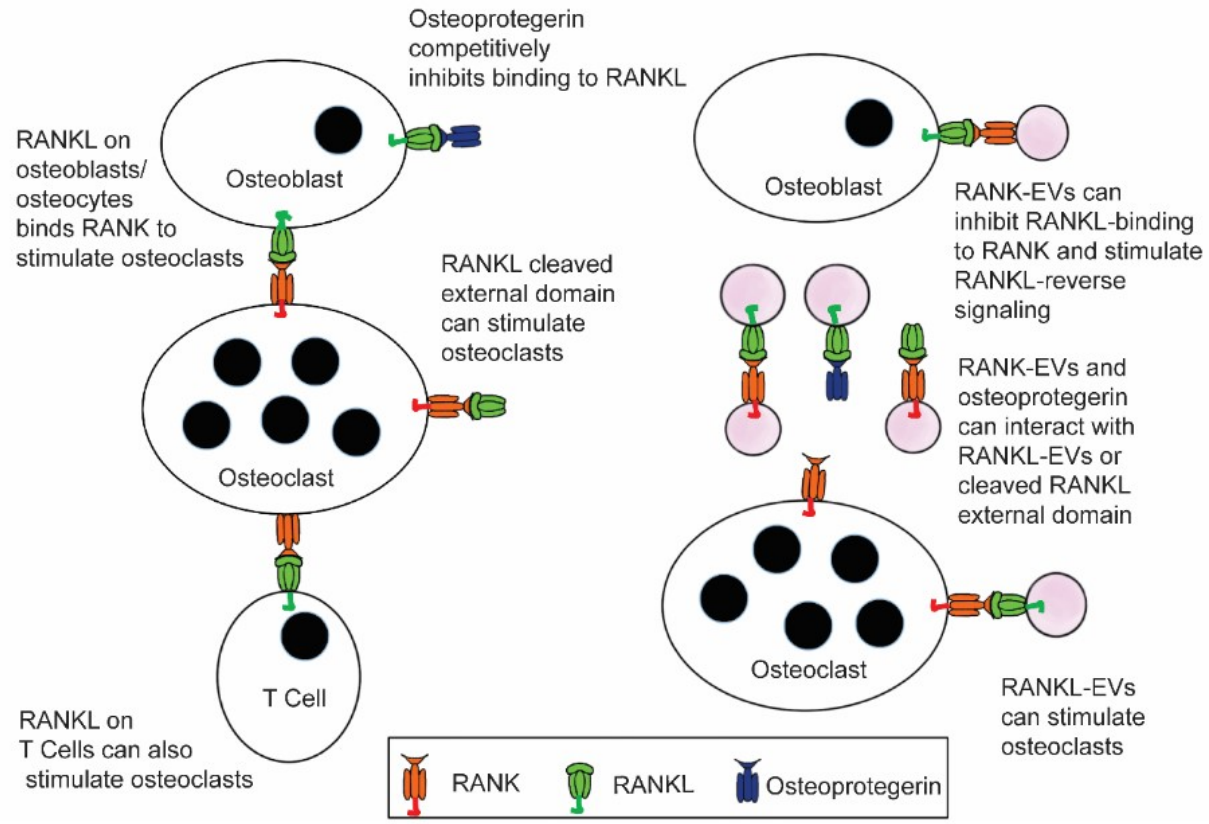


Figure 1. Extracellular vesicles containing RANKL or RANK add increased complexity to the RANKL/RANK/osteoprotegerin signaling network that is at the core of bone biology. Until recently, RANKL stimulation of RANK to stimulate osteoclast formation and bone resorption by osteoclasts, and the ability of osteoprotegerin to bind RANKL and competitively inhibit this signaling, were considered the primary features of this network. Now data suggest that RANKL EVs can stimulate osteoclast formation and bone resorption through RANK stimulation, and RANK-EVs bind to RANKL on osteoblasts to stimulate RANKL reverse signaling and bone formation. The latter serves to couple bone resorption and bone formation. It is also possible that RANK-EVs can bind RANKL or RANKL-EVs and competitively inhibit their stimulation of RANK on osteoclasts. RANKL-EVs could serve as competitive inhibitors of RANK-EV's stimulation of RANKL reverse signaling.

elegant and parsimonious evolutionary solution for regulating the coupling of bone resorption and bone formation. As this work develops, it promises deeper understanding of how bone is remodeled, and new therapeutic approaches to treat bone disease. However, numerous unanswered questions are open in this field of research.

RANKL-EVS

Osteoblast EVs: long range stimulation of osteoclastic bone resorption

RANKL is expressed by osteoblasts, osteocytes, T cells, B cells, and various other tissues during development[4,19,20]. It is also found in the brain in the lateral septal nucleus[21], and astrocytes and microglia contain RANK and respond to RANKL[22,23]. RANKL is a transmembrane protein that can also act as a receptor[24]. RANKL exists in cells as a homotrimer. The binding site for RANK (also a homotrimer) on RANKL is formed by elements of two adjacent RANKL molecules[25]. Until the discovery of RANKL in EVs, two forms of RANKL were known in biology, the intact membrane bound form, and a soluble form cleaved from the plasma membrane by exoproteases/sheddases including TNF-α converting enzyme, several a disintegrin and metalloproteinases, and matrix metalloproteinases[26-28]. A recent study showed that in mice where RANKL was altered to prevent the action of exoproteases, normal bone physiology was not

perturbed, although the mice were more susceptible to cancer invasion[29].

Data suggesting regulatory RANKL in EVs was first described in an *in vitro* system[14]. EVs isolated from a stromal/osteoblastic cell line (UAMS-32P) contained RANKL and were able to stimulate differentiation of RAW 264.7 cells into osteoclast-like cells. Immunogold transmission electron microscopy indicated that the EVs containing RANKL were large (200 nm or more), suggesting that they may be microvesicles. While this report utilized cell lines, it provided the first suggestion of functional RANKL in EVs.

These data were confirmed and extended to show that EVs released by primary osteoblasts (isolated from mouse calvaria) produced RANKL-containing EVs that stimulated primary mouse monocytes to differentiate into osteoclasts[30]. These EVs were also large, 100-200 nm in diameter, suggesting that they may be microvesicles. In addition, these RANKL-containing EVs triggered osteoclast formation *in vivo* when injected into transgenic mice in which RANKL was knocked out. By using osteoblast EVs loaded with the fluorophore chloromethyl fluorescein diacetate, it was shown that the osteoblast-derived EVs could fuse with target cells and deliver the luminal contents to the cytosol. It was then demonstrated that RANKL-containing EVs could be used to deliver anti-osteoclastic drugs to osteoclasts *in vivo*. Overactive osteoclasts were stimulated by treatment of mice with retinoic acid. These mice were then injected with RANKL-containing EVs from osteoblasts in which the osteoclast inhibitors, zoledronic acid (which blocks prenylation pathways) or dasatinib (which blocks tyrosine kinases including c-src) had been incorporated. The therapeutic efficacy of the EVs was demonstrated by the reduction of the overstimulation of osteoclasts, caused by retinoic acid, measured by both a serum marker of bone resorption and counts of osteoclasts and apoptotic osteoclasts in bone sections[30]. In a larger sense, these results suggest that in addition to stimulating RANK on the surface of the osteoclasts, the RANKL-EVs fused with and delivered the contents of the lumen of the EVs (osteoclast inhibiting pharmaceuticals) to the target cells. While in this case, the lumens contained zoledronic acid or dastinib, it suggests that under physiologic conditions, regulatory agents found in the EV lumen (microRNAs for example) are targeted to the cytosol of osteoclasts. Characterization of the composition of RANKL-EVs shed by osteoblasts will be required to identify other regulatory signals that may be present.

Recently, a zebra-fish model using fluorescently-tagged proteins for direct visualization of EVs was used to demonstrate that osteoblasts release RANKL-containing EVs that stimulate osteoclast formation *in vitro*, and also deliver luminal contents to osteoclasts *in vivo*[31]. These data confirmed the results of the studies in mammalian cells and suggest that the involvement of RANKL-containing EVs in bone signaling has ancient evolutionary origins.

Studies have suggested that EVs from osteoblasts and osteocytes are involved in communication with each other, with mesenchymal stem cells, and with myoblasts[32]. Osteoblast EVs contain various microRNAs, but the receptor-ligand binding interactions that dock the EVs have not been identified. It is also not clear whether microRNAs or other type of RNAs carried in EVs are acting in these regulatory pathways.

RANKL in osteocyte EVs

Osteocytes have been shown to release regulatory EVs[33-38] and RANKL was shown to be released by in EVs from osteocytes in response to mechanical force[34]. However, it has not yet been shown that RANKL-containing EVs shed by osteocytes stimulate biologically relevant osteoclast formation. It has emerged that most (90%) of the RANKL stimulation of osteoclasts *in vivo* in mammals is by RANKL derived from osteocytes[17]. These cells are a final differentiation step in the osteoblast lineage and are the most abundant cells in bone, inhabiting lacunae that weave through the bone. It is not clear how RANKL on the surface of

osteocytes would be accessible to osteoclasts and their precursors. One possibility is that soluble RANKL cleaved by proteases might serve to stimulate distant osteoclasts, but recent data indicating soluble RANKL is not required for normal bone physiology suggest that this is not the case[29]. It is attractive to hypothesize that RANKL-containing EVs released from osteocytes have the ability to stimulate spatially distant osteoclasts.

RANK-EVS

Osteoclast EVs: RANKL reverse signaling

RANK was first identified in EVs *in vitro* in efforts to identify the basis of differential regulatory activities of EVs released by pre-osteoclasts compared with EVs released from mature osteoclasts[15]. Several differentially expressed proteins were detected, and mass spectrometry identified RANK to be relatively abundant in EVs from osteoclasts, but not in EVs from precursors. This result was confirmed by immunoblots and by immunogold labeling of EVs from osteoclasts visualized in negative stained transmission electron microscopy. The electron microscopy indicated that RANK in EVs was concentrated in a small subset of the total EVs shed by osteoclasts. The size of the EVs in which RANK was detected was about 50-nm in diameter, which is consistent with the RANK-containing EVs being exosomes. Data were also presented that suggested that the RANK-containing EVs inhibited osteoclast formation in calcitriol-stimulated mouse marrow cultures by a paracrine mechanism. It was suggested that RANK-EVs might act as competitive inhibitors of the RANKL-RANK interaction in the manner of osteoprotegerin[15,39].

The publication showing RANK in EVs appeared concurrently with two other article describing regulatory EVs released by osteoclasts[40,41]. Both of these reports found that EVs from osteoclasts delivered microRNA-214-3p to osteoblasts and consequent reduction of target mRNA translation inhibited osteoblast formation and function. One group reported that targeting of the EVs from osteoclasts to osteoblasts was achieved by interaction between semaphorin 4D on the osteoclast EVs with plexinB1 on the osteoblast[40]. The semaphorin 4D-containing EVs then fused with the osteoblast to release luminal microRNA 214-3p into the cytosol where it inhibited translation of the mRNA coding for activating transcription factor 4. This resulted in reduced osteoblast formation and bone formation[40]. The second study found that osteoclast EVs bound to osteoblasts through ephrinA2 binding to ephrin receptor on the osteoblasts. They also reported that osteoblasts were inhibited due to the introduction of microRNA 214-3p into the osteoblast cytosol. Both of these studies found EVs from osteoclasts to be inhibitors of osteoblasts[40,41]. These studies were not consistent with EVs from osteoclasts serving as "coupling factors" that signal to balance bone formation with bone resorption.

The initial finding of RANK in EVs was subsequently confirmed by several groups[42-44]. Ikebuchi*et al.*[24] greatly expanded the understanding of RANK-containing EVs in an article in *Nature*. They showed that when RANK-containing EVs bind RANKL on osteoblasts, a RANKL reverse signaling pathway is stimulated that triggers pre-osteoblastic cells, which do not form bone, to differentiate and form bone. This reverse signaling pathway acted through phosphatidylinositol 3-kinase, mammalian target of rapamycin and runt-related transcription factor 2. They hypothesized that the RANK-containing EVs would act as "coupling factors" that could promote bone formation to replace bone resorbed by osteoclasts[45,46]. They demonstrated *in vivo* that RANK-EVs promoted bone formation in the calvarial critical size bone defect model. In an exciting addition, they found that certain cross-linked anti-RANKL antibodies mimicked RANK-containing EVs to stimulate RANKL reverse signaling. These antibodies were tested in an *in vivo* rodent model of postmenopausal osteoporosis. They blocked the ability of RANKL to stimulate RANK, like osteoprotegerin, and thus blocked bone resorption. Unlike osteoprotegerin, they also stimulated RANKL reverse signaling and bone formation. This provided a proof-of-concept for a new type of bifunctional

therapeutic agent that may have the potential to bring about transformative changes to the way osteoporosis and other bone diseases are treated.

Regulation of RANK-EVs release by osteoclasts

It is attractive to hypothesize that the shedding of RANK-containing EVs is regulated. One line of evidence supporting this idea is the finding that osteoclasts resorbing bone released 5-times more RANK than the same cells resorbing dentin, the mineralized tissue that makes up the bulk of a tooth[47]. The differential release of RANK in EVs was accompanied by differential release of numerous other components as determined by quantitative high-resolution liquid chromatograph-mass spectrometry. The difference in RANK-containing EV shedding observed in this study could have been driven by matrix-related factors. This is biologically plausible in that tooth resorption is not coupled to bone formation. Moreover, if coupled bone formation occurred associated with tooth resorption, it could lead to fusion of the alveolar bone to the tooth structure, a pathological process named ankylosis. It is of interest to identify the factors in bone that trigger RANK-containing EV release, or perhaps the factors that suppress release when the cells are resorbing dentin. Although the two mineralized tissue share many components, previous studies show that there are compositional differences[48-50].

What about other factors? For example, do specific cytokines known to regulate osteoclast formation and activity also activate, or suppress RANK-EV release? Might mechanical stress, which favors bone formation *in vivo*[51,52], stimulate RANK-EV shedding in the same way as it does with osteocytes? At a more fundamental level, what mechanisms are involved in packaging RANK into EVs? For example, are receptors incorporated into EVs, in this case exosomes, as a pathway that is part of the general receptor recycling process [Figure 2]? RANK recycling after internalization from the plasma membrane has been shown to be controlled by the retromer complex[53]. Reduction in the vps35 component of the retromer leads to more RANK on the osteoclast surface, and increased bone resorption that is uncoupled with bone formation in mice leading to osteoporosis[53]. We suggest that RANK recycling may simultaneously regulate the number of RANK molecules on the osteoclast cell surface that are available for stimulation by RANKL, and the number of RANK molecules loaded into exosomes. Regulation of the recycling process could serve as an osteoclast-centered rheostat regulating the amount of osteoclastic bone resorption and the relative amount of bone formation coupled to the bone resorption.

RANK-EVs in apoptotic bodies from osteoclasts

A number of recent studies have suggested a change in paradigm; apoptotic bodies/vesicles may serve not only as easily handled materials for cells tasked with cleaning the dead cells remains, but also as regulatory signals[13]. In particular, apoptotic vesicles have been shown to produce pro-inflammatory signals. Apoptosis of osteoclasts typically precedes the recruitment and differentiation of osteoblasts in a bone remodeling unit[45]. Ma *et al.*[54] explored whether apoptotic bodies might contain RANK and stimulate reverse RANKL signaling in the same way and RANK-containing EVs. They found that when osteoclasts were grown in culture and stimulated to undergo apoptosis with alendronate, apoptotic bodies recovered from the conditioned media contained RANK. These apoptotic vesicles were able to stimulate RANKL-reverse signaling and mineralization by osteoblasts as had been described by RANK-containing EVs[24]. The stimulation was blocked by the inclusion of recombinant soluble RANKL. Although this is an intriguing *in vitro* result, questions remain regarding whether this is a true physiologic mechanism. For example, when alendronate is given *in vivo,* it binds bone and reduces bone resorption by triggering osteoclasts that begin to resorb the treated bone to undergo apoptosis[55]. If apoptotic bodies were a significant anabolic signal, alendronate should be a potent bone anabolic agent. Instead, alendronate is able to reduce bone loss, but not stimulate increases in bone density[55]. It is possible that *in vivo*, surveilling macrophages detect and phagocytize apoptotic bodies from osteoclasts before RANK on the surface can stimulate RANKL reverse

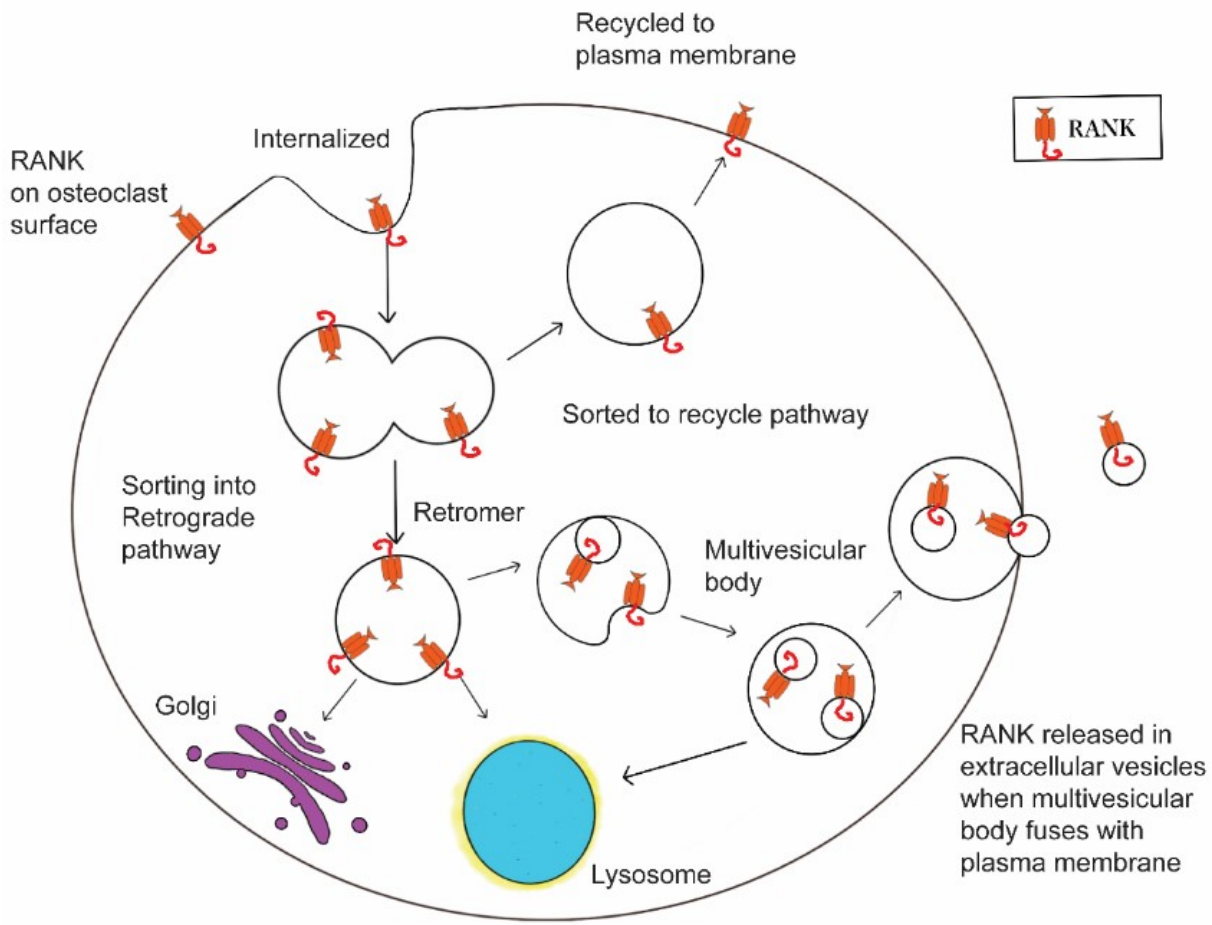


Figure 2. Regulation of RANK-containing EV production. We hypothesize that when RANK is internalized, an element of the normal receptor life cycle, it can either be recycled back to the plasma membrane, where it would be available for continued stimulation by RANKL. This would favor bone resorption. Alternatively, it could be sorted into a retrograde pathway that could lead to the Golgi for reuse of RANK, to the lysosome for degradation, or to multivesicular bodies, some of which would fuse with the plasma membrane and release RANK-EVs. All the retrograde pathways would remove RANK from the plasma membrane surface, and thus reduce stimulation by RANKL. In addition, shedding of RANK-EVs would also stimulate the RANKL reverse pathway and bone formation. If this idea is correct, shifts in the sorting of RANK could have profound effects on both bone resorption and bone formation, and understanding the underlying mechanisms may identify new strategies for treating bone disease.

signaling in cells of the osteoblast lineage[13].

RANKL-containing EVs and cancer invasion

Numerous studies have suggested that cancer cells produce EVs that facilitate the growth and invasion of cancer cells to specific niches including bone[56,57]. Certain types of cancers, including breast, prostate, and multiple myeloma are particularly dangerous because of their ability to invade bone[57]. This is accomplished at least in part by sending signals that trigger inappropriate bone resorption by osteoclasts[57]. The "seed and soil" hypothesis proposes that that resorptive activity produces factors that trigger cancer growth and invasion and the stimulation of even more inappropriate bone resorption[57]. Might such cancer cells produce EVs containing RANKL to trigger bone resorption?

A recent study reports that EVs released by the breast cancer cell line MDA-MB-231 release EVs that contain the mRNA for RANKL[58]. However, RANKL was not detected by Western blot analysis, and cytofluorometric analysis also failed to convincingly demonstrate RANKL. These EVs were able to reach the

bone microenvironment after intraperitoneal injection and be taken up by osteoclasts and osteoblasts. It was suggested that these EVs might be involved in deregulation of normal bone and endothelial physiology[58].

RANKL- and RANK- containing EVs outside of the skeletal system

As mentioned above, RANKL and RANK are found in tissues other than the skeletal system. There is some evidence that these cells may release and be regulated by EVs containing these proteins. One study identified RANKL and RANK in circulating EVs in humans[43]. They found that the level of RANK in the circulating EVs served as a biomarker for psoriatic, but not rheumatoid, arthritis. They also found that isolated circulating EVs could regulate bone cells *in vitro*. Subsequently, it was shown that circulating RANK- and RANKL- containing EVs serve as biomarkers for bone loss linked to antiviral treatment for HIV-infections[44].

Might the RANKL in circulating EVs prove to be a better biomarker than overall circulating RANKL? Numerous studies have examined RANKL in circulation and in other body fluids as a possible biomarker for disease, with limited success[59-65]. These were performed under the assumption that circulating RANKL would all be the external domain cleaved by sheddases. However, we now know that some portion of these molecules is present in EVs. The absolute amount of these molecules in EVs, or ratios, like the amount of circulating cleaved RANKL divided by RANKL in EVs, may prove useful as biomarkers.

As described above, the RANKL/RANK systems are regulators in the brain[23]. For example, injection of RANKL into the brain causes thermoregulatory disruption in female mice[21]. EVs have been shown to pass the blood brain barrier, particularly under inflammatory conditions. It is possible that EVs in circulation that are derived from the brain can be isolated based on specific neuronal, or microglial markers, and then assayed for RANKL and RANK. These may prove useful biomarkers for neuroinflammatory or neurodegenerative diseases.

Finally, circulating RANKL has been shown to be a biomarker for cardiovascular disease[63]. Detection of circulating RANKL in EVs and perhaps circulating RANK in EVs, may improve the diagnostic performance.

CONCLUSIONS

RANKL and RANK have been studied with great intensity since the discovery that RANKL was essential for stimulating osteoclast formation and bone resorption[6]. The therapeutic agent denosumab, a humanized monoclonal antibody that binds RANKL, has been successful for a decade for the treatment of osteoporosis and metastatic bone cancer[8,9]. RANKL and RANK have only recently been identified in EVs, and this area of study is still in its infancy. Already strong support for the hypothesis that RANKL and RANK-containing EVs have important roles in maintaining bone has emerged, as has evidence that these signaling molecules and their interactions can be targeted for therapeutics[24] and used diagnostically[42,44]. The most exciting result so far has been the demonstration that certain crosslinked antibodies against RANKL can both inhibit bone resorption and stimulate bone formation[24]. Such bifunctional therapeutics might transform the treatment of bone diseases.

DECLARATIONS

Authors' contributions

Wrote first draft of manuscript, performed literature searches, and made initial interpretation: Holliday LS
Constructed figures and aided in revision of manuscript: Patel SS

Critically read, interpreted, and revised manuscript: Rody WJ Jr

Availability of data and materials
Not applicable.

Financial support and sponsorship
This work was supported by National Institutes of Health/National Institute of Dental and Craniofacial Research R21 DE019862 (Holliday LS), R03 DE027504 (Rody WJ Jr), a University of Florida College of Dentistry Seed Grant (Rody WJ Jr, Holliday LS), and a University Scholar Fellowship (Patel SS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

REFERENCES
1. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys* 2008;473:139-46. DOI PubMed PMC
2. Lacey D, Timms E, Tan H, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 1998;93:165-76. DOI PubMed
3. Yasuda H, Shima N, Nakagawa N, et al. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A* 1998;95:3597-602. DOI PubMed PMC
4. Kong YY, Yoshida H, Sarosi I, et al. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 1999;397:315-23. DOI PubMed
5. Fata JE, Kong Y, Li J, et al. The Osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 2000;103:41-50. DOI PubMed
6. Nagy V, Penninger JM. The RANKL-RANK story. *Gerontology* 2015;61:534-42. DOI PubMed
7. Compston JE, Mcclung MR, Leslie WD. Osteoporosis. *Lancet* 2019;393:364-76. DOI
8. Bone HG, Wagman RB, Brandi ML, et al. 10 years of denosumab treatment in postmenopausal women with osteoporosis: results from the phase 3 randomised FREEDOM trial and open-label extension. *Lancet Diabetes Endocrinol* 2017;5:513-23. DOI PubMed
9. Lipton A, Fizazi K, Stopeck AT, et al. Effect of denosumab versus zoledronic acid in preventing skeletal-related events in patients with bone metastases by baseline characteristics. *Eur J Cancer* 2016;53:75-83. DOI PubMed
10. Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213-28. DOI PubMed
11. Mathieu M, Martin-Jaular L, Lavieu G, Théry C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol* 2019;21:9-17. DOI PubMed
12. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC
13. Caruso S, Poon IKH. Apoptotic cell-derived extracellular vesicles: more than just debris. *Front Immunol* 2018;9:1486. DOI PubMed PMC
14. Deng L, Wang Y, Peng Y, et al. Osteoblast-derived microvesicles: a novel mechanism for communication between osteoblasts and osteoclasts. *Bone* 2015;79:37-42. DOI PubMed
15. Huynh N, VonMoss L, Smith D, et al. Characterization of regulatory extracellular vesicles from osteoclasts. *J Dent Res* 2016;95:673-9. DOI PubMed PMC
16. Killock D. Bone: Osteocyte RANKL in bone homeostasis: a paradigm shift? *Nat Rev Rheumatol* 2011;7:619. DOI PubMed

17. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* 2011;17:1231-4. DOI PubMed
18. Xiong J, O'Brien CA. Osteocyte RANKL: new insights into the control of bone remodeling. *J Bone Miner Res* 2012;27:499-505. DOI PubMed PMC
19. Kong YY, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. *Immunol Cell Biol* 1999;77:188-93. DOI PubMed
20. Kong Y, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a regulator of immune responses and bone physiology. *Immunology Today* 2000;21:495-502. DOI PubMed
21. Hanada R, Leibbrandt A, Hanada T, et al. Central control of fever and female body temperature by RANKL/RANK. *Nature* 2009;462:505-9. DOI PubMed
22. Serrano EM, Ricofort RD, Zuo J, Ochotny N, Manolson MF, Holliday LS. Regulation of vacuolar H(+)-ATPase in microglia by RANKL. *Biochem Biophys Res Commun* 2009;389:193-7. DOI PubMed PMC
23. Hanada R. The role of the RANKL/RANK/OPG system in the central nervous systems (CNS). *J Bone Miner Metab* 2020. DOI PubMed
24. Ikebuchi Y, Aoki S, Honma M, et al. Coupling of bone resorption and formation by RANKL reverse signalling. *Nature* 2018;561:195-200. DOI PubMed
25. Liu C, Walter TS, Huang P, et al. Structural and functional insights of RANKL-RANK interaction and signaling. *J Immunol* 2010;184:6910-9. DOI PubMed
26. Pan B, Farrugia AN, To LB, et al. The nitrogen-containing bisphosphonate, zoledronic acid, influences RANKL expression in human osteoblast-like cells by activating TNF-alpha converting enzyme (TACE). *J Bone Miner Res* 2004;19:147-54. DOI PubMed
27. Hikita A, Yana I, Wakeyama H, et al. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. *J Biol Chem* 2006;281:36846-55. DOI PubMed
28. Lynch CC, Hikosaka A, Acuff HB, et al. MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Cancer Cell* 2005;7:485-96. DOI PubMed
29. Asano T, Okamoto K, Nakai Y, et al. Soluble RANKL is physiologically dispensable but accelerates tumour metastasis to bone. *Nat Metab* 2019;1:868-75. DOI PubMed
30. Cappariello A, Loftus A, Muraca M, Maurizi A, Rucci N, Teti A. Osteoblast-derived extracellular vesicles are biological tools for the delivery of active molecules to bone. *J Bone Miner Res* 2018;33:517-33. DOI PubMed
31. Kobayashi-Sun J, Yamamori S, Kondo M, et al. Uptake of osteoblast-derived extracellular vesicles promotes the differentiation of osteoclasts in the zebrafish scale. *Commun Biol* 2020;3:190. DOI PubMed PMC
32. Huang X, Xiong X, Liu J, Zhao Z, Cen X. MicroRNAs-containing extracellular vesicles in bone remodeling: an emerging frontier. *Life Sci* 2020;254:117809. DOI PubMed
33. Eichholz KF, Woods I, Riffault M, et al. Human bone marrow stem/stromal cell osteogenesis is regulated via mechanically activated osteocyte-derived extracellular vesicles. *Stem Cells Transl Med* 2020;9:1431-47. DOI PubMed PMC
34. Morrell AE, Brown GN, Robinson ST, et al. Mechanically induced Ca^{2+} oscillations in osteocytes release extracellular vesicles and enhance bone formation. *Bone Res* 2018;6:6. DOI PubMed PMC
35. Qin Y, Peng Y, Zhao W, et al. Myostatin inhibits osteoblastic differentiation by suppressing osteocyte-derived exosomal microRNA-218: A novel mechanism in muscle-bone communication. *J Biol Chem* 2017;292:11021-33. DOI PubMed PMC
36. Lv PY, Gao PF, Tian GJ, et al. Osteocyte-derived exosomes induced by mechanical strain promote human periodontal ligament stem cell proliferation and osteogenic differentiation via the miR-181b-5p/PTEN/AKT signaling pathway. *Stem Cell Res Ther* 2020;11:295. DOI PubMed PMC
37. Sato M, Suzuki T, Kawano M, Tamura M. Circulating osteocyte-derived exosomes contain miRNAs which are enriched in exosomes from MLO-Y4 cells. *Biomed Rep* 2017;6:223-31. DOI PubMed PMC
38. Yan Y, Wang L, Ge L, Pathak JL. Osteocyte-mediated translation of mechanical stimuli to cellular signaling and its role in bone and non-bone-related clinical complications. *Curr Osteoporos Rep* 2020;18:67-80. DOI PubMed
39. Holliday LS, McHugh KP, Zuo J, Aguirre JI, Neubert JK, Rody WJ Jr. Exosomes: novel regulators of bone remodelling and potential therapeutic agents for orthodontics. *Orthod Craniofac Res* 2017;20 Suppl 1:95-9. DOI PubMed PMC
40. Li D, Liu J, Guo B, et al. Osteoclast-derived exosomal miR-214-3p inhibits osteoblastic bone formation. *Nat Commun* 2016;7:10872. DOI PubMed PMC
41. Sun W, Zhao C, Li Y, et al. Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity. *Cell Discov* 2016;2:16015. DOI PubMed PMC
42. Marton N, Kovács OT, Baricza E, et al. Extracellular vesicles regulate the human osteoclastogenesis: divergent roles in discrete inflammatory arthropathies. *Cell Mol Life Sci* 2017;74:3599-611. DOI PubMed
43. Okada E, Nakata H, Yamamoto M, Kasugai S, Kuroda S. Indirect osteoblast differentiation by liposomal clodronate. *J Cell Mol Med* 2018;22:1127-37. DOI PubMed PMC
44. de Menezes EG, Ramallho J, Bucovsky M, Shane E, Yin MT, Norris PJ. Serum extracellular vesicles expressing bone activity markers associate with bone loss after HIV antiretroviral therapy. *AIDS* 2020;34:351-61. DOI PubMed PMC
45. Sims NA, Martin TJ. Osteoclasts Provide Coupling signals to osteoblast lineage cells through multiple mechanisms. *Annu Rev Physiol* 2020;82:507-29. DOI PubMed
46. Rody WJ Jr, Chamberlain CA, Emory-Carter AK, et al. The proteome of extracellular vesicles released by clastic cells differs based on their substrate. *PLoS One* 2019;14:e0219602. DOI PubMed PMC

47. Arana-Chavez VE, Bradaschia-Correa V. Clastic cells: mineralized tissue resorption in health and disease. *Int J Biochem Cell Biol* 2009;41:446-50. DOI PubMed
48. Wang Z, McCauley LK. Osteoclasts and odontoclasts: signaling pathways to development and disease. *Oral Dis* 2011;17:129-42. DOI PubMed
49. Linde A. Dentin matrix proteins: composition and possible functions in calcification. *Anat Rec* 1989;224:154-66. DOI PubMed
50. Bullock WA, Pavalko FM, Robling AG. Osteocytes and mechanical loading: the Wnt connection. *Orthod Craniofac Res* 2019;22 Suppl 1:175-9. DOI PubMed
51. Dodge T, Wanis M, Ayoub R, et al. Mechanical loading, damping, and load-driven bone formation in mouse tibiae. *Bone* 2012;51:810-8. DOI PubMed PMC
52. Xia WF, Tang FL, Xiong L, et al. Vps35 loss promotes hyperresorptive osteoclastogenesis and osteoporosis via sustained RANKL signaling. *J Cell Biol* 2013;200:821-37. DOI PubMed PMC
53. Bourron O, Phan F, Diallo MH, et al. Circulating receptor activator of nuclear factor kB ligand and triglycerides are associated with progression of lower limb arterial calcification in type 2 diabetes: a prospective, observational cohort study. *Cardiovasc Diabetol* 2020;19:140. DOI PubMed PMC
54. Ma Q, Liang M, Wu Y, et al. Mature osteoclast-derived apoptotic bodies promote osteogenic differentiation via RANKL-mediated reverse signaling. *J Biol Chem* 2019;294:11240-7. DOI PubMed PMC
55. Stepan JJ, Alenfeld F, Boivin G, Feyen JH, Lakatos P. Mechanisms of action of antiresorptive therapies of postmenopausal osteoporosis. *Endocr Regul* 2003;37:225-38. PubMed
56. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem* 2019;88:487-514. DOI PubMed
57. Peinado H, Zhang H, Matei IR, et al. Pre-metastatic niches: organ-specific homes for metastases. *Nat Rev Cancer* 2017;17:302-17. DOI PubMed
58. Loftus A, Cappariello A, George C, et al. Extracellular vesicles from osteotropic breast cancer cells affect bone resident cells. *J Bone Miner Res* 2020;35:396-412. DOI PubMed
59. Dovio A, Data V, Angeli A. Circulating osteoprotegerin and soluble RANKL: do they have a future in clinical practice? *J Endocrinol Invest* 2005;28 Suppl 10:14-22. PubMed
60. Eleutherakis-Papaiakovou E, Kastritis E, Gavriatopoulou M, et al. Circulating soluble receptor activator of nuclear factor kappa B ligand and C-C motif ligand 3 correlate with survival in patients with waldenström macroglobulinemia. *Clin Lymphoma Myeloma Leuk* 2018;18:431-7. DOI PubMed
61. Granchi D, Garaventa A, Amato I, Paolucci P, Baldini N. Plasma levels of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in patients with neuroblastoma. *Int J Cancer* 2006;119:146-51. DOI PubMed
62. Oh KW, Rhee EJ, Lee WY, et al. Circulating osteoprotegerin and receptor activator of NF-kappaB ligand system are associated with bone metabolism in middle-aged males. *Clin Endocrinol (Oxf)* 2005;62:92-8. DOI PubMed
63. Raaz-Schrauder D, Schrauder MG, Stumpf C, et al. Plasma levels of sRANKL and OPG are associated with atherogenic cytokines in patients with intermediate cardiovascular risk. *Heart Vessels* 2017;32:1304-13. DOI PubMed
64. Sarink D, Schock H, Johnson T, et al. Receptor activator of nuclear factor kB ligand, osteoprotegerin, and risk of death following a breast cancer diagnosis: results from the EPIC cohort. *BMC Cancer* 2018;18:1010. DOI PubMed PMC
65. Zaman T, Sun P, Narod SA, Salmena L, Kotsopoulos J. Plasma RANKL levels are not associated with breast cancer risk in. *BRCA1* 2016;10:2475-83. DOI PubMed PMC

Comfort *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:29-48
DOI: 10.20517/evcna.2020.09

Extracellular Vesicles and Circulating Nucleic Acids

Original Article

Open Access

Check for updates

Isolation and characterization of extracellular vesicles in saliva of children with asthma

Nicole Comfort[1], Tessa R. Bloomquist[1], Alex P. Shephard[2], Carter R. Petty[3], Amparito Cunningham[4], Marissa Hauptman[4,5], Wanda Phipatanakul[4,5], Andrea Baccarelli[1]

[1]Department of Environmental Health Sciences, Columbia University Mailman School of Public Health, New York, NY 10032, USA.
[2]NanoView Biosciences, Malvern Hills Science Park, Malvern, Worcestershire WR14 3SZ, UK.
[3]Brigham and Women's Hospital, Boston, MA; Boston Children's Hospital, Clinical Research Center Boston, MA 02115, USA.
[4]Harvard Medical School, Boston, MA 02115, USA.
[5]Division of Allergy and Immunology, Boston Children's Hospital, Boston, MA 02115, USA.

Correspondence to: Dr. Andrea Baccarelli, Environmental Health Sciences, Columbia University, 722 W 168th Street, New York, NY 10032, USA. E-mail: ab4303@cumc.columbia.edu

How to cite this article: Comfort N, Bloomquist TR, Shephard AP, Petty CR, Cunningham A, Hauptman M, Phipatanakul W, Baccarelli A. Isolation and characterization of extracellular vesicles in saliva of children with asthma. *Extracell Vesicles Circ Nucleic Acids* 2021;2:29-48. https://dx.doi.org/10.20517/evcna.2020.09

Received: 28 Dec 2020 **First Decision:** 4 Feb 2021 **Revised:** 21 Feb 2021 **Accepted:** 11 Mar 2021 **Available online:** 30 Mar 2021

Academic Editor: Y. Peng Loh **Copy Editor:** Yue-Yue Zhang **Production Editor:** Yue-Yue Zhang

Abstract

Aim: To confirm the presence of extracellular vesicles (EVs) in cell-free saliva (CFS) of children with asthma and describe the isolated EV population.

Methods: A pooled sample of CFS EVs isolated from 180 participants using ExoQuick-TC was examined in downstream analyses. Transmission electron microscopy (TEM) was used to confirm the presence of EVs. Nanoparticle tracking analysis (NTA) and single particle interferometric reflectance imaging sensing (SP-IRIS) with fluorescence were used for sizing, counting, and phenotyping of EVs. Capillary immunoassays were used for protein quantitation.

Results: TEM confirmed the presence of EVs of diverse sizes, indicating the prep contained a heterogeneous population of EVs. Capillary immunoassays confirmed the presence of EV-associated proteins (CD9, CD63, CD81, ICAM-1, and ANXA5) and indicated limited cellular contamination. As others have also reported, there were discrepancies in the EV sizing and enumeration across platforms. Fluorescent NTA detected particles with a mode diameter of ~90 nm, whereas SP-IRIS reported sizes of ~55-60 nm that more closely approximated the TEM results. Consistent with protein immunoassay results, SP-IRIS with fluorescence showed that the majority of these EVs were CD9- and CD63-positive, with little expression of CD81.

Conclusion: EVs from CFS can be isolated using a high-throughput method that can be scaled to large epidemiological studies. To our knowledge, we are the first to characterize CFS EVs from patients with asthma. The use of CFS EVs as potential novel biomarkers in asthma warrants further investigation and opens a new avenue of research for future studies.

Keywords: Extracellular vesicles, exosome, TEM, NTA, SP-IRIS, saliva, asthma, biomarker

INTRODUCTION

Asthma is the most common noncommunicable childhood disease, affecting approximately 8% of children worldwide and 12%-15% of urban children in the United States[1,2]. Asthma is a multifactorial disorder characterized by inflammation, airway remodeling, and airway hyperresponsiveness. While the clinical presentations may appear similar, evidence suggests that asthma may not be a single disease but rather encompasses a group of heterogeneous endotypes with various etiologies and prognoses[3,4]. Identifying biomarkers that can profile clinical subtypes early in the course of asthma is critical in applying tailored therapy, yet the utility of current biomarkers is very limited because they are either invasive or non-specific and therefore unable to faithfully identify clinically meaningful subtypes[5]. Thus, identification of objective biomarkers of asthma subtypes is a clinical research priority that may also advance our understanding of the various underlying pathologies that give rise to asthma.

Extracellular vesicles (EVs) are attractive candidates for biomarkers of asthma endotypes. EVs and their cargo have been implicated in asthma[6-9]. Also, EVs have been found in nearly all biofluids tested, including saliva[10-12]. Saliva, regarded as the "mirror of the body", is an easily accessible biofluid that harbors constituents that provide sources for monitoring of health and disease states[13,14] including asthma[15-21]. Yet, to the best of our knowledge, no study has investigated the characteristics of saliva EVs among patients with asthma. Hence, the aim of the present study is to describe the EVs isolated from cell-free saliva (CFS) of children with asthma.

METHODS

Study population

This study includes 180 children aged 4 to 14 years with asthma who were enrolled in the School Inner-City Asthma Study (SICAS). Details of the cohort have been described previously[22]. Inclusion criteria included: (1) history of physician-diagnosed asthma; (2) evidence of active asthma (defined as cough, wheezing, shortness of breath, whistling in the chest in the past year or daily controller medication use); (3) at least one unscheduled medical visit for asthma in the past year; and (4) attendance in schools that agreed to participate in the study. Written informed consent was obtained from each participant's parent or legal guardian, and assent was obtained from each participant. The protocol was approved by the Boston Children's Hospital and Columbia University Institutional Review Boards (Columbia IRB: AAAS9936).

Saliva collection

Whole saliva was collected from each participant. The collection required the participant to chew on parafilm (thin paraffin wax) and continue swallowing saliva normally for 1 min. Then, participants would "hold" saliva (i.e., stop swallowing) and spit oral fluid into a cold 50 mL falcon tube every 30 seconds until at least 5 mL saliva was collected or 15 minutes of chewing/spitting had passed, whichever occurred first. The whole saliva was aliquoted into microcentrifuge tubes and centrifuged for 20 min at 13,000 × g at 4°C to obtain CFS. Resulting CFS was removed, aliquoted (0.3-1.2 mL), and immediately stored at -80°C until EV isolation.

