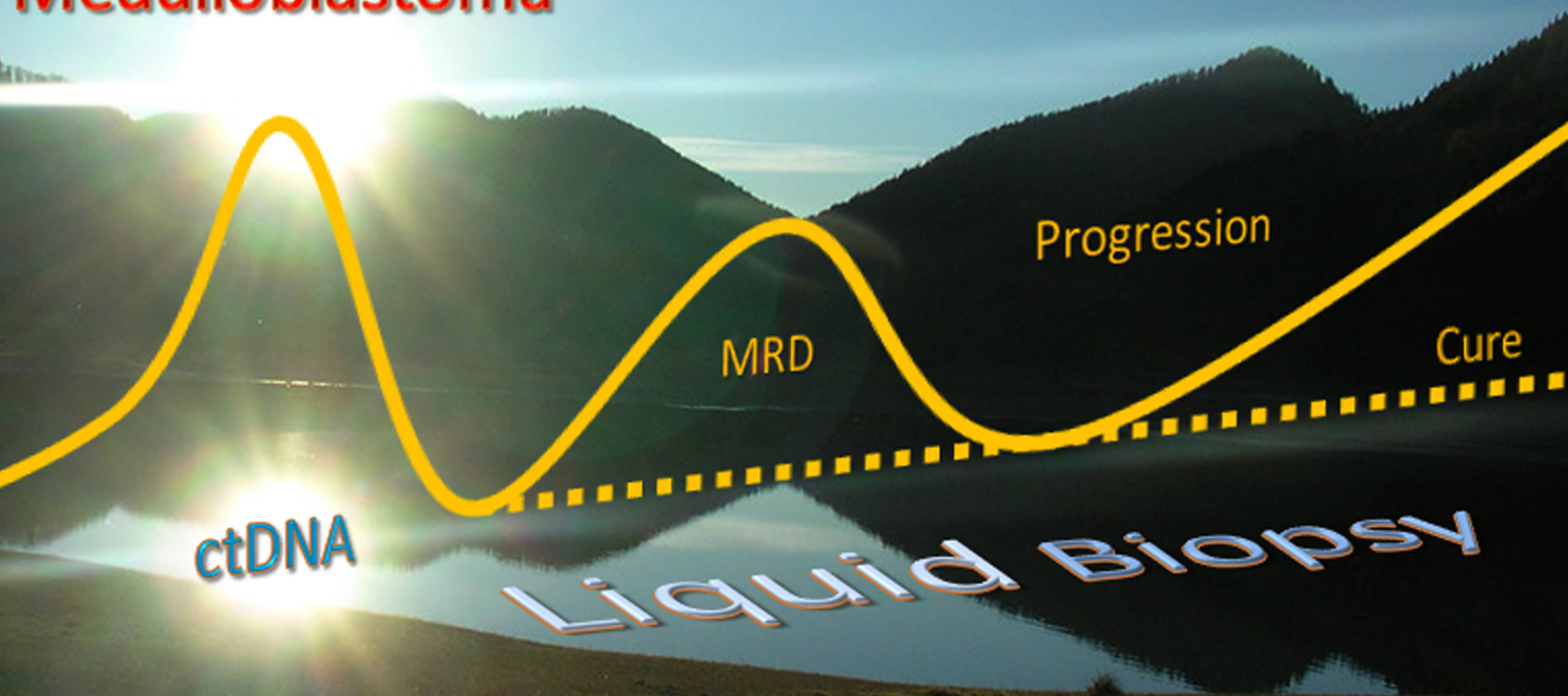


Extracellular Vesicles and Circulating Nucleic Acids

Diagnosis Prognosis Monitoring Therapy

Medulloblastoma



Cell-free DNA as a biomarker in cancer

Robert H. Eibl, Markus Schneemann

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OAE Publishing Inc.

245 E Main Street st112, Alhambra, CA 91801, USA

Website: www.oaepublish.com

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Meeting Abstracts

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Meeting abstracts of the first meeting of the American Society for Intercellular Communication 2021

Gurudutt Pendyala, Ashley E. Russell, Shilpa Buch, Susmita Sil, Emeli Chatterjee, Bojan Losic, Alissa M. Weaver, Tsuneya Ikezu, Randy Schekman, Xiaoli Yu

How to cite this article: Pendyala G, Russell AE, Buch S, Sil S, Chatterjee E, Losic B, Weaver AM, Ikezu T, Schekman R, Yu X. Meeting abstracts of the first meeting of the American Society for Intercellular Communication 2021. *Extracell Vesicles Circ Nucleic Acids* 2022;3:163-71. <http://dx.doi.org/10.20517/evcna.2022.10>

Received: 24 Feb 2022 **Accepted:** 2 Mar 2022 **Published:** 13 Jul 2022

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1. R(EV)ealing sex differences with nicotine addiction

Gurudutt Pendyala

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Smoking remains a significant health and economic concern in the United States. Furthermore, the emerging pattern of nicotine intake between sexes further adds a layer of complexity. Nicotine is a potent psychostimulant with a high addiction liability that can significantly alter brain function. However, the neurobiological mechanisms underlying nicotine's impact on brain function and behavior remain unclear. Elucidation of these mechanisms is of high clinical importance and may lead to improved therapeutics for smoking cessation. To fill in this critical knowledge gap, our recent study focused on identifying sex-specific brain-derived extracellular vesicles (BDEV) signatures in male and female rats post nicotine self-administration. Interestingly, females post nicotine self-administration showed larger BDEV sizes and impaired EV biogenesis than males. Next, using quantitative mass spectrometry-based proteomics, we identified BDEV signatures, including distinct molecular pathways, impact among males and females. We now are employing a novel technology, Single EV analyses with multiplexing (SEAM), to validate these identified sex-specific BDEV markers in the blood plasma from preclinical and clinical samples and further extend our studies into a model of relapse.

2. Cortisol's effects on iron transport proteins and EV release in placental cells

Sophie C. Anderlind, Ashley E. Russell

Department of Biology, School of Science, Pennsylvania State University, Erie, PA 16563, USA.

Although the placenta's significance is commonly unrecognized, a healthy pregnancy relies heavily on the placenta's role to function for the fetus' lungs, heart, kidney, and other vital underdeveloped organs. Extracellular vesicles (EVs) are important intracellular communicators essential to an optimally functioning placenta because they contain bioactive cargo released during both normal and pathological cellular activities. Previous work has shown that EVs are involved in maternal-to-fetal trafficking of important proteins, including iron transport proteins. While iron is an essential nutrient that transports oxygen for fetal development, a surplus or inadequate iron level can lead to negative pregnancy outcomes. The regulation of iron transport proteins is mediated by several factors, and previous research has shown that stress hormones, such as cortisol, have been observed to alter EV release. Three key iron transporting proteins in the trophoblast placental layer that facilitate nutrient absorption between the maternal and fetal bloodstream are Transferrin receptor 1 (TFR1), Ferroportin-1, and DMT-1. In the current study, we are investigating the effects of hydrocortisone (cortisol) on the cellular expression of these iron transport proteins and determining whether they are present in EVs. We are exposing a placental trophoblast cell line, BeWo, to physiologically relevant concentrations of hydrocortisone and assessing the expression of

these iron transport proteins via western blot. Additionally, we are using size exclusion chromatography to separate EVs from the conditioned cell culture media in order to examine changes in their protein composition after hydrocortisone exposure. Our results will demonstrate the effects of cortisol exposure on iron transport proteins, which may influence how iron is delivered during fetal development.

3. The effects of endoplasmic reticulum stress on oligodendrocyte derived EVs

Madison T. Jones, Samantha W. Manioci, Ashley E. Russell

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Oligodendrocytes are a type of glial cell found in the central nervous system, which form myelin sheaths that insulate neuronal axons. Myelin sheaths contain various proteins and lipids that are necessary for their formation. The proteins and membranes are synthesized, folded, and transported in the cell's endoplasmic reticulum (ER). Disruption of the ER's protein synthesis mechanisms can cause ER stress due to the accumulation of misfolded and unfolded proteins. Adequate protein production is crucial for oligodendrocytes to form their myelin sheaths properly; as such, these cells are particularly susceptible to the negative effects of ER stress. Previous work has demonstrated that ER stress induces EV release in some cell types. In the current study, we are determining how ER stress affects EV release and protein composition in oligodendrocytes. To achieve this, we are culturing human oligodendroglioma (HOG) cells and exposing them to tunicamycin to induce ER stress. To confirm the successful induction of ER stress, we are assessing the expression of three proteins that become activated in response to ER stress: inositol-requiring enzyme 1 α (IRE1 α), protein kinase R (PKR) endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). To determine how ER stress affects EV release and composition, we separate EVs from the conditioned cell culture media of control and tunicamycin treated cells by size exclusion chromatography and examining changes in their protein composition via western blot. We also exposing naïve oligodendrocytes to EVs from ER stressed oligodendrocytes to determine the role of ER stress-induced oligodendrocyte-derived EVs in ER-stress mediated pathology. These results can provide insight into our understanding of the pathophysiology of neurodegenerative diseases associated with demyelination, including amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS).

4. HIV-1 tat primes and activates microglial NLRP3 inflammasome leading to synaptodendritic injury in neurons via exosomes

Shilpa Buch, Ernest T. Chivero, Susmita Sil, Seema Singh, Abiola Oladapo, Muthukumar Kannan

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Neuroinflammation associated with HIV-1 infection affects 50% of HIV-infected individuals. NLR family pyrin domain containing 3 (NLRP3) inflammasome has been implicated in HIV-induced microglial activation; however, the mechanism(s) underlying this remain elusive. Since HIV-1 Transactivator of Transcription (Tat) protein persists despite antiretroviral therapy and, as a result, activates the NF- κ B pathway, we hypothesized that Tat protein could prime the NLRP3 inflammasome, which, in turn, could be released by the EVs, ultimately leading to synaptodendritic injury in neurons. As expected, there was an induction of NLRP3 expression in microglia exposed to Tat compared with cells not exposed to Tat. Tat exposure of microglia also time-dependently increased the expression levels of mature caspase-1 and IL-1 β as well as IL-1 β secretion. These *in vitro* findings were validated in archival postmortem brain tissues from Simian Immunodeficiency Virus (SIV)-infected and uninfected rhesus macaques. Further validation

of NLRP3 priming *in vivo* involved administration of lipopolysaccharide (LPS) to HIV transgenic (Tg) rats, followed by assessment of IL-1 β mRNA expression and inflammasome activation (ASC oligomers and mature IL-1). Intriguingly, LPS potentiated upregulation of IL-1 β mRNA and inflammasome activation in HIV-Tg rats compared with the wild-type controls. Interestingly, we found an inverse relationship in the expression of NLRP3 and its negative regulator, miR-223, suggesting a miR-223-mediated mechanism for Tat-induced NLRP3 priming. Furthermore, blockade of NLRP3 resulted in decreased IL-1 β secretion. Taken together, our findings suggest that microglial NLRP3 can be released in Tat-EVs, resulting in synaptodendritic injury in neurons. Currently, we are investigating the protective role of silencing NLRP3 in ameliorating synaptic degeneration. In summary, our findings suggest a novel role of Tat in priming and activating the NLRP3 inflammasome, which can be carried via the EVs to recipient neurons, leading to neuronal injury. NLRP3 can thus be envisioned as a therapeutic target for ameliorating Tat-mediated neuroinflammation as well as EV-mediated synaptic neurodegeneration.

Acknowledgment: This work is supported by RO1DA044586, DA040397, DA043138, DA047156, DA050545 (S. Buch).

5. Astrocyte-derived extracellular vesicles in morphine induced synaptodegeneration

Susmita Sil, Shilpa Buch

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A study from our group has revealed the role of morphine-induced neuronal autophagy in mediating synaptodendritic injury. The role of other CNS cell types, such as the astrocytes, in contributing to morphine-mediated neuronal injury, however, remains an enigma. Several studies have identified interactive crosstalk and shared molecular machinery between extracellular vesicle biogenesis & the autophagy pathway. The current study was undertaken to understand whether morphine-mediated dysregulation of autophagy in astrocytes could also mediate neuronal dysfunction via the EVs. Based on our initial findings that morphine-initiated autophagy in astrocytes while blocking the autophagy flux, we sought to understand the link between dysregulated autophagy and EV biogenesis. Our results showed that morphine exposure induced expression of NMDA-NR1 subunit in the human astrocytes, which, in turn, led to the initiation of autophagy and formation of autophagosome, and a concomitant block in the autophagic flux. Intriguingly, morphine-mediated block in autophagic flux was accompanied by increased release of astrocyte-derived EVs containing the autophagic proteins. Next, we assessed whether the neurons could take up morphine-stimulated ADEVs and if so, the extent of synaptic impairment, if any. For this, rat hippocampal neurons were exposed to morphine-ADEVs carrying the autophagic proteins and assessed for spine morphology. Exposure of neurons to the morphine-ADEVs resulted in an increase in immature dendritic spines, a reduction in excitatory synapse densities, with a concomitant increase in numbers of inhibitory synapses, leading to synaptic dysfunction. Silencing of astrocytic NMDA-NR1 not only reduced the release of ADEVs and their autophagy cargoes, but also ameliorated synaptodegeneration. This study underscores the role of autophagy cargoes in morphine-ADEVs mediated neuronal injury and synaptic alterations. Understanding how morphine hijacks the autophagy machinery to regulate EV release via the astrocytic NMDA-NR1 and how this phenomenon is involved with neurodegeneration is a novel concept, which can set the groundwork for future development of therapeutics for opiate addicts.

Acknowledgment: This work is supported by RO1DA044586, DA040397, DA043138, DA047156, DA050545 (S. Buch).

6. An organ-on-chip model to characterize extracellular vesicles as functional biomarkers in cardiorenal syndrome

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Extracellular vesicles (EVs) and their cargo have diverse roles in mediating intercellular signaling and can thus serve as potential functional circulating biomarkers. Type 1 cardiorenal syndrome (CRS) is characterized by the development of acute kidney injury in the setting of acute decompensated heart failure (ADHF) and can complicate the clinical care of HF patients. However, there is a paucity of biomarkers and mechanistic understanding of CRS. The emergence of micro-fluidic organ-on-chip platforms comprising human tissues that can faithfully recapitulate key aspects of human physiology opens the door to studying the functional role of EVs in type I CRS. In this study, we used the Kidney Proximal TubuleChip model (Emulate) to elucidate the possible role of EVs in CRS.

EVs were isolated from patients with heart failure with preserved ejection fraction (HFpEF) with or without CRS and healthy controls, followed by treatment with RNaseA and dosed on Kidney Proximal Tubule-Chips a single bolus in the vascular channel and administered for 72 hours. Each chip includes epithelial cells in the top channel and endothelial cells in the bottom channel. EV uptake was observed using fluorescent tagging of EVs prior to perfusion in the chamber. Following the treatment, RNA was isolated from both top and bottom channels of each chip, expression of kidney injury markers like Neutrophil gelatinase-associated lipocalin (NGAL) and Interleukin-18 (IL-18) were measured by qPCR.

Isolated EVs were characterized following MISEV guidelines. Dil-stained EVs from healthy control subjects were visualized after a three-day perfusion period using fluorescence microscopy, confirming successful EVs uptake on tissue chip. After treatment with EVs, differential expression levels of mRNA of NGAL and IL-18 were observed in human glomerular endothelial cells and proximal tubule epithelial cells. NGAL and IL-18 were significantly increased in both endothelial and epithelial cells in response to incubation with EVs from HF patients. The increase was higher in HF patients with CRS than those without CRS.

Our tissue on-chip study bridges the gap between *in vitro* and *in vivo* models offering new approaches to identify the role of plasma EVs as potential biomarker for CRS.

7. Unannotated small RNA clusters associated with circulating extracellular vesicles detect early stage liver cancer

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Objective Surveillance tools for early cancer detection are suboptimal, including hepatocellular carcinoma (HCC), and biomarkers are urgently needed. Extracellular vesicles have gained increasing scientific interest due to their involvement in tumor initiation and metastasis; however, most extracellular RNA (exRNA) blood-based biomarker studies are limited to annotated genomic regions. EVs were isolated with ultracentrifugation and nanoDLD and quality was assessed by electron microscopy, immunoblotting, nanoparticle tracking and deconvolution analysis. Genome-wide sequencing of the largely unexplored small exRNA landscape, including unannotated transcripts, identified and reproducibly quantified small RNA clusters (smRCs). Their key genomic features were delineated across biospecimens and EV isolation techniques in prostate cancer and HCC. Three independent exRNA cancer datasets with a total of 479 samples from 375 patients, including longitudinal samples, were used for this study.

ExRNA smRCs were dominated by uncharacterized, unannotated small RNA with a consensus sequence of 20 bp. An unannotated 3-smRC signature was significantly overexpressed in plasma exRNA of patients with HCC ($P < 0.01$, $n = 157$). An independent validation in a phase 2 biomarker case-control study revealed 86% sensitivity and 91% specificity for the detection of early HCC from controls at risk ($n = 209$) [area under the receiver operating curve (AUC): 0.87]. The 3-smRC signature was independent of alphafetoprotein ($P < 0.0001$) and a composite model yielded an increased AUC of 0.93.

These findings directly lead to the prospect of a minimally invasive, blood-only, operator-independent clinical tool for HCC surveillance, thus highlighting the potential of unannotated smRCs for biomarker research in cancer.

8. Biogenesis of RNA-containing extracellular vesicles at endoplasmic reticulum membrane contact sites

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Extracellular RNAs carried by extracellular vesicles can affect gene expression, function, and phenotypes of recipient cells. While a number of RNA binding proteins (RBPs) are known to carry RNAs into extracellular vesicles, where and how in the cell this occurs is unclear. Here, we identify VAP-A positioned endoplasmic reticulum membrane contact sites (ER MCS) as key locations for the biogenesis of RNA-containing EVs.

We used RNA-sequencing, lipidomic, confocal and transmission electron microscopy, tumor xenograft and various biochemical techniques to analyze EV biogenesis and cargo content in colon cancer cell lines molecularly engineered for molecules that control ER MCS.

RNA-Seq analysis revealed a number of small RNAs that are altered in VAP-A KD small and large EVs compared to control cells. Density gradient fractionation revealed that VAP-A regulates a select subpopulation of small EVs enriched with RNA and RBPs. Furthermore, this VAP-A-controlled small EV population is critical for transferring miR-100 to recipient cells and the growth of xenograft mouse tumors. Analysis of small and large EVs for lipid content revealed that VAP-A controls the levels of ceramide and cholesterol, two lipids involved in EV biogenesis. Furthermore, KD of the VAP-A binding ceramide and cholesterol transporters CERT and ORP1L led to similar defects in EV biogenesis.

We uncovered a novel pathway of EV biogenesis that takes place at ER MCS. These data suggest a model in which lipid transfer at ER MCS drives the biogenesis of a select subpopulation of EVs containing RNA-RBP complexes. Beyond improving our understanding of EV biogenesis, we anticipate that these findings may be helpful in future engineering of therapeutic EVs as well as exploring the functions of RNA-containing EVs.

Funding: Funding was provided by NIH grants U19CA179514 and P01CA229123; Vanderbilt CTSA grant UL1 RR024975 and UL1 TR002243.

9. Cell type-specific EV define disease-related protein networks associated with astrocyte activation in Alzheimer's disease

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Extracellular vesicles (EVs) are known to transfer pathogenic molecules in neurodegenerative diseases and are involved in disease progression. Here we investigated the proteomic profile of EVs isolated from human induced pluripotent stem cell (hiPSC)-derived neural cells. Novel cell type-specific EV protein markers are identified for excitatory neurons (ATP1A3, NCAM1), astrocytes (LRP1, ITGA6), microglia-like cells (ITGAM, CD300A) and oligodendrocyte-like cells (LAMP2, FTH1), as well as 16 pan-EV marker candidates including integrins and annexins. To further demonstrate how cell type-specific EVs may interplay in Alzheimer's disease (AD), we performed protein co-expression network analysis with cell type assessment of proteomes of brain-derived EVs from the control, mild cognitive impairment, and AD cases. A protein module enriched in astrocyte-specific EV markers was found most significantly associated with AD pathology and cognitive impairment, suggesting the important role it may play in AD progression. Finally, we confirmed that the hub protein from this module, integrin- β 1 (ITGB1), was elevated in AD astrocyte-derived EVs purified from total brain-derived EVs and associated with brain A β 42 and tau load in independent cohorts. Thus, our study provides a featured framework and rich resource for analyses of EV functions on neurodegenerative diseases in a cell type-specific manner.

10. Selective protein sorting into exosomes: a role in cell differentiation and a possible tool in genome editing

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Extracellular vesicles (EVs) are thought to mediate the transfer of cytoplasmic proteins and RNA between cells to inform the processes of differentiation, cell motility and possibly malignant transformation. We have found that undifferentiated or embryonic stem cells (ESCs), which are induced to differentiate into neural progenitor cells, secrete EVs that selectively capture proteins implicated in G1/S cell cycle progression. Further, we have found that these EVs promote the differentiation of ESCs to a neural progenitor fate and that cyclin D1 plays a rate-limiting role in that EV-mediated progression.

A challenge in genome editing *in vivo* is to devise an efficient means of delivering editing functions, preferably by a vehicle that evades immune detection. We sought a means to deliver Cas9 and a gRNA enclosed within exosomes, a subclass of EVs, as a vehicle for efficient and targeted gene editing. Cas9 was expressed in a donor cell tethered noncovalently to an integral membrane protein, CD63, enriched in exosomes. Exosomes highly enriched in Cas9 and a gRNA were isolated by buoyant density sedimentation. Isolated exosomes were incubated with reporter cells containing an integrated copy of N-luciferase behind a site that, when edited, would allow the luciferase expression. In a control experiment, expression of the Cas9/gRNA constructs directly in the reporter cell elicited a 60-70 fold increase in luciferase expression. Exosomes containing a similar level of Cas9 elicited no more than a 50% increase above the background of luciferase. The same was true of conditioned medium containing Cas9-exosomes and even of donor and acceptor cells incubated together, separated by a vesicle-permeable membrane in a transwell chamber. Thus, for these EVs, the functional uptake to promote gene expression was not observed as we found for those isolated from differentiating neurons. In contrast, donor and acceptor cells cocultured to near confluence showed a 60-fold increase in luciferase expression. Transfer of Cas9 appears to be mediated by open-end membrane tubular connections, likely dependent on membrane fusion at the junction point between a tubule from one cell and the target. Molecular dissection of the requirements for this transfer may permit the development of an efficient means for targeted delivery of Cas9/gRNA.

11. Characterizing IgG antibodies in plasma and extracellular vesicles of patients with glioblastoma and meningioma

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Glioblastoma (GBM) is the most common malignant primary brain tumor in humans. Meningioma (MMA) is a generally benign brain tumor formed from the meningeal layers of the brain; 10%-15% of these tumors become malignant. Previous findings suggest that tumor antibodies have decreased function from subtle proteolytic cleavage. Therefore, we hypothesized that immunoglobulin G (IgG) antibodies in the plasma of brain tumor patients are abnormal and may play a significant role in tumor pathogenesis. Using multiple immunoassays, we characterized IgG antibodies in plasma and plasma extracellular vesicles (EVs) from patients with GBM ($n = 82$), MMA ($n = 83$), and controls (non-tumor CNS disorders and healthy donors, $n = 50$). By capturing ELISA, we found that significantly higher levels of Fc heavy chain IgG antibodies and IgG1 subclass are present in GBM plasma compared to MMA ($P = 0.0002$ and $P = 0.0003$, respectively). Similarly, EVs purified from GBM plasma contained higher IgG Fc heavy chain levels than that of MMA. In addition, immunohistochemistry demonstrated the presence of IgG antibodies in GBM tumors tissues, concentrating around the periphery of the tumor. Importantly, we demonstrated

that the IgG antibodies in both GBM plasma and EVs produce complement-dependent cytotoxicity on a neuroblastoma cell line SH-SY5Y used as a neuronal surrogate. The higher IgG levels in the plasma and EVs in GBM patients and the high cell killing capacity suggest that GBM IgG antibodies may play an important role in tumor pathogenesis.

Review

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Exploring extracellular vesicles as mediators of clinical disease and vehicles for viral therapeutics: Insights from the COVID-19 pandemic

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How to cite this article: Craddock VD, Cook CM, Dhillon NK. Exploring extracellular vesicles as mediators of clinical disease and vehicles for viral therapeutics: Insights from the COVID-19 pandemic. *Extracell Vesicles Circ Nucleic Acids* 2022;3:172-88. <https://dx.doi.org/10.20517/evcna.2022.19>

Received: 19 Apr 2022 **First Decision:** 13 May 2022 **Revised:** 1 Jul 2022 **Accepted:** 11 Jul 2022 **Published:** 19 Jul 2022

Academic Editors: Yoke Peng Loh, Shilpa Buch **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

The COVID-19 pandemic has challenged researchers to rapidly understand the capabilities of the SARS-CoV-2 virus and investigate potential therapeutics for SARS-CoV-2 infection. COVID-19 has been associated with devastating lung and cardiac injury, profound inflammation, and a heightened coagulopathic state, which may, in part, be driven by cellular crosstalk facilitated by extracellular vesicles (EVs). In recent years, EVs have emerged as important biomarkers of disease, and while extracellular vesicles may contribute to the spread of COVID-19 infection from one cell to the next, they also may be engineered to play a protective or therapeutic role as decoys or “delivery drivers” for therapeutic agents. This review explores these roles and areas for future study.

Keywords: SARS-CoV-2, EVs, tissue factor, endothelial apoptosis

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has led to the death of millions of people worldwide and has the potential to



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continue causing illness and mortality for the foreseeable future. SARS-CoV-2 infection most frequently causes fever with upper and lower respiratory symptoms, including rhinitis, sore throat, dyspnea, and cough. In some patients, COVID-19 can progress to a severe infection that requires hospitalization due to pneumonia, acute lung injury (ALI), or acute respiratory distress syndrome (ARDS)^[1-3]. COVID-19 patients are also at risk for endothelial dysfunction and coagulopathies that increase the risk of adverse cardiovascular events^[4,5]. Moreover, people with prior SARS-CoV-2 infection are now experiencing persistent COVID-19 symptoms following a recovery that can last many months after acute infection^[6,7]. Recent studies also suggest an increased risk of developing pulmonary fibrosis, cardiovascular dysfunction, and other long-term complications following SARS-CoV-2 infection^[8-10]. In this review, we highlight the role of extracellular vesicles (EVs) as drivers of COVID-19 clinical manifestations and the importance of EVs as potential biomarkers of disease prognosis according to published and ongoing research studies^[11]. Finally, we will discuss the implications of EVs as novel therapeutics for patients with severe COVID-19 disease [Figure 1].

ACUTE AND LONG-TERM MANIFESTATIONS OF COVID-19

SARS-CoV-2 establishes infection through endocytosis following the binding of the viral spike (S) protein to the ACE2 receptor on host cells^[12,13]. In cases of severe COVID-19, SARS-CoV-2 infects type II pneumocytes, which express ACE2 in the lower respiratory tract^[13,14]. Injury to type II pneumocytes upon infection results in decreased surfactant production by these cells, contributing to diffuse alveolar damage. In addition, the injury of type II pneumocytes is caused by an overwhelming immune response that leads to increased epithelial-capillary barrier permeability. This alveolar injury can lead to ARDS, a clinical hallmark of severe COVID-19 in hospitalized patients.

In conjunction with ARDS, patients with severe acute COVID-19 may also exhibit a hypercoagulable state and, therefore, be at increased risk for acute cardiovascular incidents. Hypercoagulability is stimulated by significant increases in proinflammatory cytokines TNF- α , IL-1 β , and IL-6, which are all involved in signaling for the upregulation of coagulation factors^[15,16]. These three cytokines are overexpressed in patients with severe COVID-19 and contribute to the infamous “cytokine storm”^[3]. Simultaneously, SARS-CoV-2 infection has been suggested to induce endothelial damage either indirectly through immense inflammatory mechanisms or directly through the infection of endothelial cells^[17], which further increases the risk of blood clots during infection^[11,14,15]. A recent study by Joffe *et al.* demonstrated that sera from patients with moderate and severe COVID-19 exhibit circulating biomarkers consistent with endothelial activation and dysfunction^[17]. They further suggest from their findings that direct infection of endothelial cells by SARS-CoV-2 contributes to COVID-19-associated endotheliopathy. They confirmed by viral PCR that SARS-CoV-2 enters and replicates within human microvascular endothelial cells (HMVEC), which contributes to endothelial injury. How SARS-CoV-2 directly infects endothelial cells is still debated, with some saying that endothelial entry is mediated by ACE2 and others suggesting it is more likely to be through alternative receptors, such as neuropilin-1^[18-21]. Several previous studies have reported that ACE2 is indeed found on the surface of vascular endothelial cells, and that SARS-CoV-2 dysregulates the renin-angiotensin-aldosterone system (RAAS) by inducing changes in endothelial ACE2 expression which contributes to endotheliopathy in COVID-19^[19,22-24]. These findings suggest that SARS-CoV-2 entry into endothelial cells is at least partly responsible for the endothelial dysfunction in COVID-19 and contributes to hypercoagulability in these patients.

As the pandemic evolves, it has become clear that many COVID-19 patients experience symptoms for months following their acute infection, conditions referred to as Long COVID or Post-acute Sequelae of COVID-19 (PASC). A study by Carfi *et al.* assessed the prevalence of persistent COVID-19 symptoms in

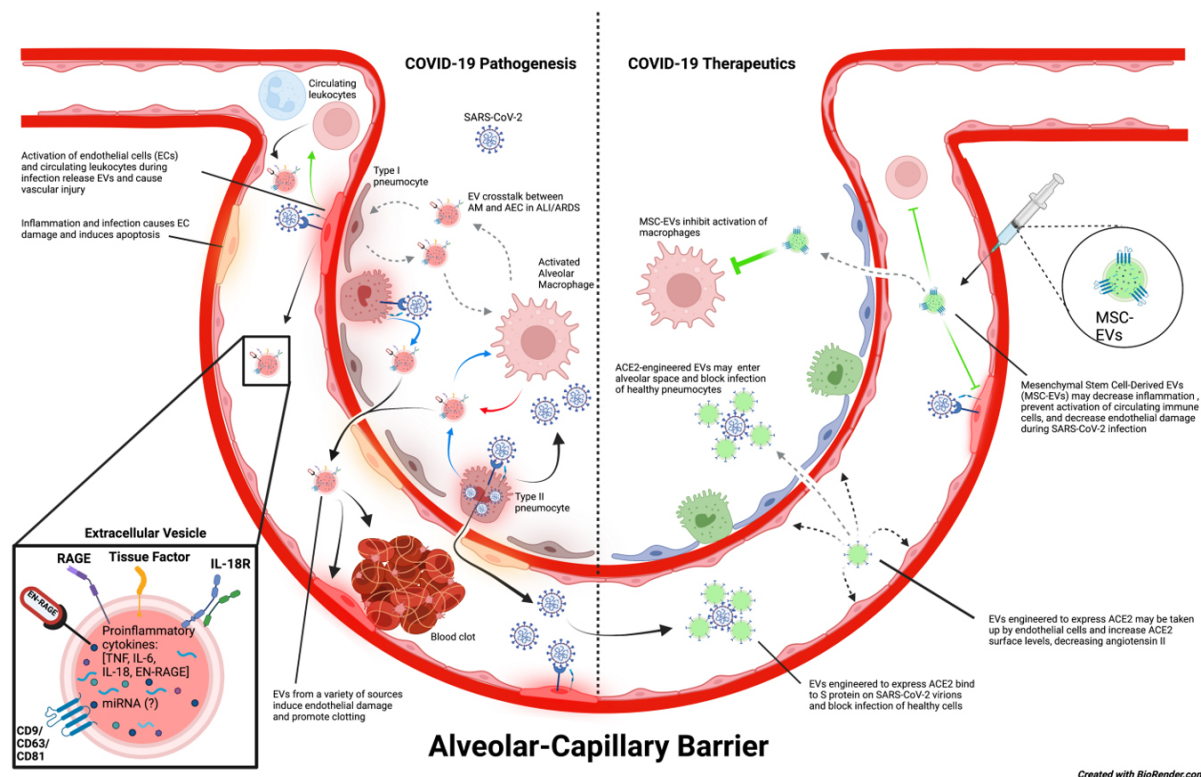


Figure 1. Potential role of EVs in COVID-19 disease pathogenesis and therapeutics. SARS-CoV-2 virions enter the host via the large airways and can travel into alveoli where they infect type II pneumocytes. Infection of type II pneumocytes by SARS-CoV-2 induces a proinflammatory response that activates alveolar macrophages and damages the alveolar epithelium. It is speculated that EV crosstalk between alveolar macrophages and alveolar epithelial cells could intensify inflammation and further enhance alveolar epithelial permeability. This may lead to increased permeability of the alveolar-capillary barrier, causing pulmonary edema and decreased functional capacity of the lungs, thereby allowing SARS-CoV-2 virions, EVs, and other inflammatory mediators to enter the bloodstream and progress to systemic infection. SARS-CoV-2 virions in the blood are suggested to infect endothelial cells and cause endothelial dysfunction, which ultimately leads to blood clots that can cause major thrombotic events as well as activation of circulating immune cells. On the contrary, engineered EVs could also be used as a therapeutic tool against COVID-19. EVs modified to express ACE2 may be injected into the bloodstream or nebulized and could potentially prevent SARS-CoV-2 from infecting healthy cells in either the bloodstream or alveolar space. These ACE2-expressing EVs may also be taken up by endothelial cells, increasing the amount of surface ACE2 expression on these cells, and maintaining the appropriate function of RAAS. In addition, injected MSC-EVs have anti-inflammatory properties that can promote tissue repair and prevent hyperinflammation during SARS-CoV-2 infection and ARDS. In COVID-19, they might be particularly useful for decreasing endothelial damage and preventing immune activation. (The copyright is retained by the authors).

patients discharged from the Gemelli University Hospital (Foundation Policlinico Universitario Agostino Gemelli) in Rome, Italy, and found that, after approximately 60 days post recovery from acute infection, 87.4% of patients in their study still experienced at least one symptom of SARS-CoV-2 infection, and a majority of patients experienced three or more symptoms^[25]. The most common long-term sequelae were fatigue and difficulty breathing^[25]. Other studies have demonstrated that post-acute COVID patients exhibit decreased alveolar gas exchange based on pulmonary function testing and signs of interstitial lung damage and fibrosis on radiographic data for up to 12 months or longer post infection^[10,26]. Long COVID patients also commonly report shortness of breath, fatigue, myalgias, sleep disturbances, malaise, mood changes, and chest pain 28 days or more after their acute infection^[27,28].

Of equal concern is the increased risk of cardiovascular disorders following SARS-CoV-2 infection. A recent large study that assessed patients within the U.S. Veterans Health Administration system found that COVID-19 was associated with an extra 23.98 incidents of major adverse cardiovascular events per 1000

people (i.e., stroke and heart attack) and 9.88 incidents of thromboembolic disorders per 1000 people, along with increased risks of many other cardiovascular abnormalities^[9]. Although patients with severe COVID-19 are more likely to suffer from cardiovascular abnormalities post infection, even patients with mild cases are at increased risk of suffering from cardiovascular dysfunction^[9]. It is anticipated that PASC will cause significant disease and mortality for years to come; therefore, it is vital to monitor post-acute COVID patients longitudinally to better understand the chronic manifestations of the disease. How the virus causes these persistent symptoms has yet to be fully understood, but it likely involves a variety of factors that may include the persistence of SARS-CoV-2 fragments in some tissues and associated inflammation for prolonged periods, reactivation of latent infections like Epstein-Barr virus, and/or worsening of preexisting health conditions^[29-31].

EXTRACELLULAR VESICLES AS CONTRIBUTORS TO COVID-19 DISEASE PATHOGENESIS

A brief description of EVs

EVs are small, lipid-enveloped nanoparticles ranging from 30-5000 nm in size found in plasma, serum, urine, and many other biophysiological fluids. They carry cargo, such as DNA fragments, RNAs (e.g., mRNA, miRNA, tRNA, or long noncoding RNAs), lipids, and proteins. These complex biomolecules are delivered between cells via EV trafficking and thus are important for intercellular communication^[32,33]. EVs are divided into three classes that are differentiated by size, biogenesis, content, and function: apoptotic bodies, microvesicles, and exosomes^[34]. Apoptotic bodies are 1-5 µm in diameter and are released from the plasma membrane by outward budding during apoptosis^[35]. Although originally thought to be “junk” material released from dying cells, cargo from apoptotic bodies has been shown to stimulate progenitor cells and initiate tissue remodeling following cell injury^[36]. Microvesicles (MVs) are large EVs that range in size from 100-1000 nm in diameter and are generated by budding from cell plasma membranes^[34]. Exosomes are small EVs that range in size from 30-150 nm in diameter and are formed by cell sorting mechanisms, such as the endosomal sorting complex required for transport (ESCRT) pathways or ESCRT independent endosomal pathways^[34,37]. Both MVs and exosomes have been studied for their involvement in intercellular communication and transporting cargo between cells. Extracellular vesicles are commonly studied for their use as biomarkers in metastatic cancer, sepsis, cardiovascular disease, COVID-19, and other conditions^[11,38-41].

Extracellular vesicles can be released by virus-infected cells, consequently transferring viral proteins, viral receptors, and proinflammatory cargo to recipient cells, thereby spreading infection and exacerbating tissue injury^[32,33,35]. The EV lipid membrane offers protection for biomolecules against proteases, DNases and RNases, and other degradation catalysts that may exist in the bloodstream or other bodily fluids. Therefore, EVs can serve as vehicles for liquid biopsies by preserving cargo that may otherwise be degraded while circulating in the bloodstream as free biomolecules. EV cargo is derived directly from the cells generating and releasing them and therefore can offer a “snapshot” of the health and status of EV-generating cells, reflecting the pathogenesis of diseases in real time. Analysis of EV cargo can shed light on the body's inflammatory and immune response to infection, provide evidence of endothelial dysfunction, and demonstrate features of coagulopathy. This has proven to be particularly helpful as we work to better understand the pathobiology of SARS-CoV-2 infection.

EVs and COVID-19-associated inflammation and immune response

Since the start of the pandemic, multiple studies have demonstrated that a profound inflammatory response to the virus plays a major role in SARS-CoV-2 pathophysiology. The innate immune response to SARS-CoV-2 is robust, with many cytokines and chemokines serving as markers of disease severity. Patients with

severe COVID-19 exhibit increased secretion of IL-2R, IL-6, and TNF- α ^[42], and elevated circulating levels of IL-6 and TNF- α are independent predictors of mortality^[43]. Recent findings from our lab demonstrate that both large and small EVs from the plasma of hypoxic patients with moderate-to-severe COVID-19 infection contain significantly elevated levels of members of the TNF superfamily and IL-6 family^[11]. IL-6 can activate the protein kinase B (PKB)/Akt, mitogen-activated protein kinases (MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling pathways, all of which can promote inflammation and cellular crosstalk between immune and stromal cells^[44]. We also observed a rise in the EV levels of RAGE and its ligand EN-RAGE (Extracellular Newly identified Receptor for Advanced Glycation End-products, a.k.a. S100A12) with the progression of COVID-19 infection, and significantly higher EV levels of EN-RAGE could distinguish patients with severe COVID-19 infection from those with moderate disease as well as deceased from critically ill patients who survived^[11]. Advanced glycation end products (AGEs) are irreversible adducts formed from the glycation of proteins, lipids, and nucleic acids. AGEs interact with cell-surface receptor RAGE (receptor for AGE), causing an inflammatory cascade that generates reactive oxygen species (ROS) and tissue injury^[45].

EVs have previously been shown to contribute to ALI and ARDS in sepsis models^[2,46], and they may be involved in COVID-19-associated lung injury as well. EVs recovered from bronchoalveolar lavage fluid (BALF-EVs) during ARDS exhibit a complex interplay of communication between alveolar epithelial cells (AECs) and alveolar macrophages. In LPS-induced ARDS mouse models, BALF-EVs were enriched with miRNA that was shown to induce proinflammatory cytokines by AECs as well as alveolar macrophages (AMs) in cell culture experiments^[46-48]. BALF-EVs containing increased amounts of miR-466g and miR-466m-5p were shown to activate the inflammasome and induce IL-1 in AMs^[48]. Additionally, BALF-EVs enriched with miR-155 and miR-146a induced overexpression of TNF- α and IL-6 in AECs. These miRNAs also decreased the expression of the tight-junction protein, ZO-1, thereby increasing the permeability of the alveolar epithelial-capillary barrier^[46,47]. Therefore, EVs have been shown to contribute to the increased alveolar vascular permeability, decreased alveolar ventilation and lung compliance, and the hyperinflammatory response that occurs in the lungs during ARDS. These disease processes broadly contribute to diffuse alveolar damage and pulmonary edema and, therefore, are highly applicable to COVID-19 ALI. Bronchoalveolar lavage (BAL) can be performed safely in patients with respiratory failure and lower respiratory tract infections^[49-51]. Bronchoscopy and BAL extraction procedures have been limited during the pandemic to protect healthcare workers from exposure to SARS-CoV-2^[49]. At the time of this writing, there is limited data on human BALF and BALF-EVs in COVID-19 pneumonia and ARDS. Nonetheless, we hypothesize that human BALF-EVs may be feasible biomarkers in COVID-19 patients on invasive mechanical ventilation, as previous studies demonstrate their role in ALI and ARDS. Since SARS-CoV-2 infection increases alveolar epithelial and vascular permeability, this might allow for BALF-EVs to “leak” into the bloodstream and further induce the production of proinflammatory cytokines that may lead to a more systemic immune response, thereby contributing to endothelial dysfunction and coagulopathy.

Apoptosis and necrosis of epithelial cells during ALI induced by SARS-CoV-2 is another important factor to consider in severe COVID-19. SARS-CoV-2 can induce cell death of lung epithelial cells by activating caspase-8, which can also contribute to immune activation through the induction of caspase-8-dependent expression of proinflammatory cytokines^[50-52]. In addition, Fas Ligand (FasL) is an activator of caspase-8-mediated apoptosis in cells that express the death receptor Fas, the receptor for FasL, and is observed on the surface of EVs in chronic lung disease^[53-55]. Cells expressing the death receptor Fas may include lung epithelial cells, virally infected cells, and T-lymphocytes which are all highly implicated in COVID-19 pathophysiology, especially during ALI/ARDS^[53]. Interestingly, soluble FasL competes with membrane-associated FasL (mFasL) for binding to the death receptor Fas and, as a result, blocks cell death, highlighting

that it is specifically mFasL that induces apoptosis^[53,56]. Microvesicles and exosomes have been reported to express mFasL on their surfaces and induce apoptosis in T-lymphocytes^[57,58]. Lymphopenia is a marker of COVID-19 severity and risk^[59], and one could speculate that EV-associated mFasL may contribute to T-cell death and serve as a potential therapeutic target in critically ill COVID-19 patients to maintain proper immune response.

Apoptotic bodies may also be important in viral-associated tissue damage. The role of apoptotic bodies is to safely compartmentalize dying cell debris which is then taken up by macrophages for digestion^[60]. In theory, the packaging of debris from a dying cell into apoptotic bodies prevents activation of host immune cells, though some propose that apoptotic vesicles may elicit an immune response by delivering cytokines and damage-associated molecular patterns, or by activating immune cells, such as CD4⁺ T cells, through MHC-II molecules^[61-63]. A very intriguing theory detailed later in this review is the potential capability of viruses to usurp apoptotic extracellular vesicles (ApoEVs) to promote their spread to healthy cells without calling attention to themselves by inducing an immune response.

EVs and COVID-19-associated endothelial dysfunction

As mentioned above, in addition to epithelial injury, COVID-19 is associated with endothelial dysfunction, coagulopathy, and major adverse cardiovascular events like heart attack and stroke^[11,64]. The endothelial damage and apoptosis could be due to direct infection of endothelial cells or indirect effects of inflammatory and thrombotic changes in circulation^[22,65]. SARS-CoV-2 enters cells most commonly by binding to ACE2 receptors on respiratory epithelial cells and maybe endothelial cells as well. Infection subsequently leads to the downregulation of ACE2 receptors. Consequently, reduced ACE2 expression on endothelial cells would result in dysregulation of RAAS and could cause endothelial dysfunction due to inflammation and vasoconstriction induced by higher levels of angiotensin II^[23,24,66]. Interestingly, EVs from patients with severe COVID-19 are observed to have lower levels of ACE2 compared to EVs from patients with a moderate disease^[11].

EVs appear to be heavily involved in COVID-19-associated endothelial activation and dysfunction. We demonstrated that both large and small EVs isolated from the plasma of patients with severe COVID-19 disease were significantly more capable of inducing apoptosis of pulmonary microvascular endothelial cells compared to EVs isolated from the plasma of healthy and asymptomatic groups^[11]. In a recent study by Sur *et al.*, the authors isolated exosomes from patients with mild-to-severe COVID-19 and observed that COVID-19 exosomes significantly induce the expression of NLRP3, caspase-1, and IL-1 mRNA in human endothelial cells^[67]. Authors did not elucidate the cause of NLRP3 inflammasome activation by COVID-19 exosomes; however, it has been noted in conditions, such as diabetic retinopathy, that exosomes may carry damage-associated molecular patterns, such as high mobility group box-1 (HMGB-1)^[68-70], and induce the NLRP3 inflammasome via the canonical pathway^[71]. Furthermore, EVs may traffic proinflammatory cytokines that activate the inflammasome via transactivation by NF- κ B and contribute to endothelial dysfunction^[72].

EV-induced endothelial dysfunction most notably results in higher blood pressure due to reduced production of nitric oxide, a potent vasodilator, and increased clotting^[73]. This causes increased resistance to flow, which can decrease perfusion to local organs and cause ischemic organ damage and increased systemic workload on the heart. We found that EV-associated ST2, a biomarker of myocardial dysfunction and organ damage, was positively correlated with increasing disease severity and length of hospitalization^[74].

Findings from Joffre *et al.*'s study, discussed earlier, might be of considerable interest in the EV field^[17]. The authors point out that it is difficult to study the vascular endothelium in real time because of a lack of non-invasive procedures that can specifically and successfully help determine the health and status of endothelial cells during systemic infection, such as in COVID-19. In addition, the vascular endothelium is a vast monolayer of cells that serves as a barrier between blood and tissue and performs distinctive functions depending on the tissue site^[75,76]. Therefore, assessing the function of the vascular endothelium systemically is very difficult with a tissue biopsy or ultrasound alone and requires one to first identify the site of endothelial damage. EVs may be the answer to this problem. EVs derived from endothelial cells (EC-EVs) express a variety of endothelial cell markers that can be captured with antibodies and isolated for further analysis^[76]. Findings from EVs in COVID-19 pathogenesis^[11] propose some of the targets that could be used as biomarkers to assess endothelial function by analyzing EC-EVs in real time.

EVs and COVID-19-associated coagulopathy

Endothelial damage from SARS-CoV-2 infection exposes connective tissue and induces the coagulation cascade following the binding of von Willebrand factor (vWF)^[77]. Importantly, EVs and their cargo can directly contribute to the induction of the coagulation cascade in COVID-19 patients. Phosphatidylserine-exposing (PS+) EVs^[78] have been observed to be higher in COVID-19 patients with mild or moderate disease compared to those with severe disease^[79,80]. Phosphatidylserine is a so-called "eat me" signal that interacts with phagocytes to stimulate phagocytosis of cells or vesicles^[81], but it is also a negatively charged molecule that facilitates activation of coagulation^[78]. Recent studies demonstrated higher levels of tissue factor (TF) on EV surface in COVID-19 patients, and the levels of tissue factor-linked EVs (EV-TF) were found to be positively correlated with COVID-19 severity and mortality^[11,80,82]. In addition, EV-linked vWF, urokinase plasminogen activator receptor (uPAR), and ADAMTS13 in COVID-19 patients correlated with the levels of thrombotic marker D-dimer, status of disease severity, and length of hospitalization.