EV isolation

The exosomal fraction from CFS aliquots was isolated using ExoQuick-TC (EQ) (System Biosciences, Palo Alto, CA, Catalog No.: EXOTC50A-1) following the manufacturer's recommendations, with some modifications for saliva. CFS was mixed with EQ in a 2:1 CFS-to-EQ volume ratio, as done previously[23]. Tubes were inverted 5 times and then incubated upright overnight at 4°C. The next day, the samples were centrifuged at 1500× g for 30 min, and then 16,000 × g for 2 min. The supernatant (SN) was removed from samples and pooled. Samples then underwent a second round of centrifugation at 1500× g for 5 min, SN was removed, and samples underwent a final centrifugation of 16,000× g for 1 min. Any residual SN was removed and discarded. The addition of short, high-speed centrifugation steps enabled creation of a denser (i.e. not easily disturbed) pellet. All centrifugation steps were performed at 4°C. The resulting pellets were re-suspended in 105 µL of 3 kDa filtered 1× Dulbecco's phosphate buffered saline (dPBS). To create a representative sample for downstream analyses, we removed 5 µL of sample from each re-suspended EV pellet and pooled to create a single total EV sample representative of the study population. The pooled EV and SN samples were both diluted at 1:1 with filtered 1× dPBS prior to downstream analyses. The EV sample was sent directly for transmission electron microscopy and then frozen at -80°C before further analyses (nanoparticle tracking analysis, Exoview, protein capillary immunoassay).

Transmission electron microscopy

5 µL of the pooled EV sample was layered onto a formvar/carbon-coated grid and allowed to settle for 60 sec. The sample was blotted and negative-stained with 4 successive drops of 1.5% uranyl acetate in water, blotting between each drop. The grids were blotted and allowed to air dry at room temperature. Grids were imaged with a JEOL JSM 1400 (JEOL, USA, Ltd, Peabody, MA) transmission electron microscope operating at 100 kV. Images were captured on a Veleta 2K × 2K CCD camera (Olympus-SIS, Munich, Germany). 10-15 images were captured from each of three randomly selected areas of each grid at 50,000× and 100,000× lens magnification. The camera magnifications were calibrated using a grid with a grating replica (EMS cata # 80050) with line spacing of 463 nm (2160 lines/mm). Scale bars reflect the magnification at the camera. Transmission electron microscopy (TEM) micrographs were analyzed manually. Rounded or "cup-shaped" particles with high-contrast edges were considered EVs. EV diameters (N = 70 vesicles) were measured using ImageJ 1.53a with Java 1.8.0_172 (64-bit).

Nanoparticle tracking analysis

NanoSight LM10

Nanoparticle tracking analysis (NTA) was performed on the NanoSight LM10 (Malvern Panalytical, Malvern, UK) equipped with a 488 nm blue laser and NTA software, Version 2.3 Build 2.3.5.0033.7-Beta7. EVs were diluted at 1:1000 in PBS (viscosity 0.97 cP) and NTA was performed at 21.4°C on the NanoSight LM10 with the capture and analysis settings described in Supplementary Table 1. Monodisperse 100 nm polystyrene latex spheres (Colloidal Metrics, Mountain View, CA, USA) were run before measurements to ensure instrument calibration [Supplementary Figure 1]. For fluorescent NTA, the sample was stained using the ExoGlow-NTA Fluorescent Labeling Kit (System Biosciences, Palo Alto, CA, USA, Catalog No.: EXONTA200A-1) following the manufacturer's protocol before measurement on the NanoSight. In brief, EVs are added to a buffer containing a proprietary labeling dye. The sample is mixed and incubated at room temperature for 3-5 min. Free dye is removed by passing the sample through a column. Then, labeled EVs are ready for NTA analysis. Three technical replicates were run for both conventional light scatter NTA and fluorescent NTA.

ViewSizer 3000

NTA was also performed using a second instrument, the ViewSizer 3000 (Horiba Ltd, Kyoto, Japan, Software version 1.9.0.3019/1.0.9 WeekBuild 2919). The optical system of the ViewSizer 3000 makes it

advantageous compared to other NTA instruments; it combines three laser light sources (450 nm, 520 nm, and 635 nm) to enable detection and recording of scattered light from individual particles simultaneously in multiple spectral bands[24]. The total EV sample was diluted at 1:8000 with 3 kDa filtered 1X dPBS and loaded into a cuvette and into the ViewSizer 3000. Twenty-five videos (30 frames per second, 300 frames per video) were recorded at 22°C with the following recording parameters: Blue laser, 210 mW; green laser, 12 mW; red laser, 8 mW; exposure, 15 ms; camera gain, 30 dB. Samples were processed with the Main Chart in "LogBinSilica" and integrated in the range [50, 1900] nm. 100 nm uniform polystyrene beads (3100A Nanosphere Size Standard, ThermoFisher Scientific, Waltham, MA, USA) were run before measurements to ensure the instrument was properly calibrated [Supplementary Figure 1]. Two technical replicates of 25 videos each were run. The number of completed tracks for all NTA measurements (on both the NanoSight LM10 and ViewSizer 3000) always exceeded the proposed minimum of 1000 to minimize artifactual spikes in the data based on single large particles[25].

Capillary western immunoassay

Protein concentration of the pooled EV sample and pooled SN sample was quantified with the Nanodrop (Implen NanoPhotometer P300, Thermo Fisher Scientific, Waltham, MA, USA). We used the ProteinSimple capillary immunoassay (Wes) method following the manufacturer's instructions (ProteinSimple, San Jose, CA, USA). Samples were frozen at -80°C until they were sent to RayBiotech (Peachtree Corners, GA) for analysis via the Auto Western testing service. The EV sample was diluted 2X so that all samples were loaded at 1 mg/mL. All target proteins and controls were detected with antibodies provided by RayBiotech (Peachtree Corners, GA, USA). The CD9 antibody used was a mouse monoclonal IgG1k antibody with immunogen mouse CD9. The CD63 antibody used was a rabbit polyclonal antibody with immunogen AA 103-203 of recombinant human CD63 (accession #: P08962). The CD81 antibody used was a rabbit polyclonal antibody with AA 113-201 of recombinant CD81 as the immunogen (accession #: NP_004347.1). Samples were loaded with no boil/no DTT.

Single particle interferometric reflectance imaging sensing

Sample preparation

Samples were analyzed using single particle interferometric reflectance imaging sensing (SP-IRIS) using the ExoView Human Tetraspanin Kit (NanoView Biosciences, USA). 1 µL of each sample was diluted in 99 µL of manufacturer supplied buffer, solution A. 35 µL of the diluted sample was incubated on one ExoView Tetraspanin Chip per sample overnight (16 h) at room temperature. Chips were washed three times in solution A prior to incubation with fluorescent tetraspanin antibodies. The same antibody clones were used for capture and fluorescent detection (CD9: HI9a; CD63: H5C6; CD81: JS-81).

Labeling antibodies consisted of anti-CD9 CF488, anti-CD81 CF555, and anti-CD63 CF647. For fluorescent staining, 250 µL of the fluorescent antibody cocktail (antibodies diluted at 1:500 in the fluorescent cocktail) was added to 250 µL of solution A which remained on the chip post-wash (resulting in a 1 in 2 dilution of the antibody cocktail on the chip, 1:1000 dilution overall). The fluorescent antibodies incubated on chips for 1 hour at room temperature. Chips were then washed in kit-supplied buffers, dried, and imaged by the ExoView R100 using nScan v2.9.3.

Measurement details

Interferometric measurements for particle sizing were acquired using a 405 nm LED. The excitation and emission [Em] wavelengths of the three fluorescent channels are shown in Supplementary Table 2. CF647 was conjugated to CD63 [Absorption (Abs) max: 650 nm; Em max: 665 nm]. CF555 was conjugated to CD81 (Abs max: 555 nm; Em max: 565 nm). CF488 was conjugated to CD9 (Abs max: 490 nm; Em max: 515 nm).

Data processing

Background scans of the chips were performed prior to sample incubation. Detected particles were subtracted from the particle counts post sample incubation to account for debris on the chip. For fluorescent analysis, the MIgG spots on each chip were used as a negative control to account for non-specific fluorescent antibody binding. The fluorescence intensity of particles present on the MIgG spots was used to set a baseline intensity value; only particles which exceed this fluorescence intensity were counted as positive. Thus, fluorescent cut offs were set relative to the MIgG control.

A 150-μm-diameter area of each capture spot was selected for analysis using an automated circle finding algorithm. The particles within this area were counted, producing a particle value that represents normalization of particle count to spot area. Each chip has the antibody capture spots in triplicate. Data were analyzed using NanoViewer 2.9.3.

Statistical analyses

Statistical analysis was performed with R software[26], version 4.0.3. As NTA sizing and concentration values derived from one sample are normally distributed, unpaired two-tailed Student's *t*-test was used to compare particle concentrations and size summary statistics with alpha set to 0.05.

RESULTS

Evidence of EVs in human saliva of children with asthma

This analysis includes 180 children with asthma aged 4 to 14 years who were enrolled in SICAS and provided a saliva sample at the study baseline visit. Details of the cohort have been described previously[22]. Table 1 presents characteristics of the study population included in this analysis. The mean age of the participants was 8.1 years, and the majority (58%) of participants identified as Hispanic/Latino. 20% of participants lived in a household that reported an annual household income of less than $25,000. 22% of participants reported a smoker at home, and a majority of participants (60%) were sensitized to at least one allergen [Table 1].

EVs were isolated from the CFS fraction of each participant (N = 180) using ExoQuick-TC (EQ). An overview of the saliva collection and EV isolation protocols is depicted in Figure 1. To create a representative sample for downstream analyses, we pooled 5 μL from each re-suspended EV pellet to create a single total EV sample representative of the SICAS study population. To assess the purity of the total EV sample and characterize single vesicles, we imaged EVs using negative-stain TEM. For the TEM analysis, the pooled EV sample was examined in the Microscopy and Image Analysis Core, Weill Cornell Medicine (see Acknowledgements). Sizing analysis on vesicles (N = 70) was performed using ImageJ. TEM confirmed the presence of EVs of diverse particle sizes. Electron micrographs show rounded, electron-dense vesicles (Figure 2, examples marked with red arrowheads) with a mean (median) size of 64.95 (55.09) nm in diameter [Figure 3, Table 2], similar to previous reports of saliva exosomes[10]. Other structures present in the background (Figure 2, examples marked with yellow arrowheads) could represent protein aggregates. In rare instances, fibrous-like shapes could be seen in the micrographs, likely resulting from carry-over of the EQ solution [Supplementary Figure 2].

Nanoparticle tracking analysis of saliva EVs

EV sizing and enumeration was performed using NTA on the NanoSight LM10 equipped with a blue laser (488 nm). Particle size distributions (PSDs) of the EV prep are depicted in Figure 4. According to the NTA measurements, the isolated particles were within the expected size range for extracellular vesicles, 70-404 nm. A summary of the EV concentration and size distribution, with or without the finite track length adjustment (FTLA) algorithm, is provided in Table 3. We also performed fluorescent NTA (fNTA), since

Table 1. Characteristics of the SICAS study population

	All study participants, no. (%)
Population	*N* = 180 *
Age (years), mean (range)	8.1 (4-14)
Female sex	85 (47%)
BMI (kg/m^2), mean (range)	19.3 (13.7-37.2)
Race/ethnicity	
Black/African American	30 (17%)
Hispanic/Latino	104 (58%)
White	26 (15%)
Mixed/other	19 (11%)
Environmental tobacco smoke	39 (22%)
Use of controller medication	103 (58%)
Atopic asthma (any allergen sensitization)	107 (60%)
Annual household income	
< $25,000	36 (20%)
≥ $25,000	84 (47%)
Missing	59 (33%)

*180 SICAS participants provided saliva samples. One participant was missing all demographic data. SICAS: School Inner-City Asthma Study.

Table 2. Summary statistics of EV* measurements

	Size (nm)
Min	16.16
Max	320.79
Mean	64.95
Median	55.09

*EVs (*n* = 70) were sized using ImageJ software. EVs: extracellular vesicles.

Table 3. NanoSight LM10 analysis of CFS EVs. Summary of EV number and size distribution, with or without the FTLA algorithm, quantified by NanoSight analysis

		Mean (nm)	Mode (nm)	SD (nm)	D10 (nm)	D50 (nm)	D90 (nm)	Total Concentration (Particles/mL)	Number of Completed Tracks	Number of Valid Tracks
Light scatter	FTLA size distribution	196.4 ± 1.7	165.0 ± 3.1	104.2 ± 7.6	106.0 ± 1.2	172.0 ± 1.2	306.0 ± 8.0	7.43E11 ± 1.89E9	26006	8663
	size distribution	194.1 ± 1.1	155.3 ± 2.4	117.7± 5.0	98.0 ± 1.2	168.7 ± 0.7	304.3 ± 2.3	7.43E11 ± 1.89E9	26006	8663
Fluorescent	FTLA size distribution	227.7 ± 5.2	93.7 ± 7.5	228.8 ± 11.3	78.0 ± 2.0	150.7 ± 1.3	469.3 ± 17.4	3.47E11 ± 4.32E9	13839	4704
	size distribution	214.3 ± 6.3	87.0 ± 7.1	235.2 ± 12.2	69.7 ± 1.2	142.7 ± 2.2	404.0 ± 17.8	3.47E11 ± 4.32E9	13839	4704

Mean and SD of three technical measurements for light scatter NTA and fluorescent NTA are shown. Concentrations adjusted for dilution factors. All values provided are batch average results from 3 technical replicates. D10 is the point in the size distribution where 10% of the sample is contained, D50 is the point where 50% of the sample is contained (median), and D90 is the point where 90% of the sample is contained. CFS: Cell-free saliva; EVs: extracellular vesicles; FTLA: finite track length adjustment; SD: standard deviation.

conventional NTA does not distinguish membranous vesicles from co-isolated non-membranous particles of similar sizes. We found that fNTA reported a significantly smaller total particle concentration ($P < 0.0001$), indicating presence of contamination in the EV preparation likely by protein aggregates and/or

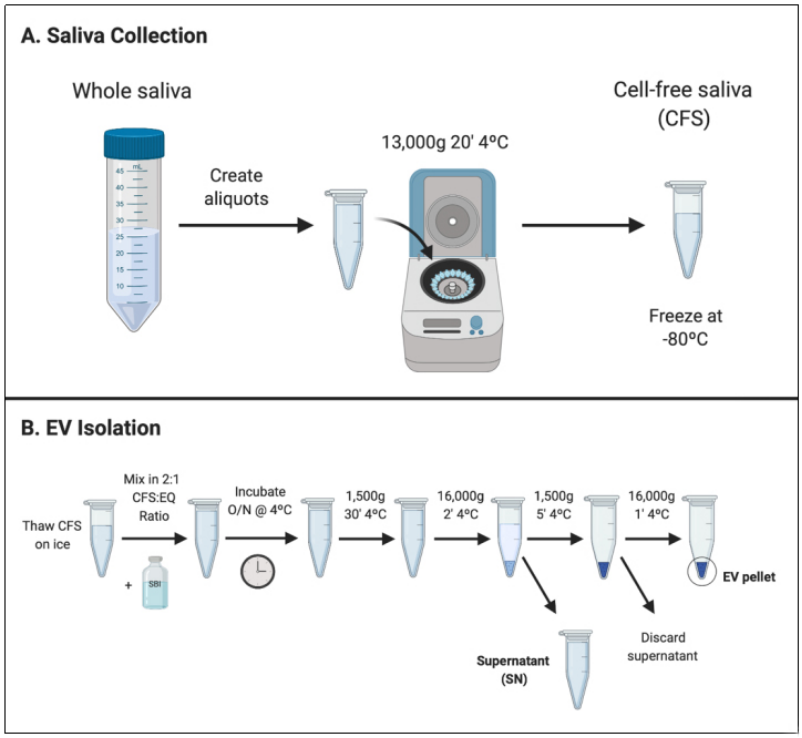


Figure 1. Graphical overview of laboratory methods for CFS collection and EV isolation. (A) CFS collection protocol. CFS was collected from *N* = 180 participants of the School Inner-City Asthma Intervention Study. Aliquots of CFS were frozen at -80ºC before shipment to Columbia University for EV isolation; (B) EV isolation protocol. EVs were isolated from CFS using a polymer-based reagent, EQ, in a 2:1 CFS-to-EQ ratio. After overnight incubation, EVs were precipitated from the solution by centrifugation. CFS: Cell-free saliva; EV: Extracellular vesicle; EQ: ExoQuick-TC; SBI: System Biosciences.

residual EQ solution. No significant differences were found between the mean and mode diameters of unlabeled particles assessed in light scatter mode and labeled EVs assessed by fNTA ($P > 0.05$) [Table 3], but there were differences in the overall PSD, with significant differences in the reported D10 ($P < 0.001$), D50 ($P = 0.004$), D90 ($P = 0.03$), and standard deviation ($P = 0.005$) between conventional NTA and fNTA.

We also performed NTA using a second platform, the ViewSizer 3000, and found results that closely approximated the fNTA [Supplementary Figure 3, Supplementary Table 3]. There were no differences in total particle concentration nor in mean and mode particle diameter comparing the NanoSight LM10 fNTA and ViewSizer 3000 light scatter NTA results ($P > 0.05$). Comparing the overall PSD (D10, D50, D90, standard deviation) between NanoSight fNTA and ViewSizer light scatter NTA, there were significant differences in D50 ($P = 0.0003$) and the standard deviation ($P = 0.009$). Comparing the NanoSight LM10 and ViewSizer 3000 light scatter data, there were no significant differences in mean, mode, D10, D90, or standard deviation in particle size, but the ViewSizer reported significantly larger D50 ($P = 0.02$) and total particle concentration ($P = 0.012$) values.

Wes protein analysis of saliva EVs

Capillary Western immunoassays performed on the Wes (ProteinSimple, San Jose, CA, USA) were used for protein quantitation[27]. The Wes method can detect proteins of various sizes with high sensitivity while only using small amounts of precious biological sample[28]. In Wes, proteins are separated by size in capillaries, where they are incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies and a chemiluminescent substrate. The chemiluminescent signal is detected, quantified, and displayed as an

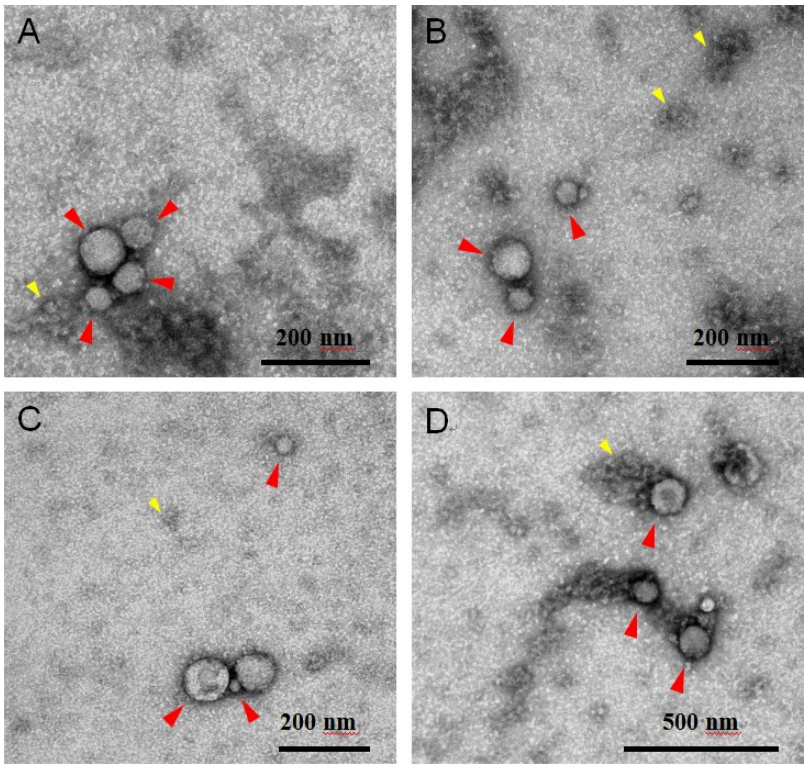


Figure 2. Representative electron microscopy negative staining images of pooled CFS EVs (independent EV isolations *N* = 180) showing round-shaped EVs. Electron micrographs show round-shaped vesicles (red arrowheads) 16-320 nm in diameter (mean 64.95 nm). Other structures seen in the background (yellow arrowheads) could be protein aggregates. Scale bars reflect the magnification at the camera. (A-C) 100k magnification, scale bar 200 nm; (D) 50k magnification, scale bar 500 nm. CFS: Cell-free saliva; EV: extracellular vesicle.

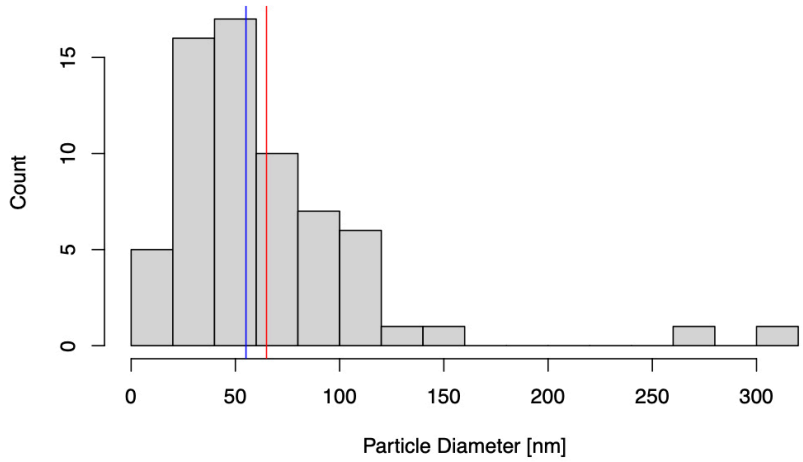


Figure 3. Histogram of transmission electron microscopy (TEM) particle diameters. Particle size distribution of *N* = 70 vesicles sized using ImageJ. Blue and red lines mark the median and mean particle diameter [nm], respectively.

electropherogram or virtual blot-like image. Wes analysis verified the presence of the tetraspanin proteins CD9, CD63, and CD81 which were enriched in the EV pellet compared to the SN in varying intensities following isolation with EQ [Figure 5A]. CD81 was enriched in the EV pellet but with low intensity. In addition, the EV-associated proteins ICAM-1 (Intercellular adhesion molecule-1) and ANXA5 (Annexin

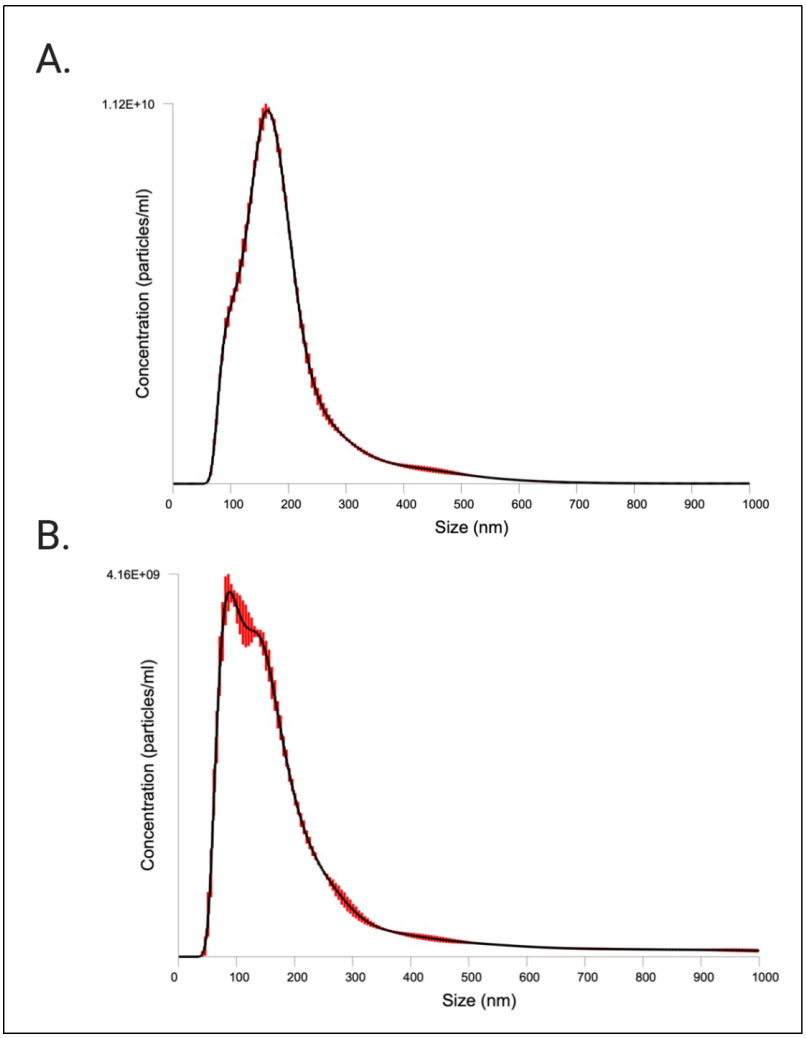


Figure 4. Characterization of CFS EVs by NTA. PSDs from NTA FTLA data on the NanoSight LM10 are shown. Red bars indicate ± standard error of the mean. The PSDs are the average size/concentration of three technical replicates. Graphs are representative of measuring samples using the optimal particle/frame rate according to the operating manual for NanoSight LM10. (A) NTA in light scatter mode (conventional NTA); (B) NTA in fluorescent mode (fNTA). CFS: Cell-free saliva; EVs: extracellular vesicles; NTA: nanoparticle tracking analysis; PSD: particle size distribution; FTLA: finite track length adjustment.

A5) were detected in the EV pellet but were barely detectable in the SN [Figure 5B]. The lack of calnexin (CANX, endoplasmic reticulum protein) and GM130 (Golgi protein) verified the purity of the EV sample [Figure 5C]. These proteins are major constituents of non-EV structures often co-isolated with EVs. Their absence in the EV prep indicates little cellular contamination[29].

Surface biomarker characteristics of saliva EVs

Biological particle counts

To obtain more elaborate results, the EV and SN samples were analyzed using single particle interferometric reflectance imaging sensing (SP-IRIS) by the ExoView R100 platform (NanoView Biosciences, Boston, MA, USA). Briefly, the ExoView combines microfluidics with immunodetection and interferometric imaging to detect unlabeled EVs based on their expression of EV marker proteins. Here, CD9, CD63, and CD81-positive immuno-captured EVs from the pooled EV sample and pooled SN sample were imaged on a single EV basis and subsequently sized [Table 4] and counted [Figure 6A]. Results show a higher number of

Table 4. Sizing of tetraspanin-positive EVs by interferometry-based label-free measurements performed on each capture spot

Marker	Mean (nm)	Median (nm)
CD9	58.2	62.5
	57.9	61.9
CD63	58.2	60.9
	58.1	58.6
CD81	56.0	54.9
	55.9	53.1

Lower limit of detection: 50 nm. Upper limit of detection: 200 nm. Means and medians (each value calculated from three spots for each capture antibody) from two technical replicates are shown. Particle sizing across the two replicates had a deviation of 0.1-0.2 nm.

tetraspanin-positive vesicles in the EV pellet compared to the SN, as expected since these transmembrane proteins are enriched in EVs. The counts of CD9, CD63, and CD81-positive EVs positively correlated (Pearson's r = 0.62) with the protein quantitation and relative intensities of the pseudo-gels obtained by Wes capillary immunoassay, including the low signal of CD81 in this EV preparation. However, while the signal intensity of CD9 was significantly greater than that of CD63 in the capillary immunoassay, SP-IRIS measured counts of CD63-positive particles that were nearly equal to the counts of CD9-positive particles. This discrepancy could be due to differences in the CD63 antibody used across assays.

Single particle phenotyping by fluorescence

In addition to direct detection of unlabeled EVs through interferometric analysis, it is also possible to label the bound EVs using secondary fluorescently labeled antibodies and detect EVs with fluorescence. Following sizing and enumeration of unlabeled EVs, the chips were incubated with labeling antibodies consisting of anti-CD9 CF-488, anti-CD63 CF-647, and anti-CD81 CF-555 for 3-color phenotyping of captured particles [Figure 6B-C]. In contrast with SP-IRIS to count unlabeled particles, which has a limit of detection of 50-200 nm diameter, the fluorescent phenotyping counts any particle that expresses a copy of the target protein, regardless of size. Compared to SP-IRIS particle counts, fluorescent phenotyping reported a 17.5-fold increase in the number of CD9-positive vesicles, a 21.6-fold increase in the number of CD63-positive vesicles, and a 35-fold increase in the number of CD81-positive vesicles, indicating there were a large number of particles present outside the 50-200 nm size range for non-fluorescent detection [Figure 6B]. In agreement with the SP-IRIS and immunoassay data, the fluorescent particle counts show that the majority of vesicles expressed CD9 and CD63 [Figure 6C]. Thus, this trend held not only for EVs 50-200 nm in diameter but also for EVs outside of this size range. No signal was detected in the CD81 channel of the SN. Since the majority of vesicles expressed more than one type of tetraspanin, we analyzed this further via colocalization analysis.

We examined co-expression of the three tetraspanins by looking at colocalization of fluorescent signals after analyzing composite images from the different fluorescent channels [Supplementary Figure 4]. The results show that the majority of vesicles (63%) express either CD9 only (28%) or both CD9 and CD63 (35%). A quarter (25%) of vesicles express CD9 and CD81 and 12% express all three tetraspanins [Figure 7].

DISCUSSION

Saliva is the most accessible and non-invasive biofluid, which makes it a very appealing biofluid to use for the detection of biomarkers of disease, especially among children. Indeed, saliva has been shown to reflect overall health status, containing robust biomarkers (including EV biomarkers) of systemic disease such as cancers and autoimmune diseases[30,31]. Saliva also contains biomarkers associated with asthma[21,32]. Yet, to our knowledge, saliva EVs from patients with diagnosed asthma have not been investigated so the potential

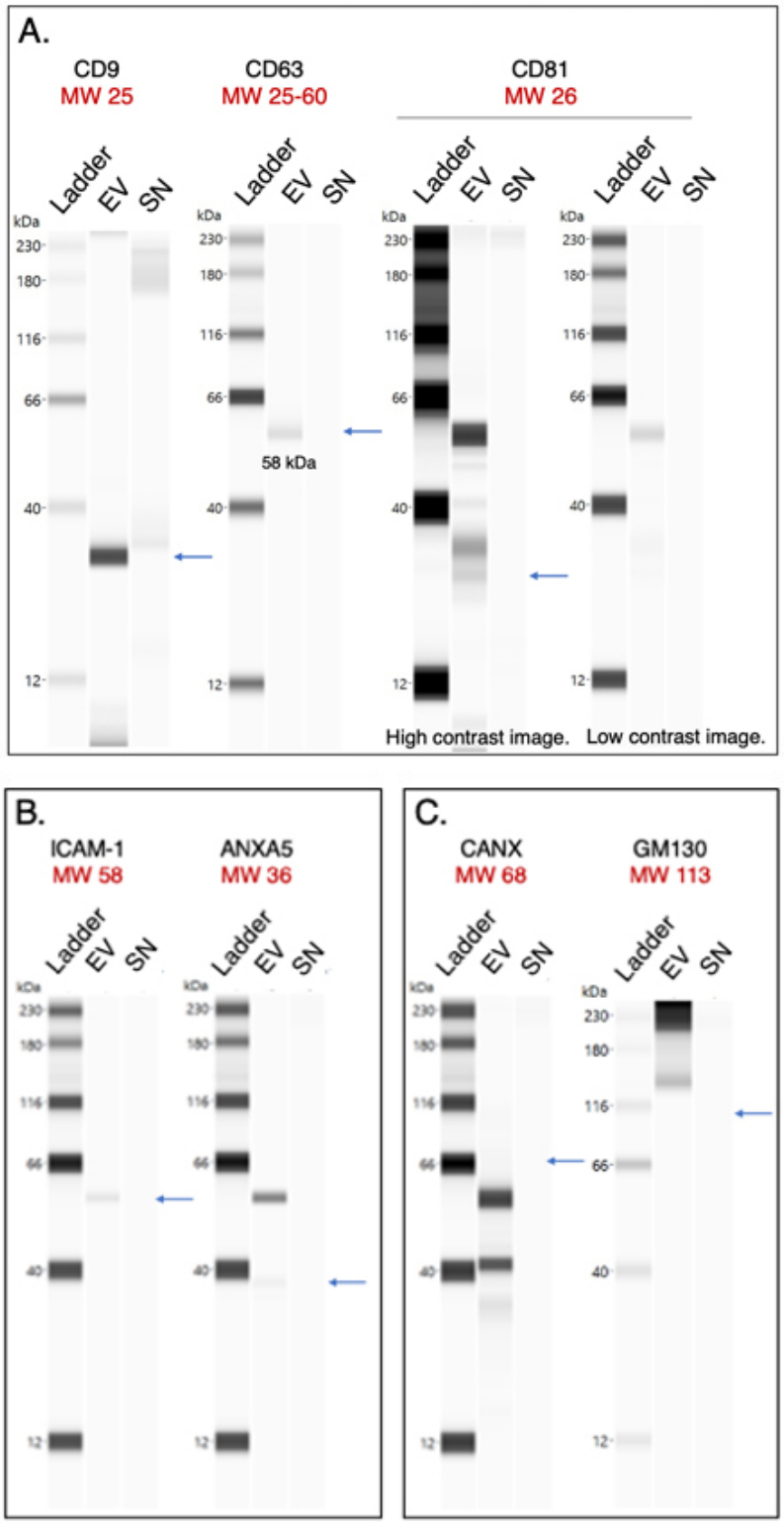


Figure 5. Protein composition of CFS EVs and SN analyzed by the Wes system (ProteinSimple). Calculated molecular weights (in kDa) are shown in red. Pseudo-gel images of representative runs shown for (A) Tetraspanins CD9, CD63, and CD81; (B) Intercellular cell adhesion molecule (ICAM-1) and annexin A5 (ANXA5); and (C) Intracellular proteins calnexin (CANX) and GM130. For all runs, the peak signal-to-noise ratio given by the software ≥ 10. CFS: Cell-free saliva; EVs: extracellular vesicles; MW: molecular weight; EV: pooled extracellular vesicle sample; SN: pooled supernatant sample.

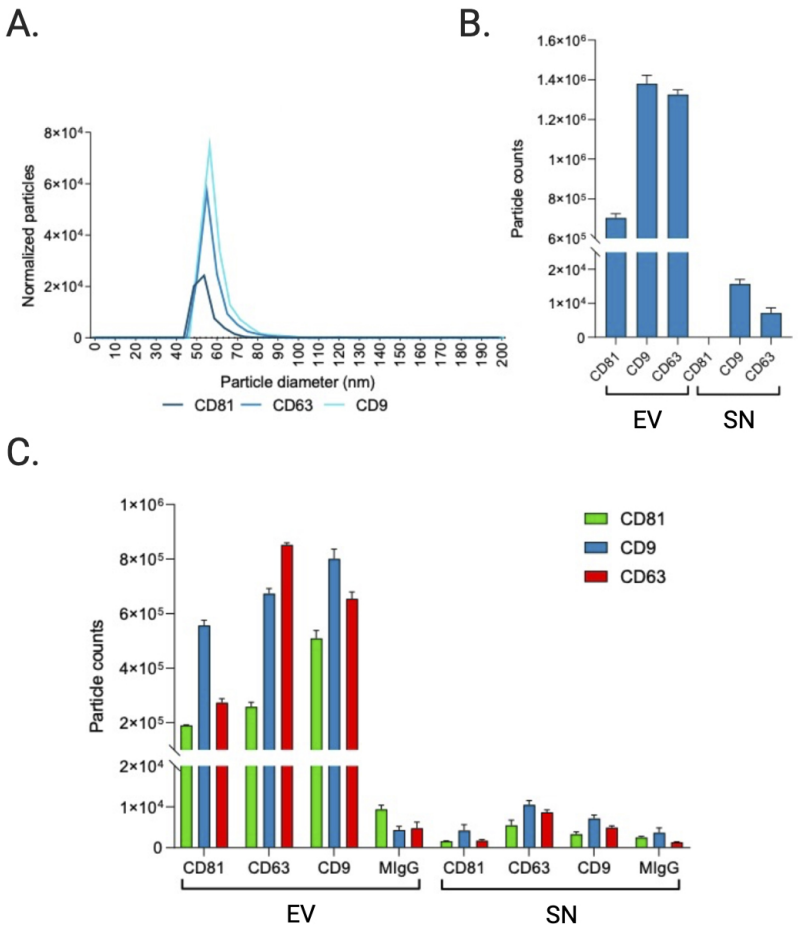


Figure 6. Sizing and enumeration of tetraspanin-positive vesicles. All data has been adjusted for dilution of the sample onto the chip. Average of three technical replicates that were run. Particle number is quantified by the number of particles in a defined area on the antibody capture spot. (A) Interferometry-based sizing and counting of label-free EVs, normalized to MIgG control. Limit of detection is 50-200 nm. (B) Quantification of the number of CD9, CD63, and CD81-positive particles after probing particles with a cocktail of fluorescent tetraspanin antibodies. Any particle that expresses a copy of the target protein and is detected by fluorescence will be counted, regardless of size. (C) Particle count by fluorescent channel shows the number of particles on each capture spot that express each fluorescent marker.

of saliva EVs as biomarkers of asthma, asthma prognoses, and asthma subtypes remains unknown. Here, we demonstrate proof of concept that we can isolate EVs from CFS of patients with asthma by characterizing a pooled EV sample representative of a cohort, SICAS, which is comprised of children with asthma living in an urban area.

We confirmed the presence of EVs using negative-stain TEM. EVs were distinguished from background and non-EV particles due to their distinct morphology and contrast properties. The EVs pooled from 180 individual samples representing the SICAS cohort appeared as electron-dense membranous structures with a mean (median) diameter of 64.95 (55.09) nm. The data were positively skewed by the presence of two microvesicles exceeding 250 nm in size. Thus, EQ isolated different EV subpopulations, with sizes consistent with that of exosomes but also larger vesicles (microvesicles). Note that while TEM is widely used to characterize EVs, the sample preparation for TEM imaging can induce changes in the EV morphology; the "cup-shaped" appearance commonly seen among EVs in TEM micrographs is an artifact of the fixation and contrast steps of the sample prep[33]. Thus, cryo-electron microscopy, which shows the lipid bilayer and

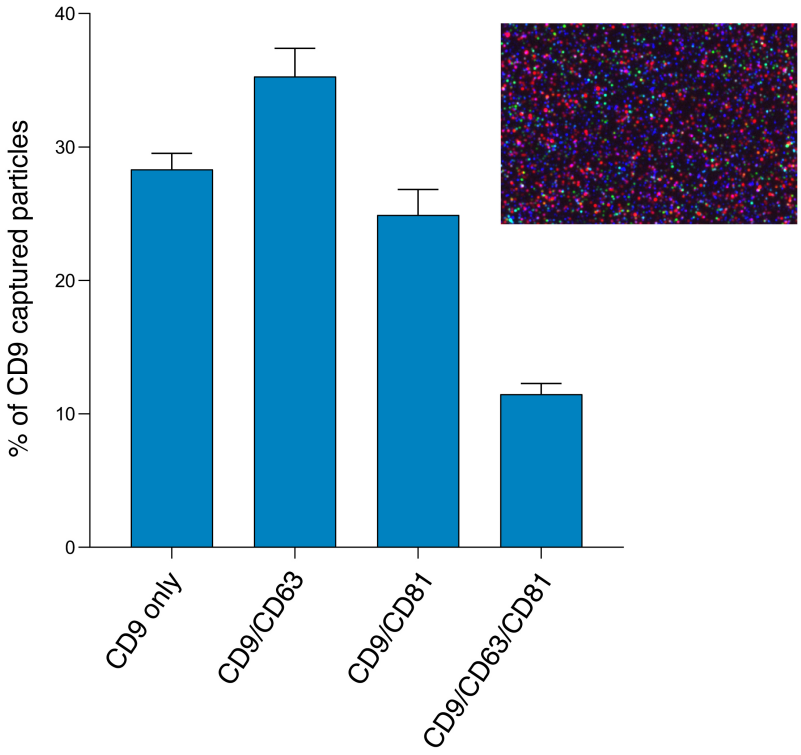


Figure 7. Colocalization of tetraspanins CD9, CD63, and CD81 detected by 3-color fluorescence measured on the ExoView R100. The fluorescent antibodies are CD9 (blue), CD63 (red), and CD81 (green). CD9 capture spot data for the pooled EV sample (average of three technical replicates). Inset shows the CD9 capture spot composite fluorescent image.

better preserves EV size, is preferred for visualization of single vesicles[34-36].

Conventional light scatter NTA analysis of EVs measured a total particle concentration of 7.43E11 ± 1.89E9 particles/mL with a mode size of 155.3 ± 2.4 nm (NanoSight LM10), while fNTA results reported a total particle concentration of 3.47E11 ± 4.32E9 particles/mL with a mode size of 87.0 ± 7.1 nm (NanoSight LM10). We interpret the raw NTA and fNTA results rather than FTLA results, as FTLA can introduce artifacts and so the interpretation of these results is not recommended[37]. Contrary to TEM which can visualize particles of the smallest sizes, NTA has a larger limit of detection (~50 nm, minimum detectable vesicle sizes ~70-90 nm on Nanosight NS500) due to the small size and relatively low refractive index of EVs, explaining the shifted PSD reported by NTA compared to TEM[37,38]. Furthermore, NTA may overestimate EV sizes because it visualizes particles in the native state, compared to TEM which, as mentioned above, can result in dehydration and shrinkage of EVs through the sample preparation process.

Unlike TEM, NTA is a non-specific particle analysis method that cannot differentiate EVs from non-EV structures such as lipoproteins or residual EQ reagent (which was present in our prep as shown in Supplementary Figure 2 and could skew results). NTA of fluorescently labeled EVs attempts to overcome this limitation. Our fNTA analysis of EVs labeled with a fluorescent lipophilic membrane dye reported a significantly smaller total particle concentration compared to conventional NTA and with a mode particle size (87.0 nm) closer to the mode reported by TEM (42.7 nm) than that reported in light scatter mode (155.3 nm). This highlights that vesicle-specific fNTA measurements should be carried out when possible to obtain more robust particle counts and PSDs. However, lacking knowledge of the stain, its optical properties, the method of staining (the fluorescent dye is a proprietary commercially available kit), and a

detergent-treated control, we can't be certain that the fNTA results are specific to intact EVs and as such, our results should be interpreted with caution. Additionally, our particle concentrations cannot be interpreted quantitatively because we lack multiple measurements at different sample dilutions. Regardless, because NTA often overestimates particle concentrations and because the true concentration of an EV sample is unknown, we avoid interpreting the absolute concentration determination and instead shift our focus to the analysis of particle sizes[25,39].