Tissue factor is a primary initiator of coagulation in the blood and is associated with life-threatening thrombosis if overexpressed^[83]. EVs have been shown to release or present TF and pro-coagulant phospholipids on their surface, promoting clot formation and accelerating fibrin polymerization^[84,85]. EV-TFs in the blood are suggested to synergize with inflammation and endothelial injury, which consequently increases TF levels to overcome the thrombotic threshold^[86]. EV-TF's effect may be further potentiated by adhering to neutrophil extracellular traps (NETs) and concentrating at the site of clot formation. We observed higher levels of TF on EVs from COVID-19 patients who experienced stroke, venous thromboembolism, splenic infarct, or vision changes due to hypercoagulability^[11], consistent with previous findings showing an association of higher levels of EV-TF with venous thromboembolism^[87,88]. Importantly, the positive correlation of EV-TF with disease severity and length of hospitalization^[11] was even stronger than the correlation of tissue injury marker lactate dehydrogenase, proinflammatory marker C-reactive protein, D-Dimer, and age with disease severity and length of hospitalization.

A study by Guervilly *et al.* similarly noted elevated EV-TF activity in COVID-19 patients and reported a positive correlation between elevated EV-TF activity and COVID-19 disease severity^[80]. They also found that EV-TF activity was higher in patients with severe COVID-19 than in patients with septic shock, indicating that COVID-19 specifically influences the concentration of circulating EV-TF compared to other causes of infection^[80]. Rosell *et al.* also reported elevated TF activity in EVs from plasma of COVID-19 patients, and further found that higher EV-TF activity correlated with a greater need for respiratory support (patients required > 5 L O₂/min)^[82]. They further found higher EV-TF activity in patients who ended up dying compared to those who survived. Although findings from our group also showed an increase in EV-TF activity in patients who died compared to those who survived, we did not find significant differences between the groups. This could be explained due to differences in the patient cohort. Our group compared

severely diseased (WHO score ≥ 5) patients with deceased, whereas most of the patients in the Rosell *et al.* study had a moderate disease with a WHO score ≤ 5 ^[82]. Nevertheless, these results suggest that EV-TF is a marker of disease severity and is associated with hypercoagulability, which could compound into severe COVID-19 disease.

Not only do COVID-19 EVs carry TF, but they also carry inflammatory molecules that induce the production of TF. Barberis *et al.* studied the exosomal proteome during COVID-19 and discovered that COVID-19 EVs traffic C-reactive protein (CRP) to distant cells and promote inflammation^[89]. Patients with severe acute COVID-19 especially had elevated CRP in EVs. CRP is a highly soluble protein and a marker of COVID-19 severity^[90,91] and can induce endothelial and smooth muscle cells to express TF^[92]. In addition, CRP causes endothelial dysfunction by inhibiting nitric oxide production, thereby preventing the normal function of vasodilatory responses that occur in response to increased pressures^[93]. Therefore, it is unclear if EV-associated CRP is more biologically significant compared to soluble CRP in COVID-19 pathobiology, but it is a contributor to COVID-19 inflammation and endothelial dysfunction.

Barberis *et al.* also discovered that COVID-19 exosomes show a two-fold increase in kininogen-1, a precursor to bradykinin^[89]. Des-Arg⁹bradykinin (DABK), the activated form of bradykinin, is a substrate of ACE2 and is of particular importance during ALI and inflammation^[94,95]. During an ALI, when ACE2 expression may be reduced on alveolar epithelial cells, especially during SARS-CoV-2 infection, bradykinin inactivation is impaired, and thus, DABK increases^[96]. DABK is a known mediator of angioedema^[97] that significantly increases capillary permeability and induces the expression of several proinflammatory cytokines^[95,96]. In fact, the kinin-kallikrein system is shown to be overactive in cases of severe COVID-19^[95,98,99]. Moreover, exosomal kininogen-1 may be yet another biomarker of thrombosis risk in COVID-19 patients, because the kinin-kallikrein system is activated by Factor XII of the coagulation cascade^[97]. Since the kinin-kallikrein system plays a role in the pathophysiology of ALI, we hypothesize that EVs carrying kininogen-1 may enter the bloodstream, contribute to systemic inflammation, and consequently increase the risk for thrombosis in COVID-19.

Finally, platelet-derived EVs (pEVs) are involved in coagulopathy in both infectious and noninfectious inflammatory conditions, including COVID-19^[100,101]. Platelets are anuclear cells derived from megakaryocytes that serve a vital function in hemostasis^[102]. The plasma-derived EVs are increased during acute SARS-CoV-2 infection^[100,103], but surprisingly, this increase is more pronounced in patients with non-severe COVID-19 compared to those in critical condition^[100]. It is speculated that pEVs might be decreased in severe disease due to consumption because of blood hypercoagulability in COVID-19 patients. Therefore, pEVs appear to be partial contributors to coagulopathy in COVID-19 patients. In addition, pEVs may be released in response to endothelial activation and dysfunction or may themselves contribute to endothelial dysfunction and contribute to thromboembolic risk factors in COVID-19^[104].

EVs and dissemination of SARS-CoV-2 infection

An emerging hypothesis that EVs aid in the spread and persistence of SARS-CoV-2 genetic material and proteins is of high interest. EVs and viruses have similar mechanisms of entry, budding, and biogenesis during infection. Previous studies have demonstrated that EVs can carry viral proteins and genetic material from infected cells to healthy cells during infections caused by CMV, HIV-1, or HSV-1^[105-108]. Recent studies on spike-expressing cells and analysis of circulating EVs from a small set of COVID-19 patients suggest that EVs can incorporate S protein-derived peptides, but it is not yet clear if the spike peptide/protein gets incorporated on the surface or within the cargo of these particles^[109,110]. S protein on EVs is shown to alter the immune response to SARS-CoV-2, which may modulate the immune response into one that is more

severe^[109,110]. A recent study by Barberis *et al.* reported the presence of low range copy numbers of SARS-CoV-2 RNA in the exosomes isolated from COVID-19 patient plasma, although in a very small number of patients^[89].

In another study by Yim *et al.*, small EVs carrying endothelial marker CD31 were also observed to carry spike S1^[111]. However, the authors did not find viral RNA in EVs by PCR detection^[111]. Nonetheless, these data support the hypothesis that SARS-CoV-2 may be capable of infecting endothelial cells. Not only this, but the virus might be exploiting EV biogenesis by incorporating spike S1 into EVs which may serve as decoys that bind to neutralizing antibodies and protect SARS-CoV-2 virions from immune detection and destruction^[111]. However, the authors went on to observe that patients with EVs carrying spike S1 also demonstrated high levels of antibodies against SARS-CoV-2 and less of an anti-inflammatory immune profile^[111]. Therefore, it is unclear if it is SARS-CoV-2 exploiting EVs to protect itself or if the incorporation of spike S1 into EVs is an intentional attempt by infected cells to warn surrounding cells of viral infiltration. Further investigation is necessary to better understand this ambiguity surrounding the biological significance of spike S1-positive EVs.

In addition, EVs released by lung epithelial cells transduced with lentivirus encoding SARS-CoV-2 proteins have been shown to transfer the viral RNA to cardiomyocytes leading to upregulation of inflammatory gene expression in the recipient cells^[112]. All these studies support the hypothesis that EVs carrying viral proteins or genetic material from SARS-CoV-2 contribute to the injury of healthy cells. This EV-facilitated crosstalk could intensify not only lung injury but also dysfunction of multiple other tissues and organs in patients with severe COVID-19^[113,114].

In a study with Syrian hamsters, S protein produced from a pseudovirus was shown to downregulate ACE2 via ACE2 receptor endocytosis by the S protein-carrying pseudovirus^[24]. Additionally, S protein from the pseudovirus in hamsters increased oxidative stress through impaired mitochondrial function in endothelial cells, further exacerbating endothelial dysfunction^[24]. Since the results of this study came from an attenuated pseudovirus, it is proposed that S protein alone can cause endothelial dysfunction and dysregulation of RAAS. All these findings suggest that S protein-positive EVs have the potential to contribute to endothelial dysfunction and disease severity in SARS-CoV-2 infections by dysregulating RAAS.

Frleta *et al.* found that HIV-1 induced cell death during acute infection and utilized apoptotic microparticles to inhibit dendritic cell activation by binding to CD44^[115]. In ALI caused by COVID-19, there is a high degree of cell death within pulmonary tissue. Since COVID-19 decreases alveolar epithelial integrity and increases capillary permeability, SARS-CoV-2 might facilitate its own spread to systemic tissue through apoptotic vesicles, similar to HIV-1^[115]. By utilizing host vesicles to conceal and traffic itself, SARS-CoV-2 may spread to other tissue and organs before the immune system can catch up. This idea is similar to the “Trojan Horse” mechanism in which viruses are thought to utilize EVs to disseminate their particles and genomes to other host cells^[106]. Not only this, but pEVs have been shown to associate with viruses, including SARS-CoV-2^[100], and contribute to viral spread and proliferation^[79]. Since platelets are constitutively present in the bloodstream and are hyperactive during SARS-CoV-2 infection^[100], it is reasonable to predict that platelets and the pEVs they release contribute to the systemic spread of the virus during acute infection. Future studies on this topic are warranted to fully elucidate the biological mechanisms of SARS-CoV-2 associations with pEVs.

A major challenge in EV research is the presence of other particles of the same size and shape, such as lipoproteins, bacteria-derived EVs, or virions and viral particles, that are often isolated with EVs. In EV

research that involves viral infection, it can be difficult to isolate pure EVs from defective viral particles. Moreover, enveloped viruses, such as SARS-CoV-2, acquire a membrane derived from host cells, which is generated by budding from the plasma membrane and may include host cell markers^[116]. To add to these challenges, viral particle biogenesis is shown to overlap with exosome biogenesis pathways, such as ESCRT, which means some enveloped viral particles may express common exosomal markers^[117]. This is demonstrated in HIV, which recruits exosomal markers Alix, TSG101, and other ESCRT-1 proteins during viral budding, and makes it more difficult to truly ascertain if one is isolating pure EVs or a mix of EVs and defective viral particles^[117-119]. Therefore, there is a possibility of co-isolation of virions along with EVs from bio-fluids using currently accepted methods of EV isolation, such as ultracentrifugation, precipitation with crowding reagents, size-exclusion chromatography, or affinity purification^[120]. This is an inevitable challenge that must be acknowledged by researchers studying EV biology in the presence of viral infection. Nevertheless, viremia in COVID-19 patients is not as frequent, and if present, the reported copy numbers in plasma during acute infection are relatively low.

EVS AS NOVEL THERAPEUTICS FOR COVID-19

One of the more exciting features of EVs is their potential usefulness for targeted therapeutics. EVs can be engineered to modulate the immune system for immunotherapies to deliver drugs or to inhibit tumor growth^[121-123]. Now, EVs have been proposed for novel therapeutics for COVID-19. In one example, EVs were engineered to express the ACE2 receptor and serve as a decoy to prevent S protein from infecting healthy cells^[124]. Coccozza *et al.* transduced 293FT cells with ACE2 and TMPRSS2, a host cell protease required for entry of SARS-CoV-2, using a lentivirus plasmid as the vector and isolated ACE2/TMPRSS2-expressing EVs released by these cells^[124]. When cultured with healthy Caco-2 cells, they found that ACE2/TMPRSS2-expressing EVs decreased the rate of SARS-CoV-2 infection of healthy Caco-2 cells by 50%^[124]. Another study proposed similar use of ACE2 EV decoys but using EVs derived from mesenchymal stem cells^[125]. If administered to patients, these ACE2-loaded EVs could competitively bind S-protein of SARS-CoV-2 and prevent cell damage as well as dysregulation of RAAS by maintaining cell surface expression of ACE2. Therefore, ACE2-positive EVs may protect against ALI and endothelial dysfunction brought about by SARS-CoV-2 infection and high angiotensin II levels when ACE2 is subsequently decreased.

Another promising novel therapeutic is EVs derived from mesenchymal stem cells (MSC-EVs). Mesenchymal stem cells (MSCs) themselves have been proposed for clinical therapies in regenerative medicine, especially in mitigating lung injury during ARDS or other acute pulmonary exacerbations^[126,127]. Therapies incorporating MSCs are attractive because MSCs are multipotential, naturally attracted to damaged tissue, and can “home” into tissues and aid in regeneration at directed sites^[126,128,129]. However, concerns over their potential to cause iatrogenic cancerous tumors following administration may outweigh their potential therapeutic benefits^[127].

EVs derived from MSCs do offer several advantages over MSCs. First, they are less complex than MSCs, not self-proliferating, and, therefore, are easier to store and maintain. Second, because they are not proliferative, they do not pose a great risk of causing iatrogenic cancerous tumors. Thirdly, the inherent function of EVs is cell communication and so little effort needs to be allocated to modifying EVs collected from MSC-conditioned media following collection. Due to their nanoscale size, EVs can move freely even through small capillaries without obstructing flow, which allows for enhanced systemic communication. Finally, MSC-EVs do not express HLA-I and II and, therefore, can be used for allogeneic transfer without concerns of inducing an adverse immune reaction^[130].

Previously, MSC-EVs have been shown to have the potential for decreasing the severity of ALI. A study by Wei *et al.* demonstrated that MSC-EVs produced by human umbilical cord MSCs (hucMSCs) attenuated LPS-induced ALI in mice^[131]. The group identified miR-377-3p, which was enriched in hucMSC-EVs, as an immunomodulator in BALF and inducer of autophagy, thereby reducing lung dysfunction and the severity of LPS-induced ALI^[131]. Another study by Liu *et al.* showed that MSC-EVs decreased the expression of TLR4 and NF- κ B as well as the production of proinflammatory cytokines TNF- α , IL-1 β , and IL-6 in lung tissue in an intestinal-ischemia reperfusion lung injury model in rats^[132]. These studies demonstrate the potential MSC-EVs have in attenuating the severity of ALI and preventing ARDS that can be induced by the “cytokine storm” in COVID-19 patients.

Sengupta *et al.* recently published a human clinical trial on ExoFlo[®], a novel therapy involving the administration of bone marrow MSC-EVs (bmMSC-EVs) and reported positive outcomes in moderate-to-severe COVID-19 patients^[133]. Following administration of a single 15 mL dose of bmMSC-EVs, ExoFlo[®] was shown to increase patient survival and reduce the need for invasive oxygen support^[133]. Additionally, because bmMSC-EVs have immunomodulatory properties, COVID-19 patients that were given ExoFlo[®] also demonstrated a significant improvement in immune function and decreased levels of harmful acute inflammation^[133]. Many other clinical trials involving the administration of anti-inflammatory and regenerative MSC-EVs are ongoing and can be found on ClinicalTrials.gov.

While MSC-EVs have strong potential for future therapeutics for many diseases, there are still several challenges to overcome. For one, the cumulative effects of MSC-EVs are far-reaching. MSC-EVs are most known for their immunomodulatory functions, but they are also shown to affect metabolism, tissue repair mechanisms, and angiogenesis^[134]. Therefore, if administered to patients, the side effects experienced could be highly heterogeneous unless MSC-EVs can be tailored to only have specific functions. Additionally, a major challenge in administering MSC-EVs to patients is the need for a high volume of conditioned media containing MSC-EVs to be given to a single patient, which is not conducive to currently approved methods of EV isolation and is also costly, though new methods, such as free-flow electrophoresis (FFE), are suggested may overcome this challenge^[135,136]. Although promising, more research in this area is necessary to ensure that these therapies are safe and reproducible.

CONCLUSION

In this review, we provide supporting evidence for EVs as biomarkers for COVID-19, EV involvement in acute and chronic COVID-19 pathologies, as well as the potential for engineered EVs and MSC-EVs as novel therapeutics in COVID-19 patients [Figure 1]. EVs are nanoparticles that clearly exhibit a diverse range of applications in science and medicine. Their inherent biological function as cell communicators allows for convenient assessments of disease pathologies via EV cargo, and their nanoscale size and non-proliferative nature make them safe and feasible for developing novel therapeutics. In addition, EVs carry a complex array of biomolecules, which can provide more information on organ and tissue function in one test compared to single biomarkers that are currently used to assess organ function. While more research is needed to develop logistical clinical diagnostics and therapeutics, EVs appear to be important mediators, promising biomarkers, and potential medicinal agents in COVID-19.

DECLARATIONS

Authors' contributions

Conceived and designed the outline of this review: Dhillon NK

Prepared the initial draft: Craddock VD, Cook CM, Dhillon NK

Designed and prepared the figure: Craddock VD, Dhillon NK
Collection and collation of literature: Craddock VD, Dhillon NK
Edited and revised the manuscript: Craddock VD, Dhillon NK
All authors read and approved the final version of the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This review was in part supported by funding from the NIH R01DA040392 to Navneet K. Dhillon.

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.

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Conference Report

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Meeting report of the 2nd Lugano ExoDay: extracellular vesicles as next-generation clinical biomarkers and therapeutic agents

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How to cite this article: Balbi C, Cretich M, Barile L. Meeting report of the 2nd Lugano ExoDay: extracellular vesicles as next-generation clinical biomarkers and therapeutic agents. *Extracell Vesicles Circ Nucleic Acids* 2022;3:189-94. <https://dx.doi.org/10.20517/evcna.2022.17>

Received: 11 Apr 2022 **Accepted:** 27 Jul 2022 **Published:** 29 Jul 2022

Academic Editors: Yoke Peng Loh, Michael W. Pfaffl **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

INTRODUCTION

The *2nd Lugano ExoDay* was the second edition of a scientific initiative in southern Switzerland with a focus on innovations, guidelines, and pitfalls of extracellular vesicle (EV) research. This periodical meeting in Lugano intends to actively involve and bring into focus EV experts and scientists at the early career stage, driving interactions and promoting collaborations. The symposium was supported by the Swiss National Foundation (SNF) under the funding scheme of "Scientific Exchanges", which is designed for researchers who want to host a highly relevant scientific event in Switzerland and invite experts from abroad. For the second year, the SNF grant was awarded to Istituto Cardiocentro Ticino-EOC Ente Ospedaliero Cantonale, who led the organization of the symposium.

The *2nd Lugano ExoDay* was a joint event with the first workshop of a pan-European consortium carrying out the MARVEL EU-funded project. The symposium benefited the consortium by disseminating the project's concept, meeting with potential end-users or companies, and networking for future collaborations.



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All participants had an in-depth overview of technical requirements and advances in isolation, characterization, and potential clinical application of EVs. Three keynote lectures of invited internationally renowned EV scientists were combined with eleven presentations of young scientists selected from the abstracts, plus a dedicated MARVEL talk giving an overview of the project. The program was framed by a poster session (displaying twelve posters), including an industrial exhibition. The best oral and poster presentations were awarded with prizes sponsored by *Extracellular Vesicles and Circulating Nucleic Acids* (EVCNA).

The *2nd Lugano ExoDay* was one of the first in-person meetings in Switzerland since the start of the pandemic outbreak. All attendees showed a natural disposition toward interpersonal interaction, thus providing an enjoyable atmosphere. Thanks to all participants, the organizing team, and the supporting staff, the meeting offered a context for productive discussions. We report on the experts' lectures and summarize the main scientific content in the following. Abstracts of oral and poster presentations are published in the present Special Issue.

Keynote lectures

Edit Buzàs (Department of Genetics, Cell- and Immunobiology, Semmelweis University Budapest, Hungary) held the first keynote lecture. Prof. Buzàs, the newly nominated President of the International Society of Extracellular Vesicles (ISEV), performed an exciting lecture on the basic biology and features of EVs. She elucidated the different mechanisms of release for different classes of vesicles and pointed out how difficult it is to define EVs' biogenesis. She then presented the new fascinating theory about the EV protein corona formation, recently published by her research group^[1]. In particular, she showed how EVs from THP1 cells (a human leukemia monocytic cell line) with an external plasma protein cargo induced an increased expression of TNF- α , IL-6, CD83, CD86, and HLA-DR of human monocyte-derived dendritic cells, while EV-free protein aggregates and "clean-EV" has no effect^[2]. Prof. Buzàs's lecture was an excellent and well-presented overview of the complexity of extracellular vesicles.

Michele De Palma (EPFL Swiss Federal Institute of Technology in Lausanne, School of Life Science, Lausanne, Switzerland) presented the second lecture. The speaker addressed basic questions regarding the role of tumor-released EVs in mediating the pro-metastatic effects of chemotherapy. He showed advanced and elegant research in his laboratory, providing evidence that two classes of cytotoxic drugs are broadly employed in preoperative (neoadjuvant) breast cancer therapy, elicit tumor-derived EVs with enhanced pro-metastatic capacity. Mechanistically he showed that chemotherapy-elicited EVs are enriched in annexin A6 (ANXA6), a Ca²⁺-dependent protein that promotes NF- κ B-dependent endothelial cell activation, Ccl2 induction, and Ly6C+CCR2+ monocyte expansion in the pulmonary pre-metastatic niche to facilitate the establishment of lung metastasis^[3]. Genetic inactivation of Anxa6 in cancer cells or Ccr2 in host cells blunts the pro-metastatic effects of chemotherapy-elicited EVs. ANXA6 is detected and potentially enriched, in the circulating EVs of breast cancer patients undergoing neoadjuvant chemotherapy.

Benedetta Bussolati (Department of Molecular Biotechnology and Health Sciences, University of Turin, Italy) held the third lecture. Prof Bussolati, the current President of the Italian Society of Extracellular Vesicles (EVIIta), talked about one of the most exciting aspects of EV research: the therapeutic application of EVs. She well explained the evidence of the role of EVs derived from mesenchymal stem cells as a therapeutic agent, in particular showing how the scientific community shifted from cell therapy to the paracrine therapy paradigm. She then presented different supporting data, in particular on the beneficial role of EVs in the acute kidney disease (AKI) model^[4].

MARVEL overview

MARVEL project (www.marvel-fet.eu), grant number 951768, started on 1 November 2020 and will end by 28 February 2023. It is coordinated by CNR (the National Research Council of Italy) and has the participation of six partners: two units, Università Vita-Salute San Raffaele (Italy) and Istituto Cardiocentro Ticino; Ente Ospedaliero Cantonale (Switzerland); and three SMEs, HansaBiomed from Estonia, PaperDropDX from Spain, and AMIRES from the Czech Republic. The main focus of MARVEL is to introduce a paradigm shift in affinity isolation of EVs from the use of antibodies to the use of peptides. Being well-established and totally synthetic molecules, peptides can overcome the major limitations inherent to using antibodies in affinity isolation, exceeding the analytical scale, such as high costs, batch-to-batch variation, and limited versatility of chemical manipulation. In particular, MARVEL is introducing membrane-sensing peptides (MSP)^[5] as novel ligands for the size-selective capturing of small EV. These ligands are “universal” because the capture of EVs is unbiased by differential protein expression and only exploits the general features of EV membranes such as high curvature, electrostatic charge, and lipid packing defects. In parallel, MARVEL is developing specific peptide probes (SPP) for EV-associated biomarkers to enrich clinically relevant EV subpopulations selectively. The versatility and modularity in scaling-up of these peptide-based systems are being demonstrated on medium/large sample volumes in two appropriate settings: (1) the manufacturing of GMP-compliant EVs as a medicinal product for cardiac repair with the collaboration of Istituto Cardiocentro Ticino; and (2) laboratory-scale urine-based liquid biopsy for bladder cancer stratification and monitoring in partnership with Università Vita-Salute San Raffaele.

MARVEL aims to impact the field of EVs by empowering the sustainability of EV use in both regenerative medicine and diagnostics. Such empowerment is expected to increment the readiness level of EV technologies and endow them with clinical-grade maturity. In line with the spirit of the “Transition” program funded by the EIC (European Innovation Council), MARVEL targets a technology readiness level (TRL) equal to 6 and envisages commercial exploitation of its results.

During the meeting, the project consortium, the concept, and the general aims were introduced by the coordinator Marina Cretich (CNR), whereas the Workpackage leader Alessandro Gori (CNR) provided details on the design and development of the peptide probes developed within MARVEL. In particular, an overview of MSP’s model of action and ways to generate a second generation of membrane binding probes was presented with emphasis on the scalability and versatility of peptide technology, which can be integrated into different EV isolation platforms including micro-analytical devices (biosensing chips and lateral flow test strips) and isolation tools such as beads, chromatography resins, and tangential flow filtration membranes.

Main scientific content

The scientific program included oral presentations that focused on potential theranostic (therapeutic and diagnostic) applications of EVs, interspersed with technical reports on innovative approaches for isolation and discrimination of different vesicle subpopulations.

Talks focusing on the therapeutic application included mainly research performed using EVs isolated from conditioned medium of mesenchymal primary human cell lines: EVs from adipose tissue (ADSC) and bone marrow (BMSC) presented similar characteristics in terms of size, concentration, and marker expression, but they exhibited different characteristics in terms of protein content and functional effects. ADSC-EVs over-expressed pro-angiogenic factors in comparison to the BMSC counterpart (Cansu Gorgun, University of Genova, Italy). MSC from the connective tissue of human umbilical cords, the so-called Wharton’s jelly, are enriched in small non-coding RNA (miRNAs) that are able to target TP53 transcript and induce

therapeutic benefit in preclinical models of premature birth-related white matter injury (Vera Tscherrig, University of Bern, Switzerland). The talk by Vera Tscherrig was selected as “Best Oral Presentation”.

Cardiac-specific mesenchymal cells cultured using a Good Manufacturing Practice (GMP)-compliant method in xeno-free conditions secrete EVs that are capable of preserving heart function in rat and pig models of acute myocardial infarction (Elena Provasi, Istituto Cardiocentro Ticino). A different therapeutical application envisioned the use of EVs as a drug delivery system. A setup for optimal EV loading strategy with chemotherapeutics was presented as a potential treatment for bladder cancer cells, thus increasing delivery efficiency while reducing toxicity (Alessia Brancolini, Università Vita-Salute San Raffaele, Milano, Italy). Not only mammalian cell-derived EVs, but also plant-derived EVs are among the most appealing next-generation biological and industrial agents. EV-like nanoparticles have recently been recognized as bioactive components of ginger (*Zingiber officinale*) and have been proven to hold health-protecting and/or health-enhancing properties in pre-clinical settings, thus prospecting novel tools for delivering effector molecules with encouraging efficacy and safety profiles. Francesca Loria from HansaBiomed Life Sciences (Tallinn, Estonia) showed how to overcome the challenges in isolation and purification of ginger-derived nanoparticles (GDNs). She investigated an enzyme-assisted ginger rhizome cell wall digestion to promote GDN extraction from the apoplast.

The diagnostic potential of circulating EVs was mainly approached by technical reports showing the implementation of a strategy for targeting and analyzing EVs directly embedded in complex biofluids such as serum, plasma, urine, *etc.*, thus allowing the exploitation of “ease-of-use” and possible “close to point-of-care” assays for the detection of the disease at the earliest possible stage. In this context, two digital detection platforms were compared side by side: the ExoView® Analyzer, which is based on the principle of single particle interferometric reflectance imaging sensing (SP-IRIS), and Quanterix Simoa® Technology, based on the single-molecule array technology (Simoa). Sensitivity in immune phenotyping of a well-characterized EV sample has been studied, as well as possible applicative implications and rationales for alternative or complementary use of the two platforms in biomarker discovery or validation. With throughput capability and level of automatization, Simoa seems to be the most suitable platform in clinical validation settings (Roberto Frigerio, Consiglio Nazionale Delle Ricerche, Milano, Italy). As an alternative method to detect EV-enclosed miRNA-638 which is associated with the risk of ischemic stroke^[6], Ana Rubio-Monterde (Paperdrop Diagnostics, Barcelona, Spain) showed the potential of a new approach consisting of an isothermal amplification followed by a read-out in a rapid diagnostic test (RDT). The isothermal amplification method used needs minimal sample preparation and worked optimally at a temperature of around 37 °C-42 °C. After 35 min of reaction time, the amplified product is applied over a test strip with a buffer, where the test result can be read after 5 min, giving a turnaround time of 40 min. An innovative approach presented during the oral section was using Fourier Transform Infrared Spectroscopy (FTIR) to directly access EV characterization for diagnostic and classification purposes using absorption bands of biomolecules. The authors showed that FTIR in the mid-IR range was used to investigate the composition of EV origin and allowed the discrimination of serum-derived EVs from patients diagnosed with hepatocellular carcinoma from healthy subjects. They showed that EVs could be classified with high accuracy, precision, specificity, and sensitivity using logistic regression and PCA based on characteristic mid-IR bands (Sabrina Romanò, Università Cattolica Del Sacro Cuore, Rome, Italy). Andrea Zendrini from the Università Degli Studi di Brescia (Brescia, Italy) showed the results of the first attempt to quantify the surface-to-bulk partition of proteins in EVs. He established a semi-quantitative model based on microstructural data to estimate the overall protein content of an EV as well as of the partition between membrane (surface) associated and lumen (bulk) contained proteins as a function of the EV size. The model was successfully tested to analyze and describe a real preparation composed of subpopulations of small EVs

and large oncosomes from human prostate cancer cells.

Technical reports on EV fractionating included the development of a protocol based on the asymmetrical flow field-flow fractionation (A4F) technique to separate different-sized EV subpopulations from the synovial fluid (SF). The authors showed that they were able to isolate four subpopulations of EVs with a radius ranging from 20 to 700 nm with a differential profile when comparing EVs isolated from the arthritic or inflamed SF and those isolated from the healthy SF (Daniele D'Arrigo, Ente Ospedaliero Cantonale, Bellinzona, Switzerland). A further innovative approach to isolating small EVs is a strategy implementing membrane-sensing proteins as convenient, easy-to-synthesize novel molecular probes for targeting highly curved membranes. Starting from a previously identified class of membrane-sensing peptides derived from Bradykinin protein, an atomic-scale molecular dynamics (MD) simulation was presented to provide an enhanced understanding of the interactions involved in membrane sensing. Using an "atom's eye view" of the system peptide/membrane, the authors carefully designed a series of simulations, which followed the peptide and the membrane in atomic detail [Alessandro Strada, National Research Council, Istituto "Giulio Natta" (SCITEC-CNR), Milan, Italy].

POSTER SESSION

Within the poster session, twelve posters were shown and presented mainly by young scientists. It was a huge challenge to select a poster for the EVCNA Prize. The topics of the posters covered the whole area of basic research. They addressed the methodology of EV purification and analysis, as well as the role of EVs as biomarkers and therapeutic agents in patho-physiological processes. The poster voting committee finally reached a decision and selected the poster entitled "Development of breakthrough liquid biopsy diagnostic via novel exosomal biomarkers for patient stratification in prostate cancer" by Alekhya Mazumdar (University Hospital of Zürich, Zürich, Switzerland) as "Best Poster Presentation".

CONCLUSION

Overall, the *2nd Lugano ExoDay* provided a fruitful platform for establishing interactions among the participants, especially for the young scientist community, who could take advantage of free registration fees that contributed to the success of the event. Intense discussions accompanied the talk and poster sessions on the molecular and functional characterization of EVs and the refinement of strategies for therapeutic applications. The meeting was an exceptional opportunity for research groups working on EVs in Switzerland to share the progress report of their own research to remain up-to-date with the advances in the field of EVs. Such initiative may pave the way for a Swiss-based task force of researchers and scientists involved in this specific field to establish future collaborative research centers focusing on EV science and the implementation of EV core facilities.

A short film of the day can be seen at (MARVEL project symposium 13 April 2022, Lugano, Switzerland - YouTube: https://www.youtube.com/watch?v=_tkoyZOB0GY)

The *Lugano ExoDay* task force, on behalf of all institutions taking part in this special event, thanks all contributors, the sponsors, and the organizing team for their efforts in making this meeting a success. We are all looking forward to our third edition, taking place in spring 2023 in Lugano.

DECLARATIONS

Acknowledgment

Yevhen Horokhovatskyi and AMIRES for collaborative support with communication activities including social posts, website, video and photos making within the frame of the MARVEL project.

Authors' contributions

All authors contributed to the manuscript equally.

Financial support and consent to participate

LB was supported by Swiss National Science Foundation IZSEZ0_209598; MC and LB were supported by the European Union's Horizon 2020 research and innovation program under grant agreements No. 951768 (project MARVEL).

Availability of data and materials

Not applicable.

Conflicts of interest

All authors declared that there are no conflict of interest.

Consent for publication

Not applicable.

Ethical approval and consent to participate

Not applicable.

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Review

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Cell-free DNA as a biomarker in cancer

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How to cite this article: Eibl RH, Schneemann M. Cell-free DNA as a biomarker in cancer. *Extracell Vesicles Circ Nucleic Acids* 2022;3:195-215. <https://dx.doi.org/10.20517/evcna.2022.20>

Received: 21 Apr 2022 **First Decision:** 31 May 2022 **Revised:** 7 Jun 2022 **Accepted:** 26 Jul 2022 **Published:** 2 Aug 2022

Academic Editors: Yoke Peng Loh, Erik A. Sistermans **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Translational research of liquid biopsy is just at the edge of routine clinical application: an emerging validity of circulating tumor DNA (ctDNA) tests suggests its use for earlier cancer detection and better monitoring of minimal residual disease (MRD) and resistance development, thus offering earlier guidance for therapy choices with the intent to cure cancer. In this review, we focus on ctDNA as an advanced and standardized validated marker in liquid biopsy. We also discuss what will be needed to reach the new milestone of personalized (precision) medicine to be used as a common standard of care. We summarize recent developments of cell-free DNA (cfDNA) and its clinical use as a biomarker in cancer.

Keywords: Cell-free DNA, cfDNA, circulating tumor DNA, ctDNA, biomarker, liquid biopsy, MRD, cancer

INTRODUCTION

Key elements of clinical routine with tumor patients include cancer detection and diagnosis as well as monitoring tumor development and treatment response. Usually, an interdisciplinary approach is necessary with complementing areas, such as medical imaging, tissue biopsy, histopathology, surgery, chemotherapy, and radiotherapy. Over decades, measurements of protein tumor markers in the blood evolved to the current standard, but translational research offers improvement: circulating cell-free DNA (cfDNA) allows easy and early access to information on tumor evolution and treatment response at low risk of injury,



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infection, or wound healing, compared to repeated tissue biopsies or delayed medical imaging, thus enabling improved therapy decisions and avoidance of over-treatment which improves patients' quality of life [Figures 1 and 2]. Circulating tumor cells (CTCs) were first described in an autopsy over 150 years ago by Ashworth, who found tumor cells in the blood microscopically identical to the tumor cells in metastatic skin lesions [Table 1]^[3]. When in 1889, over 130 years ago, the surgeon Stephen Paget presented his "seed and soil" theory, it was already known that tumor cells were able to disseminate via blood circulation. Metastatic cancer cells ("seeds") can leave their site of origin and enter the bloodstream to grow as secondary tumors in favorable environments ("soil") quite distant from the primary tumor^[4]. With his model, Paget explained a 15-fold higher frequency of breast cancer metastasis to the liver compared to the spleen. Although only a very small fraction of tumor cells within the blood gives rise to secondary tumors, it is well accepted nowadays that circulating tumor cells (CTCs) indicate tumor progression and an increased risk of metastasis [Figure 1]^[6]. Over the last two decades, the detection and analysis of these CTCs have improved considerably enough to be tested in clinical settings [Table 1]^[28]. In 2004, Allard and colleagues were able to detect and count CTCs in blood from many patients with prostate, breast, ovarian, colorectal, lung, and other cancers^[11]. In the same year, Cristofanilli and colleagues showed that an elevated number of CTCs before treatment of metastatic breast cancer can serve as an independent prognostic marker for a worse outcome, namely a shortened duration of both progression-free survival (PFS) and overall survival (OS)^[12]. Molecular profiling of DNA extracted from CTCs from non-small cell lung cancer (NSCLC) patients during treatment allowed monitoring of tumor evolution by mutations in the epidermal growth factor receptor (EGFR) gene related or unrelated to therapy resistance^[14]. As an alternative to the technical obstacles and the low sensitivity of fishing for the extremely rare CTCs, the cell-free fraction of blood can also be used to extract DNA, which then also allows the finding of tumor-specific mutations. In 1948, French scientists reported free nucleic acids in blood, but they did not relate them to any disease^[5]. In 1977, Leon and colleagues used polyclonal antiserum from patients with systemic lupus erythematosus (SLE), an autoimmune disease generating antibodies against DNA, to detect increased values of free DNA in about half of their cancer patients^[7]. Normal values for the other half of the patients were explained by the selection of patients before radiotherapy, but who already had their tumors surgically removed and/or had chemotherapy, resulting in an already reduced tumor mass. Despite significantly higher levels in patients with metastatic disease, no correlation was found regarding the size or location of the tumors. However, radiation therapy was able to decrease the levels of cfDNA significantly for lymphoma, lung, ovary, uterus, and cervical tumors, as well as to a lesser extent, glioma, breast, colon, and rectal tumors. Decreases in cfDNA levels went along with improved clinical conditions, such as lower tumor size and less pain, whereas increasing or unchanged levels correlated with worse conditions and a lack of response to treatment. The amount of ctDNA has been shown to markedly decrease after surgery or chemotherapy, suggesting the usefulness of ctDNA as a tumor marker [Figure 2 and Table 2]^[15]. The term "liquid biopsy" was originally coined in 2010 by Pantel and Alix-Panabières for several methods of CTC detection and analysis, but it was also used for ctDNA or any other analysis of tumor-derived material from biofluids, e.g. blood, cerebrospinal fluid (CSF), or urine^[18,30]. Recently, these methods of tracking cancer in liquid biopsy are considered one of the milestones in cancer research of the last two decades^[28].

Over the last two decades, liquid biopsy has included numerous methods to search for cancer-derived markers by analyzing biofluids, e.g. blood, CSF, or urine, i.e. samples taken quite distant from both the original tumor and potential metastases, generally with much easier and less risky access [Figure 1]^[31]. This led to a new definition of liquid biopsy with different circulating biomarkers in different body fluids^[30]. Basically, liquid biopsy targets circulating tumor cells (CTC), or circulating tumor DNA (ctDNA), which is part of the whole cfDNA [Table 2]. Other examples include the analysis of extracellular vesicles (EVs) and RNA, such as microRNA (miRNA, miR) or, very recently, circular RNA (circRNA)^[32]. The common goals are the use in screening, i.e. early detection and diagnosis of cancer with the chance for earlier and more

Table 1. Historical timeframe and major developments of liquid biopsy

Year	Author	Probe	Method	Tumor	Milestone
1868	Ashworth ^[3]	CTC	Microscopy, case report	Skin metastasis of unknown primary, "liquid autopsy"	First report on tumor cells in blood; post mortem; microscopically identical cells in metastatic lesions
1889	Paget ^[4]	CTC	Autopsy	Breast cancer, postulated	"Seed and soil" theory of cancer metastasis
1948	Mandel and Métais ^[5]	ctDNA	Blood analysis	Not related to cancer, healthy blood donors	First report of (cell-free) nucleic acids in blood
1975	Fidler ^[6]	CTC	Experimental metastasis assay	B16 melanoma cell lines	Only a small fraction of intravenously injected tumor cells give rise to metastasis in mouse models
1977	Leon <i>et al.</i> ^[7]	ctDNA	Radioimmunoassay for free DNA in serum patients; correlation with therapy response	Various cancers	First report on increased ctDNA levels in some cancer
1989	Lo <i>et al.</i> ^[8]	ctDNA	PCR of Y chromosome-specific sequence; cfDNA/blood	Not related to cancer	Sex determination of fetus in pregnant women
2001	Reya <i>et al.</i> ^[9]	CTC	Applying hematopoietic stem cell knowledge to heterogeneity of cancer cells	Solid tumors and leukemia, migratory CSC	Cancer stem cell theory
2003	Balaña <i>et al.</i> ^[10]	ctDNA	Methylation-specific PCR of MGMT, p16, DAPK, RASSF1A	GBM	Detection of methylated MGMT in serum highly predictive for response to BCNU chemotherapy
2004	Allard <i>et al.</i> ^[11]	CTC	CellSearch™	Prostate, breast, ovarian, CCR, lung, and other cancers	Detection of CTCs in 7.5 mL of blood samples
2004	Cristofanilli <i>et al.</i> ^[12]	CTC	CellSearch™ Amount of CTC	Metastatic breast cancer	Independent predictive marker: reduced PFS and reduced OS
2005	Diehl <i>et al.</i> ^[13]	ctDNA	dPCR, BEAMing	Advanced CRC	APC mutations in plasma
2008	Mahewaran <i>et al.</i> ^[14]	CTC	Molecular profiling EGFR mutations	NSCL	Monitoring therapy
2008	Diehl <i>et al.</i> ^[15]	ctDNA	Mutations	Colorectal cancer	Amount and presence/absence as an independent tumor marker, monitoring
2008	Cohen <i>et al.</i> ^[16]	CTC	CellSearch Clinical study	Colorectal cancer	Enumerating CTC
2008	De Bono <i>et al.</i> ^[17]	CTC	Clinical study	Prostate Cancer	Enumerating, the first demonstration that CTCs are the most accurate and independent predictor of OS in metastatic prostate cancer
2010	Pantel and Alix-Panabières ^[18]	CTC	Concept of analyzing tumor cells in body fluids	All cancers	Coined the term "liquid biopsy"
2010	Calverly <i>et al.</i> ^[19]	TEP	Platelet mRNA	Metastatic NSCL	Downregulation of gene expression in platelets
2013	Dawson <i>et al.</i> ^[20]	CTC + ctDNA (comparison)	Side-by-side disease monitoring	Metastatic breast cancer undergoing treatment	Sensitivity: ctDNA>CTC ctDNA level correlates with treatment response
2014	Betgegowda <i>et al.</i> ^[21]	ctDNA	Digital PCR, sequencing	14 tumor types	ctDNA detectable for most tumors outside the brain
2014	Sullivan <i>et al.</i> ^[22]	CTC	"Negative depletion" CTC-iChip (removing leukocytes from blood)	GBM (usually not metastatic)	Surprising and frequent detection of CTCs in brain tumors
2015	Mazel <i>et al.</i> ^[23]	CTC	CellSearch	Breast cancer	PD-L1 detection for immune checkpoint inhibition

2016	Tie <i>et al.</i> ^[24]	ctDNA	Sequencing	Colon cancer stage II	Detection of MRD after surgery; prediction of recurrence after chemotherapy
2016	Donaldson and Park ^[25]	ctDNA	Clinical studies	NSCLC	First FDA ^[29] and EMA approval to use ctDNA for EGFR-targeted therapy
2018	Cohen <i>et al.</i> ^[26]	ctDNA, plus proteins from blood	CancerSEEK, detecting mutations in 1933 loci of 16 genes; combined with protein tumor markers	8 cancer types	Blood screening test for several common cancers
2020	Lennon <i>et al.</i> ^[27]	ctDNA, protein markers plus PET-CT	Prospective 16 gene locations, 8 tumor proteins, PET-CT	Multi-cancer screening of 10,000 women with no known cancer	Multi-cancer blood testing combined with PET-CT

CRC: Colorectal cancer; CSC: cancer stem cell; CTC: circulating tumor cell; ctDNA: circulating tumor DNA; EGFR: epidermal growth factor receptor; EMA: European Medicines Agency; enumerating: counting; FDA: US Food and Drug Administration; PET-CT: positron emission tomography-computed tomography; TEP: tumor educated platelets.

curative treatments, as well as the use in monitoring after therapy and a better quality of life. The vision is a quick, reliable, and easy-to-repeat test for all patients to track tumor progression in real-time, as well as monitor the response to treatment to allow earlier detection of resistance, relapse, and metastasis and adapt clinical decisions accordingly. In addition, the presence of tumor markers after initially successful therapy should confirm minimal residual disease (MRD) in the absence of other markers and lead to additional treatments before the tumor becomes incurable^[33]. The current standard for most solid cancers consists of a more invasive tissue biopsy and/or surgical removal of all or most parts of the tumor with a thorough microscopic analysis by a tumor pathologist. Genetic molecular profiling of the primary tumor tissue may be included. In 2016, a new WHO classification of tumors of the nervous system introduced molecular and genetic profiling as standard practice for the analysis of some brain tumors such as medulloblastomas and glioblastomas^[34]. Depending on the tumor location and the multi-morbidity of a patient, obtaining tumor tissue repeatedly can be difficult and unsuitable for long-term monitoring of tumor development. Furthermore, transthoracic lung biopsies can lead to, although rare, iatrogenic dissemination of cancer cells^[35], and fine-needle biopsies have been reported to increase CTCs in prostate cancer^[36]. The snapshot of a tissue biopsy from a primary tumor may also miss minor, but progressing, tumor clones; it may also not represent metastatic clones with perhaps different molecular profiles for treatment resistances^[37]. Therefore, liquid biopsy carries the potential to be the next gold standard for monitoring cancer progression. Following various research approaches to the detection and cultivation of CTCs, also leading to a better understanding of tumor progression and metastasis, hundreds of emerging clinical studies are continuing to develop new standards for monitoring melanoma, breast, prostate, colon, and head and neck cancers^[38-46]. In this review, we focus on another part of liquid biopsy, cfDNA, which is reliably much easier to access and does not need elaborate methodologies to capture the extremely rare CTCs.

LIQUID BIOPSY MARKERS

ctDNA and CTCs are currently more established than EVs and tumor-educated platelets (TEP) as tools in liquid biopsy [Figures 1 and 2 and Table 1]. In this review, our main focus is on ctDNA and its clinical application and potential. For completion and comparison, we mention CTCs, EVs, and TEPs only briefly.

Table 2. Principles of liquid biopsy (ctDNA or CTC) in clinical settings

Liquid Biopsy	Pro	Con	Clinical utility
CTC	Sufficient sensitivity for some advanced-stage cancers, incl. metastases	Sensitivity limited: - Early-stage cancers - Cancer screening - Many advanced-stage cancers	Prognosis in many metastatic settings: - Breast - Prostate - Colorectal carcinoma
	Validated for specific applications (enumeration)	Not fully standardized, difficult methods / for many applications; rare CTCs; very few, centralized high-tech laboratories needed	Prediction of relapse after treatment
	High specificity (mutations)	Sophisticated technology, no easy and common standards	Live CTCs useful for drug screening and functional assays
ctDNA	Standardized, common methods: - blood drawing/handling (routine in hospitals AND practice) - DNA extraction (decentralized laboratories) PCR/sequencing/analysis	Sensitivity too low: - Early-stage cancers - Cancer screening - Some advanced-stage cancers	- Diagnosis - Prognosis - Monitoring: Tumor evolution Relapse Therapy resistance (e.g., EGFR mutations) (all complementing tissue biopsy, medical imaging)
	High specificity (often close to 100% with tumor-related mutations)	Limited biological and clinical relevance of many detected mutations (not every mutation is relevant or druggable)	Personalized, precision medicine: early detection of druggable mutations
	Fast/real-time monitoring of relapse or resistance after therapy Medium sensitivity for advanced cancer		

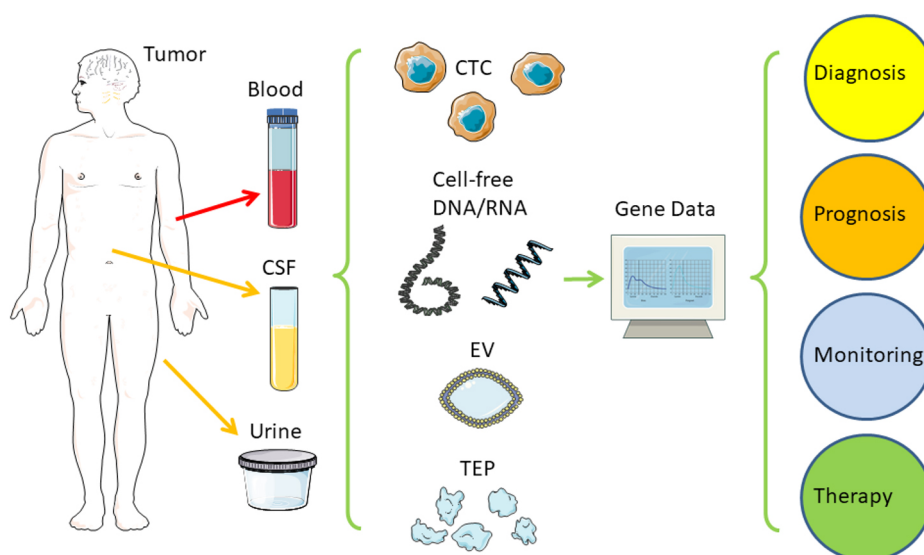


Figure 1. Liquid biopsy. Distant from the original tumor, samples from blood, CSF, or urine can serve as an easily acquired and low-risk source of tumor-derived nucleic acids (RNA and DNA) for further analysis to predict and monitor tumor progression and treatment response. CSF: Cerebrospinal fluid; CTC: circulating tumor cell; EV: extracellular vesicle; TEP: tumor educated platelets. Created/modified with SMART^[1], licensed under Creative Commons Attribution 3.0 Unported License^[2].