NTA methods are known to provide accurate results for sizing monodisperse and bimodal reference particles, which we showed here by measuring a 100 nm polystyrene latex sphere standard Supplementary Figure 1, but they fall short regarding accuracy of concentration determination and precision in general[40]. Thus, as a general guideline, it is recommended to apply more than one orthogonal method for particle size and concentration measurements of complex polydisperse samples such as EVs. Here, we confirmed our results with measurement on a second NTA instrument, the ViewSizer 3000. While not a truly orthogonal method (e.g., both instruments rely on the Stokes-Einstein equation for particle size determination), the ViewSizer 3000's optical system makes it unique from other particle tracking instruments such as the NanoSight LM10 because it includes multispectral illumination with three lasers (at 450 nm, 520 nm, and 635 nm) and detection techniques that enable video recording of scattered light from individual particles in multiple spectral bands, allowing measurement of particle sizes even in a highly polydisperse sample. Compared to conventional light scatter on the NanoSight, the ViewSizer reported a lower total particle concentration and larger median particle size, but there were no significant differences in mean and mode particle size nor D10, D90, and standard deviation. Though not significantly different, the right tail of the ViewSizer PSD (D50, D90) was more skewed to higher particle diameters (average ViewSizer D50/D90 across replicates: 198.84/466.95 nm, average NanoSight D50/D90 across replicates: 168.7/304.3 nm). The ViewSizer 3000 measures more closely approximated the fNTA results; the reported particle concentration measures on the ViewSizer were not significantly different from the particle concentrations reported by fNTA. Such differences between the reported EV concentrations and PSDs are caused mainly by differences in the minimum detectable vesicle sizes (i.e., detection limits) across the two instruments, but also on parameters such as instrument settings, device-dependent attributes, and operator bias.

Both NTA instruments measured larger EV diameters compared to TEM, as others have noted[39]. This is again due to the fact that TEM sample preparation causes shrinkage, slightly underestimating EV size, but more importantly because smaller EVs are below the limit of detection for NTA. Also, EV membrane surface proteins may impede mobility in solution resulting in inaccurate particle sizing by the Stokes-Einstein equation[41]. In our study, the NanoSight results can be considered more reliable than the ViewSizer results due to the greater number of tracked particles and additional technical replicate [Table 3, Supplementary Table 3]. Yet, given that the hardware and software of these two NTA platforms differ considerably, as well as the sample movement and standardized settings, the relatively similar particle concentration and PSD results provide higher confidence in our measures of total particle concentration and size, which overall was consistent with the expected size range of EVs.

Capillary Western immunoassay analysis of the EV sample confirmed the presence of the tetraspanins CD9, CD63, and CD81, although CD81 was not greatly expressed. These results were corroborated using SP-IRIS of CD81-positive immuno-captured EVs. This result is likely not due to low CD81 antibody efficiency, as the two methods used different capture antibodies (JS-81 mouse monoclonal antibody for ExoView, rabbit polyclonal antibody with AA 113-201 of recombinant CD81 as the immunogen [accession NP_004347.1] for Wes). This finding is important as many studies use CD81 expression to normalize levels of EV markers of interest[42,43]. We also detected the presence of EV-associated proteins ICAM-1 and ANXA5, which were

enriched in the EV sample compared to the SN, as expected. ICAM-1, involved in cell signaling events, plays a role in the targeting of EVs to recipient cells, while the lipid-binding protein ANXA5 is involved in membrane transport and fusion. Thus, the proteins described and characterized here are transmembrane proteins and cytosolic proteins with membrane-binding capacity that we expected to be present in the EV sample. The absence of CANX and GM130 (which we did not expect to be enriched in the EVs) in the pooled sample indicated that this EV isolation method did not co-isolate soluble proteins associated with intracellular compartments other than the plasma membrane and endosomes.

In addition to TEM and NTA, we also assessed the EV size and concentration using an emerging technology, SP-IRIS and fluorescent phenotyping on the ExoView R100. The particle counts of antigen-positive EVs in the EV sample and SN sample demonstrate that EQ-purified CFS EVs were significantly enriched in vesicles compared to the EQ SN, as expected, and consisted primarily of CD9 or CD63 positive vesicles. The EV counts in the SN samples were very low and also consisted primarily of CD9 and CD63 positive vesicles. Compared to SP-IRIS for particle counts, which has a dynamic range of 50-200 nm, fluorescent phenotyping reported a much higher number of tetraspanin-positive vesicles. This finding is supported by the TEM and NTA data; TEM detected particles < 50 nm, while NTA (which is unable to detect particles < 50 nm) and TEM both reported the presence of microvesicles > 200 nm. Compared to the fNTA measurements, the ExoView reported significantly smaller particle counts in the EV prep ($P < 0.001$). This may indicate that a sizable portion of EVs in the sample may not express CD9, CD63, or CD81, something that should be taken into consideration when choosing a method for EV normalization in downstream studies. However, the ExoView size measurement is specifically for tetraspanin-positive particles, compared to NTA which will measure any particles present. As there is no perfect purification technique which will remove every non-EV particle, the NTA measurement will always have the potential to be biased by non-EV particles present in the sample. More than likely, the discrepancy in fNTA particle counts and the tetraspanin-positive particle counts measured on the ExoView R100 is mainly because the lipophilic membrane dye is non-specific and non-EV particles are still being counted in fNTA.

The particle sizes of antigen-positive EVs [shown in Figure 6 and Table 4] closely approximate the median and mean EV diameter reported by TEM, although the median is more comparable in this case because the mean TEM particle diameter was skewed by the presence of few large particles exceeding 250 nm, whereas SP-IRIS sizing measurements have a dynamic range of 50-200 nm. This limited dynamic range is because particles smaller than 50 nm in diameter have a contrast which is indistinguishable from the chip background when using the standard 405 nm LED. Particles larger than 200 nm are outside the linear range of the interferometry measurement; when using a 405 nm light source, the relationship between Raman scattering intensity and particle size is nonlinear above 200 nm[44]. Our results corroborate previous findings comparing different EV analysis techniques (including NTA, TEM, and SP-IRIS/ExoView), which indicated that NTA consistently overestimates the sizes of particles compared to TEM sizing while in contrast, SP-IRIS/ExoView produced size histograms that closely mirrored the TEM size distribution[39]. However, immunocapture and immunofluorescence data rely on the specificity of the antibodies used and brightness/limit of detection of the dyes. While these antibody clones have been extensively validated, readers should be aware of the photophysical properties of dyes and their excitation and emission filters when interpreting such results, which we report in Supplementary Table 2.

In conclusion, EQ can be used to isolate bona fide EVs from CFS. Other studies have also reported the efficiency of EQ in comparison with other isolation methods such as ultracentrifugation[23,45]. This proof of concept opens a new avenue for saliva EV research for labs that may not have access to the equipment necessary for other EV isolation methods like ultracentrifugation or flow cytometry[46]. While this is the first

characterization of saliva EVs from patients with asthma, there are many different methods available to isolate EVs, each of which can greatly impact EV yield and purity, affecting interpretation of downstream analyses. Thus, these results may not be generalizable to studies that isolate CFS EVs using a different method. We suggest performing a thorough characterization of an EV preparation prior to any downstream experiments in order to accurately assess the generalizability of results. Characterization of EVs should be performed by applying multiple complementary analytical methods, because, as demonstrated here, each method has unique limitations and biases.

Saliva EVs from this cohort of children with asthma are 64.95 nm in diameter on average, with a median of 55.09 nm, as reported by TEM, and express mostly CD9 and CD63. We also detected enrichment of ICAM-1 and ANXA5. CD81 was not highly expressed in this sample. Future studies that characterize CFS EVs from individuals should assess whether this pattern persists at the individual sample level and whether it is: a result of the EV isolation method, characteristic of CFS EVs, and/or whether this finding of low CD81 expression is characteristic of CFS EVs from patients with asthma in comparison to healthy controls. Also, CFS EVs from individual patients with asthma can be compared to explore whether EV characteristics correspond with different clinical phenotypes or whether unsupervised clustering of EV proteins and other cargo can be used to identify novel underlying etiologies. In addition to pathologies such as asthma, EV release can further be modified by factors such as cellular stress. Thus, in addition to future research comparing CFS EVs of people with asthma and healthy controls, future studies can explore whether external factors such as environmental tobacco smoke or allergens influence EV characteristics. The CFS in SICAS was collected only at one timepoint, but future investigations with longitudinal saliva collections can investigate whether EV characteristics are stable over time, reflecting an underlying etiology, or whether the EVs are responsive to external factors and, if so, how quickly EVs can change in response to an exposure.

This research, however, is subject to several limitations. First, we only characterized a single total EV sample that was comprised of pooled re-suspended EV pellets from 180 individuals enrolled in SICAS with CFS collected at a single timepoint. SICAS is a cohort of predominantly minority children with asthma living in an urban area [Table 1] and thus the results may not be generalizable to other asthma cohorts. However, minority children living in urban areas experience a greater burden of asthma and greater asthma morbidity so novel biomarkers in this group are needed[47].

Second, we isolated CFS EVs using EQ (System Biosciences, Palo Alto, CA, USA), a polymer-based reagent that isolates EVs after a low-speed centrifugation step. This method results in a greater yield of EVs in comparison with other methods, but with lower purity. EQ samples contain EVs of varying sizes and high portions of salts, polymers, and other contaminants such as lipoproteins[48], although our TEM analysis demonstrated there was very little lipoprotein contamination in the EVs isolated from CFS. There was, however, carryover of the EQ reagent Supplementary Figure 2. However, EV isolation by EQ is simple and fast, only requiring an overnight incubation step and a one-step precipitation that can be completed with a benchtop centrifuge. It can also be used with very little sample volumes, an advantage for studies with limited quantities of precious biospecimens. The ease and use of standard equipment make this approach for EV isolation highly scalable to large cohort studies, although the cost of reagents should be considered.

Third, while we compared the EV pellet to the SN that remained after the EV precipitation using EQ, we were unable to make direct comparisons to untreated CFS to confirm that the EQ-treated CFS is enriched in EVs compared to CFS. Fourth, the pooled EV sample underwent a freeze-thaw cycle prior to NTA, SP-IRIS, and protein quantification after being frozen at -80°C for 4 months (due to lab closures resulting from the COVID-19 pandemic). While studies have found that the EV concentration and size were relatively stable

after a single freeze-thaw cycle and storage up to one year[49,50], more work is needed to understand the possible effect of storage conditions on EVs[36]. In this case, storage conditions may have affected the reported EV characteristics.

Fifth, our NTA measurements assessed a single dilution. At least two dilutions should be assessed to increase the effective size range, which is necessary because light scattering from smaller and larger particles differs greatly and thus different settings are needed to detect EVs of different sizes[37]. However, our confirmation of NanoSight LM10 NTA data on the ViewSizer 3000, which is equipped with three lasers that can more robustly characterize polydisperse samples, helps to overcome this limitation. Lastly, another limitation is that we calibrated both instruments with polystyrene bead standards, which can result in overestimation of particle concentrations in NTA due to their higher refractive index than EVs. Hollow organosilica beads should ideally be used as a reference for calibration, or alternatively silica beads, as their refractive index is more similar to EVs[51].

This is the first characterization of CFS EVs from patients with asthma. The strength in our approach is in the characterization of EVs across multiple platforms, including multiple different technologies to identify single EVs (TEM, NTA, SP-IRIS), providing an indication of the heterogeneity of the EV preparation examined. We also provide a general overview of the protein composition of the EV sample using a sensitive and quantitative method optimized for low protein concentrations, assessing several proteins expected to be present in the EV prep (e.g., tetraspanins, transmembrane proteins, and cytosolic proteins with membrane-binding capacity) as well as those not expected to be present (e.g., proteins associated with the endoplasmic reticulum and Golgi)[27,36]. Each platform applied here has distinct strengths and limitations. By performing multiple size and concentration measurements and characterizing EVs across different platforms, our results provide an initial assessment of CFS EVs that provide the basis for future studies.

DECLARATIONS

Acknowledgments

We acknowledge the School Inner-City Asthma Study staff and the community of parents, nurses, staff, and children who have contributed to this work. We thank Haotian Wu, Persis Ticknor-Swanson, Lisa Hu, and Samantha Kish-Levine for assistance with the cell-free saliva EV isolations. We acknowledge the contributions of Leona (Lee) Cohen-Gould and Juan Jimenez of the Electron Microscopy & Histology services of the Weill Cornell Medicine Microscopy & Image Analysis Core. The Core's transmission electron microscope was purchased with funds from NIH Shared Instrumentation Grant S10RR027699 for Shared Resources.

Authors' contributions

Made substantial contributions to conception and design of the study, performed data acquisition, analysis, and interpretation, and wrote the manuscript: Comfort N
Contributed to sample preparation, as well as provided administrative support: Bloomquist TR
Performed data acquisition and analysis: Shephard A
Performed analysis of SICAS demographic data: Petty CR
Performed administrative, technical, and material support, provided resources: Cunningham A, Hauptman M
Supervised the project and acquired funding: Phipatanakul W, Baccarelli A
All authors approved the final manuscript.

Financial support and sponsorship

This work was supported by National Institutes of Health (NIH) Grants U01AI110397 and R01ES025225, and by the NIH Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship (F31-ES030973-01A1).

Conflicts of interest

Alex P. Shephard is employed by NanoView Biosciences. All other authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Written informed consent was obtained from each participant's parent or legal guardian, and assent was obtained from each participant. The protocol was approved by the Boston Children's Hospital and Columbia University Institutional Review Boards (Columbia IRB: AAAS9936).

Consent for publication

Not applicable.

REFERENCES

1. Lancet. Controlling asthma. *Lancet* 2014;383:1521.
2. Zar HJ, Ferkol TW. The global burden of respiratory disease-impact on child health. *Pediatr Pulmonol* 2014;49:430-4. DOI PubMed
3. Ramratnam SK, Bacharier LB, Guilbert TW. Severe Asthma in Children. *J Allergy Clin Immunol Pract* 2017;5:889-98. DOI PubMed
4. Dharmage SC, Perret JL, Custovic A. Epidemiology of Asthma in Children and Adults. *Front Pediatr* 2019;7:246. DOI PubMed PMC
5. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716-25. DOI PubMed
6. Wu G, Yang G, Zhang R, et al. Altered microRNA Expression Profiles of Extracellular Vesicles in Nasal Mucus From Patients With Allergic Rhinitis. *Allergy Asthma Immunol Res* 2015;7:449-57. DOI PubMed PMC
7. Levänen B, Bhakta NR, Torregrosa Paredes P, et al. Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. *J Allergy Clin Immunol* 2013;131:894-903. DOI PubMed PMC
8. Mortaz E, Alipoor SD, Varahram M, et al. Exosomes in Severe Asthma: Update in Their Roles and Potential in Therapy. *Biomed Res Int* 2018;2018:2862187. DOI PubMed PMC
9. Sastre B, Cañas JA, Rodrigo-Muñoz JM, Del Pozo V. Novel Modulators of Asthma and Allergy: Exosomes and MicroRNAs. *Front Immunol* 2017;8:826. DOI PubMed PMC
10. Palanisamy V, Sharma S, Deshpande A, Zhou H, Gimzewski J, Wong DT. Nanostructural and transcriptomic analyses of human saliva derived exosomes. *PLoS One* 2010;5:e8577. DOI PubMed PMC
11. Sharma S, Rasool HI, Palanisamy V, et al. Structural-mechanical characterization of nanoparticle exosomes in human saliva, using correlative AFM, FESEM, and force spectroscopy. *ACS Nano* 2010;4:1921-6. DOI PubMed PMC
12. Lässer C, Alikhani VS, Ekström K, et al. Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages. *J Transl Med* 2011;9:9. DOI PubMed PMC
13. Wong DT. Salivary Diagnostics: Amazing as it might seem, doctors can detect and monitor diseases using molecules found in a sample of spit. *Am Sci* 2008;96:37-43. DOI PubMed PMC
14. Kaczor-Urbanowicz KE, Martin Carreras-Presas C, Aro K, Tu M, Garcia-Godoy F, Wong DT. Saliva diagnostics - Current views and directions. *Exp Biol Med (Maywood)* 2017;242:459-72. DOI PubMed PMC
15. Hyyppä T. Studies on immunologic and inflammatory factors in the saliva and gingiva in patients with asthma. *Journal of Clinical Periodontology* 1981;8:500-7. DOI
16. Siegler DI, Citron KM. Serum and parotid salivary IgA in chronic bronchitis and asthma. *Thorax* 1974;29:313-6. DOI PubMed PMC
17. Hyyppa T. Salivary immunoglobulins in children with asthma. *J Periodontal Res* 1980;15:227-31. DOI PubMed
18. Brasher GW. Salivary IgA children with atopic diseases. *Ann Allergy* 1971;29:422-7. PubMed
19. Butz A, Bellin MH, Bollinger ME, et al. Salivary cotinine measurement for all children with persistent asthma: spit matters. *Ann Allergy Asthma Immunol* 2016;116:463-5. DOI PubMed PMC
20. Negretti F, Casetta P. Remarkable increases of salivary IgE levels in allergic syndromes. *Int Arch Allergy Appl Immunol* 1990;92:103-4. DOI PubMed

21. Little FF, Delgado DM, Wexler PJ, et al. Salivary inflammatory mediator profiling and correlation to clinical disease markers in asthma. *PLoS One* 2014;9:e84449. DOI PubMed PMC
22. Phipatanakul W, Koutrakis P, Coull BA, et al. The School Inner-City Asthma Intervention Study: Design, rationale, methods, and lessons learned. *Contemp Clin Trials* 2017;60:14-23. DOI PubMed PMC
23. Zlotogorski-Hurvitz A, Dayan D, Chaushu G, et al. Human saliva-derived exosomes: comparing methods of isolation. *J Histochem Cytochem* 2015;63:181-9. DOI PubMed PMC
24. Comfort N, Cai K, Bloomquist TR, Strait MD, Ferrante AW, & Baccarelli AA. Novel nanoparticle tracking analysis technology and method for extracellular vesicle quantification and size determination. *J Vis Exp*. Forthcoming 2021.
25. Gardiner C, Ferreira YJ, Dragovic RA, Redman CW, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles* 2013;2:19671. DOI PubMed PMC
26. Core TeamR. R Foundation for Statistical Computing. (2018). Available from: https://www.r-project.org/index.html [Last accessed on 18 Mar 2021].
27. Harris VM. Protein Detection by Simple Western™ Analysis. In: Kurien BT, Scofield RH, editors. Western Blotting. New York: Springer; 2015. pp. 465-8. DOI PubMed
28. Chen JQ, Heldman MR, Herrmann MA, et al. Absolute quantitation of endogenous proteins with precision and accuracy using a capillary Western system. *Anal Biochem* 2013;442:97-103. DOI PubMed PMC
29. Lötvall J, Hill AF, Hochberg F, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *J Extracell Vesicles* 2014;3:26913. DOI PubMed PMC
30. Aqrawi LA, Galtung HK, Guerreiro EM, et al. Proteomic and histopathological characterisation of sicca subjects and primary Sjögren's syndrome patients reveals promising tear, saliva and extracellular vesicle disease biomarkers. *Arthritis Res Ther* 2019;21:181. DOI PubMed PMC
31. Nonaka T, Wong DT. Saliva-Exosomics in Cancer: Molecular Characterization of Cancer-Derived Exosomes in Saliva. Peptidomics of Cancer-Derived Enzyme Products. Elsevier; 2017. pp. 125-51. DOI PubMed PMC
32. Wexler PJ, Siqueira WL, Helmerhorst EJ, Blicharz TM, Hayman RB, et al. Saliva Diagnostics in Asthma. *Proceedings of the American Thoracic Society* 2009:6.
33. Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol* 2014;30:255-89. DOI PubMed
34. Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *Int J Mol Sci* 2017;18:1153. DOI PubMed PMC
35. Niel G, D'Angelo G, & Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 2018;19:213-28. DOI PubMed
36. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC
37. van der Pol E, Coumans FA, Grootemaat AE, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost* 2014;12:1182-92. DOI PubMed
38. der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R. Optical and non-optical methods for detection and characterization of microparticles and exosomes. *J Thromb Haemost* 2010;8:2596-607. DOI PubMed
39. Bachurski D, Schuldner M, Nguyen PH, et al. Extracellular vesicle measurements with nanoparticle tracking analysis - An accuracy and repeatability comparison between NanoSight NS300 and ZetaView. *J Extracell Vesicles* 2019;8:1596016. DOI PubMed PMC
40. Vogel R, Savage J, Muzard J, et al. Measuring particle concentration of multimodal synthetic reference materials and extracellular vesicles with orthogonal techniques: Who is up to the challenge? *J Extracell Vesicles* 2021;10:e12052. DOI PubMed PMC
41. Skliar M, Chernyshev VS, Belnap DM, et al. Membrane proteins significantly restrict exosome mobility. *Biochem Biophys Res Commun* 2018;501:1055-9. DOI PubMed
42. Goetzl EJ, Yaffe K, Peltz CB, et al. Traumatic brain injury increases plasma astrocyte-derived exosome levels of neurotoxic complement proteins. *FASEB J* 2020;34:3359-66. DOI PubMed PMC
43. Abner EL, Elahi FM, Jicha GA, et al. Endothelial-derived plasma exosome proteins in Alzheimer's disease angiopathy. *FASEB J* 2020;34:5967-74. DOI PubMed PMC
44. Daaboul GG, Gagni P, Benussi L, et al. Digital Detection of Exosomes by Interferometric Imaging. *Sci Rep* 2016;6:37246. DOI PubMed PMC
45. Helwa I, Cai J, Drewry MD, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* 2017;12:e0170628. DOI PubMed PMC
46. Erdbrügger U, Lannigan J. Analytical challenges of extracellular vesicle detection: A comparison of different techniques. *Cytometry A* 2016;89:123-34. DOI PubMed
47. Azmeh R, Greydanus DE, Agana MG, et al. Update in Pediatric Asthma: Selected Issues. *Dis Mon* 2020;66:100886. DOI PubMed
48. Gemoll T, Rozanova S, Röder C, et al. Protein Profiling of Serum Extracellular Vesicles Reveals Qualitative and Quantitative Differences After Differential Ultracentrifugation and ExoQuick™ Isolation. *J Clin Med* 2020;9:1429. DOI PubMed PMC
49. Jeyaram A, Jay SM. Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications. *AAPS J* 2017;20:1. DOI PubMed PMC
50. Yuana Y, Böing AN, Grootemaat AE, et al. Handling and storage of human body fluids for analysis of extracellular vesicles. *J*

Extracell Vesicles 2015;4:29260. DOI PubMed PMC

51. Varga Z, van der Pol E, Pálmai M, et al. Hollow organosilica beads as reference particles for optical detection of extracellular vesicles. *J Thromb Haemost* ;2018:1646-55. DOI PubMed

Ikezu. *Extracell Vesicles Circ Nucleic Acids* 2021;2:49-54
DOI: 10.20517/evcna.2021.08

Extracellular Vesicles and Circulating Nucleic Acids

Conference Report

Open Access

Current trends in extracellular vesicle research on neuroscience from ADPD2021 meeting

Tsuneya Ikezu

Laboratory of Molecular NeuroTherapeutics, Department of Neuroscience, Mayo Clinic Florida, Jacksonville, FL 32224, USA.

Correspondence to: Tsuneya Ikezu, MD, PhD, Department of Neuroscience, Mayo Clinic Florida, Jacksonville, FL 32224, USA. E-mail: Ikezu.Tsuneya@mayo.edu

How to cite this article: Ikezu T. Current trends in extracellular vesicle research on neuroscience from ADPD2021 meeting. *Extracell Vesicles Circ Nucleic Acids* 2021;2:49-54. https://dx.doi.org/10.20517/evcna.2021.08

Received: 15 Mar 2021 **Accepted:** 16 Mar 2021 **Available online:** 30 Mar 2021

Academic Editor: Yoke Peng Loh **Copy Editor:** Yue-Yue Zhang **Production Editor:** Yue-Yue Zhang

This year's ADPD2021 meeting (https://adpd.kenes.com) for Alzheimer's and Parkinson's disease (AD/PD) research is being held virtually in Barcelona, Spain. There are hundreds of papers for the cutting-edge research and clinical trials on AD/PD. Among them, exciting extracellular vesicle (EV)-related papers were presented by the following scientists from the US, France, Israel, and Spain. Blood neuron and astrocyte-specific EVs are useful liquid biopsy tools for the diagnosis of brain atrophy and cognitive dysfunction in AD/PD (Kapoginiasis), which was validated using multiple animal models of AD (Delgado-Peraza). PD cerebrospinal fluid (CSF)-derived EVs contains phosphorylated alpha-synuclein (a-syn) and can cause PD-like pathology and behavioral deficit after their intranasal administration (Herman). Frontotemporal dementia (FTD) CSF-derived EVs contain unique miRNAs, which can modulate neuronal autophagy activities (Cervera-Carles). Interstitial fluid (ISF)-derived EVs from tau mouse and human AD brains show potent tau seeding activities (Leroux). ISF-EVs show tau seeding activity when isolated from AD cases but not from controls, progressive supranuclear palsy (PSP) cases, or Pick disease (PiD) cases (Colin). Brain-derived EVs from prodromal AD and AD cases have potent tau propagation activity in aged mouse brain and cause neurophysiological dysfunction (Ruan). EV production is increased by aging in female but not male mouse brain, whereas mitochondria-derived EVs (coined as "mitovesicles") are commonly increased by aging (Kim). Finally, mitovesicles contain mitochondria-specific marker Tomm20 and are released by inhibition of mitochondrial activity. Both animal models and human cases of Down syndrome show enhanced production of mitovesicles in brain, suggesting the mitochondrial dysfunction (D'Acunzo).

Dimitrios Kapoginiasis at National Institute of Aging, NIH presented a comprehensive body of his work on insulin signaling biomarkers in blood exosomes of AD and PD patients. This is based on previous reports that pSer312 and pSer616 insulin receptor substrate-1 (IRS-1) is upregulated in the neurons in Alzheimer's disease and mild cognitive impairment (MCI) cases. Accordingly, blood L1CAM+ neuron-derived EVs (NEVs) contain increased amount of pIRS-1 in addition to Abeta42, pT181 tau, pT231 tau, and total tau. The team examined NEVs from 350 patients in The Baltimore Longitudinal Study of Aging cohort from 1958 to 2019. In this study, pY-IRS-1 was also significantly elevated in AD NEVs compared to control NEVs. Both pT181tau and pS312-IRS-1 in NEVs are negatively correlated with multiple cognitive functional readouts. In addition, pY-IRS-1 is positively correlated with temporal grey matter volume. This is in line with their recent study demonstrating the positive correlation of pIGF1R, pIR, pY-IRS1, pGSK3B, and p-p70S6K in NEVs with the temporal grey matter volume and negative correlation with the white matter hyperintensities volume. Finally, pY-IRS-1 in NEVs is decreased in individuals with PD and cognitive decline and is negatively correlated with motor symptom severity in PD, suggesting the broader applicability of NEVs as prognostic biomarkers for brain atrophy and cognitive function in AD and PD cases.

Shay Herman from Professor Offen's lab at Tel-Aviv University, Israel, presented her research on intranasal administration of CSF-derived exosomes from PD patients, which induces PD-like symptoms and pathology. This study was based on previous studies demonstrating the development of alpha-synuclein (a-syn) aggregation after treatment of neurons with CSF-derived exosomes from PD patients in vitro. Intravenous or intrastriatal injections of exosomes derived from PD patient serum or brain lysates of dementia with Lewy bodies were also shown to develop protein aggregation in the substantia nigra (SN) and motor deficits *in vivo*. The EVs were isolated from the patient's CSF by ultracentrifugation. Exosomal markers (CD81, CD9, and Tsg101) and a-syn (total and pSer129) were detected in the isolated CSF-EVs. Incubation of SH-SY5Y cells with CSF-EVs show significant accumulation of pSer129 a-syn and neurotoxicity in vitro. For the *in vivo* study, C57BL/6 mice received intranasal administration of PD CSF-EVs at three months of age and were tested for behavioral changes at 3-8 months after the treatment. PD CSF-EV-treated mice showed significant motor deficits, elevated anxiety, hyposmia, earlier greying of hair (possible melanin deficiency), and pSer129 a-syn aggregation in the SN. This would be the first demonstration of modeling a-syn aggregation in the SN and motor deficits after intranasal inoculation of PD CSF-EVs in mice.

Laura Cervera-Carles at Ana Maria Cuervo laboratory, Albert Einstein College of Medicine, presented her recent work on characterization of miRNAs in EVs isolated from CSF of patients with FTD, who show protein accumulation of TDP-43 or tau in the frontotemporal region of the brain. They focused on miRNAs in CSF-derived EVs as several FTD-related proteins could interfere with their biogenesis and several miRNA species have been linked to tau phosphorylation and splicing. In their study, EVs were isolated from CSF by precipitation method and verified by cryo-electron microscopy and bead-based flow cytometry for exosomal markers. In total, 752 miRNAs were analyzed by qPCR human panels and 103 miRNAs were detected in the isolated EVs. They initially detected eight potential candidate miRNAs on FTD patient CSF-derived EVs and validated the findings using CSF samples collected from 142 subjects (TDP, TDP/tau, and tau groups). miR-146 and miR-361 were upregulated in the tau FTD group, whereas miR-15 and miR-708 were downregulated or undetectable in the tau FTD group. The research team also investigated the effect of miRNAs on protein turnover using murine neuronal N2a cells expressing human WT tau or P301L mutant tau. Using two fluorescent reporter systems to monitor chaperone-mediated autophagy and macroautophagy dynamics, they concluded that protein degradation appears altered when certain miRNAs and tau forms are co-present in the cell lines, and the effect is dependent on autophagy: co-presence of

miRNAs alterations and mutant tau appear to inhibit macroautophagy, whereas P301L mutant tau seems to prevent the activation of chaperone-mediated autophagy by miRNAs. Further studies are necessary to elucidate the molecules involved in the autophagy process regulated by these specific miRNAs.

Elodie Leroux from Luc Buee laboratory at INSERM/Universite de Lillle presented the EV and prion-like spreading in AD. EVs were isolated by size-exclusion column (SEC) from interstitial fluid (ISF), CSF, and plasma. ISF was isolated from WT, Tau30, and APP/PS1 mice. Then, EVs' isolation was validated for their size by NTA and their morphology by electron microscopy and Western blotting of EV markers. The seeding activity of EVs was determined by the FRET-based tau seeding assay system. The FRET signal is significantly increased in ISF-derived EVs (ISV-EVs) from Thy-tau30 mice at three and six months of age. Immunoprecipitation of tau+ ISF-EVs by HT7 anti-tau antibody reduced the tau seeding activity. ISF-EVs were next isolated from prefrontal lobe of healthy controls and AD cases by SEC. The AD ISF-EVs only also had tau seeding activity, as determined by the FRET-based reporter system. However, AD CSF- or plasma-derived EVs showed no tau seeding activity, presumably due to the sensitivity issue. Overall, this is a significant work to demonstrate the tau seeding activity of ISF-EVs from tau mice, which is elevated by aging. These findings were also reproduced using ISF-EVs from AD cases.

Morvane Colin from Lille Neuroscience & Cognition, France, presented the role of brain EV in tauopathies. They examined the EVs isolated from human ISF (ISF-EVs) using different brain tissues of healthy controls and AD, progressive supranuclear palsy, and Pick disease cases by SEC. There was no difference in the concentration of EVs or tau levels in ISF-EVs among groups or brain regions. Significant tau seeding activity was observed in ISF-EVs isolated from prefrontal cortex, occipital cortex, or cerebellum of AD cases but not from other groups. There was no tau pathology in the cerebellum of the tested AD cases. Finally, ISF-EVs isolated from AD or control cases were injected into Thy-Tau30 mice or non-Tg littermates. MC1 tau accumulation was observed in the CA1 region of AD ISF-EV-injected Thy-Tau30 mice but not by control ISF-EV injection or in non-Tg littermates. These data demonstrate that tau is physiologically secreted in ISF-EVs in all tested groups, but only AD ISF-EVs show tau seeding activity both in vitro and *in vivo* when using Thy-Tau30 mice. Further studies are necessary to understand the difference in tau seeding activity of ISF-EVs among AD, PSP, and PiD cases.

Zhi Ruan from Ikezu lab presented the spread of tau pathology after intrahippocampal injection of EVs isolated from the frontal cortical grey matter of HC, prodromal AD (pAD), and AD cases. Both pAD and AD brain-derived EVs were enriched in tau oligomer species and globular tau, which were mostly present on the intraluminal side of EVs. Proteomic analysis and validation with Western blotting demonstrate the enrichment of glia-specific markers and reduction in neuron-specific markers in AD brain-derived EVs. Both pAD and AD EVs showed enhanced neuronal uptake, but AD EVs also exhibited higher tau seeding activity, as determined by the FRET biosensor system in vitro. Significant tau propagation was observed after hippocampal injection of pAD or AD EVs, which was mainly in GAD67+ interneurons and GluR2/3+ mossy cells in the hilus region. This was accompanied by reduced neuronal activities, as determined by the reduction in GAD67+ synaptic puncta around CA1 pyramidal neurons and c-fos expression, and the enhancement of excitatory/inhibitory activity balance, as determined by the whole-cell patch clamp recording of CA1 pyramidal neurons. This is the first comprehensive demonstration that AD brain-derived EVs propagate tau pathology and induce CA1 neurophysiological dysfunction. This work was recently published in Brain[1].

Francheska Delgado-Peraza from Kapogiannis lab at NIH presented their work demonstrating that plasma neuronal EVs from multiple AD mouse models contain high levels of total tau, pT181-tau, and Aβ42, which

are significantly correlated with their levels in the brain tissues. 2xTg-AD (APP/PS1), 5xFAD, and 3xTg-AD mouse models were used for brain tissue analysis and isolation of EVs from blood using immunoprecipitation for neuron- and astrocyte-specific EVs (NEVs and AEVs) targeting L1CAM and GLAST, respectively. The isolated NEVs were enriched with L1CAM, GRIA2, and conventional exosomal markers. They also showed that circulating astrocytic EVs contain high levels of C1q complement, reflecting brain levels. These findings are direct validation of circulating EVs as indicators of the accumulation of total tau, pT181 tau, Aβ42, and C1q using AD mouse brain and blood samples and demonstrated that NEVs and AEVs have great potential for liquid biopsy modules for AD biomarkers.

Yohan Kim from Efrat Levy lab (Nathan S. Kline Institute for Psychiatric Research, New York, US) presented how sex and aging alter EV secretion in the brain. Brain-derived EVs were isolated from male and female C57BL/6 mice at 3-24 months of age by their established protocol using sucrose gradient ultracentrifugation. The purity was assessed by EM and Western blotting of EV and non-EV markers. EM-based morphological analysis shows no obvious changes in the shape of isolated EVs by aging. Interestingly, the level of annexin-A2 was significantly increased in female but not male brain-derived EVs by aging. This was accompanied by significant increases of Alix, Tsg101, and HSC70 levels only in female brain-derived EV by aging. Another exosome marker (CD63) and a mitovesicle marker (VDAC) were significantly increased by aging in both sexes, suggesting the elevation of specific EVs only in the female brain and mitovesicles as commonly elevated EVs by aging. These data demonstrate the sexual dimorphism of age-dependent increase in EV production in female mouse brain and that mitovesicles can serve as a common indicator of age-related EVs.

Pasquale D'Acunzo from Dr. Efrat Levy's lab (Nathan S. Kline Institute for Psychiatric Research, New York, US) presented a study in which the authors described a newly identified population of mitochondria-derived EVs that they coined "mitovesicles". These EVs are fractionated at the bottom of an OptiPrep density gradient from mouse and human brain tissues and contain an electron-dense matrix and a double membrane when visualized by cryoEM. Proteomic profiling of the mitovesicle fraction showed enrichment of proteins involved in catabolic pathways, including energy production and neurotransmitter/amino acid metabolism. Western blotting analysis of isolated mitovesicles showed enrichment of specific mitochondrial markers but a lack of other common mitochondrial proteins such as Tomm20. EVs isolated from human primary fibroblasts treated with the mitochondrial inhibitor antimycin-A showed increased levels of mitochondria markers, suggesting that mitochondrial dysfunction enhances mitovesicle release. Finally, higher levels of mitovesicles were detected in EVs isolated from a mouse model of Down syndrome, the Ts2 model, and human Down syndrome brain tissues when compared to the respective controls. The work from D'Acunzo *et al.*[2], recently published in *Science Advances*, indicates that mitochondrial dysfunction can be monitored by the amount of mitovesicles *in vivo* and that the status of intracellular mitochondria can be inferred by the analysis of mitovesicle cargo.

In addition to these oral presentations, there are several interesting poster presentations for EV-related studies:

Lauritzen I at Checler Université Cote d'Azur-CNRS, France, reported enrichment of oligomeric c-terminal fragment of APP called C99 in EVs isolated from C99- or APP-expressing cells or mice.

Johansson L at Linköping University, Sweden, reported that CRISPR-mediated loss-of-function mutation in VSP35 in SH-SY5Y human neuronal cell line resulted in intracellular accumulation of Ab oligomer, reduction in exosome release, and upregulation of autophagocytic response. Exosomes collected from Ab

oligomer-treated SH-SY5Y cells with VPS35 mutation showed neurotoxicity following incubation with organotypic hippocampal slices, suggesting the potential role of the neurotoxic effect.

Yao P from Kapogiannis laboratory at NIH reported NEVs from AD cases have reduced levels of mitochondrial respiratory protein components compared to controls in two different cohorts, suggesting their reduced energy metabolism.

Another paper by Eren E from Kapogiannis laboratory reports ante-mortem NEV Ab42 levels are positively associated with ante-mortem cognition and negatively associated with AD Braak stage at autopsy.

Peng K at Nathan S. Klein Institute, NY, reported enhanced EV production in the brain of APOE2 knock-in mice compared to APOE3 knock-in mice by aging, suggesting enhanced clearance of endosomal molecules by EV secretion in APOE2 knock-in mice.

Vacchi E at Università della Svizzera Italiana, Switzerland, presented the immune profiling of EVs isolated from plasma and CSF samples of PD, atypical PD with multiple system atrophy, or tauopathies and control cases. Multiple immune makers were differentially expressed in these groups in both plasma- and CSF-derived EVs and can be utilized for discriminating patients from controls with high accuracy and sensitivity.

Balaguer JS at University of Barcelona presented a study comparing EVs isolated from cortical neurons of SD rat, C57BL/6 mouse, and RTP801 knockout mouse embryonic brains. RTP801/REDD1 is a pro-apoptotic molecule, and its upregulation has been reported in neurodegenerative disorders. EVs from WT neurons enhanced consolidation of glutamatergic synapses in recipient neurons, while deletion of RTP801 reduced EV secretion and its consolidation effect.

Finally, Clayton K from Ikezu lab at Boston University presented enhanced tau propagation from the entorhinal cortex to dentate gyrus in aged APPNL-G-F mice, which developed compact amyloid plaque formation, compared to non-tg mice. This effect was significantly diminished by microglial depletion by PLX5622. A novel microglia-specific expression of mEmerald-CD9 reporter showed enhanced EV secretion from Clec7A+ neurodegenerative microglia compared to homeostatic microglia. Microglia-specific EVs contained p-tau, as determined by super-resolution confocal microscopy and immunoelectron microscopy.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

The author declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Ruan Z, Pathak D, Venkatesan Kalavai S, et al. Alzheimer's disease brain-derived extracellular vesicles spread tau pathology in interneurons. *Brain* 2021;144:288-309. DOI PubMed PMC
2. D'Acunzo P, Pérez-González R, Kim Y, et al. Mitovesicles are a novel population of extracellular vesicles of mitochondrial origin altered in Down syndrome. *Sci Adv* 2021;7:eabe5085. DOI PubMed PMC

Xiao *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:55-79
DOI: 10.20517/evcna.2021.04

Extracellular Vesicles and Circulating Nucleic Acids

Review

Open Access

Function of exosomes in neurological disorders and brain tumors

Lan Xiao, Sangeetha Hareendran, Y. Peng Loh

Section on Cellular Neurobiology, Eunice Kennedy Shriver, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

Correspondence to: Dr. Y. Peng Loh, Section on Cellular Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Convent Drive, Bldg 49, Rm 6A-10, Bethesda, MD 20892, USA. E-mail: lohp@mail.nih.gov

How to cite this article: Xiao L, Hareendran S, Loh YP. Function of exosomes in neurological disorders and brain tumors. *Extracell Vesicles Circ Nucleic Acids* 2021;2:55-79. https://dx.doi.org/10.20517/evcna.2021.04

Received: 10 Feb 2021 **First Decision:** 4 Mar 2021 **Revised:** 15 Mar 2021 **Accepted:** 19 Mar 2021 **Available online:** 30 Mar 2021

Academic Editor: Tsuneya Ikezu **Copy Editor:** Yue-Yue Zhang **Production Editor:** Yue-Yue Zhang

Abstract

Exosomes are a subtype of extracellular vesicles released from different cell types including those in the nervous system, and are enriched in a variety of bioactive molecules such as RNAs, proteins and lipids. Numerous studies have indicated that exosomes play a critical role in many physiological and pathological activities by facilitating intercellular communication and modulating cells' responses to external environments. Particularly in the central nervous system, exosomes have been implicated to play a role in many neurological disorders such as abnormal neuronal development, neurodegenerative diseases, epilepsy, mental disorders, stroke, brain injury and brain cancer. Since exosomes recapitulate the characteristics of the parental cells and have the capacity to cross the blood-brain barrier, their cargo can serve as potential biomarkers for early diagnosis and clinical assessment of disease treatment. In this review, we describe the latest findings and current knowledge of the roles exosomes play in various neurological disorders and brain cancer, as well as their application as promising biomarkers. The potential use of exosomes to deliver therapeutic molecules to treat diseases of the central nervous system is also discussed.

Keywords: Extracellular vesicles, neurodegenerative disorders, glioblastoma, Alzheimer's disease, neurodevelopment

INTRODUCTION

Extracellular vesicles (EV) were first described in 1967 as "platelet dusts" in plasma[1]. Currently, EVs are divided into three main categories based on the origination and size: exosome derived from endosomes, ranging from 40 to 100nm in diameter; microvesicle/shedding particles from plasma membrane, which are larger than 100nm in diameter; apoptotic bodies from plasma membrane, which are 1-5 μm in diameter[2,3]. In this review, while most of the literature cited has used the term exosome, the degree of characterization of the exosomes in the various papers varied; most have met the guidelines such as size by NTA, exosomal markers and electron microscopy or zeta view, reported for exosomes in MISEV2014[4], and others with insufficient characterization to confirm specific identity as exosomes are referred to as extracellular vesicles.

Exosomes, initially described as vesicles released from various types of cultured cells[5], are microvesicles derived from endosomal membranes. Microvesicles were first described by Dr. C. Turbide in 1987 in his study of maturation of sheep reticulocyte. Vesicles obtained after 100,000x g centrifugation were found to contain some characteristic activity of the reticulocyte[6]. These vesicles were then further defined as being originated from endosomes, with a diameter from 30 to 100nm[7]. As a subtype of extracellular vesicles with a bilayer membrane that bud from cell membrane and/or are secreted, exosomes are heterogeneous and influenced by the physiological and pathological conditions of the originating cells. Exosomes are distributed broadly in human secretions and act as intercellular messengers via transferring or exchanging DNA, RNA, and proteins between cells[8,9].