Cell-free DNA and circulating tumor DNA

The total amount of non-cellular DNA in samples such as blood is called cfDNA, which for tumor patients contains all tumor-derived DNA in various amounts, i.e. ctDNA.

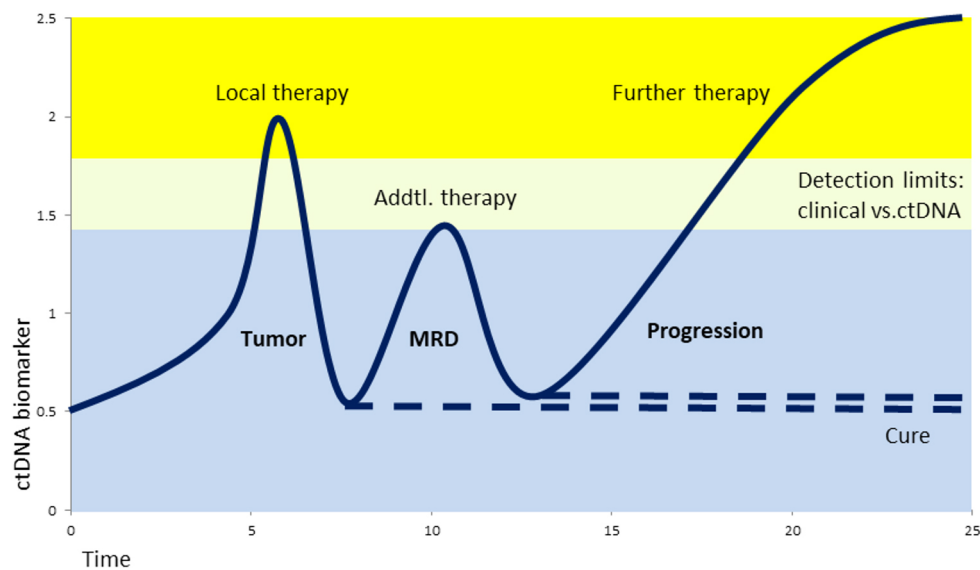


Figure 2. Hypothetical ctDNA biomarker levels during tumor development and therapy. After tumor removal, the biomarker level drops significantly and will remain low in the case the patient is cured. Early detection of minimal residual disease (MRD) by ctDNA allows additional treatment (e.g., chemo-, radio-, or hormone therapy) with the intent of cure, much earlier than with clinical imaging methods.

cfDNA in blood is highly fragmented with a peak of 166 bp length and many smaller peaks of 10 bp less or more in healthy individuals. Interestingly, the fragment size of the tumor-derived ctDNA appears to be shifted to a lower peak at 146 bp, which allows for selection strategies to increase the relative amount of tumor-derived ctDNA and to increase the sensitivity. Most of the cfDNA is considered to come from leukocytes, such as granulocytes, lymphocytes, and monocytes, and to a lesser extent from other cells (muscle cells, endothelial cells, epithelial cells, fibroblasts, mesenchymal stem cells, and hepatocytes)^[47]; interestingly, physical activity can trigger cfDNA release almost exclusively from granulocytes (up to 2-20-fold increase) and may affect liquid biopsy^[47]. Tumor-derived ctDNA can come from apoptotic or necrotic parts of the primary tumor, as well as from metastases and CTCs. ctDNA often represents only an extremely small fraction of the total cfDNA, especially at early stages and after successful therapies. Therefore, detection of low concentrations of ctDNA remains the major challenge for widespread tumor screening and treatment monitoring. Several key technologies have been developed and applied to detect potential micro-metastases and local relapse before clinical and radiological manifestation. This includes several ways of targeted sequencing of known mutations as well as non-targeted, i.e., genome-wide analysis, and fragmentomics^[48].

Targeted analysis

Targeted analysis aims to identify tumor-specific mutations or methylations in ctDNA. Techniques include digital PCR (dPCR), BEAMing (beads, emulsions, amplification, magnetics), safe-sequencing system (Safe-SeqS), cancer personalized profiling by deep sequencing (CAPP-Seq), and tagged-amplicon deep sequencing (Tam-Seq). ctDNA can also be analyzed for its relative amount from total cfDNA to serve as a surrogate for tumor burden. This allows risk assessment and staging, as well as early monitoring of therapy response or treatment failure. Tight monitoring can detect mutations as targets or escape mechanisms and supports clinical decision making based on tumor evolution and resistance development. Specific mutations in the EGFR gene in ctDNA allow treatment of NSCLC with a tyrosine kinase inhibitor (TKI)^[29,49]. Activating mutations of phosphatidylinositol-3-kinase catalytic subunit alpha (PIK3CA) are used to guide treatment in breast cancer. Resistant tumor cells can escape from therapies by gaining new mutations and

clonal selection of the fittest tumor cells. For example, KRAS mutations can develop as a resistance mechanism during EGFR-targeted therapy for patients with CRC^[50,51].

DNA methylation is an epigenetic mechanism to regulate gene expression by adding methyl groups to the DNA^[52]. In contrast to normal tissue, tumors can show a reversed hyper- and hypomethylation pattern, which can be important for tumor development and progression, as well as for clinical management of patients. Since 2003, typical methylation patterns of tumor DNA have been used to identify ctDNA in blood samples, e.g. MGMT relevant for brain tumors as an actionable target^[10,53,54]. More recently, hydroxymethylation profiling with detection of 5-hydroxymethylcytosine (5hmC) as a less known molecular marker of epigenetics was applied in liquid biopsy of lung, pancreatic, and hepatocellular cancer (HCC)^[55]. Lung cancer was characterized by a loss of 5hmC, whereas HCC and pancreatic cancer showed disease-specific changes, thus providing information about tumor type and stage.

Non-targeted analysis

Without prior information on specific mutations, non-targeted approaches try to investigate the entire genome, e.g., with whole exome sequencing (WES), whole genome sequencing (WGS), detection of copy number aberrations (CNA), and others^[56]. This also allows the detection of subclones evolving under treatment or during natural tumor progression. Unfortunately, low amounts of ctDNA from patients without relapse or metastasis affect sensitivity and utility. Some of these techniques can detect point mutations not previously found in the primary tumor, but with specific value options for treatment and prognosis^[57].

Fragmentomics

The size of tumor-derived cfDNA from plasma tends to be shorter than normal background cfDNA^[58]. These differences in fragmentation of DNA have been associated with reduced levels of a secreted DNASE1-like nuclease, DNASE1L3, in many tumor types (breast, colorectal, lung, gastric, head and neck non-squamous cell, and liver cancers)^[59,60]. Future studies will have to link and translate the biology of circulating DNA and nucleases to include fragment size, end motifs, and jagged (single-stranded) ends into clinical applications in tumor biology^[48].

Other types of liquid biopsy: CTCs, EVs, TEPs

Over the last two decades, CTCs entered clinical applications to detect and count them, as well as to determine their genetic profile for prognostics and clinical decision making [Figure 1 and Table 1]. CTCs are usually rare, but they often correlate and contribute to metastatic progression, although only a fraction of CTCs gives rise to metastasis^[6]. Despite major advantages of CTCs in diagnostics, prognostics, monitoring, and as guides to therapy choice and delivery schedule, widespread use appears to be still further away. Currently, only a few highly equipped labs supported by major research funds seem to be able to collect and analyze CTCs. A common standard is not established. With CellSearch, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved the first liquid biopsy in 2004^[29]. Originally, CellSearch was approved as a diagnostic tool to detect and count CTCs in blood samples to predict outcome (PFS and OS) only in metastatic breast cancer, but it later was expanded to monitor metastatic breast, colorectal, and prostate cancer patients.

miRNA are noncoding, 20-24 nucleotides long RNA molecules derived from just 1% of the whole genome. They are involved in the regulation of stability and translation of mRNA in health and disease. The potential effects of up to 1900 miRNAs are not fully understood. They can be found up- or downregulated in serum, EVs, and CTCs, as well as in urine^[61]. Future studies may also include further RNA molecules, such as larger

and more stable circular RNA (circRNA). circRNA can serve as an antagonistic sponge for miRNAs and can be involved in gene regulation of tumor cells. However, common standards are needed to be validated, especially since dysregulation of miRNA and circRNA may also be found in inflammatory diseases.

Tumors and normal cells can release small EVs, which contain typical proteins, DNA, and RNA. The intact cell membrane protects the enclosed compounds against degrading enzymes, such as RNases, from outside the vesicle. Therefore, EVs can be analyzed for the potential markers. Isolation of EVs from blood is the method of choice for most solid tumors. As an exception, CSF to analyze EVs from brain tumors can give better results compared to blood samples for tumors growing close to the ventricles and due to a better signal-to-noise ratio for tumor vs. non-tumor EVs^[62].

Blood platelets are derived as anucleated cytoplasmic fragments from megakaryocytes. They can react to activation of membrane receptors and outside-in signaling and have been shown to facilitate metastasis via several mechanisms, including protecting tumor cells within the circulation from immune cells and shear stress, supporting the adhesion to endothelium through adhesion receptors, and releasing of angiogenic and mitogenic growth factors at sites of metastasis. Surprisingly, tumors can alter the RNA profile of platelets, leading to the term tumor educated platelet (TEP)^[63]. The mechanism remains to be elucidated. TEPs from lung, brain, and breast cancer patients have been shown to be distinct from those with inflammatory or other diseases^[19,64].

CLINICAL STUDIES

Currently, the registry ClinicalTrials.gov from the NIH^[65] finds over 900 clinical trials related to the search term “ctDNA” (911 as of 15 March 2022), most of them are ongoing (with 648 “not yet recruiting”, “recruiting”, “enrolling by invitation”, or “active, not recruiting”) and only a minority (95) listed as “completed”, the remaining others being declared as “terminated”, “suspended”, or “unknown status”. Many of these studies are not originally intended to prove the use of ctDNA as a possible measurement of treatment response for new therapy schemes, but they just routinely integrate the ctDNA analysis as an early detection arm for success or failure of the treatment, i.e., as a complement, or when data from tissue biopsy are missing. However, some of the studies can use the potential of early detection of MRD, often months before the clinical manifestation of local relapse or metastasis [Table 3]; this lead time allows an earlier adaptation of the therapy decisions, sometimes with the intent to cure. It indicates the emerging role of ctDNA measurements in clinical settings, which may become the new gold standard, especially when such treatment studies will become new therapy regimes - and then may need or accept ctDNA as necessary control.

Breast cancer

Globally, breast cancer is the most frequent cancer and the leading cause of cancer-related death in women, although most patients can be cured at an early stage. After early surgical removal, additional treatments with chemo-, radio, and/or hormone therapy may further prevent a metastatic relapse. However, overtreatment of cancer-free patients also represents a risk of unnecessary side effects. Therefore, patients should benefit from personalized therapies adjusted for as many additional treatments as required and as few as possible. The challenge to identify MRD with ctDNA analysis from blood samples has been addressed in clinical settings.

In a pilot study in 2015, later complemented with a larger cohort in 2019, Garcia-Murillas and colleagues were able to monitor early breast cancer after initial therapy by detection of ctDNA, which was associated with a high risk of relapse in all breast cancer subtypes^[66,75]. Individual somatic mutations were identified by

Table 3. Examples of ctDNA studies for screening or monitoring different cancers and stages, as well as treatment response

Year	Author	Tumor	Method	Findings
2008	Diehl <i>et al.</i> ^[15]	CRC	Quantification of ctDNA by patient-specific mutations	Prediction/exclusion of relapse after surgery for at least a year (no long-term follow-up)
2015	Garcia-Murillas <i>et al.</i> ^[66]	Early-stage breast cancer	Monitoring of patient-specific mutations by dPCR from cfDNA	Monitor for MRD, prediction of metastatic relapse after initial therapy (pilot study)
2015	Olsson <i>et al.</i> ^[67]	Breast cancer	Whole genome sequencing of primary tumor, rearrangements by dPCR in plasma	ctDNA quantity predictive of poor survival
2016	Donaldson and Park ^[25,29]	Advanced NSCL	cobas EGFR Mutation Test v2	First ctDNA-based treatment selection EGFR mutation, exons 18-21 (for Erlotinib)
2017	Phallen ^[68]	CRC, breast, lung, ovarian, cancer	TEC-seq of cfDNA	Detection of early stage tumors
2017	Abbosh <i>et al.</i> ^[82]	Early-stage NSCLC, TRACERx	ctDNA gene profiling with multiplex-PCR NGS	Identify post-operative relapse
2017	Ng <i>et al.</i> ^[70]	CRC	PPS-ctDNA, multiplex-PCR	Monitoring after surgery
2017	Schøler <i>et al.</i> ^[71]	CRC	Patient-specific mutations for ctDNA	Postoperative detection of residual disease; prognosis for high risk of relapse; early detection of relapse and response to treatment
2017	Chaudhuri <i>et al.</i> ^[72]	NSCLC stage I, II, III	Gene panel with CAPPseq ctDNA	Lead time 5.2 months before clinical recurrence
2018	Mehra <i>et al.</i> ^[73]	mCRPC, Prostate cancer	cfDNA concentration	Independent prognostic factor for rPFS and OS in first- and second-line chemotherapy
2018	Annala <i>et al.</i> ^[74]	mPRPC	ctDNA	Disruption of TP53, BRCA2 or ATM results in worse outcomes on novel AR targeting
2019	Coombes <i>et al.</i> ^[75]	Early-stage breast cancer	Signatera assay, Personalized, 16-plex assays for patients-specific mutations in ctDNA	Lead time 8.9 months (up to 2 years) ahead of clinical relapse)
2019	Garcia-Murillas <i>et al.</i> ^[76]	Early-stage breast cancer	Monitoring of patient-specific mutations (mutation tracking) by dPCR from plasma cfDNA	Major “proof of concept” supporting the idea of clinical utility for prediction of relapse to be tested in larger studies
2020	Moding <i>et al.</i> ^[77]	NSCLC	ctDNA, CAPP-Seq	Detection of MRD with the prediction of benefit from consolidation immunotherapy
2021	McDuff <i>et al.</i> ^[78]	LARC	ctDNA	MRD measured by pre- and postoperative ctDNA predicts outcome for chemoradiation
2021	Taniguchi <i>et al.</i> ^[79]	CRC	ctDNA	Testing platform combining a prospective screening registry with two phase 3 interventional studies
2022	Gale <i>et al.</i> ^[80]	Early stage NSCLC	Monitoring of patient-specific mutations (48 amplicons)	MRD, identification of patients for further therapy
2022	Tie <i>et al.</i> ^[81]	Stage II colon cancer	Tumor-informed personalized sequencing	ctDNA-guided approach to reduce adjuvant chemotherapy without compromising recurrence risk

CAPP-Seq: Cancer personalized profiling by deep sequencing; dPCR: digital PCR; LARC: locally advanced rectal cancer; lead time, period before clinical or imaging manifestation of relapse; MRD: minimal residual disease; PPS: patient primary-tumor-specific; TRACERx: TRACing non-small cell lung Cancer Evolution through therapy (Rx); rPFS: radiological progression-free survival; mCRPC: metastatic castration-resistant prostate cancer.

sequencing of DNA from the primary tumors. The same mutations were later tracked by digital PCR (dPCR) in patient’s blood samples. Interestingly, the lead time was 10.7 months (95%CI, 8.1-19.1 months), i.e., long before the clinical manifestation of relapse.

Metastatic breast cancer is only rarely curable at a symptomatic stage. In a retrospective study and long follow-up in 2015, Olsson and colleagues combined whole-genome sequencing of primary tumors with personalized dPCR from plasma ctDNA for quantification of tumor-specific chromosomal

rearrangements^[67]. With this method, they were able to detect 0.01% of tumor DNA content or one rearranged sequence per 10,000 wild-type sequences. With an average lead time of 11 months (range 0-37 months), they were able to detect metastatic recurrence accurately before its clinical manifestation. In addition, long-term disease-free survivors had undetectable ctDNA. This supported the rationale to evaluate ctDNA in larger studies to monitor early metastasis detection, adjust therapy, and avoid overtreatment.

Mutations in the tumor suppressor protein p53 (TP53) are common among most cancers, although with varying frequencies^[82,83]. A high prevalence in a subgroup of breast cancers allowed for monitoring those mutations in ctDNA. Using massively parallel sequencing (MPS), Riva and colleagues detected patient-specific mutations in tumor tissue of non-metastatic triple-negative breast cancer (TNBC)^[84]. They tracked these patient-specific TP53 mutations in 10 mL of plasma from different time points: (1) before neoadjuvant chemotherapy (NCT); (2) after one cycle; (3) before surgery; and (4) after surgery. With dPCR of ctDNA, a sensitivity of 75% at baseline was achieved. ctDNA levels correlated not only with tumor burden but also with tumor proliferation rate (tumor grade or mitotic index). During NCT treatment, ctDNA levels dropped in all patients. After surgery, no MRD was detectable. However, a shorter survival was correlated with a slow decrease of ctDNA during NCT^[84]. Radovich and colleagues confirmed ctDNA and CTC enumeration above standard clinical parameters as independent factors for the worse outcome, i.e., distant disease-free survival (DDFS), DFS, and OS [Table 4]^[85].

Colorectal cancer

Colorectal cancer is the second and third most common cancer in women and men, respectively, with almost two million new cases each year^[90].

In 2016, Tie and colleagues investigated a prospective cohort of patients after resection of stage II colon cancer^[24]. They used massively parallel sequencing to identify patient-specific mutations from primary tumors and designed personalized Safe-SeqS assays for the identified mutations to quantify ctDNA from plasma. The presence of ctDNA in 7.9% of patients not treated with adjuvant chemotherapy was highly associated with recurrence of the disease within the median follow-up of 27 months (79% of patients). In patients who completed chemotherapy, the detection of ctDNA also correlated with a worse outcome. Therefore, ctDNA is useful to identify MRD and the high risk of recurrence in patients with stage II colon carcinoma.

Ng and colleagues developed a patient primary-tumor specific (PPS) assay to detect ctDNA in plasma samples by tracking individual mutations identified from tumor tissue^[70]. ctDNA correlated well with clinical treatment outcome: ctDNA was detectable before, but not directly after, surgery. Furthermore, ctDNA was detectable ahead of clinical manifestation of recurrence, indicating its validity to detect metastasis earlier than established methods of imaging and other markers (CEA).

Schøler and colleagues also showed that ctDNA can be useful for monitoring patients with CRC^[71]. The detection of ctDNA after surgery reflected MRD and identified patients with a very high risk of relapse. Follow-ups of three years allowed early detection of relapse with a lead time of 9.4 months compared to standard medical imaging, as well as monitoring additional treatment responses. In contrast to CEA levels, preoperative ctDNA detection rates correlated well with the stage of disease.

For example, CIRCULATIng tumor DNA-based decision for adjuvant stage II Evaluation (CIRCULATE), as an ongoing multicenter, prospective, randomized, controlled interventional trial (NCT04089631; Table 4)^[88], evaluates the adjuvant therapy in patients with colon cancer stage II. The primary outcome of

Table 4. Examples of clinical trials using ctDNA to validate screening tests or to monitor treatment response

Year	Study	Tumor	Name	Outcome measures
2014 ^[85]	NCT02101385 ^[86]	TNBC	Randomized controlled trial of genomically directed therapy in patients with TNBC	Comparison of 2-year DFS rate in patients with a genomically directed therapy or standard of care following preoperative chemotherapy
2019	NCT03934866 ^[87] Observational study (25,000 participants, 55-77 years with smoking history)	Multiple types of cancer (smoking history), lung cancer	SUMMIT - Cancer screening study using GRAIL's blood test	Validate blood tests for early detection of multiple types of cancers Examine the performance of LDCT screening
2020	NCT04089631 ^[88] Ongoing interventional clinical trial Phase 3 Drug: Capecitabine	Colon cancer stage II	CIRCULATing tumor DNA-based decision for adjuvant stage II Evaluation (CIRCULATE)	<i>Primary outcome measure:</i> DFS of ctDNApos patients; chemotherapy vs. follow-up <i>Secondary outcome measures:</i> OS in ctDNApos patients with adjuvant therapy vs. follow-up (5 yrs) DFS in ctDNAneg patients (3 yrs) OS in ctDNAneg patients; Kaplan-Meier (5 yrs) DFS and OS of ctDNApos vs. ctDNAneg (3 and 5 yrs) Site of metastasis (5 yrs); lymph node vs. peritoneal/local recurrence vs. other ctDNApos vs. ctDNAneg Sensitivity and specificity for diagnosis and follow-up
2021	NCT04931732 ^[89] Observational study	Glioma	circTelodiAG	

ctDNApos: ctDNA positive; ctDNAneg: ctDNA negative; DFS: disease-free survival; GRAIL: company name; LDCT: low-dose computed tomography; OS: overall survival; SUMMIT: name of study, not an acronym; TNBC: triple-negative breast cancer.

this phase 3 study is to compare the DFS in patients who are positive for ctDNA with vs. without the drug capecitabine. Patient-specific mutations previously identified from panel analysis of FFPE tumor blocks with a lack of microsatellite instability are used to detect ctDNA as inclusion criteria for the study, thus demonstrating the integration of ctDNA in clinical settings. The study started in June 2020 with expected primary completion in June 2023 and completion in June 2026^[88].

Lung cancer

Lung cancer is the leading cause of cancer death (18.4% of total cancer deaths) and, for both sexes combined, the most common cancer (11.6%) with about 2.1 million cases each year^[90]. In a seminal paper, Abbosh and colleagues used a phylogenetic approach to track the genetic dynamics of a tumor^[69]. They identified predictors of ctDNA in early-stage NSCLC: non-adenocarcinoma histology, necrosis, high proliferative index, and lymphovascular invasion. Chaudhuri and colleagues used a gene panel of 128 genes for CAP-Seq to predict recurrence with an average lead time of 5.2 months^[72]. In 2016, the FDA broadened the approval of the Cobas test (Roche) to be used not only in tissue sections but also as the first liquid biopsy assay for treatment selection to identify specific EGFR mutations in ctDNA of advanced NSCLC patients [Table 4]^[29].

The SUMMIT study at the University College London hospitals plans to enroll 25,000 participants 55-77 years of age who have no diagnosis of cancer but a high risk for lung cancer due to a significant smoking history^[87]. This study is an ongoing, prospective, observational, cohort study with two major aims: (1) to validate the collaborating company's (GRAIL, LLC) blood test on cell-free nucleic acids (cfNAs) for early detection of multiple types of cancer; and (2) to deliver low-dose CT (LDCT) screening for lung cancer. Participants will receive at least one low-dose chest CT at baseline. Another possible scan after 12 months will be randomized. The study aims to keep most scans below 1 mSv radiation dose (ultra-low dose) and all scans under 2 mSv. The liquid biopsy will evaluate the test performance, including sensitivity, specificity, and tissue of origin of detected cancers. The performance of the LDCT screening to detect lung cancers will be evaluated in comparison to established measures of risk prediction of lung cancers and other incidental findings. The study started in April 2019 with an estimated primary completion date of August 2023 and an estimated completion date of August 2030^[87].

Prostate cancer

Prostate cancer is the second most common non-cutaneous cancer in men worldwide with an estimated 1.3 million new cases each year with more than 350,000 deaths^[90]. Prostate cancer contributes to 3.8% of all cancer deaths and is diagnosed in 7.1% of all cancer patients. Mehra and colleagues detected the levels of cfDNA in mCRPC patients in phase 3 clinical studies with first- and second-line chemotherapies^[73]. Baseline cfDNA concentration, which is partially derived from the tumor, was an independent prognostic factor in both first- and second-line chemotherapy settings, and it correlated with known prognostic factors, shorter radiological PFS (rPFS), and OS. Higher levels of cfDNA before chemotherapy were associated with more aggressive tumors. A decrease of cfDNA within the first few weeks after initiation of chemotherapy correlated with a benefit for the patient, thus identifying a treatment response. Other studies found that cfDNA can include 15%-20% of ctDNA, depending on tumor stage and tumor burden, but localized prostate cancers typically remain below the threshold for detection of ctDNA, whereas prostate-specific antigen (PSA), a protein marker that is not uniquely an indicator for cancer, is already at a high-risk level^[91]. This implies that cfDNA may currently not be feasible to detect and monitor early state or less aggressive tumors. Annala and colleagues^[74] investigated cfDNA from mCRPC patients prior to novel AR therapy. Using whole-exome sequencing (WES) with capturing of coding regions of 72 selected genes, they correlated mutations of TP53, BRCA2, or ATM as predictors of worse outcomes on novel AR targeting, thus suggesting liquid biopsy as new guidance in AR-targeted therapy in general practice.