Recently, emerging studies have revealed that exosomes have more complicated facets. They are not just secreted as cellular wastes or by-products, but contain a variety of cargos such as proteins, lipids, and nucleic acids, and exert their function by delivering cargoes to target cells and modulating the bioactivity of recipient cells. Therefore, exosomes serve as a new mode of intercellular communication and play a critical role in biological systems, and pathogenesis of diseases, including those of the central nervous system. In addition, the ability of exosomes to cross the blood-brain barrier makes them ideal therapeutic delivery vehicles and potential biomarkers for neurological disorders[10].

Exosome structure and content

Exosomes are released from a variety of cell types, and can be found in physiological fluids such as blood[11], cerebrospinal fluid[12], saliva[13],urine[14] and breast milk[15]. Exosomes consist of a wide range of molecules such as proteins, lipids and nucleic acids [Figure 1], and reflect the pathophysiology and physiological features of parental cells.

Current studies have shown that exosome membranes are enriched in sphingomyelin, phosphatidylserine, cholesterol, and ceramides. Exosomes contain a variety of proteins such as tetraspanins (CD9, CD63, CD81), endosomal origin proteins (ALIX,TSG 101), heat-shock proteins (HSP70, HSP90), enzymes(GAPDH, nitric oxide synthase, catalase), receptor (EGFR), major histocompatibility complex I-II, adhesion proteins, integrins, cytoskeleton proteins (actin, gelsolin, myosin, tubulin) and cytosolic proteins[16,17]. Irrespective of the origin, certain proteins such as TSG101, HSP70, CD81 and C63 are exclusively involved in the biogenesis of exosomes, and thus generally used as exosome markers. However, since the purity of the exosomes isolated has not been fully assessed in some studies, it is possible that skeletal proteins e.g., actin, myosin and tubulin reported to be present in exosomes may be contaminants of the exosome-enriched fraction. In addition, lipid components within exosomes can be incorporated into recipient cells and mediate complex biological effects[2]. Moreover, RNA sequencing showed that mRNA and microRNA are also abundant in exosomes from human plasma, in addition to other species of RNA such as ribosomal RNA, small nuclear RNA, transfer RNA[2,18] and long RNA[19] that maintain critical

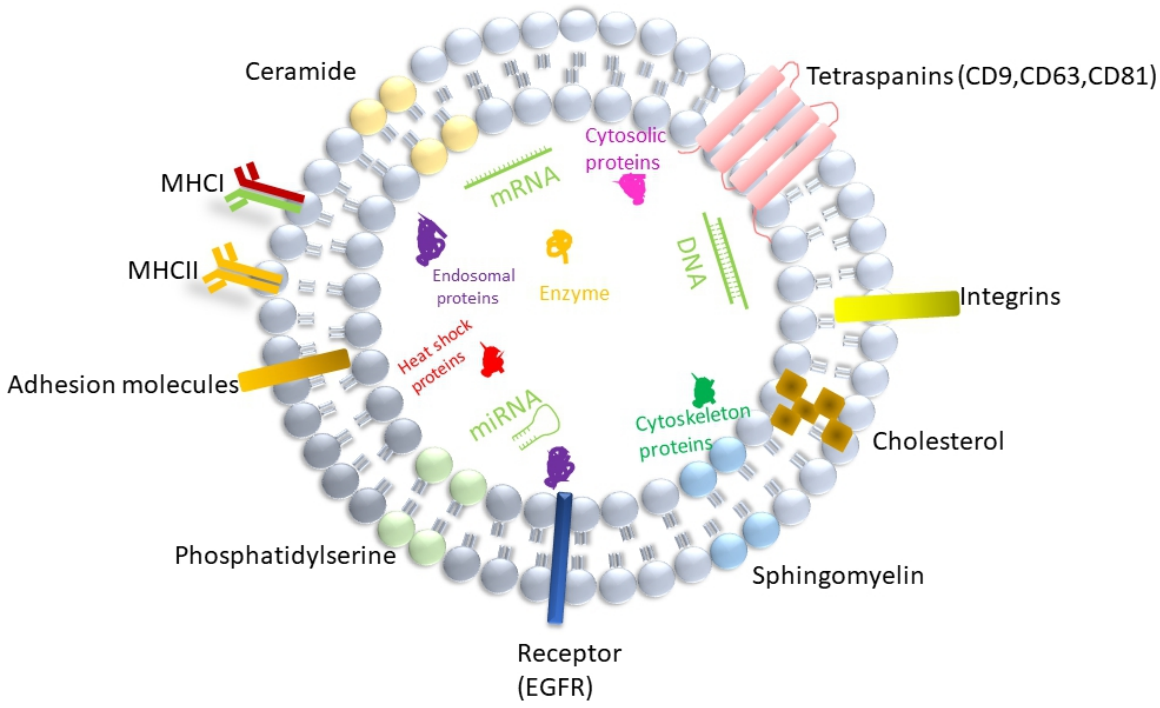


Figure 1. Structure and composition of exosome. Exosome is a lipid bilayer structure that contains lipids, proteins and nucleic acids. Sphingomyelin, phosphatidylserine, cholesterol and ceramides are highly distributed on the membrane. In addition, exosomes also contain a variety of proteins such as major histocompatibility complex I and II (MHCI and MHCII), tetraspanins (CD9,CD63,CD81), endosomal origin proteins (ALIX,Tsg101), heat shock proteins (HSP70,HSP90), enzymes(GAPDH, nitric oxide synthase, catalase), receptor(EGFR), adhesion proteins, integrins, cytoskeleton proteins (actin, gelsolin, myosin, tubulin) and cytosolic proteins, as well as RNA, miRNA and DNA.

biological functions.

Exosome biogenesis, secretion, and uptake

Exosome biogenesis is a complicated process that involves a variety of signaling cascades. Exosomes are formed by multi-vesicular bodies (MVBs) which are late endosomes. The membrane of MVB buds inward to form intraluminal vesicles (ILVs) with components derived from either endocytic pathway or secretory (ER/Golgi) pathway, into the endosomal lumen[20] [Figure 2]. Following accumulation of vesicles, MVBs will be either transported to lysosomes for degradation[21] or fused with plasma membrane to release ILVs into extracellular space as exosomes[22]. However, how the cargo is sorted to ILVs and how formation and release of exosomes are regulated by internal and external factors are still not fully understood.

Recent studies indicate that both endosomal sorting complex transport (ESCRT)-dependent and ESCRT-independent pathways are involved in the formation and secretion of exosomes[2,23]. ESCRT consists of four major protein complexes, including ESCRT0, ESCRT-I, ESCRT-II, ESCRTIII and associated AAA ATPase Vps4 Complex. In an analysis with RNA interference screen targeting 23 components of ESCRT and associated proteins, it was found that seven ESCRT proteins contributed to the release of exosomes[24]: Knockdown of ESCRT-0 proteins Hrs and TSG101, ESCRT-I protein STAM1 decreased the secretion of exosomes; in contrast, knockdown of ESCRT-III proteins CHMP4C, VPS4B,VTA1 and ALIX increased the secretion of exosomes. Further studies revealed that ESCRT-0 sequestered ubiquitinated proteins into

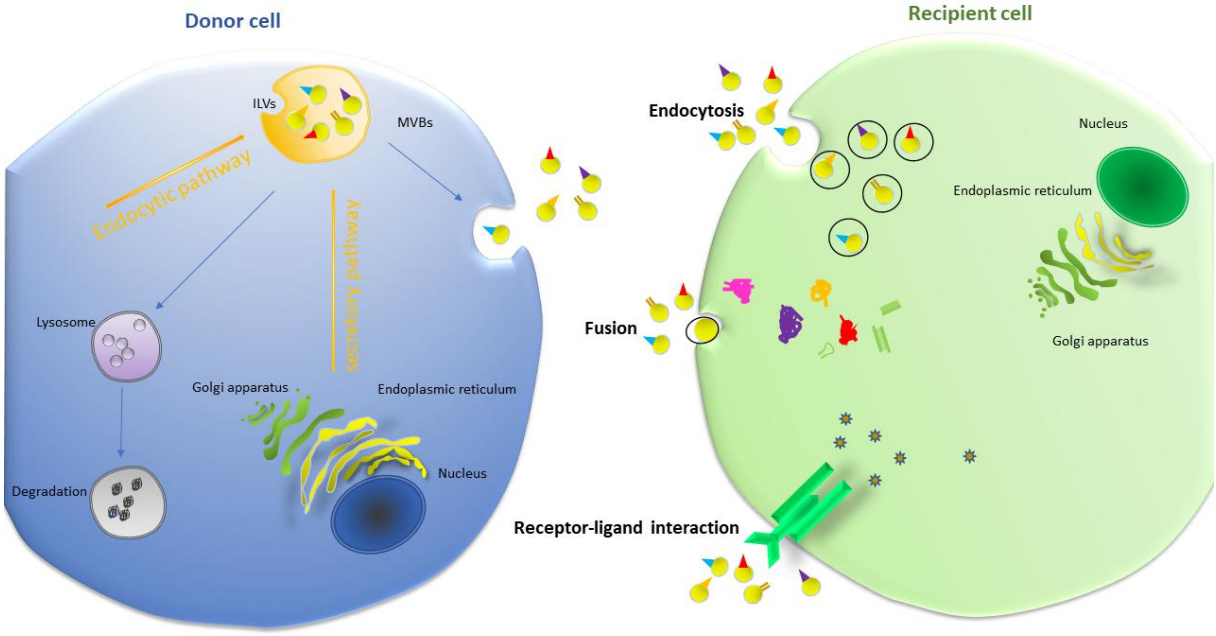


Figure 2. Biogenesis of exosome. The biogenesis of exosomes occurs when multivesicular bodies uptake intraluminal vesicles formed from either endocytic pathway or ER/Golgi secretory pathway. Then, MVBs either fuse with cellular membrane to release exosomes, or fuse with lysosomes for cargo degradation. After releasing into extracellular space, exosomes act as a mediator of intercellular communication through being taken up by recipient cells via endocytosis, fusion or receptor-ligand interaction. This process can be either paracrine or endocrine in manner. MVBs: Multivesicular bodies.

specific domains of endosomal membrane, and then combined with ESCRT-III after crosslinking with ESCRT-I and ESCRT-II complex. ESCRT-III finally promotes intraluminal vesicle formation via facilitating the budding process and separation from the MVB membrane[25]. Interestingly, syndecan heparan sulphate proteoglycans and their cytoplasmic adaptor, syntenin, have been shown to regulate exosome formation via modulating ALIX through LYPX(n)L motifs to facilitate the intraluminal budding of endosomal membranes[26]. These results suggest ESCRT is critical for cargo sorting, multivesicular body formation, and the budding process[27].

Conversely, a large amount of evidence indicates that exosomes can also be formed and released in an ESCRT-independent manner. Studies showed that when four major ESCRT complexes were simultaneously silenced, ILVs were still observed in MVBs, suggesting existence of a ESCRT-independent mechanism[28]. In addition, other proteins and lipids are also involved in the regulation of exosome biogenesis and secretion. Tetraspanins, transmembrane proteins that are highly distributed in exosomes, contributed to the ESCRT-independent exosome release[29]: overexpression of tetraspanins CD9 and CD82 increased catenin in exosomes released from HEK293 cells[30]; tetraspanin Tspan8 promoted recruitment of specific proteins and mRNA into exosomes, such as CD106 and CD49d that are critical for exosome-endothelial cell binding and internalization[31]; Tetraspanin CD63 has also been reported to be involved in exosome biogenesis as evidenced by decreased small vesicle secretion after (CRISPR)/Cas9 knockout of the CD63 gene in HEK293 cells[32]; finally, tetraspanin-enriched microdomains and tetraspanin CD81 are important for sorting specific receptors and components toward exosomes[33]. Furthermore, ceramides have been shown to enhance domain-induced budding due to its activity to promote negative curvature of endosomal membrane[34]. Rab guanosine triphosphatases (GTPases) such as Rab27a/b[35], and Rab35 and GTPase-activating proteins TBC1D10A-C have also been reported to contribute to the process of exosome secretion pathway[36].

Interestingly, cellular homeostasis also can affect exosome secretion. For example, increased intracellular Ca^{2+} induced more exosome secretion in K562 cells, a hematopoietic cell line[37]. Environmental pH has also been shown to influence exosome secretion[38]. In addition, cellular stress such as irradiation[39,40], cisplatin treatment[41], exposure to hypoxia[42] and ER stress[43] can all increase exosome release. Increased release of waste via exosomes might be a natural response to stress, but also could be an approach for cells to communicate with each other under pathological conditions. Particularly, many neurodegenerative disorders are associated with lysosomal or autophagy dysfunctions and aggregations of pathological proteins; exosomes could play a critical role in such neuropathogenesis[23].

As a critical mediator for intercellular communication, exosomes are taken up by recipient cells via three major methods: receptor-ligand interaction, fusion with plasma membrane, and endocytosis by phagocytosis[17] [Figure 2]. For receptor-ligand uptake, the molecular mechanism remains elusive. Current studies revealed that Tim1- or Tim4-expressing Ba/F3 B cells could bind exosomes via phosphatidylserine, suggesting Tim4 and Tim1 are possible phosphatidylserine receptors for exosomes[44]. Another study implied that intercellular adhesion molecule 1 (ICAM-1) is critical for mature exosomes to prime naive T cells[45]. Fusion with plasma membrane was supported by studies showing exosomes can be taken up by melanoma cells through membrane fusion[17]. Interestingly, K562 or MT4 cells-derived exosomes were internalized more efficiently by phagocytes than non-phagocytic cells, implying that phagocytosis may play a unique role in exosome-cell interactions and uptake[46].

Strikingly, numerous studies have indicated that exosomes are critical for communication between different neural cell types. Microglia could specifically internalize oligodendrocyte-derived exosomes by macropinocytosis, and most of these microglia were MHC class II negative and did not activate immunological responses[47]. Neurons have been shown to be able to internalize oligodendrocyte-derived exosomes by endocytosis[48]. In addition, crosstalk between neuron and glia also occurs through exosomes. Exosomes from stressed astrocytes that were exposed to oxygen and glucose deprivation could produce neuroprotective effects against oxidative stress in neurons and this effect was dependent on Prion protein[49]. It has been demonstrated that exosomes are internalized via several mechanisms and the uptake depends on the type of recipient cells. For example, exosomes derived from neuroblastoma bound to neurons and glial cells, but were preferentially endocytosed by glial cells; exosomes derived from cortical neurons were exclusively bound and endocytosed by neurons[50]. Indeed, a lot more studies are needed to understand the specificity and molecular mechanism of exosome uptake among different neuronal and glial cell types.

Exosome-mediated intercellular communication in the nervous system

In 1980, exosomes were still believed to be a means of disposing cell debris[51]. However, emerging studies have indicated that exosomes also play multiple roles in biological activities such as cell-to-cell communication, which was traditionally considered to be mediated by gap junction, receptor/ligand, or electrical and chemical signals[52,53]. Studies showed that exosome release was increased from cortical neurons by treatment with GABA receptor antagonist, bicucullin; however, this increase was blocked by either AMPA receptor antagonist, CNQX, or NMDA receptor antagonist, MK-801, suggesting exosome release was regulated by glutamatergic synaptic activity[54].

Oligodendrocytes secrete exosomes into extracellular space that can inhibit morphological differentiation in oligodendrocytes and myelin formation, and this effect could be blocked with inhibitors of actomyosin contractility. Interestingly, conditioned neuronal medium dramatically reduced secretion of exosomes from oligodendrocytes, suggesting interaction between neurons and oligodendrocytes during myelin

biogenesis[55]. Other studies have shown that microglia could internalize exosomes released from oligodendroglia by macropinocytosis, which was then transferred to late endosomes and lysosomes[47]. Conversely, studies have revealed that neurotransmitters could stimulate the release of exosomes from oligodendroglial, which subsequently could be internalized and utilized by neurons[48]. Mice with absence of proteolipid protein and 2',3'-cyclic nucleotide 3'-phosphodiesterase, which are enriched in oligodendroglial exosomes, exhibited axonal degeneration[56]. In addition, it was shown that Hsp/Hsc70 exiting from oligodendroglia could be taken up by squid giant axon[57], and this process is likely mediated by exosomes[58].

Neurons also regulate intercellular communication and maintain homeostasis such as neurogenesis and synaptic activity via exosomes. Studies using electron microscopy showed that exosomes were secreted from somato-dendritic compartments of mature cortical neurons, confirming neurons secrete exosomes[54]. Exosomes released from primary cortical neurons contained several functional proteins that could regulate synaptic activity, and the release of exosomes was controlled by depolarization[59]. Cystatin C was detected in exosomes released from mouse primary neurons and played a critical role in neuroprotection[60]. In addition, studies in *Drosophila* neuromuscular junction demonstrated that release of exosomal synaptotagmin 4 from presynaptic terminals was crucial for synaptic growth[61]. Co-incubation of mouse microglial cell line with PC12 cells enhanced the elimination of degenerating neurites in PC12, and treatment with PC12-derived exosomes significantly increased the pruning activity of microglia[62]. In addition, exosomes secreted from primary cortical neurons were internalized into astrocytes and upregulated GLT1 proteins[63].

Microglia can also have crosstalk with neurons and modulate neuronal activity through exosomes. Synapsin I has been observed in the exosomes released from glial cells and found to promote neurite outgrowth in hippocampal neurons and survival of cortical neurons[64]. Also a group of miRNA, including miR-146a-5p, has been detected in the extracellular vesicles released from microglia, which regulates the expression of important synaptic proteins[65].

All the evidence suggests that exosomes contribute to intercellular communication via internalization by target cells, activating downstream signaling cascades, or releasing components into the extracellular space. However, the precise understanding of the molecular mechanism underlying this process continues to evolve. Since most experiments were performed *in vitro*, further studies in animal models will open up new perspectives for understanding the function of exosomes in communication in the central nervous system[48].

Role of exosomes in neurodevelopment

Recent studies have shown that exosomes play an integral role in normal neurodevelopment such as neural plasticity, and contribute to the pathological changes in neurodevelopmental diseases[66]. For instance, embryonic cerebrospinal fluid-derived exosomes improved neural stem cell amplification through targeting the rapamycin complex 1 pathway[67]. Exosomes also seem to act as a regulator in the niche of mesenchymal stem cell and a modifier of proliferation and differentiation of neural stem cells[68]. Exosomes originated from neural progenitor cells have been shown to promote neuronal differentiation and facilitate neurogenesis through miR-21a[69]. Exosomes from human induced pluripotent stem cell (hiPSC)-derived neurons increased proliferation in human primary neural cultures *in vitro*. In parallel with *in vitro* studies, injection of exosomes purified from DIV9 rodent primary neural cultures into the lateral ventricles of P4 mouse brains increased neurogenesis in the dentate gyrus of hippocampus[70]. On the other hand, studies have shown that exosomes are not only involved in neurogenesis, but also regulate synaptogenesis and neural circuit development. For example, treatment with normal control exosomes could reduce damages

in neuronal proliferation, differentiation, synaptogenesis, and synchronized firing in methyl-CpG-binding protein 2 (MECP2)-knockdown human primary neural cultures, which is a key gene contributing to abnormal neurodevelopment in Rett syndrome. Further proteomic analysis revealed that normal (control) exosomes may contain critical factors that are crucial for neuronal maturation and synaptogenesis which are absent in MECP2LF exosomes, suggesting the involvement of exosomes in neuronal development. Interestingly, exosomes have been reported to produce therapeutic effects in neurodevelopment disorders *in vivo*. Intranasal treatment with exosomes derived from mesenchymal stem cells enhanced behavioral autistic-like phenotype such as social vocalization and reduced repetitive behaviors in Shank3B Knockout autism mouse model[71]. Recently, extracellular vesicles have been used to encapsulate CRISPR/Cas9 genome editing machinery for delivery to cells. This could be a potentially new approach for delivering Cas9/sgRNA for treating a variety of genetic diseases, including those impacting the nervous system[72,73].

Function of exosomes in neurodegenerative disorders

As a critical mediator for cell communication, exosomes have been reported to augment the progression of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Prion disease, Amyotrophic lateral sclerosis and Huntington's disease, via delivery of proteins or molecules associated with the pathology of such diseases [Table 1].

Role of exosomes in Alzheimer's disease

Alzheimer's disease (AD) is one of the most devastating neurodegenerative disorders that cause dementia and decreased cognitive function. It currently affects more than 5 million people in the United States and is expected to rise to about 13.8 million by 2050[74,75]. Accumulation of amyloid β-peptide (Aβ) plaque extracellularly and formation of neurofibrillary tangles from hyperphosphorylated tau intracellularly are pathological hallmarks of AD that generally precede the clinical symptoms[76]. Recent studies have revealed that exosomes play very complex roles in AD[76-79]. Both Aβ peptide and tau are released from exosomes and have been implicated in the propagation of aggregates of these proteins. A recent proteomic and bioinformatics study of exosomal proteins in human iPSC neurons expressing mutant Tau (mTau) revealed many differences with normal exosomes such as the presence of a PP2A phosphatase inhibitor. Their data suggest that mTau exosomes may be able to regulate propagation of phosphorylated tau *in vivo* and contribute to the neuropathology[80].

It has been reported that neuron-derived exosomes have the ability to confer conformational changes to extracellular Aβ, converting these molecules into non-toxic fibrils which promote uptake by microglia[81]. Secretion of these neuronal exosomes appears to be regulated by neutral sphingomyelinase 2 and sphingomyelin synthase 2 (SMS2). SMS2 siRNA enhanced exosome secretion and Aβ uptake into microglia and decreased extracellular Aβ[81]. Studies *in vivo* have shown that neuroblastoma-derived exosomes injected into mouse brain trapped Aβ and facilitated the internalization of Aβ into microglia. Continuous injection of these exosomes into amyloid-β precursor protein transgenic mice significantly reduced Aβ and Aβ-mediated synaptotoxicity in the hippocampus. Further studies revealed that glycosphingolipids that are highly distributed on these exosome membranes are critical for the Aβ binding[82]. Another line of study showed that N2a cell-derived exosomes could rescue Aβ-mediated disruption of synaptic plasticity via trapping Aβ with cellular prion protein[83,84]. Glycosphingolipids on these exosome are important for binding and sequestering Aβ[85]. All these studies suggest that exosomes may play an important role in the nervous system. Additionally, studies have suggested that exosomes contain a variety of components that produce neuroprotective effects such as neprilysin[86] and insulin-degrading enzyme that are important for Aβ degradation[87].

Table 1. Exosome cargo as biomarkers in neurodegenerative disorders

Name of disease	Exosome cargo	Pathology	Application	Ref.
Alzheimer's disease	Aβ	Neuronal impairment	Early diagnosis	Saman *et al.*[99] ,2012
Parkinson's disease	α-syn	Neuronal damage	Early diagnosis Monitoring severity of cognitive impairment	Shi *et al.*[116] ,2014 Stuendl *et al.*[113] ,2016 Si *et al.*[117] 2019 Jiang *et al.*[118] ,2020 Niu *et al.*[119] ,2020
Amyotrophic lateral sclerosis	TDP-43	Neuronal inflammation and damage	Early diagnosis	Chen *et al.*[137] ,2020

Other studies have provided controversial results which suggest that exosomes might play complicated roles in the development of AD. For example, in APPxPS1 transgenic AD mouse model, intracellular Aβ was found to be colocalized with raft marker flotillin-1, suggesting that Aβ accumulated within multivesicular bodies[88]. A minute fraction of Aβ was subsequently released into extracellular space in association with exosomes[89]. Similarly, other studies have shown that amyloid precursor protein (APP), APP-C-terminal fragments, and amyloid intracellular domain were all secreted from exosomes in differentiated neuroblastoma and primary neuronal culture cells[90]. In HEK-293-derived exosomes, Holo-APP, Presenilin and APP C-terminal fragments were all detected, and secretion of total APP C-terminal fragments was higher in exosomes derived from retromer deficient cells[91]. In addition, intraperitoneal injection of GW4869, a neutral sphingomyelinase 2 inhibitor, significantly decreased brain ceramide, exosome secretion from brain and serum exosome levels, as well as Aβ1-42 plaques in *5XFAD* mice[92]. In contrast, feeding female mice with ceramide showed a higher load of plaque burden and exosome secretion[93],suggesting an association with exosome levels and Aβ accumulation in plaques. Furthermore, APP, BACE1, PSEN1, PSEN2 and Adam10, and many proteases that have the capacity to splice APP, have also been reported to be released from exosomes[94]. Thus, exosomes represent a novel pathway for APP processing and secretion, and amyloid deposition in AD brain. Interestingly, current research revealed that while during early stage of AD, activation of microglia produced protective effect by increasing phagocytosis and Aβ clearance[95-97]; during late stage of AD, microglia increased the release of exosomes or EV that contained soluble toxic Aβ and facilitated the progression of AD[97,98].

Due to the unique characteristics of exosomes in that they recapitulate the features of the originating cells and are able to cross the blood brain barrier, their contents can serve as potential biomarkers for diagnosis and monitoring treatment and progression of AD. Tau[99,100], and phosphorylated Tau have been detected in exosomes isolated from AD patients[101], and can potentially serve as biomarkers for early diagnosis of AD, although further investigation is required to establish this connection. Furthermore, recent research has shown that serum-derived neuronal exosomes might be a potential biomarker for diagnosis and clinical monitoring of AD[102,103]. The use of exosomes as a delivery system for therapeutic drugs has also been extensively studied. Intranasal administration of exosome encapsulated drug led to rapid distribution of drugs into the brain[104], indicating the possibility that exosomes can cross the blood brain barrier bi-directionally. Indeed, a large number of studies have shown that injection of exosomes as a drug delivery system could reduce Aβ and other relevant pathological changes[97].

Role of exosome in Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative disorders affecting millions of people worldwide. The pathological hallmark of PD is the presence of Lewy bodies which contain misfolded α-synuclein (α-syn) that tends to aggregate, resulting in progressive loss of dopaminergic neurons in

substantia nigra and striatum[105].

Studies showed that Lewy bodies are initially found in the peripheral tissues, and then gradually spread to the brain stem, and eventually to cerebral cortex, suggesting PD progressed into the central nervous system from peripheral tissues, similar to prion-like disease[106]. Interestingly, studies revealed that exosomes play a critical role in the propagating and progression of PD[107]. First, exosomes have been found to be a carrier that can deliver pathological proteins: both newly synthesized and aggregated forms of α-syn could be released through unconventional, endoplasmic reticulum/Golgi-independent exocytosis. Intravesicular α-syn has a greater tendency to aggregate than the cytosolic protein. This secretion was enhanced by proteasomal and mitochondrial dysfunction associated with PD[108]. Further studies revealed that synaptic vesicles that contain α-syn could be sorted into early endosomes through Golgi or clathrin-mediated endocytosis[109], and then transported into MVBs and fused with membrane to secrete the exosomes[110]. Alternatively, α-syn could also be sorted into the recycling endosome system and exocytosed as secretory granules[111]. Exosomes derived from α-synuclein producing cells, are released in a calcium-dependent manner. Studies have also shown that exosomes contribute to the formation of aggregation of α-syn: monitoring the aggregation kinetics with thioflavin T fluorescence revealed that exosomes facilitated the process by providing a catalytic environment for nucleation[112]. Quantification of cerebrospinal fluid (CSF) exosome numbers and α-syn content from PD patients revealed a correlation with the severity of cognitive impairment. Interestingly, incubation of exosomes derived from CSF of patients with PD and Lewy body dementia induced oligomerization of soluble α-synuclein in recipient cells in a dose-dependent manner. One hypothesis is that a pathogenic species of α-syn in these exosomes could induce oligomerization of soluble α-syn in the recipient cells to confer disease pathology[113]. It has also been suggested that exosome-mediated release of toxic forms of oligomeric α-syn, which is more easily taken up by recipient cells than free α-syn may be a mechanism for clearing toxic α-syn oligomers when autophagy is insufficient[114].

Moreover, recent studies revealed that exosomes originated from the central nervous system could cross the blood brain barrier and carry the pathologic proteins into the blood[115]. Therefore, the cargo of serum/plasma-derived exosomes from patients with PD has been under extensive study as containing promising biomarkers in PD pathogenesis and clinical progression[116-119].

It is noteworthy that exosomes are currently exploited as a drug delivery vehicle for treating PD. Several studies have demonstrated significant neuroprotective effects using exosome-based delivery system in *in vitro* and *in vivo* models of PD[120-122]. For example, intranasal administration of catalase-loaded exosomes effectively protected dopamine neurons in the substantia nigra pars compacta against oxidative stress in PD mouse brain[120]; and intravenous treatment with dopamine-loaded serum-derived exosomes also produced significant effects in PD mouse models[122]. However, the use of exosome delivery of therapeutics to treat PD remains challenging.

Role of exosomes in Prion disease

Prion disease is a fatal neurodegenerative disease in humans and animals, caused by infectious abnormal microscopic protein particles known as prions. Prion disease is primarily characterized by assemblies of misfolded beta-sheet prion proteins in the brain and a rapid decline in cognition and cerebral and cerebellar functions[123]. Although the mechanism of prion transmission still remains unclear, studies have shown that misfolded prion proteins are associated with exosomes, and these exosomes could spread the disease[124]. Furthermore, studies showed that infection of N2a neuroblastoma cells with prions associated with scrapie could induce the release of prion proteins into the medium, predominantly via exosomes[125]. Knockdown of HRS/Vps27, a subunit of ESCRT-0 or TSG101-ESCRT-I subunit in Mov 127S cells significantly reduced

accumulation or release of infective prion, suggesting that ESCRT-dependent and independent transmission mechanisms are both involved in the regulation of exosome-mediated release of prion proteins[126]. Stimulation of exosome release with monensin increased prion infectivity; by contrast, inhibition of exosome release with GW4869 decreased prion spreading[127]. Studies have also revealed that exosomes derived from neurons infected by prion could infect normal neurons and initiate prion propagation. In addition, these exosomes could induce prion disease when inoculated into mice. Interestingly, these prion proteins were found to have undergone N-terminal modification and selection of specific glycoforms for incorporation into exosomes[128]. In line with these findings, it was also reported that exosomes derived from infected mice could spread prion disease[129]. Recent studies also revealed that some specific miRNAs such as miR-146a, miR-29b found within exosomes from prion-infected cells may play important roles at various stages of spreading of prion disease[130]. All the data support that exosome potentially contributes to the rapid colonization in the development of prion disease.

Role of exosomes in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease in humans, which is characterized by progressive muscle atrophy due to the loss of motor neurons. Approximately 10% of ALS patients are familial, and the rest of 90% are sporadic. Both environment and genetic factors such as mutation of superoxide dismutase-1 (SOD1) and nuclear TAR DNA-binding protein 43 (TDP-43) have been shown to be involved in the pathogenesis of ALS[131]. A common pathologic feature of ALS is the aggregation of misfolded cytoplasmic proteins, for instance, TDP-43, ubiquilin 2 and SOD1[132]. TDP-43 is an RNA/DNA binding protein that regulates RNA transcription and DNA repair. Hyperphosphorylated and ubiquitinated TDP-43 has been reported to contribute to the development of ALS[133]. Strikingly, recent studies have revealed that aggregated TDP-43 or SOD1 proteins could be transported by exosomes to recipient cells to induce neurotoxicity[134,135]. In clinical studies, TDP-43 levels have been reported to be much higher in exosomes derived from frozen post-mortem temporal cortices of patients with sporadic ALS, compared with controls[136]. A clinical 3- and 6-month follow up study also showed exosomal TDP-43 levels were much higher in ALS patients compared with the control group[137]. *In vitro*, TDP-43 is secreted via exosomes in Neuro2a cells, and inhibition of exosome secretion exacerbates the aggregation of TDP-43. In addition, inhibition of exosome secretion also worsens the phenotype of TDP-43^{A315T} transgenic mice[136]. Other studies showed that exosome-induced cytokine secretion is compromised in CD14++ monocytes from ALS patients, and this abnormality is modulated by exosomal TDP-43, suggesting that exosomal TDP-43 contributes to the impaired neuroinflammatory reaction in ALS pathogenesis[134].

With distinct advantages, exosomes can also be used as therapeutic delivery carriers. Exosomes isolated from adipose-derived stem cells have been shown to restore mitochondrial complex I activity, efficiency of electron transfer system and membrane potential in an *in vitro* model of ALS, NSC-34 cell line overexpressing human mutated SOD1, suggesting a potential therapy for ALS using such exosomes[138].

Role of exosomes in Huntington's disease

Huntington's disease (HD) is a progressive autosomal dominant neurodegenerative disease that is characterized by cognitive impairment and involuntary choreiform movements. Pathologically, it is caused by CAG expansion in exon 1 in Huntingtin gene that leads to production of mutant huntingtin (mHtt). Emerging research has revealed that the mutated products, polyglutamine protein could lead to severe neuronal toxicity, and CAG repeat length is positively associated with clinical symptoms[139]. To date, studies have implied that exosomes are involved in the pathogenesis and propagation of Huntington disease[140]. When SH-SY5Y cells were cultured with conditioned medium from HEK cells that overexpress GFP, GFP-mHtt-Q19 or GFP-mHtt-Q103, the exogenous mHtt proteins were detectable within SH-SY5Y cells after 5 days of exposure. In addition, after co-culturing mouse neural stem cells with exosomes derived from

fibroblast from HD patient carrying the 143 CAG repeat (HD143F) for 4 days, mHtt aggregates were detected within the neurons, suggesting mHtt could propagate from cell to cell through internalizing exosomes that contain pathological proteins. Furthermore, intraventricular injection of exosomes isolated from HD143F, resulted in the Huntington-like phenotype in mice, and mHtt was detected in the striatum[141]. In another study, both *in vivo* and *in vitro* data suggest that extracellular vesicles can transfer toxic trinucleotide repeat RNAs between cells and trigger the manifestation of HD-related behaviors and pathology in mice; however, activity of exosomes or cell-type specificity was not fully evaluated[142]. These findings support the hypothesis that exosomes contribute to the HD progression by transferring toxic proteins or RNAs from one cell to another. Data have revealed that certain types of circulating microRNAs were up or down regulated in patients with HD, but exosome-derived microRNAs as biomarkers are still under investigation[143,144]. Moreover, recent studies showed that injection of exosome-delivered miR-124 into R6/2 transgenic HD mice reduced the RE-1 Silencing Transcription Factor, which is involved in the development of HD[145]. In addition, infusion of hydrophobically modified Htt-hsiRNA-loaded exosomes into mouse striatum resulted in significant bilateral silencing of about 35% of Huntingtin mRNA[146]. Thus, the potential use of exosomes as a route for delivering various siRNAs to the brain to suppress expression of mHtt or other relevant regulatory proteins offers another approach to treating HD.

Role of exosomes in epilepsy

Epilepsy is a neurological disorder that is characterized by abnormal electrical discharge of brain neurons[147]. Status epilepticus can lead to neuron damage and gliosis[148]. Emerging studies have suggested that microvesicles such as exosomes could be released following brain injury or stimulation and serve as a biomarker for epilepsy. For example, status epilepticus induced by intra-amygdala kainic acid led to up-regulation of both ESCRT-dependent and -independent signaling pathways and thus increased exosome release in mice. This effect lasted for a long time and the enhanced secretion of exosomes was still detectable 2 weeks after status epilepticus[149]. In addition, studies from both animals and human have implied that certain types of exosomal miRNA are highly associated with epilepsy. In a rat model of chronic temporal lobe epilepsy, miR-346 and miR-331-3p were found to be decreased in extracellular vesicles of the forebrain[150]. Besides, a clinical study involving 40 patients with mesial temporal lobe epilepsy with hippocampal sclerosis (mTLE-HS) showed that two exosomal miRNAs were upregulated, while 48 miRNAs were downregulated. Among these candidates, exosomal miRNA-8071 was reported to have the sensitivity of 83.33% and the specificity of 96.67% for diagnosis of mTLE-HS[151]. In another study, exosomal circulating miRNAs, such as miR194-2-5p, miR15a-5p, miR-132-3p, and miR-145-5p, have been reported to be potential biomarkers in patients with focal cortical dysplasia and refractory epilepsy[152]. Interestingly, intranasal administration of A1-exosomes derived from human bone marrow-derived MSCs rescued neuron loss, inflammation and neurogenesis, as well as alleviated compromised memory and cognitive capacity in mice which typically occur after status epilepticus[153]. These studies demonstrate that epilepsy could alter exosome release and its miRNA content, which could be a potential biomarker for clinical diagnosis. Further studies in exosomes will be needed to identify the distinct types of epilepsy subtype to determine the specific miRNA pathophysiological significance for epileptogenesis.

Role of exosomes in multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system[154]. Currently, the etiology of MS remains elusive, and the diagnosis mainly relies on clinical symptoms. Thus, earlier diagnosis and effective clinical intervention are very important for improving patient outcomes. Recent studies have found that exosomal contents such as myelin oligodendrocyte glycoprotein[155], sphingomyelinase (SMase)[156], and a variety of microRNA[157-160] are potential diagnostic biomarkers for MS. In addition, Schwann cell-derived exosomes contain a variety of neuroprotective proteins and anti-inflammatory factors[161] that play critical roles in MS via regulating myelin membrane biogenesis and

providing trophic factors required for myelin maintenance[162]. For instance, exosomes which contain myelin and protective proteins against stress, were released from oligodendrocytes into the extracellular space in a calcium dependent manner[163]; Schwann cell-derived exosomes can improve axonal regeneration after axotomy[164], and increase nerve activity[165]. Exosomes from adipose-derived mesenchymal stem cells, bone marrow-derived mesenchymal stem cells, and umbilical cord stem cells have shown potential therapeutic effects in protecting oligodendrocyte and promoting neurite outgrowth and nerve regeneration[166-168].

Function of exosomes in stroke

Stroke has been increasing during past few decades and has become one of the major life-threatening medical conditions around the world. Thus, early diagnosis and effective monitoring of recovery phases are critical for the management of stroke patients. Compared with most biomarkers obtained from blood and body fluids, exosomes have an advantage due to their high heterogeneity[7] which reflects the pathophysiological conditions of the cells from which they originate, and thus their cargo are potential biomarkers for diagnosis and clinical evaluation. Studies have shown that exosomes can cross the blood brain barrier and enter peripheral blood and cerebral spinal fluid after stroke[169]. In addition, exosomes orchestrate a complicated process after stroke involving nerve regeneration, angiogenesis, neurogenesis, remodeling of immune response, neuronal plasticity and axon dendrite outgrowth[170]. Studies have shown that endothelial cell-derived exosomes can promote the differentiation of neural progenitor cells into oligodendrocytes for myelination; neuron and neuronal progenitor cell-derived exosomes can regulate peripheral immune response; pericyte-derived exosomes can facilitate neurogenesis; circulating endothelial progenitor cell-derived exosomes can facilitate angiogenesis by interacting with cerebral endothelial cells[7]. Further studies revealed that mesenchymal stromal cell-derived exosome enhanced neurite branch and length in rat cultured neurons after middle artery occlusion[171]. Exosomal miR-126 and miR-124 were also reported to be involved in the angiogenesis[9,172] in rats and neurogenesis in mice after stroke[173]. However, exosomes also produce some adverse effects in peripheral organs after stroke, such as increasing pro-inflammatory cytokines and activating T and B lymphocytes, thus effecting heart[174], kidney[175], and digestive intestine system[7].

Extensive studies have shown that stroke could induce a variety of changes in the contents of exosomes released from central nervous system. For example, next generation sequencing analysis showed that human neural stem cell-derived miroRNAs were altered by hypoxic condition[176]. Data from both human and animal models suggested that certain types of exosomal cargoes were altered: In animal models, plasma-derived exosomal rno-miR-122-5p was significantly downregulated and rno-miR-300-3p upregulated in ischemic rats[177]. In clinical studies, proteome analysis of microvesicles from plasma of patients with lacunar infarction revealed that brain-related proteins such as myelin basic protein, focal adhesion and coagulation related proteins were upregulated, and albumin was downregulated in patients with adverse outcomes compared with matched controls[178]. Analysis of plasma EV from patients with manifest vascular disease showed elevated protein cystatin C and CD14 levels correlated with white matter lesions and progression of brain atrophy[179]. In patients with acute ischemic stroke, the serum exosome levels of miR-9 and miR-124 were both elevated compared with healthy controls, and positively correlated with National institute of Health Stroke Scale scores (NIHSS), infarct volumes and IL-6 levels[180]. Plasma-derived exosome miR-422a and miR-125b-2-3p were both decreased during the subacute phase of ischemic stroke, with miR-422a increased in the acute phase in comparison with controls[181]. In addition, exosomal miRNA such as miR-223, miR-21-5p and miRNA-30a-5p were also reported to be highly related with occurrence and severity of stroke in several clinical studies[182,183]. These results suggest that designing a multiplex platform to assay for multiple biomarker molecules in exosomes known to be associated with stroke might be a promising approach for diagnosis and clinical progress evaluation of stroke patients, especially with the advancement

of exosome isolation and purification techniques.

Role of exosomes in traumatic brain injury

Traumatic brain injury (TBI) often leads to injury-induced death and disability around the world[184]. After TBI, brain parenchymal damage and hemorrhage and compromised blood-brain barrier, as well as associated inflammation, oxidative stress and cell death contribute to the TBI-induced pathological alterations and dysfunction. As a critical player in cell communication, exosomes have been proposed to be able to carry specific biomarkers during traumatic brain injury and can serve in early diagnosis of concussion and monitoring of clinical progress[185]. Recent studies implied that certain components such as miR-124-3p in microglial exosomes were upregulated significantly after TBI and exerted anti-inflammatory function and promoted neurite outgrowth[186]. In another study in veterans with mild traumatic brain injury, elevated exosome-derived neurofilament light chain was observed, even years after injury[187]. Studies of serum-derived neuronal exosomes from patients with acute TBI and chronic TBI showed that proteins associated with neuronal functions were significantly increased in acute TBI, while neuropathological proteins were up-regulated in both acute and chronic TBI. These results suggest that cargo in serum-derived neuronal exosomes could act as potential biomarkers for clinical diagnosis[188]. Additionally, the capacity of exosomes to cross the blood-brain barrier offer a potentially effective therapeutic approach in treatment of patients with TBI[189].

Roles of exosomes in neuropsychiatric disorders

Neuropsychiatric disorders such as major depression and schizophrenia are associated with certain changes of brain structures and neurotransmitters. Although the molecular mechanism is not fully understood, emerging studies suggest that miRNAs enriched in exosomes may be key factors in the development of neuropsychiatric disorders[190-194]. Acting as a complicated mediator of cell communications, alterations of exosomal components have been identified in patients with neuropsychiatric disorders[10]. One analysis of exosomal miRNAs from frozen postmortem prefrontal cortices of patients revealed that miR-497 was significantly elevated in schizophrenia, and miR-29c increased in bipolar disorders in comparison with control[195]. Genome-wide analysis of miRNAs from serum exosomes, with subsequent bioinformatic predictions and validations, has also indicated miRNA dysregulation in schizophrenic patients[196]. Of all the miRNAs, hsa-miR-206 was the most upregulated in these patients. Hsa-miR-206 has been reported to interact with *BDNF* mRNA directly, leading to the decreased expression of this gene and compromised cognitive function in mice[197]. In another study, in patients with depression, 12 miRNAs that regulate the neurotrophin pathway were found to be increased, and 20 miRNAs that control apoptosis, cell growth, immune and hypoxia activity were downregulated[198]. A recent study has revealed that exosomal miR-139-5p is significantly increased in patients with major depressive disorder in comparison with controls, suggesting it might be a potential biomarker for this disorder[199].

To date, studies in the role of exosomes in neuropsychiatric disorders are very limited. These findings have opened up challenging possibilities of uncovering the function of exosomes and molecules associated with them in mental disorders.

Exosomes in brain tumors

Glioblastoma multiforme (GBM) is the most aggressive and common primary tumor of the adult brain, with median survival of less than 15 months from diagnosis[200]. Regardless of patients receiving rigorous standard of care, such as surgical resection alongside chemotherapy and/or radiotherapy, this rare astrocytoma has very poor prognosis[201,202]. Among the heterogenous cell populations that form the GBM tumor mass are the cancer stem cells, which contribute to therapy resistance, tumor growth and recurrence[203-206]. Recent reports have[207] suggested that EVs including exosomes mediate critical bilateral

communication between the tumor cells and their microenvironment to sustain the growth of malignant GBM. A longitudinal time-lapse imaging study showed that glioma cells have crosstalk with non-glioma cells such as glial cells, neurons and vascular cells through EVs, to alter the tumor microenvironment and promote glioma growth *in vivo*[208]. GBM derived EVs are known to facilitate angiogenesis, tumor progression and invasion, drug resistance and immune regulation[209-212] [Figure 3]. Moreover, various GBM exosomal cargoes are involved in mediating these processes [Table 2].

Hypoxia within the GBM microenvironment promotes neo-angiogenesis, as a means to supply oxygen and nutrients to the rapidly growing tumor cells. VEGF-A carrying EVs secreted by glioma stem cells (GSC), stimulates endothelial cells to proliferate, migrate and form tubular structures, promoting vasculature[213]. In addition, GSC exosomes can transfer miRNAs such as miR-21 and miR-26a to endothelial cells, to upregulate VEGF expression and support GBM angiogenesis[214,215]. Studies using clinical samples have shown that microvesicles derived from CSF of GBM patients upregulate proliferation of cultured endothelial cells through AKT/beta-catenin pathway[216]. Oncogenic EGFRvIII and tissue transglutaminase are reportedly other protein factors transferred through EVs, which are known to induce mitogenic and/or angiogenic signaling in recipient cells[217,218]. Interestingly, exosome-mediated delivery of long non-coding RNAs such as lncCCAT-2 and lncPOU3F3 can also enhance vascularization of GBM[219,220]. On the contrary, miR-1 enriched glioma EVs have been implicated in suppressing angiogenesis and tumor growth[221]. Increased growth and aggressiveness of advanced stages of GBM is associated with the phenotypic transition from proneural to mesenchymal subtype. GSC EVs contribute towards this process, by way of transferring mRNAs, miRNAs and other regulatory RNAs, and transcription factors, which can possibly reprogram the recipient cells, alter their epigenetic signatures and render the GBM microenvironment more permissive to malignant transformation[211,222,223]. EV-mediated crosstalk in GBM involves the interaction between a chemokine receptor CCR8 on recipient cells and the glycans on the EV surface, with the CCL1 ligand acting as a bridging molecule[224]. RNA-seq and DNA methylation analyses showed that pro-angiogenic miRNA such as miR-9-5p transferred via GSC EVs can reprogram human brain endothelial cells *in vitro* to induce angiogenesis, by distinct pathways compared to those activated by vascular growth factors[225]. Similar molecular profiling studies conducted earlier using GSC EVs had revealed that the molecular subtypes and functional state of GSCs determine the tumor regulatory effect of EVs[226,227].

GBM cells interact with the surrounding astrocytes to modulate tumor growth and survival. In a study using patient tumor derived cell lines, it was shown that GBM EVs can transform normal human astrocytes to a pro-tumorigenic phenotype, exhibiting increased production of growth factors, chemokines and cytokines, to support in vitro growth of GBM cells[207]. GBM EVs regulate tumor signaling pathways such as p53 and c-MYC in astrocytes to induce a senescence associated secretory phenotype, to favor tumor progression[228]. Moreover, EVs derived from GBM cells were shown to induce podosome formation, ECM degradation and increased migration of astrocytes[207,228]. Astrocytes cultured with GBM EVs show enhanced secretion of immunosuppressive cytokines such as CSF2 and interleukins 4, 10 and 13, thus providing a tumor supportive microenvironment. EVs secreted by irradiated GBM cells have enhanced presence of CD147, which in turn stimulates increased MMP9 release from recipient astrocytes, suggesting the contribution of astrocyte signaling in promoting GBM invasiveness, particularly in response to ionizing radiation[229]. A recent study reported that GSC EVs induce metabolic reprogramming of pre-transformed astrocytes to enhance proliferation, self-renewal and tumor growth in a mouse allograft model[230].

Immune suppression fosters aggressive transformation of brain tumor. The molecular cargo transferred by GBM EVs can influence the status of tumor-associated macrophages or myeloid cells (TAMs)[231]. GBM EVs induce the *in vitro* differentiation of peripheral blood derived monocytes to anti-inflammatory M2-

Table 2. Function of EVs/exosomes in regulating glioma

Types of exosomal cargo	Parental cell	Recipient cell	Function	Ref.
VEGF-A	GSC	Endothelial cell	Promote angiogenesis	Treps *et al.*[213],2017
miR-21, miR-26a, miR-9-5p	GSC	Endothelial cell	Promote angiogenesis	Sun *et al.*[214],2017 Wang *et al.*[215], 2019 Lucero *et al.*[225], 2020
lncCCAT-2, lncPOU3F3	GBM cell	Endothelial cell	Promote angiogenesis	Lang *et al.*[219],2017 Lang *et al.*[220],2017
EGFRvIII, tissue transglutaminase	GBM cell	GBM cell	Support tumor growth	Al-Nedawi *et al.*[217],2008 Antonyak *et al.*[218],2011
CD147	Irradiated GBM cells	Astrocytes	Support tumor invasion	Colangelo *et al.*[229],2020
STAT3 proteins	GSC	Monocytes	Immunosuppression	Gabrusiewicz *et al.*[235] ,2018 Grimaldi *et al.*[236] ,2019 Ricklefs *et al.*[237],2018
miR-21	GBM cells	Microglia	Immunosuppression, tumor growth	Abels *et al.*[234], 2019 Van der Vos *et al.*[231], 2016
MGMT, APNG	GBM cells	GBM cells	Chemoresistance	Shao *et al.*[210],2015
miR-9	GBM cells	GBM cells	Chemoresistance	Munoz *et al.*[239], 2013 Munoz *et al.*[238],2015
miR-21	Tumor associated macrophages	GBM cells	Chemoresistance	Chuang *et al.*[240],2019

macrophages, which exhibit enhanced phagocytosis and secretion of IL-6 and VEGF, to support immune evasion of glioma[232]. Peripheral blood analyses of GBM patients signified the role of tumor-derived exosomes in promoting an immune evasive Th2 bias, and their ability to induce CD163 (a macrophage marker) expression on normal astrocytes[233]. EVs carrying miR-21 released by GBM cells were shown to target the expression of Btg2, an anti-proliferative protein in recipient microglia, subsequently reprogramming these cells to support tumor progression[231,234]. STAT3 pathway proteins present within GSC EVs including exosomes, also mediate immune suppressive changes of monocytes including their phenotype change to M2-macrophages, cytoskeletal reorganization, and upregulation of PD-L1 ligand, which binds to PD1 to inhibit T-Cell activation[235-237].

Studies addressing the mechanism of resistance to Temozolomide (TMZ), an alkylating agent used as the standard of care for glioma, have uncovered the possible influence of EVs in the process. Using a microfluidic chip-based analysis, it was found that sera-derived EVs from GBM patients (small cohort study) are enriched in mRNA levels of MGMT (O6-methylguanine DNA methyltransferase) and APNG (alkylpurine-DNA-N-glycosylase), primary DNA repair enzymes involved in inducing chemoresistance[210]. miR-9 is upregulated in exosomes released from TMZ resistant glioma cell lines and is implicated in increasing MDR1 (multidrug resistance mutation 1) expression and repressing Patched (PTCH1), Sonic Hedgehog receptor to confer chemoresistance[238]. Transfer of anti-miR-9 through MSC exosomes to GBM cells was shown to impart chemosensitivity and reverse multidrug transporter expression[239]. Analysis of clinical samples has revealed that Pacritinib, a STAT3 inhibitor can potentially overcome TMZ resistance by reducing miR-21 enriched exosomes secreted by GBM-associated macrophages[240].

EV cargo can be potential biomarkers for GBM diagnosis and progression. EGFRvIII is found in high levels within GBM EVs, and hence can be used as a potential biomarker[211]. A study conducted using clinical samples from GBM patients undergoing tumor resection, reported that CSF derived EVs can be developed as a diagnostic tool to assess EGFRvIII positive GBM status accurately[241]. CSF EVs of GBM patients have also been found to be enriched in miR-21 compared to non-oncologic patients, suggesting the potential use

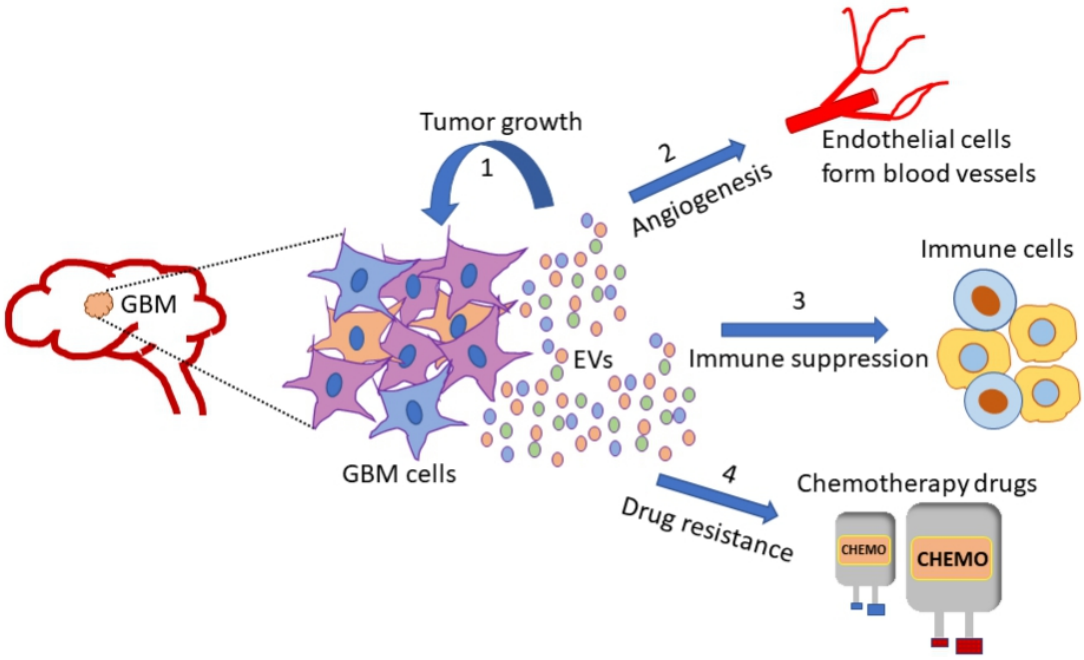


Figure 3. Glioblastoma multiforme (GBM) is one of the most aggressive tumors of the adult brain. Cells that make up the GBM tumor, release extracellular vesicles (EVs), which mediate the transfer of vital cues between tumor cells and the surrounding microenvironment. GBM tumor mass is highly heterogenous comprising differentiated tumor cells and glioma stem cells (GSCs). GSC derived EVs are particularly important players in sustaining glioma growth and invasion. Depending on their cell of origin, GBM EVs deliver unique cargo such as proteins, nucleic acids and lipids to recipient cells, to possibly alter their gene expression profile and phenotypes, and in the process favor malignant transformation. Some critical functions attributed to GBM EVs include (1) supporting tumor growth and survival; (2) promoting angiogenesis by regulating gene expression in endothelial cells; (3) mediating immune evasive phenotype changes in tumor associated immune cells: T cells, macrophages and microglia; (4) inducing resistance to chemotherapy drugs/ radiation therapy. GBM: Glioblastoma multiforme; EVs: extracellular vesicles.

of CSF derived EV miR-21 as another biomarker for GBM prognosis[242,243]. In addition, RNA/proteins with growth promoting functions such as TrkB (neurotrophin tyrosine kinase receptor-1), Timp1 (NF-κB target gene) and CLIC1 (circulatory protein), and PTEN, a tumor suppressor protein, enriched within EVs are some other potential prognostic biomarkers for GBM[209].

CONCLUSION AND PERSPECTIVES

Emerging research in the last decade suggests that exosomes and EVs are critical players in regulating physiological and pathological processes in the brain. Exosomes and EVs mediate intercellular communication by trafficking of biomolecules such as proteins, lipid, mRNA, and miRNA.

Circulating exosomes have vast potential in being developed as a source of biomarkers for various neurodegenerative disorders and brain tumors, and as vehicles for drug delivery. Neurotoxic proteins associated with AD, PD and prion disease, such as Aβ, Tau, α-synuclein and PrP respectively are encapsulated and transferred through exosomes. Oncogenic proteins (EGFRVIII, TrkB, Timp1) and miRNAs (miR-21, miR-9) carried as exosomal cargo can reprogram recipient cells in the tumor microenvironment to favor glioma progression. Besides targeting these exosomal proteins for therapeutics, the possibility of isolating exosomes readily from the circulating biofluids represents a novel and effective tool for non-invasive diagnosis and monitoring the status of various neurological conditions and glioma

progression.

Knowledge of the fundamental aspects of exosome biology (exosome biogenesis, origin, cargo sorting and targeting to specific recipient cells) and the downstream signal transduction, is key to the application of exosomes for treatment of brain disorders. Although data have indicated that various exosomal proteins or miRNAs are altered during the development of neurodegenerative or other CNS diseases, precise signaling cascade or involvement is not well understood. This might be due to the limitation of isolation and characterization techniques of exosomes that fail to specifically capture and identify exosomes from specific cell type of origin, such as neurons or microglia from limited sample volume. Therefore, more specific exosome associated biomarkers and better isolation and purification techniques for capturing specific sub-populations of exosomes will greatly advance the ability to identify biomarkers.

Research advances in areas of exosome isolation, characterization, tissue targeting and understanding of their specific biological functions would allow exosomes to impact clinical therapy of neurological diseases. Indeed, the future prospect of developing the use of exosomes for delivery of functional cargo such as miRNA, siRNA and mRNA/proteins into the brain and other regions of the nervous system, such as in axonal regeneration, opens up exciting new avenues for drug delivery applications.

DECLARATIONS

Authors' contributions

Wrote and edited the manuscript: Xiao L, Hareendran S, Loh YP

Availability of data and materials

Not applicable.

Financial support and sponsorship

This research was supported by the Intramural Research Program of The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) (ZIA HD000056), National Institutes of Health, USA.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol* 1967;13:269-88. DOI PubMed
2. Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. *Cell Biosci* 2019;9:19. DOI PubMed PMC
3. Cufaro MC, Pieragostino D, Lanuti P, et al. Extracellular Vesicles and Their Potential Use in Monitoring Cancer Progression and Therapy: The Contribution of Proteomics. *J Oncol* 2019;2019:1639854. DOI PubMed PMC
4. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750. DOI PubMed PMC

5. Trams EG, Lauter CJ, Norman Salem J, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochim Biophys Acta* 1981;645:63-70. DOI PubMed
6. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987;262:9412-20. PubMed
7. Liu W, Bai X, Zhang A, Huang J, Xu S, Zhang J. Role of Exosomes in Central Nervous System Diseases. *Front Mol Neurosci* 2019;12:240. DOI PubMed PMC
8. Malm T, Loppi S, Kanninen KM. Exosomes in Alzheimer's disease. *Neurochem Int* 2016;97:193-9. DOI PubMed
9. Osier N, Motamedi V, Edwards K, et al. Exosomes in Acquired Neurological Disorders: New Insights into Pathophysiology and Treatment. *Mol Neurobiol* 2018;55:9280-93. DOI PubMed
10. Saeedi S, Israel S, Nagy C, Turecki G. The emerging role of exosomes in mental disorders. *Transl Psychiatry* 2019;9:122. DOI PubMed PMC
11. Looze C, Yui D, Leung L, et al. Proteomic profiling of human plasma exosomes identifies PPARgamma as an exosome-associated protein. *Biochem Biophys Res Commun* 2009;378:433-8. DOI PubMed PMC
12. Street JM, Barran PE, Mackay CL, et al. Identification and proteomic profiling of exosomes in human cerebrospinal fluid. *J Transl Med* 2012;10:5. DOI PubMed PMC
13. Michael A, Bajracharya SD, Yuen PS, et al. Exosomes from human saliva as a source of microRNA biomarkers. *Oral Dis* 2010;16:34-8. DOI PubMed PMC
14. Gonzales PA, Pisitkun T, Hoffert JD, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. *J Am Soc Nephrol* 2009;20:363-79. DOI PubMed PMC
15. Admyre C, Johansson SM, Qazi KR, et al. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* 2007;179:1969-78. DOI PubMed
16. Cañas JA, Sastre B, Rodrigo-Muñoz JM, Del Pozo V. Exosomes: A new approach to asthma pathology. *Clin Chim Acta* 2019;495:139-47. DOI PubMed
17. Zhang X, Yuan X, Shi H, Wu L, Qian H, Xu W. Exosomes in cancer: small particle, big player. *J Hematol Oncol* 2015;8:83. DOI PubMed PMC
18. Huang X, Yuan T, Tschannen M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics* 2013;14:319. DOI PubMed PMC
19. Hewson C, Capraro D, Burdach J, Whitaker N, Morris KV. Extracellular vesicle associated long non-coding RNAs functionally enhance cell viability. *Noncoding RNA Res* 2016;1:3-11. DOI PubMed PMC
20. Soria FN, Pampliega O, Bourdenx M, Meissner WG, Bezard E, Dehay B. Exosomes, an Unmasked Culprit in Neurodegenerative Diseases. *Front Neurosci* 2017;11:26. DOI PubMed PMC
21. Sahu R, Kaushik S, Clement CC, et al. Microautophagy of cytosolic proteins by late endosomes. *Dev Cell* 2011;20:131-9. DOI PubMed PMC
22. Record M. Intercellular communication by exosomes in placenta: a possible role in cell fusion? *Placenta* 2014;35:297-302. DOI PubMed
23. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci* 2018;75:193-208. DOI PubMed PMC
24. Colombo M, Moita C, van Niel G, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci* 2013;126:5553-65. DOI PubMed
25. Henne WM, Buchkovich NJ, Emr SD. The ESCRT pathway. *Dev Cell* 2011;21:77-91. DOI PubMed
26. Baietti MF, Zhang Z, Mortier E, et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol* 2012;14:677-85. DOI PubMed
27. Hurley JH. ESCRTs are everywhere. *EMBO J* 2015;34:2398-407. DOI PubMed PMC
28. Stuffers S, Sem Wegner C, Stenmark H, Brech A. Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic* 2009;10:925-37. DOI PubMed
29. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 1998;273:20121-7. DOI PubMed
30. Chairoungdua A, Smith DL, Pochard P, Hull M, Caplan MJ. Exosome release of β-catenin: a novel mechanism that antagonizes Wnt signaling. *J Cell Biol* 2010;190:1079-91. DOI PubMed PMC
31. Nazarenko I, Rana S, Baumann A, et al. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res* 2010;70:1668-78. DOI PubMed
32. Hurwitz SN, Conlon MM, Rider MA, Brownstein NC, Meckes DG Jr. Nanoparticle analysis sheds budding insights into genetic drivers of extracellular vesicle biogenesis. *J Extracell Vesicles* 2016;5:31295. DOI PubMed PMC
33. den Boorn JG, Dassler J, Coch C, Schlee M, Hartmann G. Exosomes as nucleic acid nanocarriers. *Adv Drug Deliv Rev* 2013;65:331-5. DOI PubMed
34. Castro BM, Prieto M, Silva LC. Ceramide: a simple sphingolipid with unique biophysical properties. *Prog Lipid Res* 2014;54:53-67. DOI PubMed
35. Ostrowski M, Carmo NB, Krumeich S, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 2010;12:19-30; sup pp 1. DOI PubMed
36. Hsu C, Morohashi Y, Yoshimura S, et al. Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-

C. *J Cell Biol* 2010;189:223-32. DOI PubMed PMC
37. Savina A, Furlán M, Vidal M, Colombo MI. Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J Biol Chem* 2003;278:20083-90. DOI PubMed
38. Parolini I, Federici C, Raggi C, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;284:34211-22. DOI PubMed PMC
39. Lehmann BD, Paine MS, Brooks AM, et al. Senescence-associated exosome release from human prostate cancer cells. *Cancer Res* 2008;68:7864-71. DOI PubMed PMC
40. Beer L, Zimmermann M, Mitterbauer A, et al. Analysis of the Secretome of Apoptotic Peripheral Blood Mononuclear Cells: Impact of Released Proteins and Exosomes for Tissue Regeneration. *Sci Rep* 2015;5:16662. DOI PubMed PMC
41. Xiao X, Yu S, Li S, et al. Exosomes: decreased sensitivity of lung cancer A549 cells to cisplatin. *PLoS One* 2014;9:e89534. DOI PubMed PMC
42. King HW, Michael MZ, Gleadle JM. Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer* 2012;12:421. DOI PubMed PMC
43. Kanemoto S, Nitani R, Murakami T, et al. Multivesicular body formation enhancement and exosome release during endoplasmic reticulum stress. *Biochem Biophys Res Commun* 2016;480:166-72. DOI PubMed
44. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. *Nature* 2007;450:435-9. DOI PubMed
45. Segura E, Nicco C, Lombard B, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. *Blood* 2005;106:216-23. DOI PubMed
46. Feng D, Zhao WL, Ye YY, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 2010;11:675-87. DOI PubMed
47. Fitzner D, Schnaars M, van Rossum D, et al. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *J Cell Sci* 2011;124:447-58. DOI PubMed
48. Frühbeis C, Fröhlich D, Krämer-Albers EM. Emerging roles of exosomes in neuron-glia communication. *Front Physiol* 2012;3:119. DOI PubMed PMC
49. Guitart K, Loers G, Buck F, Bork U, Schachner M, Kleene R. Improvement of neuronal cell survival by astrocyte-derived exosomes under hypoxic and ischemic conditions depends on prion protein. *Glia* 2016;64:896-910. DOI PubMed
50. Chivet M, Javalet C, Laulagnier K, Blot B, Hemming FJ, Sadoul R. Exosomes secreted by cortical neurons upon glutamatergic synapse activation specifically interact with neurons. *J Extracell Vesicles* 2014;3:24722. DOI PubMed PMC
51. Gurunathan S, Kang MH, Jeyaraj M, Qasim M, Kim JH. Review of the Isolation, Characterization, Biological Function, and Multifarious Therapeutic Approaches of Exosomes. *Cells* 2019;8:307. DOI PubMed PMC
52. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int* 2010;78:838-48. DOI PubMed
53. Gurunathan S, Kang MH, Kim JH. A Comprehensive Review on Factors Influences Biogenesis, Functions, Therapeutic and Clinical Implications of Exosomes. *Int J Nanomedicine* 2021;16:1281-312. DOI PubMed PMC
54. Lachenal G, Pernet-Gallay K, Chivet M, et al. Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci* 2011;46:409-18. DOI PubMed
55. Bakhti M, Winter C, Simons M. Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *J Biol Chem* 2011;286:787-96. DOI PubMed PMC
56. Edgar JM, Nave KA. The role of CNS glia in preserving axon function. *Curr Opin Neurobiol* 2009;19:498-504. DOI PubMed
57. Tytell M, Greenberg S, Lasek R. Heat shock-like protein is transferred from glia to axon. *Brain Research* 1986;363:161-4. DOI PubMed
58. Tytell M. Release of heat shock proteins (Hsps) and the effects of extracellular Hsps on neural cells and tissues. *Int J Hyperthermia* 2005;21:445-55. DOI PubMed
59. Fauré J, Lachenal G, Court M, et al. Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006;31:642-8. DOI PubMed
60. Ghidoni R, Paterlini A, Albertini V, et al. Cystatin C is released in association with exosomes: a new tool of neuronal communication which is unbalanced in Alzheimer's disease. *Neurobiol Aging* 2011;32:1435-42. DOI PubMed PMC
61. Korkut C, Li Y, Koles K, et al. Regulation of postsynaptic retrograde signaling by presynaptic exosome release. *Neuron* 2013;77:1039-46. DOI PubMed PMC
62. Bahrini I, Song JH, Diez D, Hanayama R. Neuronal exosomes facilitate synaptic pruning by up-regulating complement factors in microglia. *Sci Rep* 2015;5:7989. DOI PubMed PMC
63. Morel L, Regan M, Higashimori H, et al. Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. *J Biol Chem* 2013;288:7105-16. DOI PubMed PMC
64. Wang S, Cesca F, Loers G, et al. Synapsin I is an oligomannose-carrying glycoprotein, acts as an oligomannose-binding lectin, and promotes neurite outgrowth and neuronal survival when released via glia-derived exosomes. *J Neurosci* 2011;31:7275-90. DOI PubMed PMC
65. Prada I, Gabrielli M, Turola E, et al. Glia-to-neuron transfer of miRNAs via extracellular vesicles: a new mechanism underlying inflammation-induced synaptic alterations. *Acta Neuropathol* 2018;135:529-50. DOI PubMed PMC
66. Yamada J, Jinno S. Promotion of synaptogenesis and neural circuit development by exosomes. *Ann Transl Med* 2019;7:S323. DOI PubMed PMC

67. Feliciano DM, Zhang S, Nasrallah CM, Lisgo SN, Bordey A. Embryonic cerebrospinal fluid nanovesicles carry evolutionarily conserved molecules and promote neural stem cell amplification. *PLoS One* 2014;9:e88810. DOI PubMed PMC
68. Bátiz LF, Castro MA, Burgos PV, et al. Exosomes as Novel Regulators of Adult Neurogenic Niches. *Front Cell Neurosci* 2015;9:501. DOI PubMed PMC
69. Ma Y, Li C, Huang Y, Wang Y, Xia X, Zheng JC. Exosomes released from neural progenitor cells and induced neural progenitor cells regulate neurogenesis through miR-21a. *Cell Commun Signal* 2019;17:96. DOI PubMed PMC
70. Sharma P, Mesci P, Carromeu C, et al. Exosomes regulate neurogenesis and circuit assembly. *Proc Natl Acad Sci U S A* 2019;116:16086-94. DOI PubMed PMC
71. Perets N, Oron O, Herman S, Elliott E, Offen D. Exosomes derived from mesenchymal stem cells improved core symptoms of genetically modified mouse model of autism Shank3B. *Mol Autism* 2020;11:65. DOI PubMed PMC
72. Elsharkasy OM, Nordin JZ, Hagey DW, et al. Extracellular vesicles as drug delivery systems: Why and how? *Adv Drug Deliv Rev* 2020;159:332-43. DOI PubMed
73. Gee P, Lung MSY, Okuzaki Y, et al. Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping. *Nat Commun* 2020;11:1334. DOI PubMed PMC
74. Hebert LE, Weuve J, Scherr PA, Evans DA. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology* 2013;80:1778-83. DOI PubMed PMC
75. Alzheimer's disease facts and figures. *Alzheimers Dement* ;2020:391-460. DOI PubMed
76. Reza-Zaldivar EE, Hernández-Sapiéns MA, Minjarez B, Gutiérrez-Mercado YK, Márquez-Aguirre AL, Canales-Aguirre AA. Potential Effects of MSC-Derived Exosomes in Neuroplasticity in Alzheimer's Disease. *Front Cell Neurosci* 2018;12:317. DOI PubMed PMC
77. Xiao T, Zhang W, Jiao B, Pan CZ, Liu X, Shen L. The role of exosomes in the pathogenesis of Alzheimer' disease. *Transl Neurodegener* 2017;6:3. DOI PubMed PMC
78. DeLeo AM, Ikezu T. Extracellular Vesicle Biology in Alzheimer's Disease and Related Tauopathy. *J Neuroimmune Pharmacol* 2018;13:292-308. DOI PubMed PMC
79. Pérez M, Avila J, Hernández F. Propagation of Tau via Extracellular Vesicles. *Front Neurosci* 2019;13:698. DOI PubMed PMC
80. Podvin S, Jones A, Liu Q, et al. Dysregulation of Exosome Cargo by Mutant Tau Expressed in Human-induced Pluripotent Stem Cell (iPSC) Neurons Revealed by Proteomics Analyses. *Mol Cell Proteomics* 2020;19:1017-34. DOI PubMed PMC
81. Yuyama K, Sun H, Mitsutake S, Igarashi Y. Sphingolipid-modulated exosome secretion promotes clearance of amyloid-β by microglia. *J Biol Chem* 2012;287:10977-89. DOI PubMed PMC
82. Yuyama K, Sun H, Sakai S, et al. Decreased amyloid-β pathologies by intracerebral loading of glycosphingolipid-enriched exosomes in Alzheimer model mice. *J Biol Chem* 2014;289:24488-98. DOI PubMed PMC
83. An K, Klyubin I, Kim Y, et al. Exosomes neutralize synaptic-plasticity-disrupting activity of Aβ assemblies in vivo. *Mol Brain* 2013;6:47. DOI PubMed PMC
84. Falker C, Hartmann A, Guett I, et al. Exosomal cellular prion protein drives fibrillization of amyloid beta and counteracts amyloid beta-mediated neurotoxicity. *J Neurochem* 2016;137:88-100. DOI PubMed
85. Yuyama K, Sun H, Usuki S, et al. A potential function for neuronal exosomes: sequestering intracerebral amyloid-β peptide. *FEBS Lett* 2015;589:84-8. DOI PubMed
86. Katsuda T, Tsuchiya R, Kosaka N, et al. Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* 2013;3:1197. DOI PubMed PMC
87. Bulloj A, Leal MC, Xu H, Castaño EM, Morelli L. Insulin-degrading enzyme sorting in exosomes: a secretory pathway for a key brain amyloid-beta degrading protease. *J Alzheimers Dis* 2010;19:79-95. DOI PubMed PMC
88. Langui D, Girardot N, El Hachimi KH, et al. Subcellular Topography of Neuronal Aβ Peptide in APPxPS1 Transgenic Mice. *Am J Pathol* 2004;165:1465-77. DOI PubMed PMC
89. Rajendran L, Honsho M, Zahn TR, et al. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 2006;103:11172-7. DOI PubMed PMC
90. Vingtdeux V, Hamdane M, Loyens A, et al. Alkalizing drugs induce accumulation of amyloid precursor protein by-products in luminal vesicles of multivesicular bodies. *J Biol Chem* 2007;282:18197-205. DOI PubMed
91. Sullivan CP, Jay AG, Stack EC, et al. Retromer disruption promotes amyloidogenic APP processing. *Neurobiol Dis* 2011;43:338-45. DOI PubMed PMC
92. Dinkins MB, Dasgupta S, Wang G, Zhu G, Bieberich E. Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* 2014;35:1792-800. DOI PubMed PMC
93. Dinkins MB, Dasgupta S, Wang G, et al. The 5XFAD Mouse Model of Alzheimer's Disease Exhibits an Age-Dependent Increase in Anti-Ceramide IgG and Exogenous Administration of Ceramide Further Increases Anti-Ceramide Titers and Amyloid Plaque Burden. *J Alzheimers Dis* 2015;46:55-61. DOI PubMed PMC
94. Bellingham SA, Guo BB, Coleman BM, Hill AF. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? *Front Physiol* 2012;3:124. DOI PubMed PMC
95. Bolmont T, Haiss F, Eicke D, et al. Dynamics of the microglial/amyloid interaction indicate a role in plaque maintenance. *J Neurosci* 2008;28:4283-92. DOI PubMed PMC
96. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci* 2008;28:8354-60. DOI PubMed PMC
97. Song Z, Xu Y, Deng W, et al. Brain Derived Exosomes Are a Double-Edged Sword in Alzheimer's Disease. *Front Mol Neurosci*

2020;13:79. DOI PubMed PMC
98. Trotta T, Panaro MA, Cianciulli A, Mori G, Di Benedetto A, Porro C. Microglia-derived extracellular vesicles in Alzheimer's Disease: A double-edged sword. *Biochem Pharmacol* 2018;148:184-92. DOI PubMed
99. Saman S, Kim W, Raya M, et al. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *J Biol Chem* 2012;287:3842-9. DOI PubMed PMC
100. Guix FX, Corbett GT, Cha DJ, et al. Detection of Aggregation-Competent Tau in Neuron-Derived Extracellular Vesicles. *Int J Mol Sci* 2018;19:663. DOI PubMed PMC
101. Asai H, Ikezu S, Tsunoda S, et al. Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nat Neurosci* 2015;18:1584-93. DOI PubMed PMC
102. Fiandaca MS, Kapogiannis D, Mapstone M, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimers Dement* 2015;11:600-7.e1. DOI PubMed PMC
103. Goetzl EJ, Mustapic M, Kapogiannis D, et al. Cargo proteins of plasma astrocyte-derived exosomes in Alzheimer's disease. *FASEB J* 2016;30:3853-9. DOI PubMed PMC
104. Zhuang X, Xiang X, Grizzle W, et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* 2011;19:1769-79. DOI PubMed PMC
105. Reich SG, Savitt JM. Parkinson's Disease. *Med Clin North Am* 2019;103:337-50. DOI PubMed
106. Olanow CW, Brundin P. Parkinson's disease and alpha synuclein: is Parkinson's disease a prion-like disorder? *Mov Disord* 2013;28:31-40. DOI PubMed
107. Emmanouilidou E, Melachroinou K, Roumeliotis T, et al. Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J Neurosci* 2010;30:6838-51. DOI PubMed PMC
108. Lee HJ, Patel S, Lee SJ. Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. *J Neurosci* 2005;25:6016-24. DOI PubMed PMC
109. Alvarez-Erviti L, Seow Y, Schapira AH, et al. Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol Dis* 2011;42:360-7. DOI PubMed PMC
110. Cabin DE, Shimazu K, Murphy D, et al. Synaptic Vesicle Depletion Correlates with Attenuated Synaptic Responses to Prolonged Repetitive Stimulation in Mice Lacking α-Synuclein. *J Neurosci* 2002;22:8797-807. PubMed PMC
111. Gedalya T, Loeb V, Israeli E, Altschuler Y, Selkoe DJ, Sharon R. Alpha-synuclein and polyunsaturated fatty acids promote clathrin-mediated endocytosis and synaptic vesicle recycling. *Traffic* 2009;10:218-34. DOI PubMed PMC
112. Grey M, Dunning CJ, Gaspar R, et al. Acceleration of α-synuclein aggregation by exosomes. *J Biol Chem* 2015;290:2969-82. DOI PubMed PMC
113. Stuendl A, Kunadt M, Kruse N, et al. Induction of α-synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies. *Brain* 2016;139:481-94. DOI PubMed PMC
114. Danzer KM, Kranich LR, Ruf WP, et al. Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol Neurodegener* 2012;7:42. DOI PubMed PMC
115. Hornung S, Dutta S, Bitan G. CNS-Derived Blood Exosomes as a Promising Source of Biomarkers: Opportunities and Challenges. *Front Mol Neurosci* 2020;13:38. DOI PubMed PMC
116. Shi M, Liu C, Cook TJ, et al. Plasma exosomal α-synuclein is likely CNS-derived and increased in Parkinson's disease. *Acta Neuropathol* 2014;128:639-50. DOI PubMed PMC
117. Si X, Tian J, Chen Y, Yan Y, Pu J, Zhang B. Central Nervous System-Derived Exosomal Alpha-Synuclein in Serum May Be a Biomarker in Parkinson's Disease. *Neuroscience* 2019;413:308-16. DOI PubMed
118. Jiang C, Hopfner F, Katsikoudi A, et al. Serum neuronal exosomes predict and differentiate Parkinson's disease from atypical parkinsonism. *J Neurol Neurosurg Psychiatry* 2020;91:720-9. DOI PubMed PMC
119. Niu M, Li Y, Li G, et al. A longitudinal study on α-synuclein in plasma neuronal exosomes as a biomarker for Parkinson's disease development and progression. *Eur J Neurol* 2020;27:967-74. DOI PubMed
120. Haney MJ, Klyachko NL, Zhao Y, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* 2015;207:18-30. DOI PubMed PMC
121. Chen HX, Liang FC, Gu P, et al. Exosomes derived from mesenchymal stem cells repair a Parkinson's disease model by inducing autophagy. *Cell Death Dis* 2020;11:288. DOI PubMed PMC
122. Qu M, Lin Q, Huang L, et al. Dopamine-loaded blood exosomes targeted to brain for better treatment of Parkinson's disease. *J Control Release* 2018;287:156-66. DOI PubMed
123. Bonda DJ, Manjila S, Mehndiratta P, et al. Human prion diseases: surgical lessons learned from iatrogenic prion transmission. *Neurosurg Focus* 2016;41:E10. DOI PubMed PMC
124. Fevrier B, Vilette D, Archer F, et al. Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* 2004;101:9683-8. DOI PubMed PMC
125. Alais S, Simoes S, Baas D, et al. Mouse neuroblastoma cells release prion infectivity associated with exosomal vesicles. *Biol Cell* 2008;100:603-15. DOI PubMed
126. Vilette D, Laulagnier K, Huor A, et al. Efficient inhibition of infectious prions multiplication and release by targeting the exosomal pathway. *Cell Mol Life Sci* 2015;72:4409-27. DOI PubMed
127. Guo BB, Bellingham SA, Hill AF. Stimulating the Release of Exosomes Increases the Intercellular Transfer of Prions. *J Biol Chem* 2016;291:5128-37. DOI PubMed PMC
128. Vella LJ, Sharples RA, Lawson VA, Masters CL, Cappai R, Hill AF. Packaging of prions into exosomes is associated with a novel

pathway of PrP processing. *J Pathol* 2007;211:582-90. DOI PubMed

129. Cervenakova L, Saá P, Yakovleva O, et al. Are prions transported by plasma exosomes? *Transfus Apher Sci* 2016;55:70-83. DOI PubMed
130. Cheng L, Zhao W, Hill AF. Exosomes and their role in the intercellular trafficking of normal and disease associated prion proteins. *Mol Aspects Med* 2018;60:62-8. DOI PubMed
131. Kiernan MC, Vucic S, Cheah BC, et al. Amyotrophic lateral sclerosis. *Lancet* 2011;377:942-55. DOI PubMed
132. Brown RH, Al-Chalabi A. Amyotrophic Lateral Sclerosis. *N Engl J Med* 2017;377:162-72. DOI PubMed
133. Prasad A, Bharathi V, Sivalingam V, Girdhar A, Patel BK. Molecular Mechanisms of TDP-43 Misfolding and Pathology in Amyotrophic Lateral Sclerosis. *Front Mol Neurosci* 2019;12:25. DOI PubMed PMC
134. Zondler L, Feiler MS, Freischmidt A, et al. Impaired activation of ALS monocytes by exosomes. *Immunol Cell Biol* 2017;95:207-14. DOI PubMed
135. Grad LI, Pokrishevsky E, Silverman JM, Cashman NR. Exosome-dependent and independent mechanisms are involved in prion-like transmission of propagated Cu/Zn superoxide dismutase misfolding. *Prion* 2014;8:331-5. DOI PubMed PMC
136. Iguchi Y, Eid L, Parent M, et al. Exosome secretion is a key pathway for clearance of pathological TDP-43. *Brain* 2016;139:3187-201. DOI PubMed PMC
137. Chen PC, Wu D, Hu CJ, Chen HY, Hsieh YC, Huang CC. Exosomal TAR DNA-binding protein-43 and neurofilaments in plasma of amyotrophic lateral sclerosis patients: A longitudinal follow-up study. *J Neurol Sci* 2020;418:117070. DOI PubMed
138. Calabria E, Scambi I, Bonafede R, et al. ASCs-Exosomes Recover Coupling Efficiency and Mitochondrial Membrane Potential in an. *in vitro* ;13:1070. DOI PubMed PMC
139. Pagan F, Torres-yaghi Y, Altshuler M. The diagnosis and natural history of Huntington disease. *Handb Clin Neurol* 2017;144:63-7. DOI PubMed
140. Wang JKT, Langfelder P, Horvath S, Palazzolo MJ. Exosomes and Homeostatic Synaptic Plasticity Are Linked to Each other and to Huntington's, Parkinson's, and Other Neurodegenerative Diseases by Database-Enabled Analyses of Comprehensively Curated Datasets. *Front Neurosci* 2017;11:149. DOI PubMed PMC
141. Jeon I, Cicchetti F, Cisbani G, et al. Human-to-mouse prion-like propagation of mutant huntingtin protein. *Acta Neuropathol* 2016;132:577-92. DOI PubMed PMC
142. Zhang X, Abels ER, Redzic JS, Margulis J, Finkbeiner S, Breakefield XO. Potential Transfer of Polyglutamine and CAG-Repeat RNA in Extracellular Vesicles in Huntington's Disease: Background and Evaluation in Cell Culture. *Cell Mol Neurobiol* 2016;36:459-70. DOI PubMed PMC
143. Díez-Planelles C, Sánchez-Lozano P, Crespo MC, et al. Circulating microRNAs in Huntington's disease: Emerging mediators in metabolic impairment. *Pharmacol Res* 2016;108:102-10. DOI PubMed
144. Kumar S, Vijayan M, Bhatti J, Reddy P. MicroRNAs as Peripheral Biomarkers in Aging and Age-Related Diseases. *Prog Mol Biol Transl Sci* 2017;146:47-94. DOI PubMed
145. Lee ST, Im W, Ban JJ, et al. Exosome-Based Delivery of miR-124 in a Huntington's Disease Model. *J Mov Disord* 2017;10:45-52. DOI PubMed PMC
146. Didiot MC, Hall LM, Coles AH, et al. Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol Ther* 2016;24:1836-47. DOI PubMed PMC
147. Moshé SL, Perucca E, Ryvlin P, Tomson T. Epilepsy: new advances. *Lancet* 2015;385:884-98. DOI PubMed
148. Betjemann JP, Lowenstein DH. Status epilepticus in adults. *Lancet Neurol* 2015;14:615-24. DOI PubMed
149. Batool A, Hill TDM, Nguyen NT, et al. Altered Biogenesis and MicroRNA Content of Hippocampal Exosomes Following Experimental Status Epilepticus. *Front Neurosci* 2019;13:1404. DOI PubMed PMC
150. Gitaí DLG, Dos Santos YDR, Upadhya R, Kodali M, Madhu LN, Shetty AK. Extracellular Vesicles in the Forebrain Display Reduced miR-346 and miR-331-3p in a Rat Model of Chronic Temporal Lobe Epilepsy. *Mol Neurobiol* 2020;57:1674-87. DOI PubMed
151. Yan S, Zhang H, Xie W, et al. Altered microRNA profiles in plasma exosomes from mesial temporal lobe epilepsy with hippocampal sclerosis. *Oncotarget* 2017;8:4136-46. DOI PubMed PMC
152. Chen SD, Pan HY, Huang JB, et al. Circulating MicroRNAs from Serum Exosomes May Serve as a Putative Biomarker in the Diagnosis and Treatment of Patients with Focal Cortical Dysplasia. *Cells* 2020;9:1867. DOI PubMed PMC
153. Long Q, Upadhya D, Hattiangady B, et al. Intranasal MSC-derived A1-exosomes ease inflammation, and prevent abnormal neurogenesis and memory dysfunction after status epilepticus. *Proc Natl Acad Sci U S A* 2017;114:E3536-45. DOI PubMed PMC
154. Nicholas R, Rashid W. Multiple sclerosis. *Am Fam Physician* 2013;87:712-4. PubMed
155. Galazka G, Mycko MP, Selmaj I, Raine CS, Selmaj KW. Multiple sclerosis: Serum-derived exosomes express myelin proteins. *Mult Scler* 2018;24:449-58. DOI PubMed
156. Pieragostino D, Cicalini I, Lanuti P, et al. Enhanced release of acid sphingomyelinase-enriched exosomes generates a lipidomics signature in CSF of Multiple Sclerosis patients. *Sci Rep* 2018;8:3071. DOI PubMed PMC
157. Kimura K, Hohjoh H, Fukuoka M, et al. Circulating exosomes suppress the induction of regulatory T cells via let-7i in multiple sclerosis. *Nat Commun* 2018;9:17. DOI PubMed PMC
158. Selmaj I, Cichalewska M, Namiecinska M, et al. Global exosome transcriptome profiling reveals biomarkers for multiple sclerosis. *Ann Neurol* 2017;81:703-17. DOI PubMed
159. Ebrahimkhani S, Vafaee F, Young PE, et al. Exosomal microRNA signatures in multiple sclerosis reflect disease status. *Sci Rep* 2017;7:14293. DOI PubMed PMC