Brain tumors

Cancers of the brain and nervous system represent only 1.6% of all cancers and contribute to 2.5% of all cancer deaths. The critical location within the brain and the lack of major improvements for one of the most devastating cancers, glioblastoma, for over a century represent major challenges. Recently, Eibl and Schneemann published a review on liquid biopsy of primary brain tumors^[31]. In contrast to tumors outside of the nervous system, the blood-brain barrier (BBB) may add another challenge for detecting low-level ctDNA in the blood. CSF-although also protected by the BBB-appears to be a much better source due to the normally diminished number of leukocytes as a background source for cfDNA. However, earlier studies were able to confirm specific mutations known from tissue biopsy also in the serum^[10,21,92,93], plasma^[94-96], or both^[21]. In 1991, one of the authors described the very first TP53 mutations in primary medulloblastoma tissue biopsies^[82]. This finding supports a model of histologically indistinguishable from primitive neuroectodermal tumors (PNET)^[97,98]. Others were unable at that time to detect TP53 mutations in tissue biopsies or in xenografts of human medulloblastoma, except in only one cell line^[99]; however, this mutation may have been developed as a selective advantage during cell culture. Similar brain tumor models helped to elucidate and confirm several other oncogenic pathways in human brain tumors^[98,100-107]. Meanwhile, TP53 mutations in medulloblastomas are well established and can be used as a prognostic and diagnostic marker:

only recently, in 2016^[34] and with an update in 2021^[108], the World Health Organization (WHO) introduced four new diagnostic groups of this childhood brain tumor based solely on molecular genetic features. The correlation between different biological behavior and personalized risk assessment may allow preventing harmful radiation when not necessary or useful. The first two groups refer to different oncogenic signaling pathways, namely wingless/Integration-1 (WNT)-activated (Group 1) and sonic hedgehog (SHH)-activated (Group 2). WNT is a portmanteau for the *Drosophila* gene “wingless” (Wg), detected in mutants lacking wings, and the homologous mouse gene, integration 1 (Int-1), which was found earlier to cause tumors by insertional mutagenesis with a retrovirus; SHH refers to the hedgehog gene (hh) found in *Drosophila* mutants with spikes, reminiscent of a hedgehog (SHH is a vertebrate homolog and named after a character in a video game, Sonic the Hedgehog). WNT-activated medulloblastoma shows the highest five-year survival and a low prevalence of metastatic diseases. SHH-activated medulloblastoma can be further separated into two different subgroups, TP53-mutant or TP53-wildtype. SHH-activated, TP53-mutant occurs primarily in older children and has a very poor prognosis, whereas SHH-activated, TP53-wildtype, which is most common in adolescents and young children, has a good prognosis. The other two groups are non-WNT/non-SHH, Groups 3 and 4, respectively (also known as Groups C and D). Group 3 shows an increased prevalence of metastatic disease with the poorest five-year survival, whereas Group 4 has an increased prevalence of metastatic disease with a moderate five-year survival. TP53 mutations in SHH medulloblastomas are associated with poor survival and treatment failures^[34]. Several subgroups have been associated with TP53 and other mutated genes: for WNT-activated, CTNNB1 and APC; for SHH-activated, TP53, PTCH1, SUFU, SMO, MYCN, and GLI2 (methylome); and for non-WNT/non-SHH, MYC, MYCN, PRDM6, and KDM6A (methylome). Since the WHO classification suggests that the diagnosis from molecular profiling of a tissue biopsy is even superior to classical histopathology, at least for brain tumors, it appears reasonable to use ctDNA-based liquid biopsy for monitoring such mutations in brain tumor patients to avoid repeated and risky neurosurgical biopsies^[109]. Newer studies successfully used panels of genes. For brain tumors, CSF offers another chance to find ctDNA with a higher sensitivity than plasma or serum^[110-114]. ctDNA from CSF even represents the genomic mutations better than plasma; CSF shows an increased sensitivity for putative actionable mutations and CNA (copy number aberrations; EGFR, PTEN, ESR1, IDH1, ERBB2, and FGFR2)^[115]. Glioblastoma (glioblastoma multiforme, GBM) represents the most malignant brain tumor. Even after complete surgical removal, the tumor always relapses due to locally infiltrating cells. Chemo- and radiotherapy treatments can help temporarily, but they also trigger the evolution of the tumor to escape all current treatments. Repeated resection and biopsies are generally not indicated. Therefore, a real-time liquid biopsy with ctDNA from CSF offers an alternative at lower risk to monitor the molecular adaptations of the tumor. CSF as a source for ctDNA also provides an additional chance to investigate brain metastases from tumors from outside of the brain better, since they may contribute to the ctDNA in the blood to a lesser extent than metastases from outside of the brain.

ctDNA TESTS

Several ctDNA tests have been developed over the past few years. The spectrum of applications includes early screening, detection of targetable mutations to predict treatment response, MRD, and monitoring after treatment with early detection of resistance [Table 5]^[132]. After extraction of DNA from plasma, the obtained cfDNA is typically used with qualitative PCR or sequencing methods. Such tests can be highly personalized and target varying numbers of tumor-associated or actionable mutations; they can use a custom-built approach to profiling tumor tissue first for patient- and tumor-specific mutations and later use dPCR techniques to monitor tumor development and treatment response from repeated ctDNA probes.

Tumor screening

In a large study, the “Detecting cancers Earlier Through Elective mutation-based blood Collection and Testing” (DETECT-A) blood test demonstrated the proof-of-concept to use ctDNA for tumor screening.

Table 5. Examples of ctDNA tests for tumor screening, monitoring, and guiding treatment

Test	Method	Genes	Cancer type	Comments
Bluestar Genomics ^[116-118]	Hydroxymethylome, NGS, AI	Abnormal genomic/epigenomic signature; 5hmC	Pancreatic cancer (breast and lung cancer)	Screening of patients with new-onset of diabetes, BDD
CancerSEEK ^[126]	ctDNA plus 8 protein markers	ctDNA: 61 Amplicons	Ovary, breast, liver, and others	Sensitivity of about 70% for all 8 cancer types (33%-100% variability)
CellMax-LBx ^[119]	NGS	Mutation profile of 73 genes	Solid tumor	Associated with cancer treatment and tumor response
Circulogene ^[120]	NGS	Approximately 3,000 mutations in > 50 genes	A broad range of tumors	CAP, CLIA
Cobas EGFR mutation test v2 ^[29,49] - (Roche)	PCR, actionable EGFR mutations (exons18-21)	cfDNA	Metastatic NSCLC	FDA approved for plasma/liquid biopsy 2016 to identify patients for the first Erlotinib treatment
DETECT-A ^[27] – Thrive (developed into: MCED - Exact Sciences) ^[121]	Early version of CancerSEEK ^[26] ctDNA plus protein markers	ctDNA	Lung, ovarian, CRC and others	1 yr prospective study detecting 26 cancers first in blood
FoundationOne Liquid CDx (Roche Foundation Medicine) ^[122]	NGS	> 300 genes	NSCLC Prostate, ovarian, breast cancer	FDA approved companion diagnostic for treatment
Guardant360 CDx ^[123,124]	NGS	74 genes	Any advanced solid tumors	FDA approved (for 55 genes) tumor mutation profiling
Invitae ^[125,126] (former ArcherDX)	NGS	ctDNA	Most tumor types	Originally for FFPE, also for ctDNA
Oncomine Pan-Cancer Cell-Free Assay ^[127,128] - ThermoFisher	NGS	cfDNA 52 genes	Various tumors	CE, Europe
RaDaR - Inivata ^[80,129]	NGS	Up to 48 genes	Early stage cancer, NSCL	Predicts early relapse, MRD, BDD
Signatera ^[82,130] - Natera	NGS	ctDNA	A broad range of solid tumors	Custom-built personalized MRD assay; BDD
Target Selector - Biocept ^[131]	NGS	18 hotspot genes; 10 (breast) and 11 (lung)	Lung and breast cancer	Focus on actionable genes
Therascreen PIK3CA (Qiagen) ^[52]	PCR	11 activating mutations in exons 7, 9, and 20 of PIK3CA gene	Breast cancer	FDA approved, Identification of patients eligible for PIQRAY (alpelisib)

5hmC: 5-hydroxymethylcytosine; AI: artificial intelligence (machine learning); BDD: breakthrough device designation (by U.S. FDA, Federal Drug Administration); CAP: College of American Pathologists; CE: Communauté Européenne; cfDNA: cell-free DNA; CLIA: Clinical Laboratory Improvement Amendments; DETECT-A: detecting cancers earlier through elective mutation-based blood collection and testing; FFPE: formalin-fixed paraffin-embedded; MCED: multi-cancer early detection; PIK3CA: phosphatidylinositol-3-kinase catalytic subunit alpha; RaDaR: residual disease and recurrence.

The DETECT-A test represents an early version of the CancerSEEK test [Table 5] with minor differences, such as using pre-defined thresholds for each DNA and protein biomarker instead of employing artificial intelligence (AI)/machine learning for increasing sensitivity, or for confirming or enhancing specificity^[27]. With a cohort of about 10,000 women between 65 and 75 years of age with no history of cancer and one year of follow-up, 26 cancer patients were considered with positive blood testing to be “first detected by blood testing” after confirmation by positron emission tomography-computed tomography (PET-CT): nine lung cancers, six ovarian cancers, and two colorectal cancers - most of them localized or regional. Overall, 14 of the 26 cancers had elevated ctDNA levels, 11 had elevated protein marker levels, and 1 had both elevated ctDNA and protein markers. This test was not designed for regulatory approval, but it addressed fundamental issues of feasibility and safety of multi-cancer blood tests. Indeed, the authors confirmed that a minimally invasive ctDNA-based test was able to safely detect several types of cancer in patients who had no

previously known cancer. This allowed early treatment with the intent to cure. The test also produced 101 false positives and 46 false negatives, which still may allow its use as complementary to standard screening methods, although it cannot be used as a stand-alone test. With the use of AI and technology evolution, further improvements appear to be possible.

Tumor monitoring and treatment guidance

Only a few ctDNA tests are FDA approved for guiding treatment choices to identify cancer patients for mutation-specific treatments: Cobas, FoundationOne Liquid CDx, Guardant 360 CDx, and Therascreen PIK3CA [Table 5]. As an example of detecting targetable mutations in cancer patients, the Cobas EGFR mutation test v2 (Roche) aims to detect by PCR from plasma in less than 4 h specific and actionable mutations in the EGFR gene exons 18-21^[49]. The FDA approved this test in 2016 for use in liquid biopsy to identify patients for first-line EGFR-targeted therapy (e.g., erlotinib, a tyrosine kinase inhibitor (TKI)) of metastatic NSCLC patients. A similar test was approved earlier for FFPE samples from tumor tissue. In contrast to more recently developing ctDNA tests, the first CTC test approved by the FDA, in 2004, was CellSearch (Veridex). That test uses size, density, electrical properties, and immune surface markers (EpCAM+, Cytokeratins+, and CD45-) for selection and has been cleared for breast, colorectal, and prostate cancer to predict outcome^[12].

VISION AND CHALLENGES

As a new milestone in precision medicine, ctDNA-based liquid biopsy has shown its principal utility and safety for an emerging new era of clinical applications. Nevertheless, a few major challenges need to be addressed within the next few years to reach a widespread benefit for cancer patients: the sensitivity of ctDNA diagnostics may increase by technology improvements, thus allowing even earlier detection and, hopefully, easy-to-use multi-cancer screening platforms for at least the most common cancers. A focus will lie on those as well as developing better treatment options including with the intent to cure. Simple tests, such as detecting only actionable mutations, e.g. EGFR exon mutations, may be further developed for not yet used genes, for example, rare and tumor-specific CD44v variant exon combinations^[133]. Although many studies describe a significant “lead time” to detect a relapse before any clinical methods or radiologic imaging, one should reflect that any of these clinical methods may also improve over time, e.g., a 3 or 7 Tesla (T) MRI should have a better resolution and detection capacity than a 1.5 T MRI, thus pointing to the uncertainty of comparisons over time. Hundreds of recent and ongoing clinical trials already apply ctDNA methods to monitor new treatments in cancer patients^[65,134]. Large multicenter studies will further show how to best standardize and incorporate the use of ctDNA in clinical guidelines. Other arms of liquid biopsy may also be improved in parallel. Living CTCs should be further characterized by not yet applied technologies, such as functional assays with quartz-crystal microbalance (QCM)^[135] or atomic force microscopy (AFM), e.g., to check for tumor-specific cell adhesion and pharmacology reactions at the single-molecule level, both applied and pioneered by one of the authors^[136-144]. Sharing pharmacological and migratory data on CTCs, and making them findable, accessible, interoperable, and reusable (FAIR), will allow meta-analysis, data integration, and data mining to accelerate such new approaches in liquid biopsy^[145]. Some of these experimental technologies may be tested in established models first to allow better reproducibility^[146,147]. CellSearch with counting CTCs became clinically validated as the first CTC marker for worse outcomes in early breast cancer^[148]. It appears reasonable to combine such CTC tests with ctDNA methods to improve liquid biopsy, perhaps similarly to how CancerSEEK successfully combined ctDNA mutations with protein markers.

CONCLUSION

Depending on tumor type and treatment options, ctDNA has been shown to be a valid and independent biomarker for confirming and serial monitoring of cancer. With its very high specificity, ctDNA can be used to predict the early recurrence of a wide variety of cancers after initial therapy. This enables guidance to earlier and better treatment options, including the intent to cure, but also to prevent patients from unnecessary treatments, when there is no indication for MRD or early detection of resistance development. Numerous larger and ongoing clinical studies with ctDNA as a marker for monitoring MRD or treatment response will increase our knowledge on how to apply the best strategies.

Unfortunately, sensitivity often appears to be too low to use ctDNA for general tumor screenings, and not all patients can be monitored reliably. Further improvement in technology is needed to increase sensitivity and standardization. Although ctDNA appears to be much closer to widespread routine use than the other arms of liquid biopsy (CTCs, EVs, and TEPs), perhaps, a combination of ctDNA data with functional studies on living CTCs can soon close the gap for a faster shift to the new paradigm of liquid biopsy in personalized medicine for the benefit of most cancer patients at any stage of diseases. ctDNA as a valid biomarker is ready to enter clinical routine.

DECLARATIONS

Acknowledgments

We thank C. Alix-Panabières for discussion and comments on the manuscript. Since this is such a rapidly emerging field between basic research and clinical applications, we apologize for not being able to include all interesting studies in this review.

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Eibl RH, Schneemann M

Performed data acquisition, as well as provided administrative, technical, and material support: Eibl, RH, Schneemann M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

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.

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Review

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Expanded knowledge of cell-free DNA biology: potential to broaden the clinical utility

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How to cite this article: Che H, Stanley K, Jatsenko T, Thienpont B, Vermeesch JR. Expanded knowledge of cell-free DNA biology: potential to broaden the clinical utility. *Extracell Vesicles Circ Nucleic Acids* 2022;3:216-34.

<https://dx.doi.org/10.20517/evcna.2022.21>

Received: 30 Apr 2022 **First Decision:** 5 Jul 2022 **Revised:** 29 Jul 2022 **Accepted:** 2 Aug 2022 **Published:** 10 Aug 2022

Academic Editors: Yoke Peng Loh, Erik A. Sistermans **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Noninvasive sampling of an individual's body fluids is an easy means to capture circulating cell-free DNA (cfDNA). These small fragments of DNA carry information on the contributing cell's genome, epigenome, and nuclease content. Analysis of cfDNA for the assessment of genetic risk has already revolutionized clinical practice, and a compendium of increasingly higher-resolution approaches based on epigenetic and fragmentomic cfDNA signatures continues to expand. Profiling cfDNA has unlocked a wealth of molecular information that can be translated to the clinic. This review covers the biological characteristics of cfDNA, recent advances in liquid biopsy and the clinical utility of cfDNA.

Keywords: Cell-free DNA, liquid biopsy, biomarker

INTRODUCTION

Invasive diagnostic tests, such as tissue biopsies, are limited by procedure-associated risks and sometimes sampling difficulties. There is a strong need for noninvasive or minimally invasive biomarkers that do not rely on targeted sampling and expedite the often lengthy process of identifying disease. Cell-free DNA (cfDNA) has emerged as a vital biomarker for detecting and monitoring disease. Assessment of cfDNA has



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provided new opportunities to noninvasively obtain information from source tissues of interest. The use of cfDNA has been explored in different fields and implemented to varying extents. In obstetrics, for example, multiple large-scale clinical studies have successfully used cfDNA for prenatal screening of certain genetic conditions, underscoring the clinical value of cfDNA analysis. Specifically, common fetal aneuploidy screening is of high clinical relevance for pregnancy management. In oncology, the use of cfDNA for early cancer detection, prognosis, and treatment is under particularly extensive investigation. Moreover, there is a rising interest in broadening the diagnostic scope of cfDNA both in terms of disease and methodology. In this review, we have summarized current understandings of cfDNA and the clinical utility of cfDNA in different medical fields.

Cell-free DNA

Cell-free DNA (cfDNA) is ubiquitous in human body fluids, including blood, urine, cerebrospinal fluid, sputum, ascites and pleural effusion^[1,2]. Serum and plasma cfDNA have been extensively studied. It is generally accepted that cfDNA in the blood of healthy individuals is derived primarily from apoptotic hematopoietic cells^[3,4]. Hematopoietic cell lineages have a fast turnover rate and short half-life^[5]. In line with this, studies have shown that the main cellular origin of cfDNA is from hematopoietic cells^[3,6]. The fragment size distribution of cfDNA is referred to as the apoptotic ladder because it matches the progression of nucleosome units with successive peaks at ~167 bp corresponding to the length of DNA wrapped around a mononucleosome (147 bp) plus the linker regions (20 bp)^[7,8], at ~330 bp for the di-nucleosome and at ~500 bp for the tri-nucleosome^[9-11]. DNA fragments below 167 bp exhibit a series of smaller peaks at a periodicity of ~10 bp, likely reflecting the DNA helical repeat and cleavage at groove regions where DNA bends sharply around the nucleosome^[12,13] [Figure 1]. Other types of cfDNA release mechanisms have also been suggested under certain pathological conditions^[14]. For instance, the presence of DNA fragments over 10 kilobases in cancer patients may be indicative of necrosis^[10]. NETosis, pyroptosis and active secretion have also been proposed as putative sources of cfDNA^[15,16]. In addition to cfDNA release, studies have revealed impaired cfDNA clearance in a number of (patho)physiological states^[17-19]. Extracellular nucleases from the deoxyribonuclease (DNase) family are capable of digesting internucleosomal linker regions and enzymatically clearing free and protein-bound DNA^[20,21]. CfDNA is then eliminated from circulation through organs, such as the liver, spleen, and kidney^[22]. Abnormal DNase activity and insufficient clearance of cellular debris have been associated with elevated cfDNA concentration in patients with autoimmune diseases^[23,24] and cancer^[25]. Altogether, cfDNA reflects a heterogeneous, complex and dynamic landscape of dying cells in an individual and holds great promise for noninvasive molecular testing.

Cell-free fetal DNA

During pregnancy, fetal DNA fragments are detected in the maternal plasma DNA population. Y chromosomal DNA fragments from a male fetus were observed in the cfDNA of a pregnant individual as early as 1997^[26]. It is well-recognized that cell-free fetal DNA (cffDNA) mainly originates from placental trophoblast cells. This conclusion has been drawn from the observation that cffDNA was detectable in anembryonic pregnancies and at early gestational weeks before fetal organ development^[27,28]. Moreover, cffDNA shares methylation signatures common to trophoblast cells^[29-31] and, in the event of confined placental mosaicism, cffDNA reflects the genotype of the placenta rather than the fetus proper^[32,33]. Though the cffDNA concentration increases as gestation advances, the placental contribution to the pool of cfDNA in maternal plasma, often described as the fetal fraction (FF), remains a minor fraction, usually 10%-20% throughout the first and second trimester^[34]. The release of cffDNA is tightly linked to placental morphogenesis. Consequently, placental dysfunction can directly affect circulating cffDNA levels. For example, in pregnancies with preeclampsia or at risk of developing preeclampsia, elevated cffDNA levels have been reported^[35-37]. Maternal conditions, such as obesity, can lead to lower FF due to higher maternal

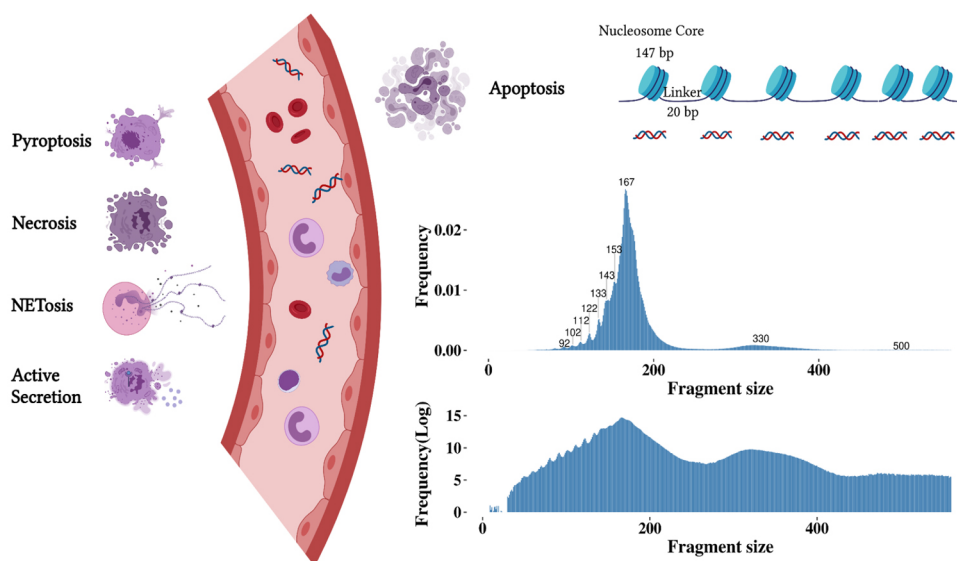


Figure 1. Illustration of cfDNA release mechanisms and the resulting fragment size (in bp) from apoptosis. Different forms of cfDNA release have been suggested, these primarily include apoptosis, pyroptosis, necrosis, the release of neutrophil extracellular traps (NETosis) and active secretion. Apoptosis is considered the primary source of cfDNA, resulting in non-random fragmentation. Other mechanisms of cell death, such as, pyroptosis that is inflammatory-regulated, necrosis that occurs due to accidental cell death, and NETosis that is neutrophil-specific, can also contribute to the release of cfDNA. In addition to cell death, cfDNA might be derived from active cellular secretions. The fragment size distribution of cfDNA shows peaks in sizes below 167 bp and peaks corresponding to nucleosome units. The log-transformed fragment size distribution demonstrates the di- and tri-nucleosome peak.

contributions^[38,39]. CffDNA is rapidly removed from maternal circulation after delivery and the estimated half-life of cffDNA clearance is about 1 h^[40]. Fragment size of cffDNA is generally shorter than the maternal cfDNA and peaks at around 143 bp^[9] [Figure 2]. The shorter size of cffDNA may be related to nucleosome organization and chromatin accessibility in placental tissue^[41]. Assay for Transposase-Accessible Chromatin (ATAC) sequencing has revealed that chromatin is more accessible in placental tissue and may indicate that DNA is more likely to be cut close to nucleosome cores resulting in shorter fragments^[41]. The basis for investigating the fetal genotype noninvasively is that the entire placental genome is present in the maternal plasma. Lo *et al.* showed that the proportion of cffDNA in maternal plasma is constant across the genome^[9]. The coverage of cffDNA fragments across the genome can, however, fluctuate from region to region, potentially indicative of nucleosomal degradation patterns in the tissue of origin^[42,43].