160. Manna I, Iaccino E, Dattilo V, et al. Exosome-associated miRNA profile as a prognostic tool for therapy response monitoring in multiple sclerosis patients. *FASEB J* 2018;32:4241-6. DOI PubMed
161. Wei Z, Fan B, Ding H, et al. Proteomics analysis of Schwann cell-derived exosomes: a novel therapeutic strategy for central nervous system injury. *Mol Cell Biochem* 2019;457:51-9. DOI PubMed PMC
162. Domingues HS, Falcão AM, Mendes-Pinto I, Salgado AJ, Teixeira FG. Exosome Circuitry During (De)(Re)Myelination of the Central Nervous System. *Front Cell Dev Biol* 2020;8:483. DOI PubMed PMC
163. Krämer-Albers EM, Bretz N, Tenzer S, et al. Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons? *Proteomics Clin Appl* 2007;1:1446-61. DOI PubMed
164. Lopez-Verrilli MA, Picou F, Court FA. Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. *Glia* 2013;61:1795-806. DOI PubMed
165. Hu M, Hong L, Liu C, et al. Electrical stimulation enhances neuronal cell activity mediated by Schwann cell derived exosomes. *Sci Rep* 2019;9:4206. DOI PubMed PMC
166. Bucan V, Vaslaitis D, Peck CT, Strauß S, Vogt PM, Radtke C. Effect of Exosomes from Rat Adipose-Derived Mesenchymal Stem Cells on Neurite Outgrowth and Sciatic Nerve Regeneration After Crush Injury. *Mol Neurobiol* 2019;56:1812-24. DOI PubMed PMC
167. Xiao Y, Geng F, Wang G, Li X, Zhu J, Zhu W. Bone marrow-derived mesenchymal stem cells-derived exosomes prevent oligodendrocyte apoptosis through exosomal miR-134 by targeting caspase-8. *J Cell Biochem* ;2018:2109-18. DOI PubMed
168. Thomi G, Joerger-Messerli M, Haesler V, Muri L, Surbek D, Schoeberlein A. Intranasally Administered Exosomes from Umbilical Cord Stem Cells Have Preventive Neuroprotective Effects and Contribute to Functional Recovery after Perinatal Brain Injury. *Cells* 2019;8:855. DOI PubMed PMC
169. Otero-Ortega L, Laso-García F, Gómez-de Frutos M, et al. Role of Exosomes as a Treatment and Potential Biomarker for Stroke. *Transl Stroke Res* 2019;10:241-9. DOI PubMed
170. Zhang ZG, Chopp M. Exosomes in stroke pathogenesis and therapy. *J Clin Invest* 2016;126:1190-7. DOI PubMed PMC
171. Xin H, Li Y, Buller B, et al. Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 2012;30:1556-64. DOI PubMed PMC
172. Chen F, Du Y, Esposito E, et al. Effects of Focal Cerebral Ischemia on Exosomal Versus Serum miR126. *Transl Stroke Res* 2015;6:478-84. DOI PubMed
173. Yang J, Zhang X, Chen X, Wang L, Yang G. Exosome Mediated Delivery of miR-124 Promotes Neurogenesis after Ischemia. *Mol Ther Nucleic Acids* 2017;7:278-87. DOI PubMed PMC
174. Seifert HA, Offner H. The splenic response to stroke: from rodents to stroke subjects. *J Neuroinflammation* 2018;15:195. DOI PubMed PMC
175. Makin SD, Cook FA, Dennis MS, Wardlaw JM. Cerebral small vessel disease and renal function: systematic review and meta-analysis. *Cerebrovasc Dis* 2015;39:39-52. DOI PubMed PMC
176. Zhang G, Chen L, Guo X, et al. Comparative Analysis of microRNA Expression Profiles of Exosomes Derived from Normal and Hypoxic Preconditioning Human Neural Stem Cells by Next Generation Sequencing. *J Biomed Nanotechnol* 2018;14:1075-89. DOI PubMed
177. Li DB, Liu JL, Wang W, et al. Plasma Exosomal miRNA-122-5p and miR-300-3p as Potential Markers for Transient Ischaemic Attack in Rats. *Front Aging Neurosci* 2018;10:24. DOI PubMed PMC
178. Datta A, Chen CP, Sze SK. Discovery of prognostic biomarker candidates of lacunar infarction by quantitative proteomics of microvesicles enriched plasma. *PLoS One* 2014;9:e94663. DOI PubMed PMC
179. Kanhai DA, de Kleijn DP, Kappelle LJ, et al; SMART Study Group. Extracellular vesicle protein levels are related to brain atrophy and cerebral white matter lesions in patients with manifest vascular disease: the SMART-MR study. *BMJ Open* 2014;4:e003824. DOI PubMed PMC
180. Ji Q, Ji Y, Peng J, et al. Increased Brain-Specific MiR-9 and MiR-124 in the Serum Exosomes of Acute Ischemic Stroke Patients. *PLoS One* 2016;11:e0163645. DOI PubMed PMC
181. Li DB, Liu JL, Wang W, et al. Plasma Exosomal miR-422a and miR-125b-2-3p Serve as Biomarkers for Ischemic Stroke. *Curr Neurovasc Res* 2017;14:330-7. DOI PubMed
182. Chen Y, Song Y, Huang J, et al. Increased Circulating Exosomal miRNA-223 Is Associated with Acute Ischemic Stroke. *Front Neurol* 2017;8:57. DOI PubMed PMC
183. Wang W, Li DB, Li RY, et al. Diagnosis of Hyperacute and Acute Ischaemic Stroke: The Potential Utility of Exosomal MicroRNA-21-5p and MicroRNA-30a-5p. *Cerebrovasc Dis* 2018;45:204-12. DOI PubMed
184. McKee CA, Lukens JR. Emerging Roles for the Immune System in Traumatic Brain Injury. *Front Immunol* 2016;7:556. DOI PubMed PMC
185. Taylor DD, Gercel-Taylor C. Exosome platform for diagnosis and monitoring of traumatic brain injury. *Philos Trans R Soc Lond B Biol Sci* 2014;369:20130503. DOI PubMed PMC
186. Huang S, Ge X, Yu J, et al. Increased miR-124-3p in microglial exosomes following traumatic brain injury inhibits neuronal inflammation and contributes to neurite outgrowth via their transfer into neurons. *FASEB J* 2018;32:512-28. DOI PubMed
187. Guedes VA, Kenney K, Shahim P, et al; CENC Multisite Observational Study Investigators. Exosomal neurofilament light: A prognostic biomarker for remote symptoms after mild traumatic brain injury? *Neurology* 2020;94:e2412-23. DOI PubMed PMC
188. Goetzl EJ, Elahi FM, Mustapic M, et al. Altered levels of plasma neuron-derived exosomes and their cargo proteins characterize acute and chronic mild traumatic brain injury. *FASEB J* 2019;33:5082-8. DOI PubMed PMC

189. Beard K, Meaney DF, Issadore D. Clinical Applications of Extracellular Vesicles in the Diagnosis and Treatment of Traumatic Brain Injury. *J Neurotrauma* 2020;37:2045-56. DOI PubMed PMC
190. Gruzdev SK, Yakovlev AA, Druzhkova TA, Guekht AB, Gulyaeva NV. The Missing Link: How Exosomes and miRNAs can Help in Bridging Psychiatry and Molecular Biology in the Context of Depression, Bipolar Disorder and Schizophrenia. *Cell Mol Neurobiol* 2019;39:729-50. DOI PubMed
191. Baudry A, Mouillet-Richard S, Schneider B, Launay JM, Kellermann O. miR-16 targets the serotonin transporter: a new facet for adaptive responses to antidepressants. *Science* 2010;329:1537-41. DOI PubMed
192. Beveridge NJ, Tooney PA, Carroll AP, et al. Dysregulation of miRNA 181b in the temporal cortex in schizophrenia. *Hum Mol Genet* 2008;17:1156-68. DOI PubMed
193. Muiños-Gimeno M, Espinosa-Parrilla Y, Guidi M, et al. Human microRNAs miR-22, miR-138-2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways. *Biol Psychiatry* 2011;69:526-33. DOI PubMed
194. Rong H, Liu TB, Yang KJ, et al. MicroRNA-134 plasma levels before and after treatment for bipolar mania. *J Psychiatr Res* 2011;45:92-5. DOI PubMed
195. Banigan MG, Kao PF, Kozubek JA, et al. Differential expression of exosomal microRNAs in prefrontal cortices of schizophrenia and bipolar disorder patients. *PLoS One* 2013;8:e48814. DOI PubMed PMC
196. Du Y, Yu Y, Hu Y, et al. Genome-Wide, Integrative Analysis Implicates Exosome-Derived MicroRNA Dysregulation in Schizophrenia. *Schizophr Bull* 2019;45:1257-66. DOI PubMed PMC
197. Lee ST, Chu K, Jung KH, et al. miR-206 regulates brain-derived neurotrophic factor in Alzheimer disease model. *Ann Neurol* 2012;72:269-77. DOI PubMed
198. role of microRNAs in major depressive disorder: diagnosis and therapeutic implications. *Dialogues Clin Neurosci* 2014;16:43-61. PubMed PMC
199. Liang JQ, Liao HR, Xu CX, et al. Serum Exosome-Derived miR-139-5p as a Potential Biomarker for Major Depressive Disorder. *Neuropsychiatr Dis Treat* 2020;16:2689-93. DOI PubMed PMC
200. Wen PY, Weller M, Lee EQ, et al. Glioblastoma in adults: a Society for Neuro-Oncology (SNO) and European Society of Neuro-Oncology (EANO) consensus review on current management and future directions. *Neuro Oncol* 2020;22:1073-113. DOI PubMed PMC
201. Davis ME. Glioblastoma: Overview of Disease and Treatment. *Clin J Oncol Nurs* 2016;20:S2-8. DOI PubMed PMC
202. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 2009;10:459-66. DOI PubMed
203. Patel AP, Tirosh I, Trombetta JJ, et al. Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 2014;344:1396-401. DOI PubMed PMC
204. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004;64:7011-21. DOI PubMed
205. Chen J, Li Y, Yu TS, et al. A restricted cell population propagates glioblastoma growth after chemotherapy. *Nature* 2012;488:522-6. DOI PubMed PMC
206. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432:396-401. DOI PubMed
207. Oushy S, Hellwinkel JE, Wang M, et al. Glioblastoma multiforme-derived extracellular vesicles drive normal astrocytes towards a tumour-enhancing phenotype. *Philos Trans R Soc Lond B Biol Sci* 2018;373:20160477. DOI PubMed PMC
208. Gao X, Zhang Z, Mashimo T, et al. Gliomas Interact with Non-glioma Brain Cells via Extracellular Vesicles. *Cell Rep* 2020;30:2489-2500.e5. DOI PubMed
209. Mondal A, Kumari Singh D, Panda S, Shiras A. Extracellular Vesicles As Modulators of Tumor Microenvironment and Disease Progression in Glioma. *Front Oncol* 2017;7:144. DOI PubMed PMC
210. Shao H, Chung J, Lee K, et al. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. *Nat Commun* 2015;6:6999. DOI PubMed PMC
211. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008;10:1470-6. DOI PubMed PMC
212. Simon T, Jackson E, Giamas G. Breaking through the glioblastoma micro-environment via extracellular vesicles. *Oncogene* 2020;39:4477-90. DOI PubMed PMC
213. Treps L, Perret R, Edmond S, Ricard D, Gavard J. Glioblastoma stem-like cells secrete the pro-angiogenic VEGF-A factor in extracellular vesicles. *J Extracell Vesicles* 2017;6:1359479. DOI PubMed PMC
214. Sun X, Ma X, Wang J, et al. Glioma stem cells-derived exosomes promote the angiogenic ability of endothelial cells through miR-21/VEGF signal. *Oncotarget* 2017;8:36137-48. DOI PubMed PMC
215. Wang ZF, Liao F, Wu H, Dai J. Glioma stem cells-derived exosomal miR-26a promotes angiogenesis of microvessel endothelial cells in glioma. *J Exp Clin Cancer Res* 2019;38:201. DOI PubMed PMC
216. Liu S, Sun J, Lan Q. Glioblastoma microvesicles promote endothelial cell proliferation through Akt/beta-catenin pathway. *Int J Clin Exp Pathol* 2014;7:4857-66. PubMed PMC
217. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 2008;10:619-24. DOI PubMed

218. Antonyak MA, Li B, Boroughs LK, et al. Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc Natl Acad Sci U S A* 2011;108:4852-7. DOI PubMed PMC
219. Lang HL, Hu GW, Chen Y, et al. Glioma cells promote angiogenesis through the release of exosomes containing long non-coding RNA POU3F3. *Eur Rev Med Pharmacol Sci* 2017;21:959-72. PubMed
220. Lang HL, Hu GW, Zhang B, et al. Glioma cells enhance angiogenesis and inhibit endothelial cell apoptosis through the release of exosomes that contain long non-coding RNA CCAT2. *Oncol Rep* 2017;38:785-98. DOI PubMed PMC
221. Bronisz A, Wang Y, Nowicki MO, et al. Extracellular vesicles modulate the glioblastoma microenvironment via a tumor suppression signaling network directed by miR-1. *Cancer Res* 2014;74:738-50. DOI PubMed PMC
222. Verhaak RG, Hoadley KA, Purdom E, et al; Cancer Genome Atlas Research Network. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 2010;17:98-110. DOI PubMed PMC
223. Suvà ML, Rheinbay E, Gillespie SM, et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. *Cell* 2014;157:580-94. DOI PubMed PMC
224. Berenguer J, Lagerweij T, Zhao XW, et al. Glycosylated extracellular vesicles released by glioblastoma cells are decorated by CCL18 allowing for cellular uptake via chemokine receptor CCR8. *J Extracell Vesicles* 2018;7:1446660. DOI PubMed PMC
225. Lucero R, Zappulli V, Sammarco A, et al. Glioma-Derived miRNA-containing Extracellular Vesicles Induce Angiogenesis by Reprogramming Brain Endothelial Cells. *Cell Rep* 2020;30:2065-74.e4. DOI PubMed PMC
226. Spinelli C, Montermini L, Meehan B, et al. Molecular subtypes and differentiation programmes of glioma stem cells as determinants of extracellular vesicle profiles and endothelial cell-stimulating activities. *J Extracell Vesicles* 2018;7:1490144. DOI PubMed PMC
227. Wei Z, Batagov AO, Schinelli S, et al. Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nat Commun* 2017;8:1145. DOI PubMed PMC
228. Hallal S, Mallawaaratchy DM, Wei H, et al. Extracellular Vesicles Released by Glioblastoma Cells Stimulate Normal Astrocytes to Acquire a Tumor-Supportive Phenotype Via p53 and MYC Signaling Pathways. *Mol Neurobiol* 2019;56:4566-81. DOI PubMed PMC
229. Colangelo NW, Azzam EI. Extracellular vesicles originating from glioblastoma cells increase metalloproteinase release by astrocytes: the role of CD147 (EMMPRIN) and ionizing radiation. *Cell Commun Signal* 2020;18:21. DOI PubMed PMC
230. Zeng A, Wei Z, Rabinovsky R, et al. Glioblastoma-Derived Extracellular Vesicles Facilitate Transformation of Astrocytes via Reprogramming Oncogenic Metabolism. *iScience* 2020;23:101420. DOI PubMed PMC
231. van der Vos KE, Abels ER, Zhang X, et al. Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain. *Neuro Oncol* 2016;18:58-69. DOI PubMed PMC
232. de Vrij J, Maas SL, Kwappenberg KM, et al. Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. *Int J Cancer* 2015;137:1630-42. DOI PubMed
233. Harshyne LA, Nasca BJ, Kenyon LC, Andrews DW, Hooper DC. Serum exosomes and cytokines promote a T-helper cell type 2 environment in the peripheral blood of glioblastoma patients. *Neuro Oncol* 2016;18:206-15. DOI PubMed PMC
234. Abels ER, Maas SLN, Nieland L, et al. Glioblastoma-Associated Microglia Reprogramming Is Mediated by Functional Transfer of Extracellular miR-21. *Cell Rep* 2019;28:3105-3119.e7. DOI PubMed PMC
235. Gabrusiewicz K, Li X, Wei J, et al. Glioblastoma stem cell-derived exosomes induce M2 macrophages and PD-L1 expression on human monocytes. *Oncoimmunology* 2018;7:e1412909. DOI PubMed PMC
236. Grimaldi A, Serpe C, Chece G, et al. Microglia-Derived Microvesicles Affect Microglia Phenotype in Glioma. *Front Cell Neurosci* 2019;13:41. DOI PubMed PMC
237. Ricklefs FL, Alayo Q, Krenzlin H, et al. Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles. *Sci Adv* 2018;4:eaar2766. DOI PubMed PMC
238. Munoz JL, Rodriguez-Cruz V, Ramkissoon SH, Ligon KL, Greco SJ, Rameshwar P. Temozolomide resistance in glioblastoma occurs by miRNA-9-targeted PTCH1, independent of sonic hedgehog level. *Oncotarget* 2015;6:1190-201. DOI PubMed PMC
239. Munoz JL, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, Rameshwar P. Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. *Mol Ther Nucleic Acids* 2013;2:e126. DOI PubMed PMC
240. Chuang HY, Su YK, Liu HW, et al. Preclinical Evidence of STAT3 Inhibitor Pacritinib Overcoming Temozolomide Resistance via Downregulating miR-21-Enriched Exosomes from M2 Glioblastoma-Associated Macrophages. *J Clin Med* 2019;8:959. DOI PubMed PMC
241. Figueroa JM, Skog J, Akers J, et al. Detection of wild-type EGFR amplification and EGFRvIII mutation in CSF-derived extracellular vesicles of glioblastoma patients. *Neuro Oncol* 2017;19:1494-502. DOI PubMed PMC
242. Akers JC, Ramakrishnan V, Kim R, et al. MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. *PLoS One* 2013;8:e78115. DOI PubMed PMC
243. Akers JC, Ramakrishnan V, Kim R, et al. miRNA contents of cerebrospinal fluid extracellular vesicles in glioblastoma patients. *J Neurooncol* 2015;123:205-16. DOI PubMed PMC

Zhao *et al. Extracell Vesicles Circ Nucleic Acids* 2021;2:80-103
DOI: 10.20517/evcna.2021.07

Extracellular Vesicles and Circulating Nucleic Acids

Review

Open Access

Isolation and analysis methods of extracellular vesicles (EVs)

Zheng Zhao[1,2], Harshani Wijerathne[3], Andrew K. Godwin[6], Steven A. Soper[1,2,4,5,6,7]

[1]Bioengineering Program, University of Kansas, Lawrence, KS 66045, USA.
[2]Center of BioModular Multiscale Systems for Precision Medicine, Lawrence, KS 66045, USA.
[3]Department of Mechanical Engineering, Temple University, Philadelphia, PA 19122, USA.
[4]Department of Chemistry, University of Kansas, Lawrence, KS 66045, USA.
[5]Department of Mechanical Engineering, University of Kansas, Lawrence, KS 66045, USA.
[6]KU Cancer Center, University of Kansas Medical Center, Kansas City, KS 66160, USA.
[7]Ulsan National Institute of Science & Technology, Ulju-gun, Ulsan, 44919, South Korea.

Correspondence to: Steven A. Soper, Department of Chemistry, Department of Mechanical Engineering, The University of Kansas, 1567 Irving Hill Road, Lawrence, KS 66045, USA. E-mail: ssoper@ku.edu

How to cite this article: Zhao Z, Wijerathne H, Godwin AK, Soper SA. Isolation and analysis methods of extracellular vesicles (EVs). *Extracell Vesicles Circ Nucleic Acids* 2021;2:80-103. https://dx.doi.org/10.20517/evcna.2021.07

Received: 1 Mar 2021 **First Decision:** 17 Mar 2021 **Revised:** 22 Mar 2021 **Accepted:** 25 Mar 2021 **Available online:** 30 Mar 2021

Academic Editor: Yoke Peng Loh **Copy Editor:** Yue-Yue Zhang **Production Editor:** Yue-Yue Zhang

Abstract

Extracellular vesicles (EVs) have been recognized as an evolving biomarker within the liquid biopsy family. While carrying both host cell proteins and different types of RNAs, EVs are also present in sufficient quantities in biological samples to be tested using many molecular analysis platforms to interrogate their content. However, because EVs in biological samples are comprised of both disease and non-disease related EVs, enrichment is often required to remove potential interferences from the downstream molecular assay. Most benchtop isolation/enrichment methods require > milliliter levels of sample and can cause varying degrees of damage to the EVs. In addition, some of the common EV benchtop isolation methods do not sort the diseased from the non-diseased related EVs. Simultaneously, the detection of the overall concentration and size distribution of the EVs is highly dependent on techniques such as electron microscopy and Nanoparticle Tracking Analysis, which can include unexpected variations and biases as well as complexity in the analysis. This review discusses the importance of EVs as a biomarker secured from a liquid biopsy and covers some of the traditional and non-traditional, including microfluidics and resistive pulse sensing, technologies for EV isolation and detection, respectively.

Keywords: Extracellular vesicles, molecular cargo, microfluidics, nanoparticle tracking analysis, resistive pulse sensing

An Erratum is published on 15 Sep 2021. You can check it here.

INTRODUCTION

Biomarkers secured from a liquid biopsy are generating significant interest in the research and medical communities due to the minimally invasive nature of acquiring them and the fact that they can enable precision medicine, which seeks to manage a variety of diseases using molecular signatures unique to the patient[1,2]. EVs are one of the many liquid biopsy markers that can be secured from a clinical sample, such as whole blood, saliva, urine, and cerebral spinal fluids.

Biological cells release vesicles of varying sizes through both the endosomal pathway or budding/blebbing from the plasma membrane. These vesicles are known by different names, including microvesicles (MVs), exosomes, and apoptotic bodies,which are collectively called EVs[3] [Figure 1A]. The particular subtype classification of EVs is based on their cellular origin and biogenesis[4]. MVs are heterogeneous, membrane-bound vesicles generated by budding/blebbing from the plasma membrane[5], and range from 100 nm to 1 μm in size. On the other hand, exosomes are the smallest category in the EV family with sizes ranging from 30-150 nm and are released into the extracellular environment after the fusion of late endosomes/multivesicular bodies with the plasma membrane. Finally, apoptotic bodies are generated due to programmed cell death called apoptosis, and range from 1-5 μm in size. Figure 1B shows the size variations of the different types of EVs[6].

EVs contain variable components including lipids, carbohydrates, cytokines, proteins, and nucleic acids, in particular RNAs[7]. Both the surface and intra-vesicle material of EVs originate from their host cells making EVs suitable biomarkers for disease management, such as diagnosis, monitoring response to therapy, and determining disease recurrence[6]. However, before analyzing EVs they must typically be "enriched" from the clinical sample because they are typically a vast minority in a mixed population.

There is now a pressing need to "enumerate" EV biomarkers and analyze their molecular contents to provide relevant information for disease detection and management. The challenge with liquid biopsy markers is the mass limits they imposed on the molecular assay. Even though EVs are high in numbers (10^6 -10^{13} EVs per mL of plasma), their small size limits the molecular content within a single EV. For example, a 150 nm (diameter) EV may contain approximately 10,000 nucleotides of nucleic acids. In addition, components present in a sample may interfere with the molecular processing, and enrichment can obviate this issue.

Enrichment and detection techniques can take advantage of either the physical properties of the EVs (size, density, electrical properties, and morphology) or their biological properties (antigen expression). The next few sections will focus on reviewing EVs' physical properties, intra-vesicle contents, diagnostic and therapeutic applications, isolation methods, and direct detection methods.

TYPES OF EVS

Microvesicles

Microvesicles are heterogeneous, membrane-bound vesicles that are 100 nm to 1 μm in size and are released from the surface of many cell types, including embryonic stem cells, neurons, and astrocytes, under both physiological and disease conditions[8]. MV biogenesis takes place through direct outward blebbing and pinching of the plasma membrane[8]. Platelets, red blood cells, and endothelial cells have been verified as a

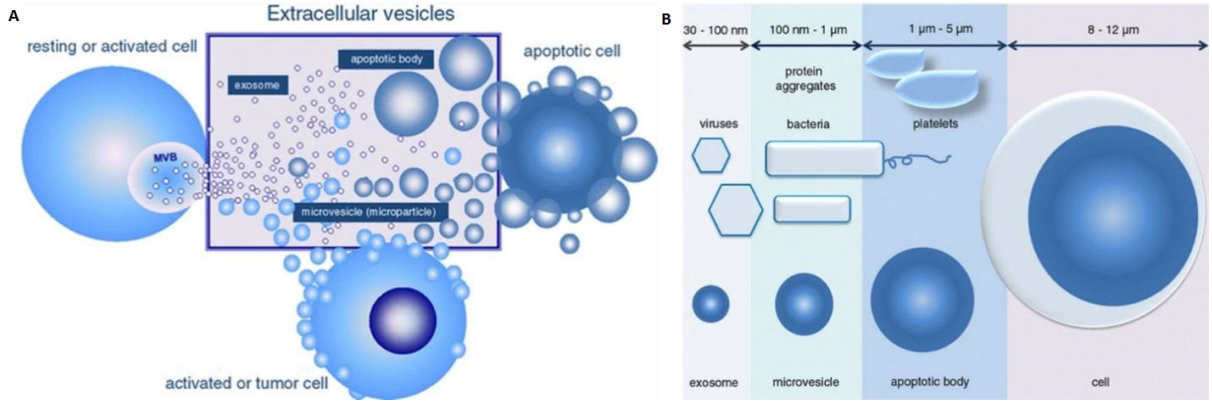


Figure 1. (A) Sub-types of extracellular vesicles including microvesicles, exosomes, and apoptotic bodies. (B) Size ranges of the three sub-types of extracellular vesicles of which exosomes are the smallest with a range from 30 to 150 nm. Microvesicles range from 100 to 1000 nm in size, but the size ranges from 100 to 400 nm when they are present in the circulatory system. Apoptotic bodies range from 1 µm up to 5 µm in size (Reproduced from[10]).

significant source of MV secretion, and tumor cells also constantly release MVs[9,10]. MVs are important in altering the extracellular environment, intracellular signaling, and facilitating cell invasion through cell-independent matrix proteolysis[11]. MVs can also contribute to the pro-invasive character of tumors and increase oncogenic intercellular transformation[12,13]. Differential centrifugation and flow cytometry are the commonly used isolation and detection methods, respectively[10,14].

Exosomes

Exosomes were first discovered by the Stahl and Johnstone groups in 1983[15,16]. Exosomes are small EVs with a size from 30-150 nm and can be produced by a majority of living cells[17,18]. Exosomes are secreted by exocytosis of multivesicular bodies and released into the intercellular environment[19]. As Figure 2 shows, hallmarks of exosomes include the tetraspanins (CD9, CD81, and CD63), ALG-2-interacting protein X (ALIX), and tumor susceptibility gene 101 (TSG101) protein[20]. The tetraspanins can serve as surface markers for exosome immuno-affinity isolation, and ALIX and TSG101 are commonly intravesicle biomarkers of exosomes[21,22]. In addition, exosomes are involved in many cellular functions such as metabolism and receptor transportation[20,23], horizontal transfer of mRNA and miRNA[24], and as a vector for oncogenic transfer[10]. Studies focused on exosomes include isolation and purification[25-28], surface and intra-vesicle protein marker analysis[29-32], cargo mRNA and miRNA analysis[6,33,34], secretion and uptake pathways[35-37], surface and cargo modification[38-41], drug delivery[42-44], and disease diagnosis and management[45-47].

Apoptotic bodies

Apoptotic bodies are generated as a result of programmed cell death and are primarily produced by cells undergoing apoptosis. Apoptosis occurs during cell-damaging or aging with the purpose of homeostasis. Cells can also show characteristic morphologies, including cell blebbing and shrinkage, nuclear fragmentation, and condensation/fragmentation of genetic material. Apoptotic bodies that are 500-1,000 nm in size are released as a product of apoptotic cell disassembly[8]. Like other types of EVs, apoptotic bodies contain protein, RNA, DNA, and other cellular fragments[48-51]. However, the only marker to recognize apoptotic bodies is phosphatidylserine (PS)[52].Apoptotic bodies coordinate many cellular membrane molecular patterns, including high-mobility group box 1, heat shock protein 90, and interleukin-33 to facilitate cell blebbing[53]. Also, the caspase-mediated activation of pannexin 1 (PANX1) signal pathway serves as a "find-me" signal for phagocytosis and further apoptotic cell removal[53]. The receptor locating

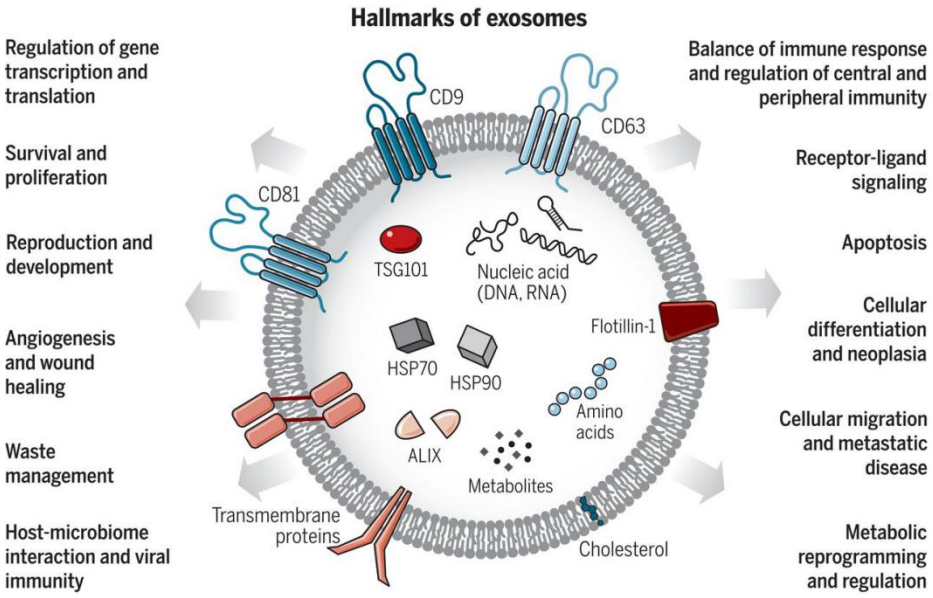


Figure 2. Exosomes are small EVs (sEVs) with the size range from 30 to 150 nm. Exosomes carry various types of molecules originating from the cell-of-origin including proteins, nucleic acids, lipids, and metabolites. Exosomes also play essential roles in cellular communication and regulation (Reproduced from[20]).

[PANX1[54], CX_3C-chemokine ligand 1 (CX_3CL1)][55], and uptaking [phosphatidylserine (PtdSer)[56], calreticulin (CRT)[57]] signaling pathway are well studied, but the detailed pathway on how cells are divided into small apoptotic bodies remains unraveled[48,49,53].

EVS AND THEIR MOLECULAR CONTENT

EV cargo mainly consists of various types of proteins and RNAs. Commonly found proteins in EVs are cytoskeletal, cytosolic, plasma membrane, and proteins that show post-translational modifications[58]. In addition, the tetraspanins, such as CD9, CD63, CD81, and CD82, have been found to be present in exosomes[58]. These transmembrane proteins are usually found on the surface of small EVs and can be used as targets for both small EV isolation and detection. However, recent studies have found that the tetraspanins can also be expressed on the surface of large EVs, including MVs and apoptotic bodies[59,60]. EVs can be secreted by most living cells, particularly tumor cells because of the continuous release and transfer of oncogenic information[17,18]. With the feature of containing host cells' hallmark proteins, tumor-related markers can be expressed on both EV surfaces and within the vesicle[61-63].

The EV membrane also contains different types of receptors or ligands to trigger intracellular signaling pathways via a simple interaction in order to initiate an uptake process to deliver the enclosed information into target cells. The well-studied receptors and ligands pairs for EV uptake include the C-type to P-selectin glycoprotein ligand-1[35,64], Galectins to Glycans[65,66], mucins to galectin-3[66,67], and PANX1 to purinergic receptor[53,55].

RNA is also an important biomarker for disease management because of the function RNAs play in genetic regulation. The RNA content of EVs has been studied using such techniques as next generation RNA sequencing and RT-qPCR[68]. Many different types of RNAs have been found in EVs, including mRNA, non-coding RNA, miRNA, and tRNA[58]. mRNA is a widely studied RNA type found in EVs. Although cellular mRNA has about 400-12,000 molecules, EV mRNA typically has < 700 molecules and can be

fragmented sections of mRNA and not full length transcripts[69,70]. Publications have shown that some types of mRNAs are only found inside EVs, but not expressed in the parental cells[24,71,72].

miRNAs are small non-coding RNAs of about 22 nt in length. miRNAs are best known as gene silencing agents of complementary mRNAs and serve to regulate gene expression[73,74]. Because miRNA is associated with gene expression regulation, upregulated mRNAs may not be translated into the expected protein due to miRNA interference[75]. miRNA has been found in body fluids with complementary RNA-binding proteins that prevent enzymatic degradation[76-78]. With the same purpose as carrying mRNA, EVs also serve as vectors to transport miRNA to recipient cells[68]. EV-related miRNAs have been studied for cancer, such as miR-21 and miR-210[79-81], and post-radiotherapy-related miRNAs such as miR-130a-3pand miR-92a-3p[34,82]. Understanding the RNA composition of EVs has become a critical endeavor for disease management.

DIAGNOSTIC POTENTIAL OF EVS

Due to the valuable cargo EVs can carry, they have been widely studied as potential biomarkers for different diseases[83-85]. However, processes such as anticoagulation and endotoxin tube contamination can affect EV concentration in blood, which complicates enumeration data[86-88]. One advantage of EVs as a biomarker over many soluble molecules in the blood like hormones and cytokines is the inherent protection of the EV cargo from degradation, thus keeping the cargo intact and functional. Hence, EVs can be released from any location and into the bloodstream making them easily accessible for liquid biopsies. Additionally, the literature has shown that EV quantity, phenotype, or cargo content can change during disease progression[89-92]. Because tumor cells constantly release EVs, tumor-related EVs in plasma are at higher concentrations compared to normals[89,93]. Therefore, understanding tumor-related EV molecular profiles can help provide a fingerprint for precision medicine.

EVs have also been studied as biomarkers for many non-cancer diseases, including diseases of the central nervous system[94], liver (liver damage in viral hepatitis, hepatocyte injury in alcoholic, drug-induced, and inflammatory liver diseases)[95], kidney (intrinsic kidney disease)[96], brain (stroke)[97], lung (Asthma)[98], arteries (atherosclerosis)[99] and radiation injury[34].

CONVENTIONAL METHODS OF EV ISOLATION

In order to analyze EVs' cargo, EVs of interest must be isolated in high purity and high yields from body fluids because non-diseased cells also generate EVs that can mask subtle molecular signatures of disease. With increasing studies conducted on EVs, many techniques have been developed to isolate EVs from liquid biopsies. Some of these isolation techniques select the entire EV sub-types irrespective of the cells of origin and others can be specific so as to isolate only the disease-related EVs. In the next few sections, different conventional isolation strategies will be discussed.

Precipitation and spin columns

Hydrophilic polymers, such as polyethylene glycol (PEG), reduce solubility by lowering the hydration of EVs and lead to precipitation[100] [Figure 3A]. These kits can be used to separate EVs using lower spin speeds with higher yields compared to ultracentrifugation (UC). Upon addition of precipitation reagents, the solubility of proteins is also decreased[101], and thus the isolate can contain protein impurities that can have a detrimental effect on downstream processing. Some of the advantages of precipitation reagents include preservation of EV integrity, no need for extensive equipment, selection pH close to the physiological range, and the possibility to process a large number of samples simultaneously[102]. However, poor reproducibility, impurities, and retention of polymer are a few drawbacks[103-105].

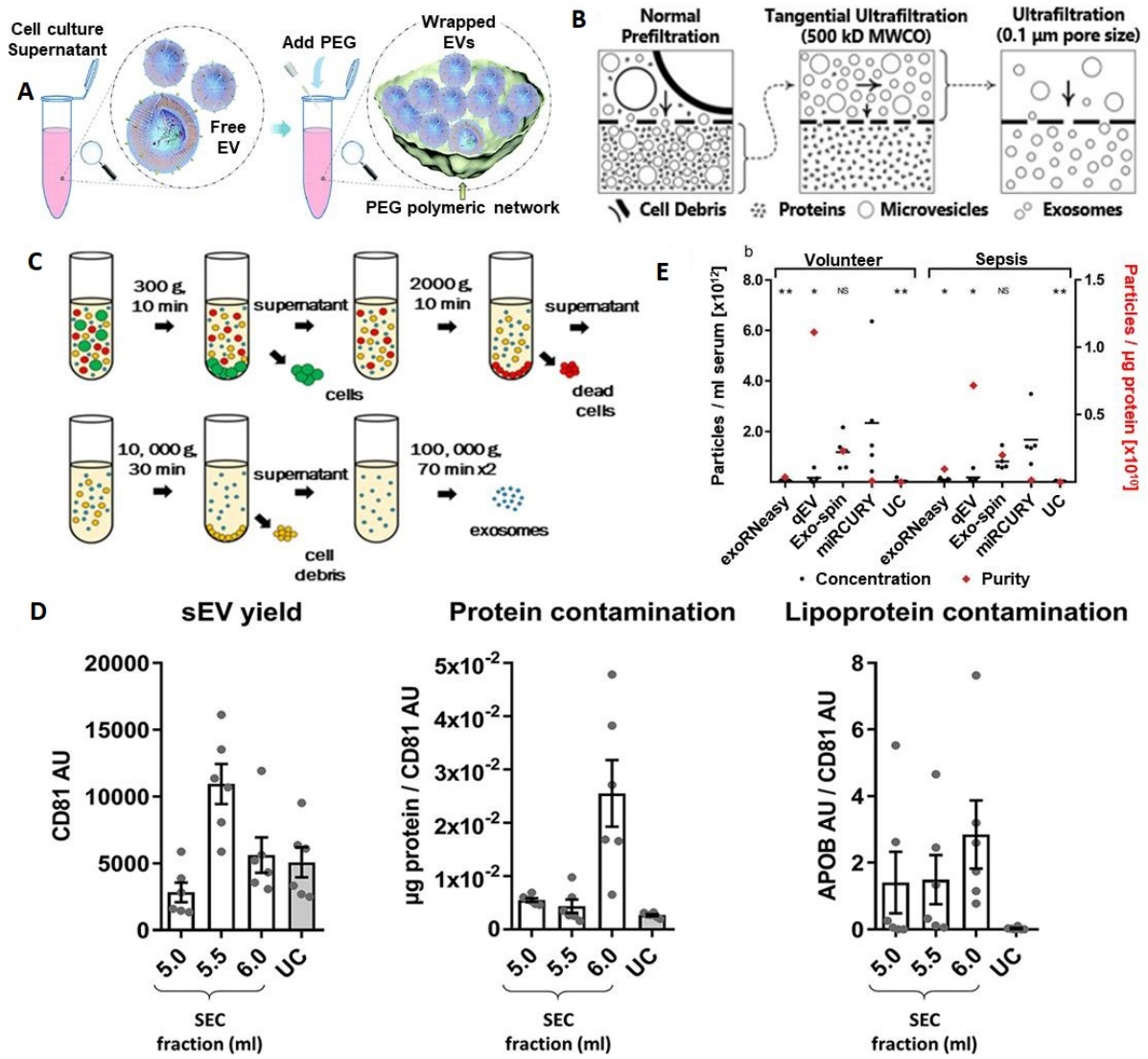


Figure 3. Conventional methods for EV enrichment. (A) Polymer-based enrichment: Precipitation with polyethylene glycol (PEG) (reproduced from Reference 100). (B) Filtration and ultrafiltration for EV isolation: normal prefiltration can collect sEVs and particles into the bottom layer of the culture dish. The bottom layer needs to be processed through tangential ultrafiltration, and the retentate is collected. Further ultrafiltration with expected pore size can be further processed and the EVs with a size smaller than the pore size will be present in the permeate (reproduced from[28]). (C) Ultracentrifugation for EV isolation (Reproduced from[111]). (D) Summary of yield and purity of sEVs isolated by SEC or UC: Normalization of APOB signal to CD81 content as an estimate of sEV purity from lipoproteins, also demonstrated almost 60 times higher APOB/CD81 ratio in the peak sEV fraction of SEC (5.5 ml) compared to the UC samples. SEC resulted in a higher yield of sEVs but with marked contamination by soluble protein and lipoproteins (reproduced from[116]). (E) Analysis of EVs by NTA demonstrates differences in size distribution. Black bars indicate the absolute number of vesicles isolated from 1 ml of serum; red diamonds plotted against the right x-axis represent vesicle purity defined as the particle to protein ratio. While precipitation most efficiently isolated EVs from serum, SEC-based isolation yielded fewer but more pure vesicles. Asterisks indicate significant differences in particle numbers compared to miRCURY. *$P < 0.05$; **$P < 0.01$; NS: not significant. All data are mean ±SD for five volunteers and five sepsis patients (reproduced from[117]). NTA: Nanoparticle tracking analysis.

Filtration

Filtration has been used as an isolation method for small particles based on size using nanomembranes[28,106]. Many times a sequential filtration or combined filtration with ultracentrifugation is used to provide high-grade exosomes [Figure 3B]. A modified polyethersulfone membrane is used for the pre-filtration of cell culture media, which can pass through the membrane. Then, tangential flow filtration

with a 500 kD molecular weight cut off hollow fiber filter is used to filter out proteins. A final step with a low-pressure filtration can only make the desired size (smaller than pore size) of particles present in the retentate. Sequential filtration can generate a throughput of 0.96 mL/h, and the size distribution of isolated EVs can be controlled. However, clogging and shear stress can be applied to the particle, damaging the EV particle[107,108].

Ultracentrifugation

Ultracentrifugation is based on separation of particles according to their buoyant density. To affect the enrichment of EVs, several UC steps are typically undertaken. First, the particles with high buoyant density like cells (300-400g), cell debris (2000g), aggregates of biopolymers, apoptotic bodies, and other structures with a density higher than EVs are sedimented [Figure 3C]. The resulting supernatant with EVs is ultracentrifuged at > 100,000g for 2 h, which yields an EV pellet[109-111].

In density gradient UC, a continuous density gradient including a sucrose or iodixanol density gradient and differential centrifugation is used[111]. In some cases, enriched EVs are further purified using filtration (0.1, 0.22 or 0.45 μm) or subsequent washing steps, which increases the purity of EVs but decreases the yield[112,113]. While UC can isolate EVs from large volumes of sample, some drawbacks include long isolation times (140-600 min), non-exosomal impurities, low reproducibility, and efficiency affected by the type of rotor, force, and sample type and only six samples can be processed in a cone ultracentrifuge[109,113,114]. Although UC methods yield low EV quantity compared to many other EV enrichment methods, Alvarez *et al.*[115] has reported that UC with a sucrose density gradient yielded high purity. EVs of the size range 20-250 nm can be isolated by UC with the isolated EVs appropriate for assaying RNA and miRNA[112]. UC and size-exclusion chromatography (SEC) have been systematically compared for isolating small EVs (sEVs; exosomes) in rat plasma and results [Figure 3D] revealed that SEC-sEVs had higher particle number, protein content, particle/protein ratios and sEV marker signals than UC-sEVs. However, SEC-sEVs also contained greater amounts of $APOB^+$ lipoproteins and large quantities of non-sEV protein[116].

Comparison of different EV isolation techniques

Comparison of different EV isolation kits revealed that the total number of particles isolated from serum was the highest for miRCURY (precipitation), followed by Exo-spin (Size-exclusion chromatography), qEV (Size-exclusion chromatography), UC, and exoRNeasy [Figure 3E]. Also, SEC-based isolation yielded EVs with significantly higher particle-to-protein ratios than all other methods, indicating less co-isolation of soluble proteins. Isolates derived from precipitation and UC, on the other hand, displayed the lowest ratios due to increased protein contamination[117]. Side-by-side analysis of four kits also showed differences in performance. The size distribution of the isolated particles was appropriate (40-150 nm), and ExoQuick™ Exosome Precipitation Solution (EXQ) generated a relatively high yield of exosomes. However, albumin impurity was abundant for all the evaluated kits. There was significant correlation of the exosomal miRNA profile and specific miRNAs between kits, but with differences depending on methods. ExoRNeasy Serum/Plasma Midi Kit and EXQ performed better in the specific exosomal miRNAs recovery[118].

Affinity selection

EVs can contain protein makers that represent the cells from which the EVs originated. Tumor-derived EVs can express essential tumor-related proteins used for cancer disease diagnosis or progress monitoring[58,119]. By targeting specific proteins on the surface of EVs using immunoaffinity-based approaches, a specific type of EV can be collected. A variety of proteins can be targeted as biomarkers for EV isolation including the tetraspanins such as CD9, CD81, CD63, and cancer-related markers such as EpCAM, CD24, and CA125. Antibodies can be immobilized on a substrate such as the surface of a microplate or beads, and bind the EVs onto their surfaces only if they express an antigen specific to the capture antibody. Using immunoaffinity,

the isolation can result in high specificity and purity for a particular EV subtype[120,121]. However, due to the cost of affinity-based assays, the isolation can only be applied with a small volume of sample, and EV-related proteins or RNA yields can be limited[120,122].

The primary advantage of affinity isolation of EVs is that if the correct targeting surface antigen is used, the isolated EVs can be associated predominately to those that are disease-associated that can be advantageous for downstream molecular analysis. However, if the affinity isolation uses the tetraspanins, all EVs, in particular the exosomes and MVs, will be in the isolate.

MICROFLUIDICS FOR EV ENRICHMENT

Many of the recently reported platforms for the isolation of EVs have been based on the use of microfluidics for several reasons including their ability to be integrated to post-enrichment processing steps such as enumeration and/or molecular profiling of the EV cargo. The enriched EVs can be enumerated[123-128], surface and cargo proteins analyzed[29,123,124,129-131], RNA profiled[33,125,128], or diagnostics performed[132-134]. By including the appropriate micro-or nanoscale structures within the chip, approaches including affinity selection, filtration, centrifugation, viscoelasticity, and acoustic waves can be used for EV isolation using a microfluidic.

Affinity enrichment

Affinity enrichment can enrich primarily disease-associated EVs, improving the quality of the molecular data secured from the isolate[135]. The ExoChip is an early example of a microfluidic used for affinity enriching EVs[124]. The ExoChip was fabricated using soft lithography and polydimethylsiloxane (PDMS) with surface-attached antibodies targeting CD63. Clinical serum samples were analyzed with immune-electron-microscopy and Western blotting used to confirm isolation of the EVs.

Many microfluidic devices used EV-specific markers, such as the tetraspanins because in some cases disease-specific can be downregulated during disease progression. A newer version of the ExoChip (^{new} ExoChip) used phosphatidylserine for enrichment[131] [Figure 4A]. PS is expressed in the lipid bilayer of cancer-related EVs. The ^{new}ExoChip achieved 90% capture efficiency of cancer-related EVs with the affinity-captured EVs released by Ca^{2+} chelation.

A graphene oxide/polydopamine (GO/PDA) nano-interface was used to increase the EV capturing surface area[123] [Figure 4B]. The capture antibody targeting CD81 and detection antibodies targeting CD81, CD63, and EpCAM were used to characterize the EVs and remove interferences from the sample. The assay provided a detection limit of 10^6 particles/mL. Compared to the direct surface modification of GO or PDA only, the GO/PDA nano-matrix increased antibody capture efficiency of EVs by ~2-fold.

An approach was reported using multiscale integration by designed self-assembly (MINDS) 3D nanostructures as the capture surface for EVs[129] [Figure 4C]. With MINDS, flow streams can pass through a bumper structure and a nanostructured herringbone (nano-HB) results in enhanced contact time of the EVs with the capture surface. This offered a limit-of-detection of 10 EVs/μL and a total minimum detectable particle number of 200 per assay. For verification of the platform, 20 ovarian cancer patients and 10 non-cancer control plasma samples were processed, and differences were achieved between the two groups in terms of the number of enriched EVs.

It is difficult to mass-produce PDMS-based microfluidic devices[136]. As an alternative, thermoplastics are attractive because of their ability to be mass-produced and the simple modification protocols that can be

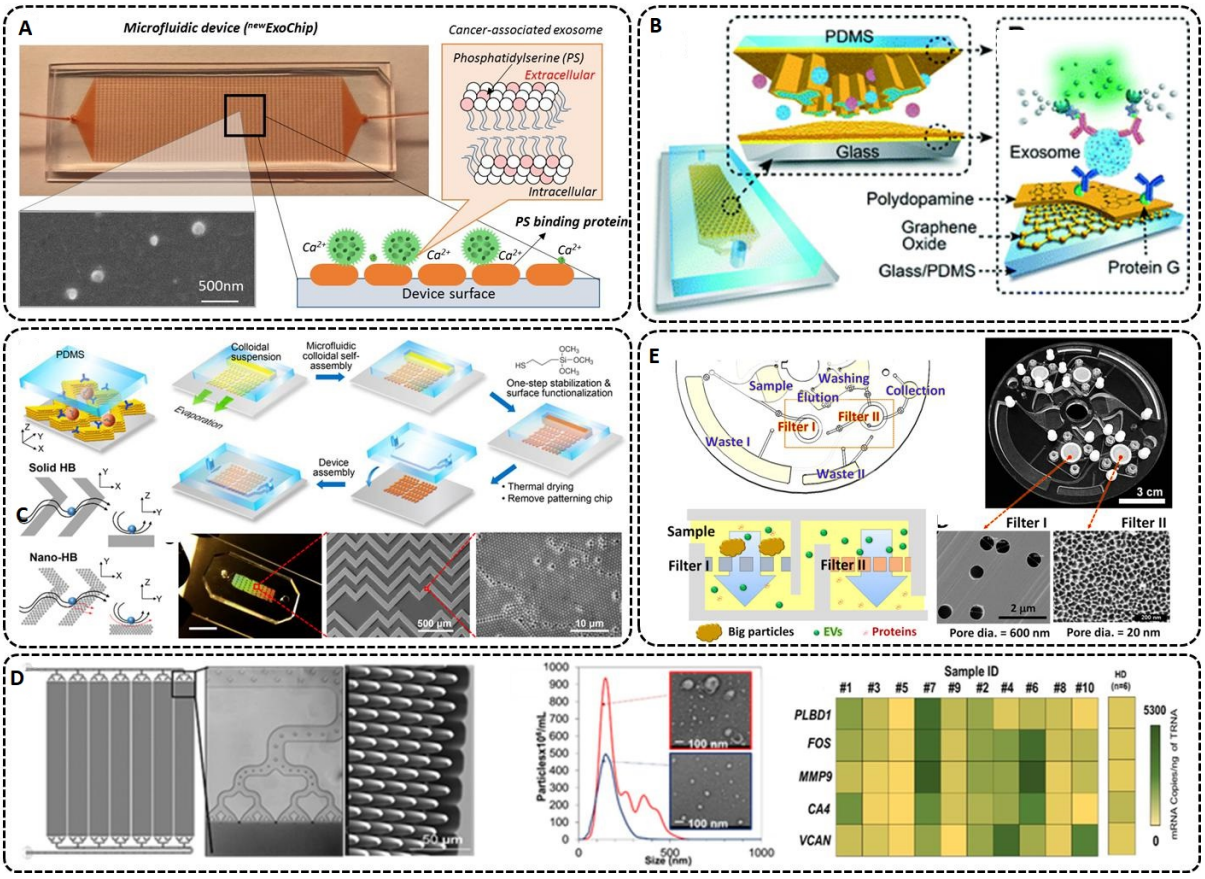


Figure 4. (A) newExoChip design, which features 30 × 60 circular patterns with a diameter of 500 μm in standard glass microscope slides. The mechanism of capture and release of cancer-associated exosomes using Ca^{2+}-dependent binding between PS and annexin V and ethylenediaminetetraacetic acid (EDTA)-based Ca^{2+} chelation. The micrograph shows capture and released exosomes (reproduced from[131]). (B) Nano-interfaced microfluidic exosome platform (nano-IMEX). Schematic of a single-channel PDMS/glass device with expanded-view highlighting the coated PDMS chip containing an array of Y-shaped microposts. The surface of the channel and microposts coated with graphene oxide (GO) and polydopamine (PDA) as a nanostructured interface for the sandwich ELISA with fluorescence signal amplification (reproduced from[123]). (C) 3D herringbone nanopatterns are designed on a microfluidic device with the ability to detect tumor-associated EVs in plasma with a minimum of 200 vesicles per 20 μL. The nano-structures were used to increase the surface area, content mass transfer, and EV capturing speed, and reduce the hydrodynamic resistance (reproduced from[129]). (D) Microfluidic device made from cyclic olefin polymer (COP), which allows for high-rate production at a low cost to accommodate diagnostic applications. CAD drawing of a 7-bed EV Microfluidic Affinity Purification (EV-MAP) showing the distribution channels and the diamond-shaped micropillars of the device. NTA and TEM images of EVs isolated from a clinical sample by PEG precipitation and affinity selected with anti-CD8 mAb using the EV-MAP device. Heat map analysis of clinical samples (marked with numbers) and healthy donor for 5 genes whose up-regulation is associated with acute ischemic stroke (reproduced from[139]). (E) ExoDisc integrated system that combines a sequential filtration and centrifugation steps used for low viscosity fluids. The EVs are collected between filter I and filter II. The filters can be replaced with different pore sizes for different expected size range selection (reproduced from[33]).

employed to change their surface chemistry[31,137,138]. A cyclic olefin copolymer EVHB-chip was manufactured with micro-injection molding and was designed to isolate tumor-specific EV-RNAs[125]. The herringbone structure was compared to a flat channel surface and the results indicated that the herringbone device captured ~60% more EVs. The device could process a wide range of sample volumes (100 μL to 5 mL) with a limit-of-detection of 100 EVs/μL.

Another group developed a microfluidic device using thermoplastics made via micro-injection molding[139]. A 7-bed EV Microfluidic Affinity Purification (EV-MAP) device contained diamond-shape pillars [Figure 4D] with a 10 μm diameter and 10 μm spacing to allow for high throughput processing for enriching EVs

via affinity selection (1.5 million pillars per chip). The device was used for diagnosing acute ischemic stroke patients using exosomal mRNA. mRNA expression of CD8+ EVs indicated that for genes upregulated during an ischemic stroke event, the EV-MAP device was successful in enriching EVs from clinical plasma samples, and gene profiling the EVs via droplet digital PCR for identifying stroke patients with a total processing assay time of 220 min. When the EVs were isolated using PEG precipitation, which isolates the entire EV subtypes, mRNA expression differences for stroke patients were not observed.

Centrifugation and filtration enrichment

Filtration can be used as an EV microfluidic isolation method. An Exodisc was reported using a combination of centrifugal forces and nano-filtration[33] [Figure 4E]. With a centrifugal force limit of 500g, EV sizes of 20-600 nm could be collected between two nano-filters. Filter I (600 nm pore size) was used to remove large particles, and Filter II (20 nm pore size) was used to enrich the EVs and exclude free proteins. The entire EV population was collected in 30 min with a recovery of 95%. Another platform with a combination of centrifugal force and filters was reported for inline EV detection by flow cytometry[126]. The EVs were isolated by anti-CD81 antibodies and with affinity microbead incubation, the enriched EVs could be concentrated and stained with a fluorescent dye.

Contactless EV enrichment methods

Researchers have also focused on contactless methods for EV enrichment using microfluidics, which takes advantage of the fluid associated with a microchannel and/or microstructures in the channel to affect the EV enrichment process. A microfluidic viscoelastic flow was developed for size-dependent isolation of EVs[127]. Poly(oxyethylene), PEO, was added into a sheath fluid at a concentration of 0.1% to maintain the feed solution at a particular viscosity. The particles were driven by an elastic force that situated particles in certain flow lines based on the size of the particle with larger particles traveling towards the center of the channel. The authors were able to demonstrate sEV recovery of ~80%.

Microfluidic viscoelastic flow was also developed using an acousto-fluidic device for EV isolation[128]. The platform included two unique surface acoustic wave modules that were operated at 19.6 MHz for cell isolation and 39.4 MHz for EV isolation. The acoustic isolation was based on size because of the deflection caused by the acoustic pressure. The cell removal rate was > 99.999%, which resulted in 75% to 90% reduction of red blood cells. Using the modules in series, the isolation of 110 nm particles from whole blood yielded > 99% recovery, and the purity of the sEVs was ~98.4%.

METHODS FOR EV DETECTION

Following isolation/enrichment of EVs, the EVs must be enumerated and their molecular content analyzed in many cases. For molecular cargo determinations, methods that can be used for protein or nucleic acid determinations include Western blotting, ELISA, RT-qPCR, and next generation sequencing. These methods rely on the disassembly of the EVs so as to analyze their intra-vesicular content. In spite of the high numbers of EVs found in clinical samples there are challenges when attempting to analyze their molecular content. For example, in spite of the exponential amplification of cDNA following reverse transcription, a certain mass of mRNA or miRNA must be secured to see a detectable signal. This is further complicated by the fact that most EVs do not contain full-length transcripts, and as such, the polyadenylated tail used for priming for the reverse transcription step may not be present and the yield of cDNA would be low. However, using random hexamer primers for reverse transcription as opposed to poly dT primers that bind to the polyadenylated tail of full length mRNA transcripts can address partially this challenge[140,141].

Because the molecular assay requires lysis of the EV to release the intra-vesicular content, the population and morphological properties of the EVs must be determined in advance of the molecular analyses. Therefore, it is necessary to conduct assays to assess population and morphological properties of the enriched EV fractions prior to the molecular assay.

A challenge with intact EV analysis includes the diverse size range of the vesicles (30-1000 nm), their low mass loads (for a 150 nm diameter vesicle, may contain ~10,000 nucleotides of various nucleic acids, and 10-100 protein molecules), and their relatively high particle numbers. As opposed to biological cells, which are 1-100 μm in diameter, special types of techniques must be used to characterize and count the intact vesicles due to their small size. For example, while conventional flow cytometry can be used for biological cells, variants of flow cytometry must be considered for enumerating EVs. In addition, while conventional Coulter counters can be used to enumerate biological cells, nano-Coulter counters must be used to enumerate EVs.

Current methods that can directly analyze EVs from a physical perspective include: (1) size and concentration analysis Nanoparticle tracking analysis (NTA), resistive pulse sensing (RPS), which can provide information on the size distribution of EVs and estimate concentrations; (2) surface protein expression analysis of EVs, which can determine the type and amount of protein expression by labeling with specific antibodies and fluorescent reporters that can permit the use of nano-flow cytometry; and (3) electron imaging of EVs. Direct imaging techniques include Transmission electron microscopy (TEM), Scanning electron microscopy (SEM), and atomic force microscopy (AFM), which can visualize the overall structure of the EVs including their size. In the sections that follow, a discussion on NTA, electron microscopies, nano-flow cytometry, and RPS will be provided for performing concentration and morphological analysis of EVs [Table 1].

Nanoparticle tracking analysis

NTA is a commonly used method for size and concentration determinations of EV samples[142-144]. Both dynamic light scattering and Brownian motion are the essential processes used to determine the size and concentration of particles using NTA. Figure 5 shows the measurement principles of NTA[145]. A laser beam illuminates the sample cell and the scattered laser beam travels through the objective of the microscope, which is analyzed by a CCD camera. The Brownian motion of each particle can be recorded and analyzed by the Stokes-Einstein equation [Figure 5], where D is the diffusion coefficient and calculated by the mean-square of particle movement, K_B is the Boltzmann's constant, T is temperature, η is the solution viscosity, and d_h is the particle diameter. From this equation, the particle's d_h can be calculated if the solution viscosity and temperature are known. In addition, by analyzing the particle presenting scattered radiation event frequency in each of the CCD image frames, concentration information can also be secured.

Considering the calculation is based on particle diffusion, NTA is typically useful for analyzing small particles with a size between 10 and 1,000 nm in diameter. NTA performance for monodispersed and polydispersed homogeneous particles has been confirmed in previously published work, while the performance for non-homogeneous particles, such as EVs or biological vesicles, is still under development[146].

In past studies, researchers have found that the introduction to a variety of parameters can increase the variability of results by up to 50%, including the threshold setting of the camera, the source of the EV sample, small vibrations, and even the method of operation[143,144,147]. Some state that sample dilution, camera grade, version of the analysis software, and the sample's size distribution should also be considered for an

Table 1. Comparison of EV detection techniques

EV Detection Technique	Principle	Potential Advantage	Potential Disadvantage
Nanoparticle Tracking Analysis (NTA)	Dynamic light scattering and Brownian motion	Straight forward operation; Both size variation and concentration information can be collected; Available addon parts for fully automatic operation	Sensitive to vibration; Contamination particles can also be included; High cost for the instrument and addon parts
Electron microscopies.	Electrons as the source of illumination	High-resolution images; Direct illumination for EV morphology	High cost for the instrument; Not appropriate for quantitative analysis; EV morphology may be damaged by the sample preparing steps
Atomic force microscopies	Scanning cantilever over the surface	High-resolution images; Ture 3D image with surface topology determinations	High cost for the instrument; EV morphology may be damaged by the scanning cantilever
High-Resolution Flow Cytometry	Light scattering or fluorescent excitation	Sub-type EV labeling and detection; Principle is applicable in micro/nano-fluidic technology for better sensitivity	Sensitivity limitation for particles size < 200 nm
Resistive pulse sensing (RPS)	Nanopore blockage with changes of current or potential	Higher sampling frequency comparing to optical sensing; Principle is applicable in micro/nano-fluidic technology for better sensitivity	Fabrication with intricate nano-structures, Small sampling efficiency; Calibration is required for each nanopore design

accurate EV size and concentration determination[143,145,146]. A study encompassing the detection and analysis of EV samples, microvesicle samples, artificial vesicle samples, polystyrene latex beads, and silica microspheres with NTA has been undertaken[143] [Figure 6]. For artificial vesicles and polystyrene beads, the size variation and concentration were 3% and 9%, respectively. However, differences in the size of the EVs ranged from 1% to 6% and concentration varied from 5% to 18%. NTA also has some other drawbacks, such as a large sample size requirement (> 250 μL), an limited dynamic range (10^6-10^9 particles/mL), and only low viscosity samples can be analyzed, and need vibration free environment for analysis.

Electron and Atomic force microscopies

Electron microscopy can be used to image nanoscale samples, including EVs. In some cases, a perception bias may be introduced with imaging location selection, and it is also challenging to get an overall population estimation when the imaging areas are manually selected. However, electron microscopy, which includes TEM and SEM, is still a primary option when the morphology of EVs needs to be determined. Both electron microscopies use a beam of electrons, while TEM produces images using electrons transmitted through the sample and SEM analyzes the scattered electrons. TEM is most often used to collect information from the internal structure of the EV, while SEM can be used to interrogate surface structure. The resolution of both TEM and SEM can be as small as 1 nm[148]. However, the high-resolution advantage of TEM can be circumvented by sample preparation needs for EVs, which requires fixation and dehydration before imaging. Unlike cells with a cytoskeleton, EVs do not have an internal supporting structure. When the EV sample is dehydrated, the vesicle can form a cup-shape with loss of original morphology[47,129,149,150] [Figure 7A and C]. Several studies have shown that EVs have a sphere-shaped morphology[151] [Figure 7B]. Other papers have reported that EVs in SEM still show a cup-shaped morphology because the EV samples also undergo the same fixation and dehydration process [Figure 7C]. To overcome sample deformation, cryo-TEM can be used. For cryo -TEM, the sample can be placed in vitreous ice at the temperature of liquid nitrogen to eliminate the fixation and dehydration steps[152,153].

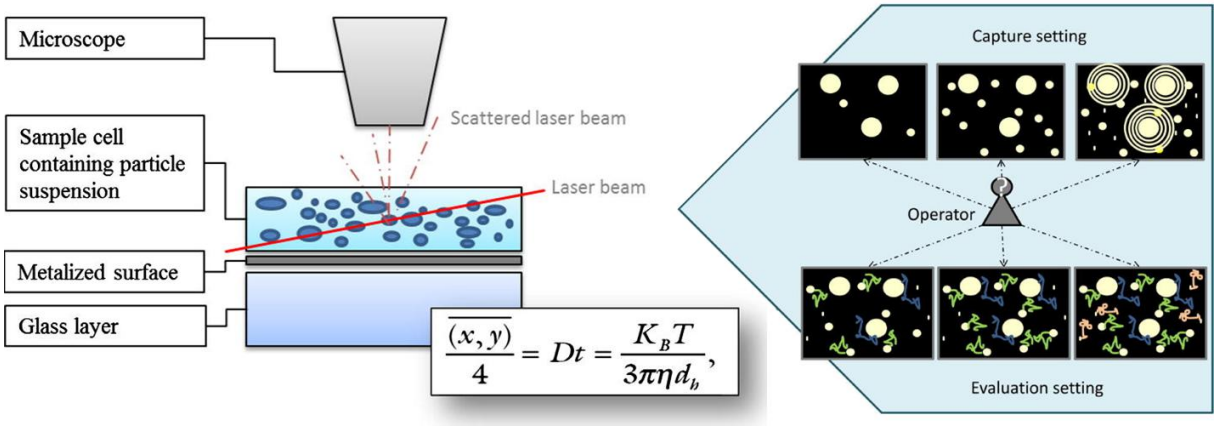


Figure 5. The principle of NTA measurements and the Stokes-Einstein equation for the analysis of particle size (reproduced from[145]).

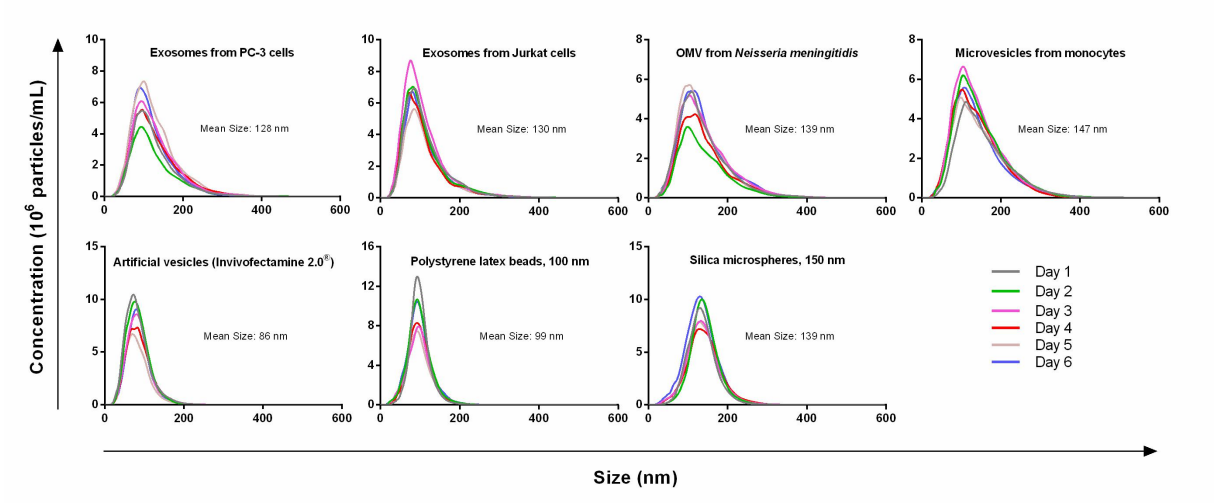


Figure 6. Concentration variations for different types of samples including EVs from PC-3 cell culture media, EVs from Jurkat cell culture media, Outer membrane vesicle from *Neisseria meningitidis*, microvesicles from monocytes, article vesicles, polystyrene latex beads (100 nm), and silica microspheres (150 nm). The samples were tested on 6 different days and the variation is from 1% to 18% (Reproduced from[143]).

AFM can record surface structure using a probe and laser reflection. A cantilever (i.e., probe) can deform while it interacts with the surface of the sample and the deformation of the probe caused by surface morphological changes can be sensed by laser reflection using position-sensitive photodiodes. AFM can obtain a true 3D image of surface structure and is commonly used for surface topology determinations[154] [Figure 7D]. However, because EVs do not have an internal supporting structure, the vesicles tend to deform during sample preparation and imaging. For EV sample probe scans with monoclonal antibody immobilization are usually combined for better imaging quality[155,156].

High-resolution flow cytometry

Flow cytometry (FC) is frequently used for cell analysis providing the quantitative information of markers on the surface and internally to the cell. Conventional FC is typically used to analyze particles with a size > 300 nm. As Figure 8A shows, FC uses a laser beam with a specific wavelength, which impinges on a sample stream consisting of single particles arranged in a single file line generated by a sheath flow. The particles in

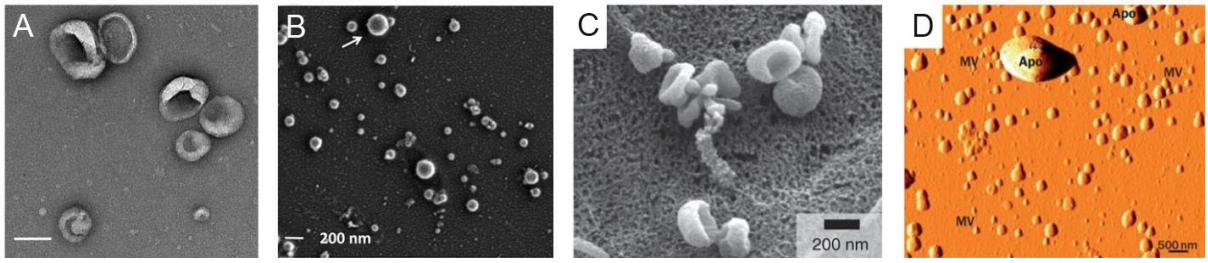


Figure 7. (A) Transmission electron microscopy image of EVs (scale bar = 100 nm). (B) Scanning electron microscope image of EVs showing the circular shape of the EVs (reproduced from[151]). (C) Scanning electron microscope image of EVs, which shows cup-shaped EVs (reproduced from[150]). (D) Atomic force microscope image for EVs (reproduced from[156]).

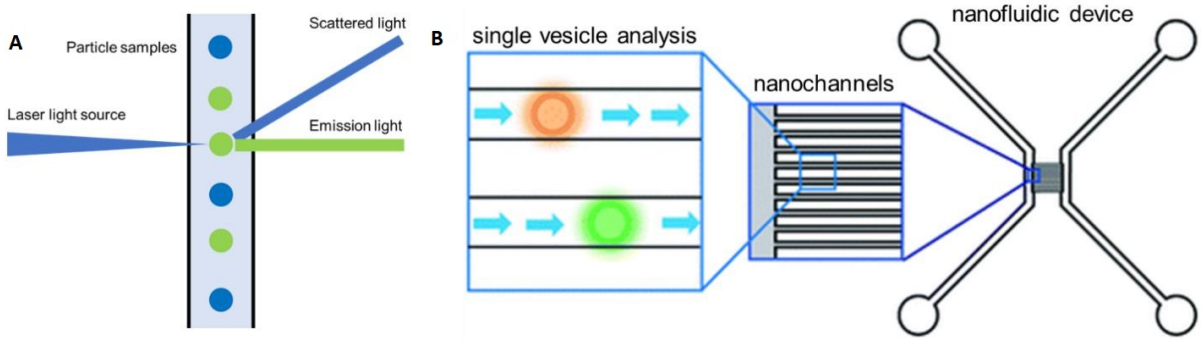


Figure 8. (A) The principle of flow cytometry. (B) A flow cytometry platform designed by Friedrich *et al.*[164] The nanofluidic device contained 100 nanochannels with a width of 300 nm and the dye-labeled EVs could be sensed and recorded by a fluorescent microscope (reproduced from[164]).

the stream can scatter light from which critical information can be secured. For example, the scattered light can be used to determine particle size. Another functional mode of FC is fluorescence readout, which is typically produced by labeling certain cellular organelles or molecules with fluorescent labels. Because the specific biomarker is dye-labeled, FC can collect information that includes the expression level of the marker of interest. For example, FC can be used to analyze the cytotoxic T-lymphocyte related immune response by labeling CD8 expressing cells.

In recent years, FC has also been applied for quantitative analysis of EVs. However, FC has a sensitivity limitation when it is applied to particles with a size smaller than 200 nm[157-159]. To overcome this drawback, the EV membrane is usually over labeled with a lipophilic dye, such as PKH26 or PKH74 to increase signal intensity[160,161]. The EV can also be analyzed indirectly when an adapter is applied[162,163]. The adapter typically carries a large quantity of fluorescent molecules to enable detection. Instead of directly sensing the EV, a well-calibrated adapter can provide higher intensity readouts by FC.

Friedrich *et al.*[164] developed a nanofluidic device to analyze EVs using FC [Figure 8B]. In this case, the sensing component consisted of a fluorescence microscope and an array of nanofluidic channels used as the flow cell. The device contained ~100 nanochannels with a size of 300 nm (width) × 300 nm (depth). Each nanochannel served as an individual FC sheath flow sampling unit and only 20 μL of sample was necessary for a typical measurement. The dynamic range of the nanofluidic device was from 10^{10} particles/mL to 10^{14} particles/mL. However, with this FC format, only concentration information of the EV sample was provided, but needed highly specific pre-isolation before sample readout[159,165].

Resistive pulse sensing

RPS was first developed in 1976 for viral particle detection and characterization[166,167]. In 1996, Kasianowicz *et al.*[168] utilized the biological nanopore as a Coulter counter for single-stranded DNA, and soon the RPS principle was applied to DNA sequencing due to different signal shapes of the four canonical DNA bases[169,170]. In recent years, RPS with flexible pore sizes and shapes has been used for EV concentration determinations and size analysis[147,171] [Figure 9].

Equation 1.

$$\Delta E = \frac{E d^3 \left[1 - 0.8(d/D)^3\right]^{-1}}{LD^2(1 + 4\rho/D\rho_s)(1 + \alpha)} \longrightarrow \Delta E = \frac{d^3}{\left[1 - 0.8(d/D)^3\right]} \cdot Constant$$

The RPS principles generate an output that can either be a change in potential or current measured across the nanopore structure. Whenever a particle moves through the nanopore, a proportion of the carrier electrolyte is replaced, which creates a change in the resistivity across the pore[172] [Figure 10A]. The change in voltage across the pore can be described using Equation 1, where ΔE is the voltage change between the occupied and unoccupied pore, E is the applied potential, ρ_s is the pore surface resistivity, α is the pore resistance to load resistance, L is the effective length of the nanopore, d is the particle diameter, D is the pore diameter, and ρ is the fluid resistivity[166,167]. For most cases, the majority of the parameters remain constant when a rigid pore and a homogeneous electrolyte are used for the RPS measurement. Thus, the size (d) of the particles in the sample can be determined by analyzing the amplitude of the electrical event (ΔE). In addition, with a known flow rate and event number, the concentration of the particles in the sample can be obtained as well. Equation 1 is primarily applicable to non-conductive particles because additional parameters must be considered for conductive particles including surface charge, particle charge density, and the permeability coefficient[167,173]. Also, for permeable biological vesicles, the particle resistivity may be lower than the carrier electrolyte due to the internal composition of the particle. As a result, some particles can produce the opposite polarity of signal compared to non-conductive particles[173-175].

Synthetic RPS sensors can be fabricated in a controllable fashion, which generates the possibility of unique measurement opportunities compared to naturally occurring (i.e., biological) nanopores, such as altering the nanopore shape or placing nanopores in-series or in-parallel. The nanopore in-series can provide additional information about particle movement and generate the zeta potential of the particle. When the nanopores are placed in-series for monodispersed samples, the system can provide precise flow rate feedback, which can help to control the stream flow in real time[176]. When the nanopore in-series is used for polydisperse samples with a known flow rate, particles with different charge densities can provide different event duration. Nanopores in-parallel are another design strategy that can be used to increase sampling efficiency and throughput. When the nanopores are set up in parallel with individual electrodes, each nanopore will provide information simultaneously from the output circuit[177-179]. It is also feasible to couple the RPS with an EV isolation microfluidic chip that can be used to analyze the EV sample on-chip negating the need for off-loading the enriched EVs for analysis by NTA. With real-time electrical signal readout, RPS can provide EV sample information during the isolation/elution phases of the assay[172,175].

Compared to optical sensing methods for EV quantification, such as NTA or flow cytometry, RPS can overcome some of their inherent drawbacks. For example, RPS can provide a faster sampling rate, up to 1000× higher[143,172,180]. For optical sensing, the exposure time has to be optimized to the millisecond or

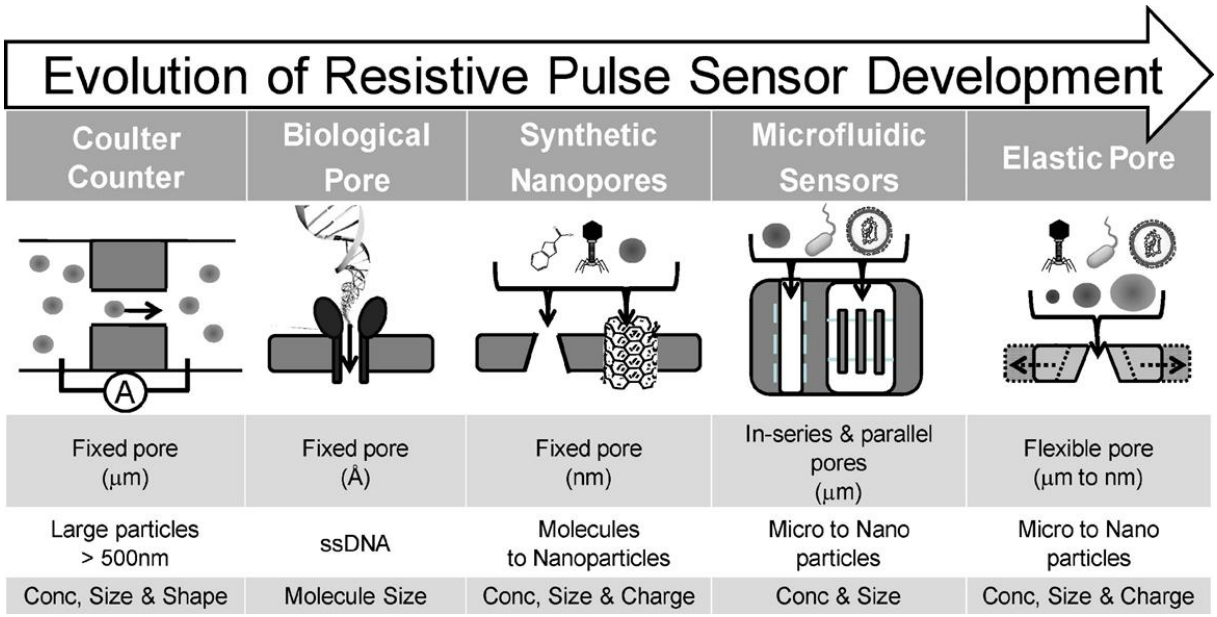


Figure 9. The evolution of resistive pulse sensing (RPS) from fixed pore with micro-scale to flexible pore with micro- to nano-scale sizes. RPS can also be applicable as a Coulter counter for EV analysis with the proper sized pore (reproduced from[171]).

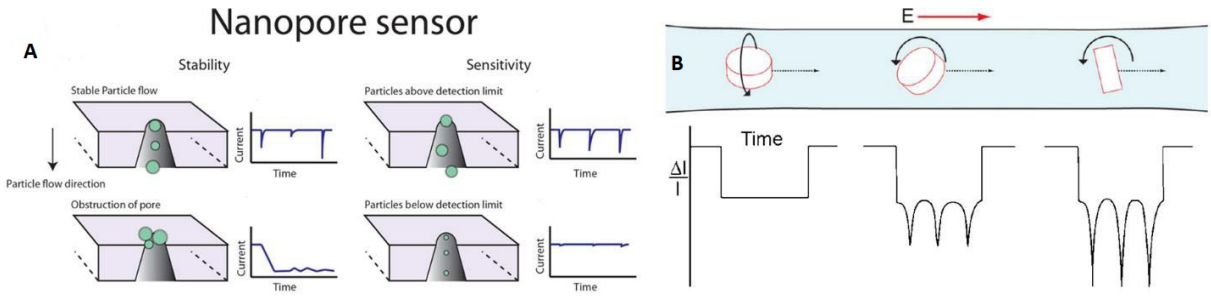


Figure 10. (A) The principle of RPS measurements with constant potential or current clamping across the measuring pore. The amplitude profile shows a relationship with particle size. The particle size distribution information can be determined by analyzing the event amplitude, and the event frequency can be analyzed for concentration information (reproduced from[172]). (B) The RPS is also used to study the particle shape, movement, and interaction with the solvent. The event can express the particle shape and also the rotation level and axis (Reproduced from[183]).

second timescale, which can make the sampling frequency ~1000 per second. The electrical signal recording for RPS can typically be set to 500 kHz[181]. In addition, the broad range of sampling frequencies for RPS can increase the dynamic range of the assay, from 10^5 to 10^{14} particles/mL[173,181,182]. RPS is also used to collect information about particle shape and movement profile. Figure 10B shows a particle shape and movement signal trace demonstration that includes a disc-shaped particle translocation with different levels and axis of rotation[173,183].

RPS does have limitations for the detection of nanoscale particles. Firstly, because of the nanostructure the sampling efficiency and detection speed can be small with the majority of RPS platforms processing samples in the nanoliter to picolitre volume scale[173,184]. The nanopore in-parallel does make it possible to overcome this limitation, which can linearly increase the processed sample volume based on the number of pores in parallel[173,185]. On the other hand, increasing the through-pore transport speed or decreasing the sampling frequency and bandwidth can decrease the measurement sensitivity.

CONCLUSIONS

As liquid biopsies are growing tremendously for applications in a variety of disease management scenarios, EVs are becoming an important target due to their biological and physical properties, including their relatively high abundance and the molecular information they carry[186-189]. EVs are rich in proteins and RNAs associated with their cell of origin, and these molecular markers have been shown to be useful for screening patients as well as being used to track disease progression[30,190,191]. As particles secreted by cells, EVs can easily pass through a series of biological barriers and travel throughout the circulatory system to their intended location[18,134,192,193]. This property allows EVs to be used as vehicles for drug delivery as well. EVs have also been studied as biomarkers for many non-cancer diseases, including the central nervous system[94], liver (liver damage in-viral hepatitis, hepatocyte injury in alcoholic, drug-induced, and inflammatory liver diseases)[95], kidney (intrinsic kidney disease)[96], brain (stroke)[97], lung (asthma)[98], arteries (atherosclerosis)[99], and radiation injury[34]. In any case, EV isolation/enrichment and quantification have become an important topic for both disease diagnostics and therapeutics. This arises from the fact that EVs are not just released from diseased cells, but non-diseased cells.

The challenge is that EVs must be enriched from a clinical sample prior to analysis of their molecular content and current methods for EV isolation are sometimes inefficient because they require a large volume of sample (UC) or alter the overall structure of the EV[120,194-196]. In addition, most traditional methods of EV isolation enrich the entire EV population consisting of both diseased and non-diseased EVs that can complicate the molecular analysis phase of the assay. Thus, the type of enrichment must be judicially chosen to match the application need. For example, if gene expression of the mRNA cargo from EVs is used for the application, endogenous expression of specific gene transcripts that may be found in non-diseased EVs must be taken into consideration because this may mask the gene expression from the mRNA found in diseased EVs only. Therefore, selection of EVs from the clinical sample using a highly specific disease-associated affinity agent may be required instead of using a non-specific enrichment protocol, such as UC. In addition, the clinical sample type and variation in sample collection and preservation may affect the quality of EVs selected and/or their yield during enrichment. As such, the EV field may need more detailed and clarified standardization protocols to minimize the variation between results emanating from different research laboratories[197].

In many protein or nucleic acid assays using EVs for diagnostics (e.g., ELISA, PCR), high purity of the input sample is required to secure the necessary clinical information[198-200]. By applying micro/nanofluidic technology, high throughput and precise enrichment using affinity capture can secure a higher purity of disease associated EVs compared to many conventional or benchtop methods that isolate the entire EV population irrespective of cell-of-origin. However, these technology platforms are only capable of processing microliter sized sample inputs indicating that sampling statistics may be a concern or securing sufficient molecular cargo to feed into conventional molecular processing pipelines, such as NGS or even droplet digital PCR. Some microfluidic platforms that use label-free or contactless technologies can compensate for this shortfall of total process volume but at the expense of sample purity[127,128,201].

In most cases, whatever molecular processing strategy is used, the intact EVs must be characterized including concentration, size distribution, and morphology, which are primarily based on electron microscopy, AFM, FC or NTA[27,143]. Recently, RPS has also been used for EV size and concentration determinations[157,202]. RPS, because it can be integrated into a microfluidic chip, can allow for straightforward analysis of particle physical characteristics and enumeration data following enrichment into a single device for potential point-of-care testing applications that use EV-based liquid biopsies.

DECLARATIONS

Authors' contributions

Wrote and reviewed the manuscript: Zhao Z, Wijerathne H, Godwin AK, Soper SA

Availability of data and materials

Not applicable.