Circulating tumor DNA

The surge of interest in cfDNA was not only sparked by the discovery of cffDNA, but also by the discovery of circulating tumor DNA (ctDNA) in cancer patients decades ago^[44-46]. Studies have shown that genetic alterations observed in ctDNA reflect copy number aberrations and somatic mutations in the primary tumor or multiregional tumor biopsies^[47-49]. Analysis of nucleosome maps of plasma cfDNA^[50] and methylation signatures^[4] in cancer patients have provided further evidence that ctDNA is derived from the malignant cells. It has also been postulated that non-malignant cells that are part of the tumor microenvironment, such as stromal, endothelial, lymphocytes and other immune cells, may contribute to plasma DNA of cancer patients^[1,51]. The amount of ctDNA in the circulation, also termed tumor fraction, is likely to be associated with tumor mass/burden, cell turnover and disease stage. Increased amounts of ctDNA were found in cancer patients with advanced stage compared to early stage of diseases^[52]. Changes in tumor fractions also reflect treatment responses over the course of therapy^[53,54]. Nevertheless, tumor fraction in plasma DNA can remain very low even in metastatic diseases, with ctDNA levels varying across different tumor types and even subtypes^[52,55,56]. The anatomical location of the tumor

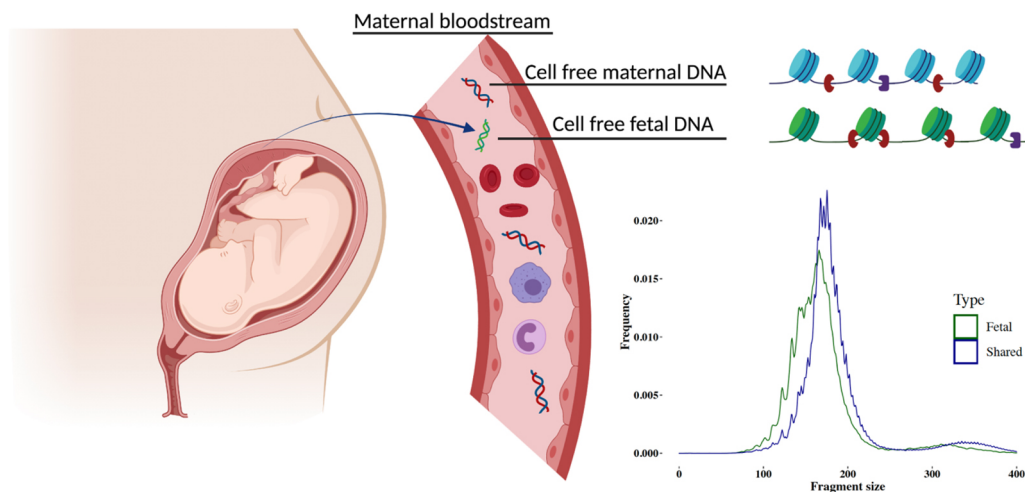


Figure 2. Illustration of maternal plasma DNA and an example of the fragment size (in bp) distribution of fetal-specific DNA (green) and shared (maternal and fetal; blue) DNA. cffDNA has shifted size distribution, being overall shorter than the maternal counterparts.

can also influence the amount of ctDNA that is shed into body fluid [Figure 3]. As an example, in patients with bladder cancer, ctDNA is more easily detected in urine compared to in blood^[57,58]. Lastly, recent works using paired-end sequencing suggest that malignant cell-derived DNA fragments are in general shorter than those of non-malignant DNA^[50,59].

Strategies to analyze plasma cfDNA

Quantitative and qualitative analyses have been developed to interrogate cfDNA. Pre-analytical factors can affect properties of cfDNA. Studies have evaluated the impact of variables, including blood collection, processing, plasma isolation and storage. Recommendations and development of standardized protocols to enhance test performance are advised^[60-62]. Quantitative assessment usually involves the extraction of DNA from plasma or other fluids for concentration quantification. CfDNA concentration as a biomarker has been investigated widely in prenatal, cancer and autoimmune disease applications^[63-65], as levels of total cfDNA are known to increase under certain conditions and fluctuate in accordance with disease state. In addition to levels of total cfDNA, levels of cffDNA and ctDNA in the plasma of pregnant women and cancer patients, respectively, may also differ from those of controls. By making use of Y chromosome- or placental-specific markers, studies have shown that cffDNA levels in complicated pregnancies appear to be higher^[36,66]. Likewise, ctDNA level, measured by a mutation template, has been explored as a biomarker for cancer detection and treatment response^[52,67].

While the abundance of total cfDNA and its sub fractions can indicate the presence of (patho)physiological states, a more comprehensive view is obtained by qualitative analyses. Those include the detection of genetic variations, methylation signatures and fragmentation patterns. Next generation sequencing (NGS) technologies have enabled analysis at unprecedented throughput and resolution. The presence of placenta- or tumor-derived DNA in cfDNA pools can be determined through the detection of genetic and epigenetic variations [Figure 4]. The limited absolute number of DNA molecules and low cffDNA or ctDNA concentration against high maternal or non-malignant backgrounds in plasma samples directly affects the signal identification. Though the biological limitations pose obstacles to robust detection of variations, approaches have been developed to address these challenges. One of the first methods that demonstrated sensitive detection of fetal aneuploidies relied on massive parallel sequencing to profile genomic representations of cfDNA^[68,69]. Quantifying the statistical deviations of a profile from an external reference

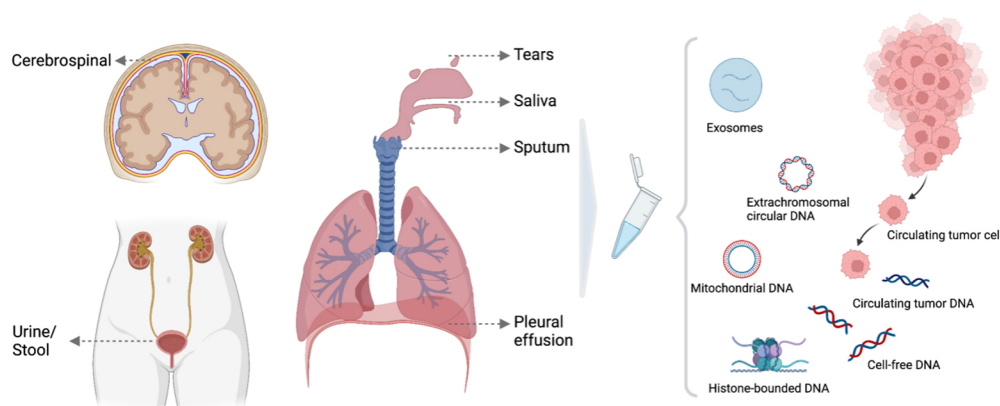


Figure 3. Illustration of ctDNA derived from other body fluids other than blood. Different forms of markers present in biological fluids, including circulating tumor cells, circulating cell-free DNA and circulating tumor DNA, histone-bound DNA, exosomes, extrachromosomal circular DNA and mitochondrial DNA.

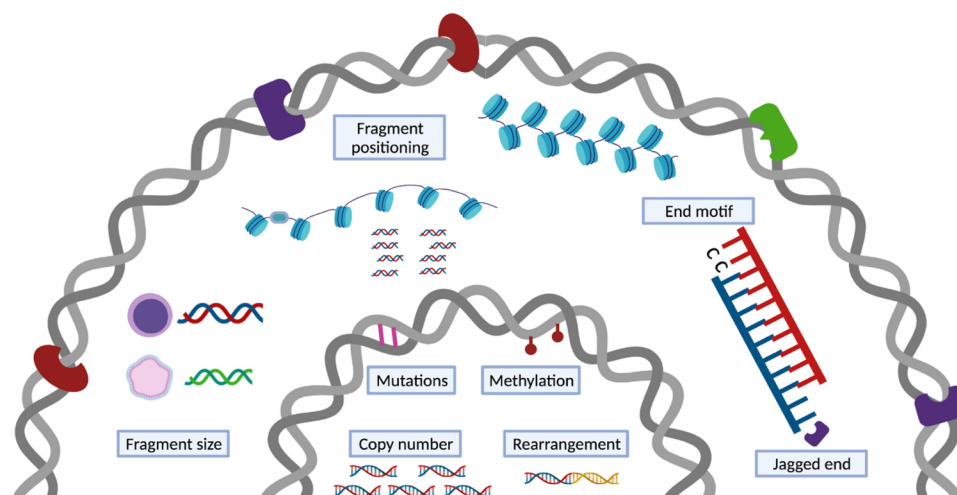


Figure 4. cfDNA carries (epi)genetic and fragmentation signatures. A catalog of genetic and non-genetic signatures that might be used for disease detection. The genetic signatures, including copy number changes, single nucleotide variations, and rearrangements, and epigenetic signatures, mainly methylation, are vastly investigated and used in clinical applications. The non-genetic changes that encompass several dimensions of characteristics resulted from cfDNA release mechanisms are under exploration.

profile comprised of normal controls or the deviation of one region from other regions within the same sample allows robust detection of common fetal aneuploidies - an approach that has been validated by multiple large-scale clinical studies^[70-72]. The strategy has been shown to capture copy number aberrations within ctDNA as well^[73,74]. Deep sequencing of single-nucleotide polymorphisms and, in the case of cancer, somatic point mutations, can also be performed for the quantification of cff- and ct-DNA, respectively. In cancer studies, to compensate for the low number of ctDNA source molecules, larger numbers of mutations are tested to improve sensitivity^[75]. Using unique molecular identifiers and analytical error corrections can further facilitate variant or mutation detection^[76-78]. A recently developed approach that leverages two or more mutations occurring on the same strand of DNA has further pushed the detection limit. Using a personalized panel, the approach allowed identification of variants with a tumor fraction as low as 1/1,000,000^[79]. Another type of genetic alteration - rearrangements - have been tested as part of NGS solutions specific for ctDNA detection, and a high concordance between cfDNA and tumor tissue was obtained for selected gene fusion analyses^[80,81].

Epigenetic approaches have been exploited extensively in recent years, among which, DNA methylation has been widely adopted for cfDNA analysis in cancer. Shortly after the discovery of tumor- and fetal-derived DNA in plasma samples, tumor and fetal specific methylation patterns were also observed in cfDNA^[82,83]. Early in disease development, DNA methylation can exhibit divergent patterns across different tissues/cells^[4,6]. Methylation changes thus offer the potential to predict and monitor disease states. Early investigative approaches were limited to DNA methylation marks on individual genes^[29,82,84], but have expanded to genome-wide assays^[85,86] aimed at improving sensitivity and specificity. For example, approaches that leverage multiple informative CpG markers^[87] or methylation haplotype blocks that exhibit highly coordinated methylation across consecutive CpG sites^[88] have been developed. The main method to analyze cytosine methylation at single nucleotide resolution is bisulfite conversion. The method makes use of sodium bisulfite treatment, which converts unmethylated cytosine residues to uracil and leaves 5-methylcytosine (5mC) unaffected. This approach has a major drawback in that it causes degradation of the DNA. To overcome this limitation, new methods for methylation analysis are being developed. Such methods include cell-free methylated DNA immunoprecipitation and high-throughput sequencing (cfMeDIP-seq)^[89], enzymatic methyl-seq (EM-Seq)^[90,91], and ten-eleven translocation (TET)-assisted pyridine borane sequencing (TAPS)^[92], which have all been successfully applied for methylation analysis in cfDNA. Furthermore, third-generation sequencing, including single-molecule real-time sequencing by Pacific Biosciences and nanopore sequencing by Oxford Nanopore Technologies, has opened new avenues for cfDNA analysis, enabling simultaneous real-time analysis of DNA sequence as well as nucleotide modifications. Though sequencing fragmented cfDNA is still challenging, proof-of-concept studies have demonstrated the potential of using these new technologies for the development of noninvasive disease management tools^[93]. Beyond methylation patterns, chromatin immunoprecipitation and sequencing (ChIP-seq) has been developed to investigate histone modifications, allowing cell-of-origin determination and disease-specific transcriptional programs to be identified^[94].

In addition to genetic and epigenetic changes, various analyses that rely on cfDNA fragmentation patterns have come into view. Fragmentation of cfDNA is non-random. It is mechanically related to modes of cell death and nuclease cleavage, and cellularly associated with the nucleosome organization, chromatin structure and gene expression of the tissue of origin. Fragmentation-based signatures or fragmentomics therefore reflect multiple processes that may provide additional markers for clinical use. CfDNA fragment size is one the most thoroughly assessed fragmentation signatures. It has long been recognized from the fragments' ladder-like patterns that cfDNA bears a signature of caspase-dependent apoptosis^[7,10]. More recent data has shown size differences between contributing tissues. Even though the exact cause of size differences between fragments from different tissues remains to be clarified, fragment size patterns have been leveraged as a single metric or as part of a series of informative markers. CfDNA fragment size was demonstrated as an approach for fetal fraction estimation, fetal aneuploidies, and tumor detection^[95,96]. Shorter fragments have been selected *in vitro* or *in silico* in order to enrich for DNA that is more likely to be derived from a tissue of interest. Enriching for fragments from a specific tissue based on the fragment size can also enhance genetic and epigenetic signals in downstream qualitative analyses^[97-99].

Beyond size profiling, the genomic location of cfDNA fragments reflects nuclear DNA architecture and gene expression in source tissues and can be used as the basis of tissue-of-origin analyses. Fragment position is known to reflect nucleosome protection of DNA from digestion. Nucleosome linker regions are more likely to be cleaved and thus play a role in preferred cfDNA fragment ends. Based on this knowledge, nucleosome occupancy maps were constructed by identifying regions with pile ups of fragment endpoints and therefore predicted to be nucleosome-free. These analyses demonstrated periodic coverage densities and nucleosome spacing that varied with chromatin state and gene activity^[43,50]. Therefore, the contribution

of different cell types can be ranked based on the correlation of their gene activity with the observed nucleosome spacing in gene bodies^[47,100]. Different preferred end sites were found in fragments of fetal and maternal origin, and in those of malignant and non-malignant origin in liver cancer^[41]. When quantifying the preferred end signature in open chromatin regions, the measure of pooled density differences of directional DNA ends could be used to trace the tissue of origin of cfDNA fragments^[101]. In addition to preferred end sites, other fragmentation characteristics include end motifs and jagged ends. At the nucleotide resolution, the 5' fragment 4-mer end motif was found to hold tissue-of-origin information as well^[102]. A catalog of end motifs have altered frequencies in cancer, pregnant and transplantation subjects when compared with healthy controls and the diversity of the end motifs present in cfDNA has been used in cancer detection^[102]. In cancer patients, these differences were ascribed to a deficiency in the DNASE1L3 nuclease^[103,104]. Lastly, Jiang *et al.* explored the single-stranded overhangs by methylation analysis to infer deliberate cleavage events^[105]. DNA end repair could introduce unmethylated cytosine using CpG sites or methylated cytosine using CH sites to newly synthesized ends. Interrogating the methylation levels along the DNA fragments could provide traces of enzymatic cutting of cfDNA fragments and their tissue-specific degradation patterns. The presence of such jagged or protruding ends corroborates the non-random fragments generated by endonucleases in plasma DNA and these jagged ends may be used as an indicator for relative activities of the molecular scissors, for example DNASE1 and DNASE1L3^[106,107].

Clinical diagnostic applications of cfDNA

Noninvasive prenatal screening for aneuploidies

The landmark finding of cfDNA in maternal plasma paved the way for the clinical translation and commercialization of cfDNA analysis for the noninvasive prenatal screening (NIPS) of common aneuploidies. NIPS is offered to pregnant women at high risk of carrying a fetus with a common trisomy 21, 18, or 13 in many countries. In Belgium and the Netherlands, NIPS is offered as a first-tier test to all pregnant women. In a recent analysis of NIPS performance in the general obstetric population, sensitivities and specificities were 98.91% and 99.98% for trisomy 21, 97.47% and 99.99% for trisomy 18, and 100% and 99.97% for trisomy 13, respectively. The positive predictive values (PPVs) were 92.39% for trisomy 21, 84.62% for trisomy 18 and 43.95% for trisomy 13^[108]. NIPS for common aneuploidies has demonstrated superior performance. In addition to the test performance. Not only does it perform well, NIPS is usually done starting from 10 weeks of pregnancy, which is earlier than or within the same time frame as invasive and first trimester combined tests. The widespread introduction of NIPS into routine prenatal care has raised concerns about disability rights, equitable access to the test, and reproductive choices^[109]. Despite these ethical concerns and ongoing debate about the clinical utility, it is likely that NIPS will continue to be adopted as a first-tier screening test.

Expanded NIPS that offers additional indications for rare autosomal trisomies (RATs) and structural anomalies is available, though PPVs (ranging from 0 to 21%) for detecting RATs or structural anomalies are lower than those of the common trisomies^[110]. With that, the PPVs of expanded NIPS is considerably higher than the First Trimester Combined Test. While the clinical implementation of expanded NIPS is still under debate, the prevalence of RATs and structural anomalies is as high as those of trisomy 18 and 13^[111], which has motivated larger prospective studies to shed light on the clinical significance. Constitutive RATs rarely result in a viable pregnancy, however, in some cases mosaic RATs can result in adverse pregnancy outcomes, including growth impairment^[112,113]. Determining the impact of these events early on in gestation could have clinical value and could support the adoption of expanded NIPS. False or inconclusive results can be the result of multiple biological factors, including confined placental mosaicism, the presence of a vanishing twin, maternal health conditions, and medication^[114,115]. Continuous efforts have been made to investigate the clinical utility of the secondary findings relevant to maternal health, leading to updates in clinical management recommendations in NIPS^[114].

Noninvasive prenatal screening for monogenic disorders

Noninvasive prenatal screening for monogenic diseases (NIPS-M) or noninvasive prenatal diagnosis is gaining interest in the clinic. Complimentary to NIPS for aneuploidies, NIPS-M can determine the mutational status of a fetus at high risk for a single-gene disorder using maternal plasma cfDNA analysis [Figure 5]. The test is most often offered for *de novo* or paternally inherited mutation diagnosis by counting the dosage of mutant and wild-type alleles or by reconstructing haplotypes. The UK National Health Service Laboratory clinically implemented NIPS-M for inherited conditions (e.g., achondroplasia, thanatophoric dysplasia and cystic fibrosis)^[116,117]. A recent clinical study reported robust performance in detecting dominant monogenic diseases^[78]. Though detection of recessive or maternally inherited conditions mostly remain in the research phase, a number of technologies that readily detect a range of dominant and recessive diseases have been developed^[118-120]. NIPS-M has been more slowly implemented in the clinic compared to NIPS for aneuploidy, which may be due to the technical difficulty of designing bespoke tests for each family based on their genetic information.

Liquid biopsy in cancer

The clinical potential of liquid biopsy for cancer management has been considerably illustrated. Different ctDNA-based biomarkers or assays have been approved by the Food and Drug Administration (FDA). These include two single-cancer detection tests, namely the cobas *EGFR* Mutation Test v2 (Roche Molecular Diagnostics) for lung cancer and Epi proColon (Epigenomics AG) for colorectal cancer, and two targeted NGS-based pan-cancer tests: FoundationOne Liquid CDx (Foundation Medicine) and Guardant360 CDx (Guardant Health), which have been introduced as companion diagnostics to identify biomarkers in patients with advanced diseases. The noninvasive nature of cfDNA allows routine analysis of tumors that are unsafe or infeasible to biopsy [Figure 6]. Moreover, cfDNA may better reflect tumor heterogeneity harbored by distinct clonal populations. Therapeutic guidance is among the most clinically relevant current utilization of ctDNA, particularly for patients for whom standard tumor biopsies fail to yield sufficient material for analysis^[121,122]. Studies have demonstrated that ctDNA testing may reveal actionable genomic mutations to guide treatment decisions^[123,124]. In addition, liquid biopsy provides a way to monitor tumor burden and the development of therapy resistance in real-time. It has been shown that levels of patient-specific tumor alterations in cfDNA correlate with the overall tumor burden through serial ctDNA testing^[67,125,126]. Biological resistance can be a consequence of tumor heterogeneity. ctDNA monitoring permits detection of secondary mutations associated with treatment resistance^[52,127,128]. Furthermore, the longitudinal analysis of ctDNA may be extended after completion of treatment and may also serve as a means of detecting postsurgical minimal residual disease (MRD) in patients with clinically undetectable disease. Several groups have explored using postoperative ctDNA to identify patients with MRD and continued to optimize technologies^[79,129,130]. A fraction of malignancies is associated with human viral infection. For the infection-induced malignancies, such as nasopharyngeal carcinoma and oropharyngeal cancer with viral DNA integrated into the host genome, plasma viral cfDNA can be quantified. Monitoring the viral cfDNA has shown prognostic potential for MRD detection^[131,132]. One of the most impactful applications of ctDNA could be the potential for early cancer detection in asymptomatic populations. Early detection of cancer could increase the chances of survival. While governmental screening programs are in place for some of the cancers (e.g., breast), for many cancers, there are no screening tests available. One study evaluated analysis of Epstein-Barr virus DNA in plasma samples to screen nasopharyngeal carcinoma in asymptomatic populations, and it revealed higher positive predictive value than existing blood-based markers. A significantly higher proportion of participants with the early stage disease was identified, demonstrating the potential for early detection and early treatment intervention^[133]. By far the biggest commercially driven endeavour for multi-cancer detection, the Galleri™ test, was developed by the company GRAIL^[87,134]. The test was developed based on 50 different cancer types, and large-scale trials are currently ongoing to evaluate its performance for cancer screening. The combined test that uses

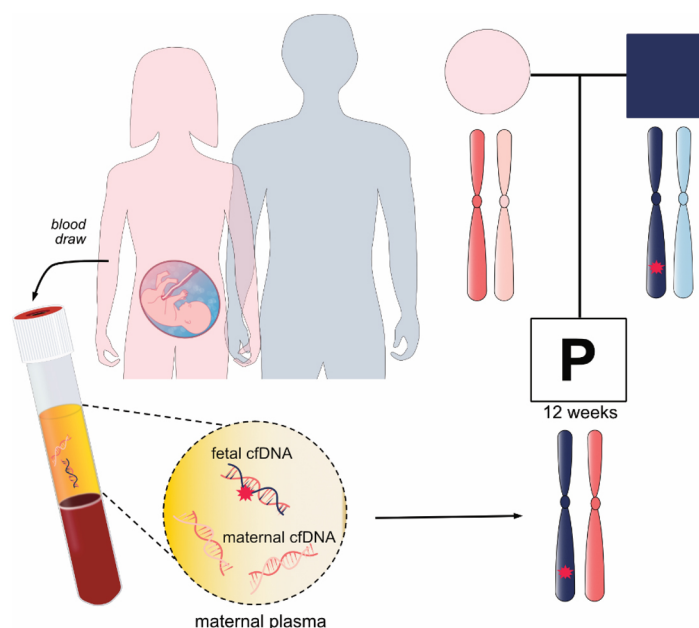


Figure 5. Noninvasive prenatal screening for monogenic disorders or noninvasive prenatal diagnosis. A family in which the father affected with an autosomal dominant disease opts for noninvasive prenatal diagnosis. Maternal plasma is taken to examine whether the fetus inherits the disease-associated allele.