Financial support and sponsorship

The authors thank the NIH for financial support of this work (NIBIB P41-EB020594 and P20-GM130423) as well as Tina's Wish Foundation.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

REFERENCES

1. Macías M, Alegre E, Díaz-Lagares A, et al. Liquid biopsy: from basic research to clinical practice. *Adv Clin Chem* 2018;83:73-119. DOI PubMed
2. Jeffrey SS, Toner M. Liquid biopsy: a perspective for probing blood for cancer. *Lab Chip* 2019;19:548-9. DOI PubMed
3. Tkach M, Théry C. Communication by extracellular vesicles: where we are and where we need to go. *Cell* 2016;164:1226-32. DOI PubMed
4. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular vesicles: composition, biological relevance, and methods of study. *Bioscience* 2015;65:783-97. DOI PubMed PMC
5. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373-83. DOI PubMed PMC
6. Wei Z, Batagov AO, Schinelli S, et al. Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nat Commun* 2017;8:1145. DOI PubMed PMC
7. He M, Zeng Y. Microfluidic Exosome Analysis toward Liquid Biopsy for Cancer. *J Lab Autom* 2016;21:599-608. DOI PubMed PMC
8. Revenfeld AL, Bæk R, Nielsen MH, Stensballe A, Varming K, Jørgensen M. Diagnostic and prognostic potential of extracellular vesicles in peripheral blood. *Clin Ther* 2014;36:830-46. DOI PubMed
9. Smalley DM, Sheman NE, Nelson K, Theodorescu D. Isolation and identification of potential urinary microparticle biomarkers of bladder cancer. *J Proteome Res* ;7:2088-96. DOI PubMed
10. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;68:2667-88. DOI PubMed PMC
11. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013;200:373-83. DOI PubMed PMC
12. Giusti I, D'Ascenzo S, Millimaggi D, et al. Neoplasia 2008;10:481-8. DOI PubMed PMC
13. Antonyak MA, Li B, Boroughs LK, et al. Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. *Proc Natl Acad Sci U S A* 2011;108:4852-7. DOI PubMed PMC
14. Momen-Heravi F, Balaj L, Alian S, et al. Current methods for the isolation of extracellular vesicles. *Biol Chem* 2013;394:1253-62. DOI PubMed PMC
15. Harding C, Stahl P. Transferrin recycling in reticulocytes: pH and iron are important determinants of ligand binding and processing. *Biochem Biophys Res Commun* 1983;113:650-8. DOI PubMed
16. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 1983;33:967-78. DOI PubMed
17. Stickney Z, Losacco J, McDevitt S, Zhang Z, Lu B. Development of exosome surface display technology in living human cells. *Biochem Biophys Res Commun* 2016;472:53-9. DOI PubMed

18. Zhu Q, Heon M, Zhao Z, He M. Microfluidic engineering of exosomes: editing cellular messages for precision therapeutics. *Lab Chip* 2018;18:1690-1703. DOI PubMed PMC
19. Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2002;2:569-79. DOI PubMed
20. Kalluri R, LeBleu VS. function**,** and biomedical applications of exosomes. *Science* 2020;367:eaau6977. DOI PubMed PMC
21. Willms E, Johansson HJ, Mäger I, et al. Cells release subpopulations of exosomes with distinct molecular and biological properties. Sci Rep 2016;6:22519. DOI PubMed PMC
22. Aliotta JM. Tumor exosomes: a novel biomarker? *J Gastrointest Oncol* 2011;2:203-5. DOI PubMed PMC
23. Al-Nedawi K, Meehan B, Micallef J, et al. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat Cell Biol* 2008;10:619-24. DOI PubMed
24. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654-9. DOI PubMed
25. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol* 2015;1295:179-209. DOI PubMed
26. Street JM, Koritzinsky EH, Glispie DM, Yuen PST. Urine Exosome Isolation and Characterization. *Methods Mol Biol* 2017;1641:413-23. DOI PubMed
27. Helwa I, Cai J, Drewry MD, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One* 2017;12:e0170628. DOI PubMed PMC
28. Heinemann ML, Ilmer M, Silva LP, et al. Benchtop isolation and characterization of functional exosomes by sequential filtration. *J Chromatogr A* 2014;1371:125-35. DOI PubMed
29. Zhao Z, Yang Y, Zeng Y, He M. A microfluidic ExoSearch chip for multiplexed exosome detection towards blood-based ovarian cancer diagnosis. *Lab Chip* 2016;16:489-96. DOI PubMed PMC
30. Li W, Li C, Zhou T, et al. Role of exosomal proteins in cancer diagnosis. *Mol Cancer* 2017;16:145. DOI PubMed PMC
31. Jackson JM, Witek MA, Hupert ML, et al. UV activation of polymeric high aspect ratio microstructures: ramifications in antibody surface loading for circulating tumor cell selection. *Lab Chip* 2014;14:106-17. DOI PubMed PMC
32. . Harshani Wijerathne, M. A. W., Mateusz L. Hupert, Joshua M. Jackson, Steven A. Soper, Microfluidic Device for EV and T-cell Isolation. 2019.
33. Woo HK, Sunkara V, Park J, et al. Exodisc for Rapid, Size-Selective, and Efficient Isolation and Analysis of Nanoscale Extracellular Vesicles from Biological Samples. *ACS Nano* 2017;11:1360-70. DOI PubMed
34. Yentrapalli R, Merl-Pham J, Azimzadeh O, et al. Quantitative changes in the protein and miRNA cargo of plasma exosome-like vesicles after exposure to ionizing radiation. *Int J Radiat Biol* 2017;93:569-80. DOI PubMed
35. Tian T, Zhu YL, Zhou YY, et al. Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. *J Biol Chem* 2014;289:22258-67. DOI PubMed PMC
36. Fares J, Kashyap R, Zimmermann P. Syntenin: Key player in cancer exosome biogenesis and uptake? *Cell Adh Migr* 2017;11:124-6. DOI PubMed PMC
37. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci* 2018;75:193-208. DOI PubMed PMC
38. Tian Y, Li S, Song J, et al. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 2014;35:2383-90. DOI PubMed
39. Nakase I, Futaki S. Combined treatment with a pH-sensitive fusogenic peptide and cationic lipids achieves enhanced cytosolic delivery of exosomes. *Sci Rep* 2015;5:10112. DOI PubMed PMC
40. Hu L, Wickline SA, Hood JL. Magnetic resonance imaging of melanoma exosomes in lymph nodes. *Magn Reson Med* 2015;74:266-71. DOI PubMed PMC
41. Wang J, Li W, Zhang L, et al. Chemically Edited Exosomes with Dual Ligand Purified by Microfluidic Device for Active Targeted Drug Delivery to Tumor Cells. *ACS Appl Mater Interfaces* 2017;9:27441-52. DOI PubMed
42. Hood JL, Scott MJ, Wickline SA. Maximizing exosome colloidal stability following electroporation. *Anal Biochem* 2014;448:41-9. DOI PubMed PMC
43. Shtam TA, Kovalev RA, Varfolomeeva EY, Makarov EM, Kil YV, Filatov MV. Exosomes are natural carriers of exogenous siRNA to human cells in vitro. Cell Commun Signal. 2013 Nov 18;11:88. DOI PubMed PMC
44. Wahlgren J, De L Karlson T, Brisslert M, et al. Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. *Nucleic Acids Res* 2012;40:e130. DOI PubMed PMC
45. Besse B, Charrier M, Lapierre V, et al. Dendritic cell-derived exosomes as maintenance immunotherapy after first line chemotherapy in NSCLC. *Oncoimmunology* 2016;5:e1071008. DOI PubMed PMC
46. Viaud S, Ploix S, Lapierre V, et al. Updated technology to produce highly immunogenic dendritic cell-derived exosomes of clinical grade: a critical role of interferon-γ. *J Immunother* 2011;34:65-75. DOI PubMed
47. Zhao Z, McGill J, Gamero-Kubota P, He M. Microfluidic on-demand engineering of exosomes towards cancer immunotherapy. *Lab Chip* 2019;19:1877-86. DOI PubMed PMC
48. Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: disease message and therapeutic target potentials. *Biosci Rep* 2019;39:BSR20180992. DOI PubMed PMC
49. Hauser P, Wang S, Didenko VV. Apoptotic Bodies: Selective Detection in Extracellular Vesicles. *Methods Mol Biol* 2017;1554:193-

200. DOI PubMed
50. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007;35:495-516. DOI PubMed PMC
51. Fleisher TA. Apoptosis. *Ann Allergy Asthma Immunol* 1997;78:245-9; quiz 249. DOI PubMed
52. Atkin-Smith GK, Tixeira R, Paone S, et al. A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. *Nat Commun* 2015;6:7439. DOI PubMed PMC
53. Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* 2014;14:166-80. DOI PubMed PMC
54. Elliott MR, Chekeni FB, Trampont PC, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* 2009;461:282-6. DOI PubMed PMC
55. Truman LA, Ford CA, Pasikowska M, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* 2008;112:5026-36. DOI PubMed
56. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature* 2002;417:182-7. DOI PubMed
57. Gardai SJ, McPhillips KA, Frasch SC, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* 2005;123:321-34. DOI PubMed
58. Witwer KW, Buzás EI, Bemis LT, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;2:20360. DOI PubMed PMC
59. Tauro BJ, Greening DW, Mathias RA, Mathivanan S, Ji H, Simpson RJ. Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. *Mol Cell Proteomics* 2013;12:587-98. DOI PubMed PMC
60. Crescitelli R, Lässer C, Szabó TG, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles* 2013;2:20677. DOI PubMed PMC
61. Dörsam B, Reiners KS, von Strandmann EP. Cancer-derived extracellular vesicles: friend and foe of tumour immunosurveillance. *Philos Trans R Soc Lond B Biol Sci* 2018;373:20160481. DOI PubMed PMC
62. Sehrawat TS, Arab JP, Liu M, et al. Circulating Extracellular Vesicles Carrying Sphingolipid Cargo for the Diagnosis and Dynamic Risk Profiling of Alcoholic Hepatitis. *Hepatology* 2021;73:571-85. DOI PubMed PMC
63. Choi DS, Lee J, Go G, Kim YK, Gho YS. Circulating extracellular vesicles in cancer diagnosis and monitoring: an appraisal of clinical potential. *Mol Diagn Ther* 2013;17:265-71. DOI PubMed
64. Yuan D, Zhao Y, Banks WA, et al. Macrophage exosomes as natural nanocarriers for protein delivery to inflamed brain. *Biomaterials* 2017;142:1-12. DOI PubMed PMC
65. Barrès C, Blanc L, Bette-Bobillo P, et al. Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. *Blood* 2010;115:696-705. DOI PubMed
66. Lakshminarayan R, Wunder C, Becken U, et al. Galectin-3 drives glycosphingolipid-dependent biogenesis of clathrin-independent carriers. *Nat Cell Biol* 2014;16:595-606. DOI PubMed
67. Merlin J, Stechly L, de Beaucé S, et al. Galectin-3 regulates MUC1 and EGFR cellular distribution and EGFR downstream pathways in pancreatic cancer cells. *Oncogene* 2011;30:2514-25. DOI PubMed
68. Kim KM, Abdelmohsen K, Mustapic M, Kapogiannis D, Gorospe M. RNA in extracellular vesicles. *Wiley Interdiscip Rev RNA* 2017;8:e1413. DOI PubMed PMC
69. Batagov AO, Kurochkin IV. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. *Biol Direct* 2013;8:12. DOI PubMed PMC
70. Chen YY, Lee MB, Chang CM, Liao SC. Methods of suicide in different psychiatric diagnostic groups. *J Affect Disord* 2009;118:196-200. DOI PubMed
71. Mitsuhashi M, Taub DD, Kapogiannis D, et al. Aging enhances release of exosomal cytokine mRNAs by Aβ1-42-stimulated macrophages. *FASEB J* 2013;27:5141-50. DOI PubMed PMC
72. Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther* 2005;11:435-43. DOI PubMed
73. Martellucci S, Orefice NS, Angelucci A, Luce A, Caraglia M, Zappavigna S. Extracellular Vesicles: New Endogenous Shuttles for miRNAs in Cancer Diagnosis and Therapy? *Int J Mol Sci* 2020;21:6486. DOI PubMed PMC
74. Vlaeminck-Guillem V. Extracellular Vesicles in Prostate Cancer Carcinogenesis, Diagnosis, and Management. *Front Oncol* 2018;8:222. DOI PubMed PMC
75. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet. 2011 Feb;12(2):99-110. DOI PubMed
76. Nishida-Aoki N, Ochiya T. Interactions between cancer cells and normal cells via miRNAs in extracellular vesicles. *Cell Mol Life Sci* 2015;72:1849-61. DOI PubMed PMC
77. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 2008;105:10513-8. DOI PubMed PMC
78. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* 2010;56:1733-41. DOI PubMed PMC
79. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* 2008;110:13-21. DOI PubMed
80. Langhe R, Norris L, Saadeh FA, et al. A novel serum microRNA panel to discriminate benign from malignant ovarian disease. *Cancer Lett* 2015;356:628-36. DOI PubMed
81. Jeppesen DK, Hvam ML, Primdahl-Bengtson B, et al. Comparative analysis of discrete exosome fractions obtained by differential

centrifugation. *J Extracell Vesicles* 2014;3:25011. DOI PubMed PMC

82. Acharya SS, Fendler W, Watson J, et al. Serum microRNAs are early indicators of survival after radiation-induced hematopoietic injury. *Sci Transl Med* 2015;7:287ra69. DOI PubMed PMC
83. Tian J, Casella G, Zhang Y, Rostami A, Li X. Potential roles of extracellular vesicles in the pathophysiology, diagnosis, and treatment of autoimmune diseases. *Int J Biol Sci* 2020;16:620-32. DOI PubMed PMC
84. Jiang WY, Xun YH. [Value of detection of extracellular vesicles in the diagnosis of nonalcoholic fatty liver disease]. *Zhonghua Gan Zang Bing Za Zhi* 2020;28:92-6. DOI PubMed
85. Shaimardanova AA, Solovyeva VV, Chulpanova DS, James V, Kitaeva KV, Rizvanov AA. Extracellular vesicles in the diagnosis and treatment of central nervous system diseases. *Neural Regen Res* 2020;15:586-96. DOI PubMed PMC
86. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol* 2008;10:1470-6. DOI PubMed PMC
87. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta* 2012;1820:940-8. DOI PubMed
88. Aziz N, Detels R, Quint JJ, Li Q, Gjertson D, Butch AW. Stability of cytokines, chemokines and soluble activation markers in unprocessed blood stored under different conditions. *Cytokine* 2016;84:17-24. DOI PubMed PMC
89. Johnsen KB, Gudbergsson JM, Andresen TL, Simonsen JB. What is the blood concentration of extracellular vesicles? *Biochim Biophys Acta Rev Cancer* 2019;1871:109-16. DOI PubMed
90. Ruhen O, Meehan K. Tumor-Derived Extracellular Vesicles as a Novel Source of Protein Biomarkers for Cancer Diagnosis and Monitoring. *Proteomics* 2019;19:e1800155. DOI PubMed
91. Brenner AW, Su GH, Momen-Heravi F. Isolation of Extracellular Vesicles for Cancer Diagnosis and Functional Studies. *Methods Mol Biol* 2019;1882:229-37. DOI PubMed
92. Zhang Q, Bansal A. Role of Extracellular Vesicles in the Diagnosis and Pathogenesis of Barrett's Esophagus: A Mini-Review. *Dig Dis Sci* 2021;66:705-13. DOI PubMed
93. Rahbarghazi R, Jabbari N, Sani NA, et al. Tumor-derived extracellular vesicles: reliable tools for Cancer diagnosis and clinical applications. *Cell Commun Signal* 2019;17:73. DOI PubMed PMC
94. Kawikova I, Askenase PW. Diagnostic and therapeutic potentials of exosomes in CNS diseases. *Brain Res* 2015;1617:63-71. DOI PubMed PMC
95. Masyuk AI, Masyuk TV, Larusso NF. Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. *J Hepatol* 2013;59:621-5. DOI PubMed PMC
96. Spanu S, van Roeyen CR, Denecke B, Floege J, Mühlfeld AS. Urinary exosomes: a novel means to non-invasively assess changes in renal gene and protein expression. *PLoS One* 2014;9:e109631. DOI PubMed PMC
97. Couch Y, Akbar N, Davis S, et al. Inflammatory Stroke Extracellular Vesicles Induce Macrophage Activation. *Stroke* 2017;48:2292-6. DOI PubMed PMC
98. Levänen B, Bhakta NR, Torregrosa Paredes P, et al. Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. *J Allergy Clin Immunol* 2013;131:894-903. DOI PubMed PMC
99. Hoefer IE, Steffens S, Ala-Korpela M, et al; ESC Working Group Atherosclerosis and Vascular Biology. Novel methodologies for biomarker discovery in atherosclerosis. *Eur Heart J* 2015;36:2635-42. DOI PubMed
100. Weng Y, Sui Z, Shan Y, et al. Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling. *Analyst* 2016;141:4640-6. DOI PubMed
101. Atha DH, Ingham KC. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. *J Biol Chem* 1981;256:12108-17. PubMed
102. Andreu Z, Rivas E, Sanguino-Pascual A, et al. Comparative analysis of EV isolation procedures for miRNAs detection in serum samples. *J Extracell Vesicles* 2016;5:31655. DOI PubMed PMC
103. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* 2017;7:789-804. DOI PubMed PMC
104. Salih M, Zietse R, Hoorn EJ. Urinary extracellular vesicles and the kidney: biomarkers and beyond. *Am J Physiol Renal Physiol* 2014;306:F1251-9. DOI PubMed
105. Taylor DD, Zacharias W, Gercel-Taylor C. Exosome isolation for proteomic analyses and RNA profiling. *Methods Mol Biol* 2011;728:235-46. DOI PubMed
106. Lamparski HG, Metha-damani A, Yao J, et al. Production and characterization of clinical grade exosomes derived from dendritic cells. *J Immunol Methods* 2002;270:211-26. DOI PubMed
107. Li X, Corbett AL, Taatizadeh E, et al. Challenges and opportunities in exosome research-Perspectives from biology, engineering, and cancer therapy. *APL Bioeng* 2019;3:011503. DOI PubMed PMC
108. Tauro BJ, Greening DW, Mathias RA, et al. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods* 2012;56:293-304. DOI PubMed
109. Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006;Chapter 3:Unit 3.22. DOI PubMed
110. Sunkara V, Woo HK, Cho YK. Emerging techniques in the isolation and characterization of extracellular vesicles and their roles in cancer diagnostics and prognostics. *Analyst* 2016;141:371-81. DOI PubMed
111. Yoo YK, Lee J, Kim H, Hwang KS, Yoon DS, Lee JH. Toward Exosome-Based Neuronal Diagnostic Devices. *Micromachines (Basel)* 2018;9:634. DOI PubMed PMC

112. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed Res Int* 2018;2018:8545347. DOI PubMed PMC
113. Livshits MA, Khomyakova E, Evtushenko EG, et al. Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. *Sci Rep* 2015;5:17319. DOI PubMed PMC
114. Abramowicz A, Widlak P, Pietrowska M. Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation. *Mol Biosyst* 2016;12:1407-19. DOI PubMed
115. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK. Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int* 2012;82:1024-32. DOI PubMed
116. Takov K, Yellon DM, Davidson SM. Comparison of small extracellular vesicles isolated from plasma by ultracentrifugation or size-exclusion chromatography: yield, purity and functional potential. *J Extracell Vesicles* 2019;8:1560809. DOI PubMed PMC
117. Buschmann D, Kirchner B, Hermann S, et al. Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing. *J Extracell Vesicles* 2018;7:1481321. DOI PubMed PMC
118. Ding M, Wang C, Lu X, et al. Comparison of commercial exosome isolation kits for circulating exosomal microRNA profiling. *Anal Bioanal Chem* 2018;410:3805-14. DOI PubMed
119. Whiteside TL. Tumor-Derived Exosomes and Their Role in Tumor-Induced Immune Suppression. *Vaccines (Basel)* 2016;4:35. DOI PubMed PMC
120. Ayala-Mar S, Donoso-Quezada J, Gallo-Villanueva RC, Perez-Gonzalez VH, González-Valdez J. Recent advances and challenges in the recovery and purification of cellular exosomes. *Electrophoresis* 2019;40:3036-49. DOI PubMed PMC
121. Serrano-Pertierra E, Oliveira-Rodríguez M, Rivas M, et al. Characterization of Plasma-Derived Extracellular Vesicles Isolated by Different Methods: A Comparison Study. *Bioengineering (Basel)* 2019;6:8. DOI PubMed PMC
122. Cvjetkovic A, Lötvall J, Lässer C. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. *J Extracell Vesicles* 2014;3:23111. DOI PubMed PMC
123. Zhang P, He M, Zeng Y. Ultrasensitive microfluidic analysis of circulating exosomes using a nanostructured graphene oxide/polydopamine coating. *Lab Chip* 2016;16:3033-42. DOI PubMed PMC
124. Kanwar SS, Dunlay CJ, Simeone DM, Nagrath S. Microfluidic device (ExoChip) for on-chip isolation, quantification and characterization of circulating exosomes. *Lab Chip* 2014;14:1891-900. DOI PubMed PMC
125. Reátegui E, van der Vos KE, Lai CP, et al. Engineered nanointerfaces for microfluidic isolation and molecular profiling of tumor-specific extracellular vesicles. *Nat Commun* 2018;9:175. DOI PubMed PMC
126. Dudani JS, Gossett DR, Tse HT, Lamm RJ, Kulkarni RP, Carlo DD. Rapid inertial solution exchange for enrichment and flow cytometric detection of microvesicles. *Biomicrofluidics* 2015;9:014112. DOI PubMed PMC
127. Liu C, Guo J, Tian F, et al. Field-Free Isolation of Exosomes from Extracellular Vesicles by Microfluidic Viscoelastic Flows. *ACS Nano* 2017;11:6968-76. DOI PubMed
128. Wu M, Ouyang Y, Wang Z, et al. Isolation of exosomes from whole blood by integrating acoustics and microfluidics. *Proc Natl Acad Sci U S A* 2017;114:10584-9. DOI PubMed PMC
129. Zhang P, Zhou X, He M, et al. Ultrasensitive detection of circulating exosomes with a 3D-nanopatterned microfluidic chip. *Nat Biomed Eng* 2019;3:438-51. DOI PubMed PMC
130. He M, Crow J, Roth M, Zeng Y, Godwin AK. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. *Lab Chip* 2014;14:3773-80. DOI PubMed PMC
131. Kang YT, Purcell E, Palacios-Rolston C, et al. Isolation and Profiling of Circulating Tumor-Associated Exosomes Using Extracellular Vesicular Lipid-Protein Binding Affinity Based Microfluidic Device. *Small* 2019;15:e1903600. DOI PubMed PMC
132. Zhang J, Huang H, Xue L, et al. On-demand drug release nanoplatform based on fluorinated aza-BODIPY for imaging-guided chemo-phototherapy. *Biomaterials* 2020;256:120211. DOI PubMed
133. Haney MJ, Klyachko NL, Zhao Y, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* 2015;207:18-30. DOI PubMed PMC
134. El-Andaloussi S, Lee Y, Lakhal-Littleton S, et al. Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat Protoc* 2012;7:2112-26. DOI PubMed
135. Ko J, Carpenter E, Issadore D. Detection and isolation of circulating exosomes and microvesicles for cancer monitoring and diagnostics using micro-/nano-based devices. *Analyst* 2016;141:450-60. DOI PubMed PMC
136. Becker H. It's the economy. *Lab Chip* 2009;9:2759-62. DOI PubMed
137. ONeil CE, Jackson JM, Shim SH, Soper SA. Interrogating Surface Functional Group Heterogeneity of Activated Thermoplastics Using Super-Resolution Fluorescence Microscopy. *Anal Chem* 2016;88:3686-96. DOI PubMed PMC
138. O'Neil CE, Taylor S, Ratnayake K, Pullagurla S, Singh V, Soper SA. Characterization of activated cyclic olefin copolymer: effects of ethylene/norbornene content on the physiochemical properties. *Analyst* 2016;141:6521-32. DOI PubMed PMC
139. Wijerathne H, Witek MA, Jackson JM, et al. Affinity enrichment of extracellular vesicles from plasma reveals mRNA changes associated with acute ischemic stroke. *Commun Biol* 2020;3:613. DOI PubMed PMC
140. Enderle D, Spiel A, Coticchia CM, et al. Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method. *PLoS One* 2015;10:e0136133. DOI PubMed PMC
141. Haque S, Vaiselbuh SR. Exosomes molecular diagnostics: Direct conversion of exosomes into the cDNA for gene amplification by two-step polymerase chain reaction. *J Biol Methods* 2018;5:e96. DOI PubMed PMC
142. Soo CY, Song Y, Zheng Y, et al. Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology* 2012;136:192-7. DOI PubMed PMC

143. Vestad B, Llorente A, Neurauter A, et al. Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *J Extracell Vesicles* 2017;6:1344087. DOI PubMed PMC
144. Gardiner C, Ferreira YJ, Dragovic RA, Redman CW, Sargent IL. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *J Extracell Vesicles* 2013;2:19671. DOI PubMed PMC
145. Gross J, Sayle S, Karow AR, Bakowsky U, Garidel P. Nanoparticle tracking analysis of particle size and concentration detection in suspensions of polymer and protein samples: Influence of experimental and data evaluation parameters. *Eur J Pharm Biopharm* 2016;104:30-41. DOI PubMed
146. Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. *Pharm Res* 2010;27:796-810. DOI PubMed PMC
147. Maas SL, de Vrij J, van der Vlist EJ, et al. Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *J Control Release* 2015;200:87-96. DOI PubMed PMC
148. . Ludwig Reimer, HK, Transmission Electron Microscopy: Physics of Image Formation. Springer: 2008; p. 487.
149. Peak TC, Praharaj PP, Panigrahi GK, et al. Exosomes secreted by placental stem cells selectively inhibit growth of aggressive prostate cancer cells. *Biochem Biophys Res Commun* 2018;499:1004-10. DOI PubMed PMC
150. Shao H, Chung J, Balaj L, et al. Protein typing of circulating microvesicles allows real-time monitoring of glioblastoma therapy. *Nat Med* 2012;18:1835-40. DOI PubMed PMC
151. Enderle D, Spiel A, Coticchia CM, et al. Characterization of RNA from Exosomes and Other Extracellular Vesicles Isolated by a Novel Spin Column-Based Method. *PLoS One* 2015;10:e0136133. DOI PubMed PMC
152. Koifman N, Biran I, Aharon A, Brenner B, Talmon Y. A direct-imaging cryo-EM study of shedding extracellular vesicles from leukemic monocytes. *J Struct Biol* 2017;198:177-85. DOI PubMed
153. Cizmar P, Yuana Y. Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy. *Methods Mol Biol* 2017;1660:221-232. DOI PubMed
154. Buzas EI, György B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol* 2014;10:356-64. DOI PubMed
155. Sharma S, Gillespie BM, Palanisamy V, Gimzewski JK. Quantitative nanostructural and single-molecule force spectroscopy biomolecular analysis of human-saliva-derived exosomes. *Langmuir* 2011;27:14394-400. DOI PubMed PMC
156. Yuana Y, Oosterkamp TH, Bahatyrova S, et al. Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost* 2010;8:315-23. DOI PubMed
157. van der Pol E, Coumans FA, Grootemaat AE, et al. Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *J Thromb Haemost* 2014;12:1182-92. DOI PubMed
158. Nolan JP, Jones JC. Detection of platelet vesicles by flow cytometry. *Platelets* 2017;28:256-62. DOI PubMed PMC
159. Szatanek R, Baj-Krzyworzeka M, Zimoch J, Lekka M, Siedlar M, Baran J. The Methods of Choice for Extracellular Vesicles (EVs) Characterization. *Int J Mol Sci* 2017;18:1153. DOI PubMed PMC
160. Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, et al. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. *Cancer Immunol Immunother* 2006;55:808-18. DOI PubMed
161. Zhang J, Li S, Li L, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics* 2015;13:17-24. DOI PubMed PMC
162. Morales-Kastresana A, Jones JC. Flow Cytometric Analysis of Extracellular Vesicles. *Methods Mol Biol* 2017;1545:215-225. DOI PubMed PMC
163. Suárez H, Gámez-Valero A, Reyes R, et al. A bead-assisted flow cytometry method for the semi-quantitative analysis of Extracellular Vesicles. *Sci Rep* 2017;7:11271. DOI PubMed PMC
164. Friedrich R, Block S, Alizadehheidari M, et al. A nano flow cytometer for single lipid vesicle analysis. *Lab Chip* 2017;17:830-41. DOI PubMed
165. Koritzinsky EH, Street JM, Star RA, Yuen PS. Quantification of Exosomes. *J Cell Physiol* 2017;232:1587-90. DOI PubMed PMC
166. DeBlois RW, Wesley RK. Sizes and concentrations of several type C oncornaviruses and bacteriophage T2 by the resistive-pulse technique. *J Virol* 1977;23:227-33. DOI PubMed PMC
167. Deblois RW, Uzgiris EE, Cluxton DH, Mazzone HM. Comparative measurements of size and polydispersity of several insect viruses. *Analytical Biochemistry* 1978;90:273-88. DOI PubMed
168. Kasianowicz JJ, Brandin E, Branton D, Deamer DW. Characterization of individual polynucleotide molecules using a membrane channel. *Proc Natl Acad Sci U S A* 1996;93:13770-3. DOI PubMed PMC
169. Astier Y, Braha O, Bayley H. Toward single molecule DNA sequencing: direct identification of ribonucleoside and deoxyribonucleoside 5'-monophosphates by using an engineered protein nanopore equipped with a molecular adapter. *J Am Chem Soc* 2006;128:1705-10. DOI PubMed
170. Deamer DW, Akeson M. Nanopores and nucleic acids: prospects for ultrarapid sequencing. *Trends in Biotechnology* 2000;18:147-51. DOI PubMed
171. Lobb RJ, Becker M, Wen SW, et al. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *J Extracell Vesicles* 2015;4:27031. DOI PubMed PMC
172. Anderson W, Lane R, Korbie D, Trau M. Observations of Tunable Resistive Pulse Sensing for Exosome Analysis: Improving System Sensitivity and Stability. *Langmuir* 2015;31:6577-87. DOI PubMed
173. Kozak D, Anderson W, Vogel R, Trau M. Advances in Resistive Pulse Sensors: Devices bridging the void between molecular and

microscopic detection. *Nano Today* 2011;6:531-45. DOI PubMed PMC

174. Lan W, Kubeil C, Xiong J, Bund A, White HS. Effect of Surface Charge on the Resistive Pulse Waveshape during Particle Translocation through Glass Nanopores. *J Phys Chem C* 2014;118:2726-34. DOI
175. Pan R, Hu K, Jiang D, Samuni U, Mirkin MV. Electrochemical Resistive-Pulse Sensing. *J Am Chem Soc* 2019;141:19555-9. DOI PubMed
176. Wu Y, Benson JD, Critser JK, Almasri M. MEMS-based Coulter counter for cell counting and sizing using multiple electrodes. *J Micromech Microeng* 2010;20:085035. DOI
177. Jagtiani AV, Zhe J, Hu J, Carletta. Detection and counting of micro-scale particles and pollen using a multi-aperture Coulter counter. *Meas Sci Technol* 2006;17:1706-14. DOI
178. Zhe J, Jagtiani A, Dutta P, Hu J, Carletta J. A micromachined high throughput Coulter counter for bioparticle detection and counting. *J Micromech Microeng* 2007;17:304-13. DOI
179. Jagtiani AV, Carletta J, Zhe J. An impedimetric approach for accurate particle sizing using a microfluidic Coulter counter. *J Micromech Microeng* 2011;21:045036. DOI
180. Lannigan J, Erdbruegger U. Imaging flow cytometry for the characterization of extracellular vesicles. *Methods* 2017;112:55-67. DOI PubMed
181. Rosenstein JK, Wanunu M, Merchant CA, Drndic M, Shepard KL. Integrated nanopore sensing platform with sub-microsecond temporal resolution. *Nat Methods* 2012;9:487-92. DOI PubMed PMC
182. Li J, Stein D, McMullan C, Branton D, Aziz MJ, Golovchenko JA. Ion-beam sculpting at nanometre length scales. *Nature* 2001;412:166-9. DOI PubMed
183. Stanley-Wood NG, Lines RW, eds. Particle Size Analysis. Royal Society of Chemistry, 1992. DOI
184. Song Y, Zhang J, Li D. Microfluidic and Nanofluidic Resistive Pulse Sensing: A Review. *Micromachines (Basel)* 2017;8:204. DOI PubMed PMC
185. Vaclavek T, Prikryl J, Foret F. Resistive pulse sensing as particle counting and sizing method in microfluidic systems: Designs and applications review. *J Sep Sci* 2019;42:445-57. DOI PubMed
186. Cui S, Cheng Z, Qin W, Jiang L. Exosomes as a liquid biopsy for lung cancer. *Lung Cancer* 2018;116:46-54. DOI PubMed
187. Mader S, Pantel K. Liquid Biopsy: Current Status and Future Perspectives. *Oncol Res Treat* 2017;40:404-8. DOI PubMed
188. Poulet G, Massias J, Taly V. Liquid Biopsy: General Concepts. *Acta Cytol* 2019;63:449-55. DOI PubMed
189. Chen M, Zhao H. Next-generation sequencing in liquid biopsy: cancer screening and early detection. *Hum Genomics* 2019;13:34. DOI PubMed PMC
190. Manna I, Iaccino E, Dattilo V, et al. Exosome-associated miRNA profile as a prognostic tool for therapy response monitoring in multiple sclerosis patients. *FASEB J* 2018;32:4241-6. DOI PubMed
191. Alimirzaie S, Bagherzadeh M, Akbari MR. Liquid biopsy in breast cancer: A comprehensive review. *Clin Genet* 2019;95:643-60. DOI PubMed
192. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev* 2016;106:148-56. DOI PubMed
193. Batrakova EV, Kim MS. Using exosomes, naturally-equipped nanocarriers, for drug delivery. *J Control Release* 2015;219:396-405. DOI PubMed PMC
194. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. *Theranostics* 2017;7:789-804. DOI PubMed PMC
195. Konoshenko MY, Lekchnov EA, Vlassov AV, Laktionov PP. Isolation of Extracellular Vesicles: General Methodologies and Latest Trends. *Biomed Res Int* 2018;2018:8545347. DOI PubMed PMC
196. Livshits MA, Khomyakova E, Evtushenko EG, et al. Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol. *Sci Rep* 2015;5:17319. DOI PubMed PMC
197. Deun J, Mestdagh P, Agostinis P, et al; EV-TRACK Consortium. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. *Nat Methods* 2017;14:228-32. DOI PubMed
198. Tang YT, Huang YY, Zheng L, et al. Comparison of isolation methods of exosomes and exosomal RNA from cell culture medium and serum. *Int J Mol Med* 2017;40:834-44. DOI PubMed PMC
199. Chen BY, Sung CW, Chen C, et al. Advances in exosomes technology. *Clin Chim Acta* 2019;493:14-9. DOI PubMed
200. Nath Neerukonda S, Egan NA, Patria J, et al. Comparison of exosomes purified via ultracentrifugation (UC) and Total Exosome Isolation (TEI) reagent from the serum of Marek's disease virus (MDV)-vaccinated and tumor-bearing chickens. *J Virol Methods* 2019;263:1-9. DOI PubMed
201. Lee K, Shao H, Weissleder R, Lee H. Acoustic purification of extracellular microvesicles. *ACS Nano* 2015;9:2321-7. DOI PubMed PMC
202. Franz C, Böing AN, Montag M, et al. Extracellular vesicles in human follicular fluid do not promote coagulation. *Reprod Biomed Online* 2016;33:652-5. DOI PubMed

AUTHOR INSTRUCTIONS

1. Submission Overview

Before you decide to publish with *Extracellular Vesicles and Circulating Nucleic Acids* (*EVCNA*), please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

1.1 Topic Suitability

The topic of the manuscript must fit the scope of the journal. Please refer to Aims and Scope for more information.

1.2 Open Access and Copyright

The journal adopts Gold Open Access publishing model and distributes content under the Creative Commons Attribution 4.0 International License. Copyright is retained by authors. Please make sure that you are well aware of these policies.

1.3 Publication Fees

The publication fee for each submission is $299. There are no additional charges based on color, length, figures, or other elements. OAE provides expense deduction for authors as appropriate. For more details, please refer to OAE Publication Fees.

1.4 Language Editing

All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smoothly and efficiently.

If needed, authors are recommended to consider the language editing services provided by Charlesworth to ensure that the manuscript is written in correct scientific English before submission. Authors who publish with OAE journals enjoy a special discount for the services of Charlesworth via the following two ways.

Submit your manuscripts directly at http://www.charlesworthauthorservices.com/~OAE;

Open the link http://www.charlesworthauthorservices.com/, and enter Promotion Code “OAE” when you submit.

1.5 Work Funded by the National Institutes of Health

If an accepted manuscript was funded by National Institutes of Health (NIH), the authors may inform Editors of the NIH funding number. The Editors are able to deposit the paper to the NIH Manuscript Submission System on behalf of the authors.

2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc.*

Here is a guideline of a cover letter for authors’ consideration:

In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc.*) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;

In the second paragraph: concisely explain what was done, the main findings and why they are significant;

In the third paragraph: indicate why the manuscript fits the Aims and Scope of the journal, and why it would be attractive to readers;

In the fourth paragraph: confirm that the manuscript has not been published elsewhere and not under consideration of any other journal. All authors have approved the manuscript and agreed on its submission to the journal. Journal’s specific requirements have been met if any.

If the manuscript is contributed to a special issue, please also mention it in the cover letter.

If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

2.2 Types of Manuscripts

There is no restriction on the length of manuscripts, number of figures, tables and references, provided that the manuscript is concise and comprehensive. The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

The abstract should be a single paragraph with word limitation and specific structure requirements (for more details please refer to Types of Manuscripts). It usually describes the main objective(s) of the study, explains how the study was done, including any model organisms used, without methodological detail, and summarizes the most important results and their significance. The abstract must be an objective representation of the study: it is not allowed to contain results which are not presented and substantiated in the manuscript or exaggerate the main conclusions. Citations should not be included in the abstract.

2.3.1.4 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether the aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

2.3.3.2 Authors' Contributions

Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

Please use Surname and Initial of Forename to refer to an author's contribution. For example: made substantial contributions to conception and design of the study and performed data analysis and interpretation: Salas H, Castaneda WV; performed data acquisition, as well as provided administrative, technical, and material support: Castillo N, Young V.

If an article is single-authored, please include "The author contributed solely to the article." in this section.

2.3.3.3 Availability of Data and Materials

In order to maintain the integrity, transparency and reproducibility of research records, authors should include this section in their manuscripts, detailing where the data supporting their findings can be found. Data can be deposited into data repositories or published as supplementary information in the journal. Authors who cannot share their data should state that the data will not be shared and explain it. If a manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.4 Financial Support and Sponsorship

All sources of funding for the study reported should be declared. The role of the funding body in the experiment design, collection, analysis and interpretation of data, and writing of the manuscript should be declared. Any relevant grant numbers and the link of funder's website should be provided if any. If the study is not involved with this issue, state "None." in this section.

2.3.3.5 Conflicts of Interest

Authors must declare any potential conflicts of interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there are no conflicts of interest, please state "All authors declared that there are no conflicts of interest." in this section. Some authors may be bound by confidentiality agreements. In such cases, in place of itemized disclosures, we will require authors to state "All authors declare that they are bound by confidentiality agreements that prevent them from disclosing their conflicts of interest in this work.". If authors are unsure whether conflicts of interest exist, please refer to the "Conflicts of Interest" of *EVCNA* Editorial Policies for a full explanation.

2.3.3.6 Ethical Approval and Consent to Participate

Research involving human subjects, human material or human data must be performed in accordance with the Declaration of Helsinki and approved by an appropriate ethics committee. An informed consent to participate in the study should also be obtained from participants, or their parents or legal guardians for children under 16. A statement detailing the name of the ethics committee (including the reference number where appropriate) and the informed consent obtained must appear in the manuscripts reporting such research.

Studies involving animals and cell lines must include a statement on ethical approval. More information is available at Editorial Policies.

If the manuscript does not involve such issue, please state "Not applicable." in this section.

2.3.3.7 Consent for Publication

Manuscripts containing individual details, images or videos, must obtain consent for publication from that person, or in the case of children, their parents or legal guardians. If the person has died, consent for publication must be obtained from the next of kin of the participant. Manuscripts must include a statement that a written informed consent for publication was obtained. Authors do not have to submit such content accompanying the manuscript. However, these documents must be available if requested. If the manuscript does not involve this issue, state "Not applicable." in this section.

2.3.3.8 Copyright

Authors retain copyright of their works through a Creative Commons Attribution 4.0 International License that clearly states how readers can copy, distribute, and use their attributed research, free of charge. A declaration "© The Author(s) 2021." will be added to each article. Authors are required to sign License to Publish before formal publication.

2.3.3.9 References

Preferably original research articles that directly support the statements should be cited. Review articles could be cited when they specifically address the statement made in the manuscript. An abstract should not be used as a reference. Non-specific citations should be avoided.

References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. If the number of authors is less than or equal to six, we require to list all authors' names. If the number of authors is more than six, only the first three authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published

should be cited in the text as "Unpublished material" with written permission from the source.

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

2.3.3.10 Supplementary Materials

Additional data and information can be uploaded as Supplementary Materials to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English and limited to a size of 500 MB.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

Manuscripts must be written in English.

2.4.4 Multimedia Files

The journal supports manuscripts with multimedia files. The requirements are listed as follows:
Videos or audio files are only acceptable in English. The presentation and introduction should be easy to understand. The frames should be clear, and the speech speed should be moderate.
A brief overview of the video or audio files should be given in the manuscript text.
The video or audio files should be limited to a size of up to 500 MB.
Please use professional software to produce high-quality video files, to facilitate acceptance and publication along with the

submitted article. Upload the videos in mp4, wmv, or rm format (preferably mp4) and audio files in mp3 or wav format.

2.4.5 Figures

Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;
Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;
Figure caption is placed under the Figure;
Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, *etc.*) should be editable in word, excel or powerpoint format. Non-English information should be avoided;
Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background;
Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;
Internal scale (magnification) should be explained and the staining method in photomicrographs should be identified;
All non-standard abbreviations should be explained in the legend;
Permission for use of copyrighted materials from other sources, including re-published, adapted, modified, or partial figures and images from the internet, must be obtained. It is authors' responsibility to acquire the licenses, to follow any citation instruction requested by third-party rights holders, and cover any supplementary charges.

2.4.6 Tables

Tables should be cited in numeric order and placed after the paragraph where it is first cited;
The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);
Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);
Abbreviations and symbols used in table should be explained in footnote;
Explanatory matter should also be placed in footnotes;
Permission for use of copyrighted materials from other sources, including re-published, adapted, modified, or partial tables from the internet, must be obtained. It is authors' responsibility to acquire the licenses, to follow any citation instruction requested by third-party rights holders, and cover any supplementary charges.

2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc*., can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs*., *et al., etc*., *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

Submit an article via https://oaemesas.com/login?JournalId=evcna.

www.oaepublish.com

Extracellular Vesicles and Circulating Nucleic Acids (*EVCNA*)
Los Angeles Office
245 E Main Street ste122, Alhambra,
CA 91801, USA
E-mail: editorial@evcna.com
Website: https://evcna.com