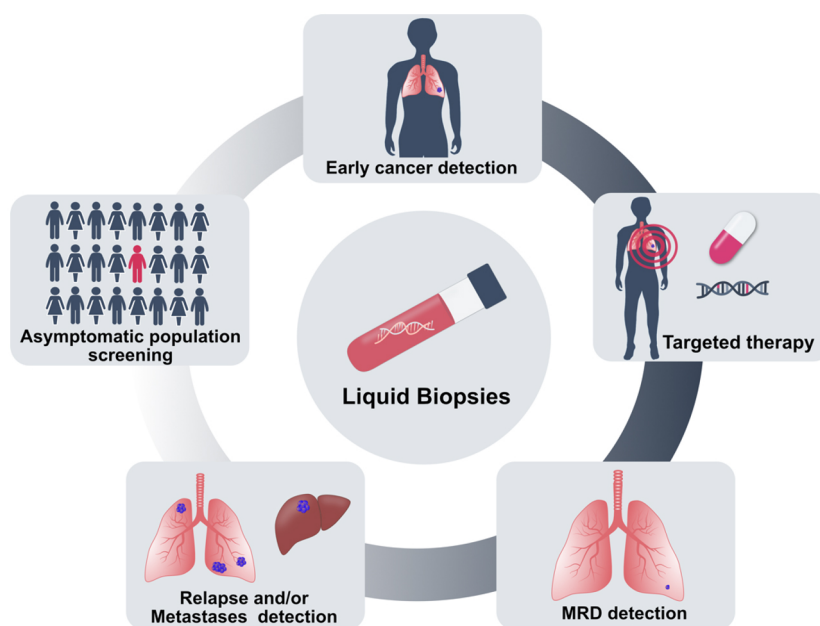


Figure 6. Clinical applications of cfDNA analysis in cancer. Analysis of cfDNA has been used to develop early cancer detection tools and ctDNA mutation analysis can guide therapy choice. After treatment, minimal residual disease (MRD) can be monitored by cfDNA analysis, and in case of resistance, cfDNA-based tests allow detection of relapse and tumor metastases. CfDNA analysis has the potential to screen cancers in the asymptomatic population.

multiparameter or multianalyte has also emerged as a key player in cancer detection^[135-137]. The feasibility and safety of liquid biopsy for multi-cancer screening was largely addressed by a pioneering large clinical trial study using the DETECT-A test which combined ctDNA and protein biomarkers and performed

positron emission tomography-computed tomography in patients with a positive blood test^[138]. In addition to the general population, several studies have reported incidental findings of maternal malignancies during routine NIPS^[139,140]. These findings have prompted large-scale or nationwide evaluations of occult maternal malignancy detection following abnormal NIPS profiles and emphasized the need to establish multidisciplinary care for clinical management of cancer in pregnancy^[141,142]. Additionally, the utility of the NIPS platform - analyzing plasma cfDNA with low-pass whole-genome sequencing - for cancer detection has also been explored in high risk asymptomatic populations^[74].

Other applications for cfDNA analysis

The increasing interest in the use of cfDNA is not just within obstetrics and oncology. Transplantation and infectious diseases are active research areas. Based on the rationale that graft rejection entails injury and leads to increased cell death in the allograft, donor-derived cell-free DNA (dd-cfDNA) as a noninvasive marker of graft rejection has been established in solid organ and bone marrow transplants^[143-145]. Quantifying the concentration of total cfDNA after transplantation, as well as the proportion of the dd-cfDNA subfraction using genotypes, allows detection of allograft injury and immunosuppression. Methyome profiling of cfDNA has also been shown to capture tissue damage in the kidney allograft^[146]. Serial dd-cfDNA monitoring can provide timely views of transplant health and immunosuppressive drug effects^[147].

Infection can be another major complication affecting the health of patients receiving allograft transplantation. A number of assays that concurrently monitor posttransplant rejection and infection by sequencing cfDNA have been developed^[146,148,149]. Coupled with NGS, the identification of pathogen DNA in plasma of patients with sepsis, invasive fungal infection and cytomegalovirus^[150-152] has broadened our ability to systematically detect clinically relevant pathogens in a noninvasive way. Viral DNA detection during NIPS has been demonstrated as well^[153,154]. Blauwkamp and colleagues developed and validated a microbial cfDNA (mcfDNA) sequencing assay - the Karius test that allows detection of a wide range of infections^[155]. Though this test is not able to detect RNA viruses, it has been used to identify secondary and co-infections in patients with COVID-19^[156]. While tracing microbial pathogens is feasible, the analytical sensitivity could be limited by the scarcity of mcfDNA against the dominating host background^[157,158]. Additionally, pathogen DNA interpretation may be complicated by reagents used for processing, sample contamination and organisms of uncertain clinical significance^[158].

Virus infection can cause damage to multiple organs and tissues. Deconvoluting the cell type mixtures in cfDNA methylation profiles from COVID-19 patients, studies found elevated cfDNA levels and mapped tissue injuries particular to lung and liver compared with healthy controls or patients with other respiratory infections^[159,160]. Interestingly, both studies highlighted a change in the hematopoietic cell signal as an informative marker for COVID-19 mortality prediction. While Cheng *et al.* observed an increase signal from erythroblasts (red blood progenitor cells) that may involve anemia and inflammation or dysregulation of erythropoiesis in COVID-19 patients^[159], Andargie *et al.* underscored the major role of neutrophils (white blood cells) that might aggravate pulmonary inflammation and erythropoiesis as a second contributor in elevated cfDNA levels^[160]. These observations may have an implication in other diseases as well. The hallmark of immune-mediated disorders is inflammation. Plasma DNA might act as an index of inflammation and damage and therefore relate to the altered genomic cfDNA seen in some patients.

“New” components of cfDNA

Along with cell-free nuclear DNA, cell-free mitochondrial DNA (cf-mtDNA) is detectable in plasma and other fluids. While cf-mtDNA research has fallen behind that of cell-free nuclear DNA, recent investigations into mitochondrial function and cf-mtDNA have elucidated its potential biological

significance. Mitochondrial DNA (mtDNA) is prone to oxidative stress and may vary from dozens to thousands of copies depending on tissue origin^[161]. Different forms of mitochondrial DNA, including free, particle-associated and respiratory competent mitochondria, were reported in plasma samples^[162,163]. Cf-mtDNA copy number did not appear to be correlated with mtDNA copy number in cellular leukocytes, with the former being more sensitive to cellular damage and the latter reflective of cellular energetics^[164]. Unlike cell-free nuclear DNA, cf-mtDNA size profile lacks nucleosome-size peaks and shows an enrichment of short fragments smaller than 100 bp^[9] due to the lack of histones. Though limited by different pre-analytical protocols and mixed conclusions, cf-mtDNA has been found to be elevated in multiple pathologies, including trauma, sepsis, neurological disorders, and cancer^[164-168]. A highlight of recent cf-mtDNA research comes from the identification of both intact circular and fragmented linear cf-mtDNA in plasma DNA. Of particular interest, the authors observed that non-hematopoietically derived cf-mtDNA was predominantly in a linear form, whereas hematopoietically derived cf-mtDNA was primarily circular using a transplantation model^[169]. It is noteworthy that analogous observations regarding fetal and maternal cf-mtDNA in surrogate pregnancies have subsequently been reported by the group^[170]. The fetal-derived cf-mtDNA mainly existed in a linear form, but about half of maternal-derived cf-mtDNA appeared to be in a circular form. Although there is a major knowledge gap between the triggers that connect pathophysiological states to the cf-mtDNA release into circulation and different biological forms of cf-mtDNA, these findings might prompt the development of cf-mtDNA assays.

Another circular DNA - extrachromosomal circular DNA (eccDNA) has been known for a long time. The recent development of an assay to quantify eccDNA in plasma DNA (referred to as cf-eccDNA throughout the text) entices renewed interest. This circular DNA exists as small (200 to 400 bp), non-amplified eccDNA in normal cells^[171] and as large (1.29 Mb EGFRvIII were detected), copy number-amplified extrachromosomal circular DNA (referred to as ecDNA to differentiate from eccDNA) that is primarily found in cancer cells^[172]. Recent studies demonstrated that ecDNA is common in human cancer cells (up to hundreds of ecDNA molecules per cell), with the highest abundance in brain tumors. Support for the presence of cf-eccDNA in circulation was first shown in two studies that enriched circular DNA by exonuclease digestion of the background linear DNA^[173,174]. These studies demonstrated that cf-eccDNA harbors characteristics of eccDNA that tends to be generated from regions with high GC content, gene density, active chromatin marks and a high frequency of direct repeats flanking eccDNA junctions. Furthermore, the size of cf-eccDNA tended to be longer when comparing pre- to post-surgery in lung and ovarian cancer patients, possibly implying that cancer-associated eccDNAs are longer than the normal counterparts^[173]. It has been found that fetal-derived eccDNA is present in the plasma of pregnant women. The cf-eccDNA fragment size distribution was shown to have a small peak at 202 bp, another major peak at 338 bp and a 10 bp periodicity in the vicinity of this peak. The fetal cf-eccDNA tended to be shorter than the maternal counterparts, consistent with the phenomenon in linear nuclear cfDNA^[175]. Reminiscent of nuclear cfDNA patterns, fetal cf-eccDNA shows relatively lower methylation levels than those of the maternal cf-eccDNA and clears rapidly after delivery with a half-life of 30 min. Longer cf-eccDNA fragments appears to carry higher methylation densities in plasma of both pregnant and non-pregnant subjects^[176]. Furthermore, nuclease activity on cf-eccDNA was reported, showing that DNASE1L3 affected eccDNA characteristics^[177].

New frontiers in cfDNA biology

CfDNA has long been considered as cell death debris. However, growing evidence points to potential functional aspects of cfDNA, including its role in horizontal genetic transfer, cellular signaling, oxidative stress response, and innate immunity^[1,178,179]. Immunological properties of cfDNA can be particularly relevant to certain disease pathologies. Interplaying with nuclease biology, the presence of endogenous DNase allows proper DNA degradation and prevents inflammatory stimulation, whereas DNase-deficient

mice were prone to develop autoimmune pathologies^[180] and exhibited cfDNA fragmentation aberrations^[103]. Indeed, DNase1 activity was found to be substantially lower in patients with systemic lupus erythematosus (SLE)^[181]. In pathological conditions like lupus, cfDNA complexed with other proteins may act as proinflammatory stimulants of toll-like receptors (TLR) that elicit the inflammatory response^[107]. Such mechanisms of recognizing extracellular DNA as damage-associated by the innate immune system has been shown in the case of cf-mtDNA in patients with trauma^[165]. Triggered by the innate immune response, NETosis that initiates the formation of Neutrophil Extracellular Traps (NETs) could promote thrombosis and mediate tissue damage^[182]. Formed as extracellular web-like structure with antimicrobial proteins, the NETs have been suggested to play a role in the pathogenesis of multiple diseases, including lupus, sepsis and tumor progression^[183-186]. Remarkably, studies have found that host DNase1 and DNase1L3 act as dual protection systems to degrade NETs and in the absence of DNase1 and DNase1L3, intravascular NETs form clots and result in organ damage^[187]. The aforementioned studies that map tissue injury in COVID-19 patients may imply potential immunological effects in cfDNA. Additional pertinent evidence reported strong correlation between levels of cfDNA and traditional inflammatory markers, in particular, absolute neutrophil count and myeloperoxidase-DNA that is regarded as a specific marker of NETs in hospitalized COVID-19 patients^[188]. In transplantation, elevated dd-cfDNA in the presence of allograft injury may indicate its active role in immunological processes as well^[189]. Rather than showing immunostimulatory characteristics itself, cfDNA may be a non-specific (pro)inflammation and (pro)coagulation marker in numerous inflammatory and cell death pathways. The question is to what extent the immunological effects are reflected in cfDNA and how the changes can be effectively captured.

Concluding remarks

Fragmented DNA constantly enters our circulation following passive and active release mechanisms. The composition of tissues contributing to cfDNA differs from one disease to another, and across different physiological states. With the rapid maturity of next generation sequencing and bioinformatics analysis, cfDNA analysis has led to robust copy number detection tools in prenatal testing and tumor liquid biopsy. Yet clinical applications lag behind our expanding understanding of cfDNA biology. In particular, epigenetic and fragmentomic characteristics of cfDNA are an untapped source of potential clinical markers in a wide range of diseases. Novel experimental and computational methods capable of simultaneously capturing and integrating cfDNA modalities present attractive opportunities to push analyses further and may allow for synergistic effects.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Che H, Stanley K

All authors revised and reviewed the manuscript.

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Research Foundation-Flanders (FWO) G080217N to JRV, FWO-SBO-MICADO (S003422N) and KU Leuven funding (no. C1/018 to JRV).

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.

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Review

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Towards responsible ctDNA-based multi-cancer screening: a preliminary exploration and discussion of ethically relevant aspects

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How to cite this article: Dondorp W, de Wert G. Towards responsible ctDNA-based multi-cancer screening: a preliminary exploration and discussion of ethically relevant aspects. *Extracell Vesicles Circ Nucleic Acids* 2022;3:235-43. <https://dx.doi.org/10.20517/evcna.2022.23>

Received: 8 May 2022 **First Decision:** 30 Jul 2022 **Revised:** 7 Aug 2022 **Accepted:** 12 Aug 2022 **Published:** 16 Aug 2022

Academic Editors: Yoke Peng Loh, Erik A. Sistermans **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

While testing for easily accessible biomarkers in the circulation (“liquid biopsy”) has found its way to clinical cancer care, a further expected development is its use as a “universal” early detection test in population screening for cancer. A promising marker for such screening is circulating cell-free fragments of tumor DNA, shed into the circulation during tumor cell turnover. Several blood-based “multicancer early detection (MCED) tests” have recently been developed - but still need validation in large-scale studies involving non-patient populations. In this paper, we proactively explore the ethical aspects of this development. We refer to an often quoted synthesis of the internationally accepted framework of principles for responsible screening as first drawn up for the World Health Organisation (WHO) by Wilson and Junger 50 years ago and further developed and fine-tuned ever since. As our analysis suggests, some specific ethical issues and concerns about potential MCED screening connect to the fact that cancer is not just one disease. As a consequence, not all findings will have the same clinical utility. We discuss this against the background of earlier debates pertaining to broad scope forms of screening in other contexts, specifically newborn and reproductive genetic screening. We highlight the guidance provided by some of the criteria from the screening framework that seems most relevant in this connection: the need for screening objectives to be defined at the outset, the need for mechanisms to minimize potential risks, and the requirement that, for those participating in the screening, the overall benefits outweigh the harm.



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Keywords: Cancer, population screening, cfDNA, ctDNA, MCED, ethics, public health, screening criteria

INTRODUCTION

As one of the leading causes of death in developed countries, cancer is a global public health problem. The high mortality burden is a consequence of most cancers being detected at a stage where treatment options have limited effectiveness. Population screening aimed at finding cancer at a pre-symptomatic stage may help change this unfortunate course of events by enabling timely treatment. However, there are many different kinds of cancer and only for some of them have so far made it possible to develop screening strategies for non-patient populations. In the Netherlands, for instance, national screening programs currently only exist for breast, cervical and colorectal cancer, targeting higher-risk age groups (of the relevant sex) in the general population^[1]. A pilot for lung cancer screening offered to those with a history of heavy smoking (as also recommended in some other countries) has just started^[2]. For each individual type of cancer, the challenge is to develop a test that is not only easily applicable in pre-symptomatic stages, but also sufficiently accurate and affordable for a population screening program to have a positive benefit to harm and cost-utility ratios. Moreover, with each additional screening for a further type of cancer, logistical burdens and costs will increase and motivation to participate may suffer from screening fatigue, especially in lower-risk populations.

Against this background, the concept of “universal” (or multi-organ) cancer screening based on easily accessible tumor markers in the circulation may well be revolutionary. This approach would allow simultaneous screening for, in principle, all types of cancer at pre-symptomatic stages using a minimally invasive blood test^[3-5]. A promising marker for such screening is circulating cell-free fragments of tumor DNA. Also, in healthy individuals, all types of cells release genetic material (cell-free DNA; cfDNA) into the bloodstream during the course of normal cell turnover. This finding has already led to the successful development and implementation in many countries of prenatal screening for fetal chromosomal abnormalities based on the presence of cell-free fetal DNA (cffDNA; actually stemming from the placenta) in the maternal circulation^[6]. Cell-free tumor DNA (ctDNA) specifically derives from tumor cell turnover. For detection and analysis, state-of-the-art technologies are being used that allow distinguishing the genomic features of the relevant DNA fragments from background signals in the circulation. The idea of using ctDNA, in combination with other biomarkers, as a test for population screening builds on the recent development and clinical use of “liquid biopsy” as a tool in cancer diagnosis, prognosis and treatment, for instance, allowing personalized treatment based on pharmacogenetic profiling, or as a powerful marker in tumor monitoring and follow-up after cancer treatment^[4,7]. However, using this approach for population screening is more challenging as it requires finding cancer at an early stage in a lower-risk population with sufficient accuracy^[8].

Several blood-based “multicancer early detection (MCED) tests” have recently been developed - but still need validation in large-scale studies involving non-patient populations^[5,9]. The limited evaluations so far suggest a relatively low sensitivity, especially for early-stage cancer^[10,11]. As a consequence, MCED screening should not be seen as an alternative but as an add-on to current cancer screening programs. Modeling has suggested that this add-on approach might potentially be an efficient cancer screening strategy based on finding additional breast, colorectal, cervical, lung and prostate cancers either missed in current screening programs for those conditions, or in people outside the target population criteria used in those programs or also in those who decline current cancer screening but would agree to MCED testing^[9,12]. To further evaluate this idea, the GALLERI test is currently piloted for the British NHS. The pilot (2021-2023) comprises a randomized controlled trial for which 140.000 non-patient participants aged 50 to 79 will be recruited^[13].

The aim is “to establish if screening with the Galleri test reduces the incidence of late-stage cancer when used in an asymptomatic population in combination with existing NHS cancer screening programs”^[14].

While the research is still in the test-validation phase, it is also important to pro-actively consider the ethical aspects of this development. In this exploratory paper, we refer to the internationally accepted principles for responsible population screening as a background for further agenda setting. As we will argue, the concept of MCED screening comes with specific ethical challenges that need to be considered. These connect to different ways in which such screening may not only have a potential for benefit but also for harm.

FRAMEWORK FOR RESPONSIBLE SCREENING

From an ethical perspective, a crucial distinction is between medical testing that is clinically available to patients and those are offered to non-patients in a screening context^[15]. Whereas clinical testing is performed in response to a complaint or other kind of individual indication on the part of the patient, screening is systematically offered on the initiative of health care professionals or screening authorities to people without a relevant complaint or indication. Leaving aside the special situation of screening in contexts where societal interests justifiably take precedence (for instance, where screening would be necessary as a means to control the spread of a pandemic), the non-indicated nature of screening and the locus of the initiative require a high level of evidence that, on balance, testing is indeed beneficial for at least a significant proportion of those to whom it is offered^[16,17].

This would always be the case, which is far from obvious. As famously stated by the former director of the UK National Screening Committee, sir Muir Gray, “[a]ll screening programs do harm; some do good as well, and, of these, some do more good than harm at reasonable cost”^[18]. Harms of screening may range from psychosocial effects such as induced anxiety or false reassurance to iatrogenic damage as a consequence of overdiagnosis and -treatment. The benefits of screening, mostly understood in terms of health gains, should clearly outweigh these harms. This requires not just that screening outcomes are sufficiently accurate, but that they meaningfully enable altering the clinical outcome in those with positive findings through effective early interventions^[19]. Moreover, where screening is paid for from public or collective funds, opportunity costs require budgetary justification^[16,17].

The acknowledgment of the precariousness of the balance between these different elements is behind the internationally accepted framework of principles for responsible screening, as first drawn up for the World Health Organisation (WHO) by Wilson and Junger in 1968 and further developed and fine-tuned also for specific types of screening (e.g. reproductive and/or genetic screening) by several expert groups and national screening authorities^[17,19]. An often quoted synthesis of these “emerging screening criteria” was drawn up by Andermann (WHO) and colleagues at the 40th anniversary of the Wilson & Jungner principles in 2008^[20] (see [Table 1](#)). Highlighting the ethical nature of this internationally endorsed framework, the final and summarizing requirement on their list is that “[t]he overall benefits of screening should outweigh the harm”.

RELEVANT ETHICAL ASPECTS OF MCED SCREENING

In a recent review on behalf of the International Society of Liquid Biopsy, Serrano and colleagues explicitly refer to this framework and remark that cancer screening programs based on MCED testing could well fulfill these criteria^[21]. We agree that there is no apriori reason why this would not be the case, given the large potential health benefits that this approach may yield. However, whether specific screening protocols do indeed meet the criteria for responsible screening and may then (depending on the health system) be either recommended by professional societies and/or incorporated into national screening programs, will have to be determined on the basis of further evidence about relevant factors.

Table 1. Screening criteria (Andermann WHO 2008)**Synthesis of emerging screening criteria proposed over the past 40 years**

- The screening programme should respond to a recognized need.
- **The objectives of screening should be defined at the outset**
 - There should be a defined target population
 - There should be scientific evidence of screening programme effectiveness
 - The programme should integrate education, testing, clinical services and programme management
- **There should be quality assurance, with mechanisms to minimize potential risks of screening**
 - The programme should ensure informed choice, confidentiality and respect for autonomy
 - The programme should promote equity and access to screening for the entire target population
 - Programme evaluation should be planned from the outset
- **The overall benefits of screening should outweigh the harm**

-Reproduced with permission from: Andermann A, Blancquaert I, Beauchamp S, Déry V. Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years. *Bull World Health Organ.* 2008 Apr;86:317-9

-Criteria rendered in bold are specifically discussed in the present text

As with all screening programs, test accuracy is an essential quality parameter. A relatively low sensitivity not only affects screening performance; it may also lead to false reassurance. Given that, precisely in the light of lower expected sensitivity, MCED screening would serve as an add-on to, rather than as a replacement for, current single-organ cancer screening programs, there is a potential risk that false reassurance induced by negative blood tests may lead to a lower uptake of current screening. Clearly, this would be an adverse effect of simply adding MCED screening to the current landscape. While such effects can only be determined in actual practice, the remaining importance of current single-organ cancer screening may need to be proactively addressed in the screening information for the relevant target groups. This also suggests that the best way of fitting MCED testing into a wider approach to cancer screening will require more precise attention.

Another ethically relevant issue with test accuracy is the false-positive rate. Even at less than 1% (the rate that some reviewers seem to regard as a benchmark^[9]), a screening offer to potentially the whole population in a wide age range will lead to many people ending up with a positive MCED test but without radiological or clinical confirmation of cancer. These people will have to be offered further monitoring, for which “pathways should be developed”^[9]. However, this is more than a logistical issue, as it may induce high levels of anxiety as well as procedure-related harms, especially if the precise focus of the necessary monitoring may not be obvious.

This connects to an important further issue: the screening should not just be able to accurately detect the presence of ctDNA in the circulation, but also to localize the tumor of origin in the correct organ system, ideally using the same blood sample. If not, this might lead to diagnostic delay as well as to unnecessary diagnostic procedures and biopsies, causing psychological distress and possibly also physical harm^[22]. In order to be able to identify the tumor of origin, MCED tests rely on additional biomarkers beyond ctDNA, such as proteins, or methylation profiles. A recent review of different tests gives rates of accurate tumor of origin identification that at 82%-93% are far from perfect^[9]. Moreover, this may differ per cancer type. In a study with the CancerSEEK test, predictions were significantly less accurate for lung and liver cancer compared to colorectum and ovary cancer^[23].

Moreover, MCED screening may lead to overdiagnosis. This would be the case if screening reveals that part of the cancers are “indolent” rather than a potentially lethal type. Overdiagnosis is problematic as it comes with unnecessary anxiety and may lead to iatrogenic harm due to unnecessary follow-up procedures. The reason why public health authorities in many countries have been reluctant to recommend prostate cancer screening is precisely that the often less aggressive nature of these cancers and the potential for harm make it questionable if such screening would on balance be beneficial. How the proposed MCED tests would relate to the concern about overdiagnosis is as yet unclear. It has been argued that precisely because more aggressive cancers shed more ctDNA into the circulation, findings (including for prostate cancer) will more likely be of a clinically relevant type^[9]. Even if that mitigates the concern, overdiagnosis may remain a drawback of MCED screening. Clearly, the rejoinder (in a report from the “Information Technology & Innovation Foundation”, a US science and technology policy think tank^[24]) that “the underdiagnosis of cancers” is a much greater problem than overdiagnosis is not a very helpful response to this concern.

Finally, MCED screening may find cancers for which there are as yet no effective treatment options, such as pancreatic cancer. Early detection of a disease that has no potential of changing the clinical outcome for the patient should be considered a problematic outcome of screening, as in such cases, there are no clear health benefits compensating for the inevitable harms of screening. Especially in a screening context, where tests are proposed on the initiative of health care professionals or screening authorities to people without a relevant complaint or indication, there is every reason to hold on to a more traditional understanding of “clinical utility” as “(referring) to the likelihood that the test will lead to an improved health outcome”^[25]. This is ignored in rejoinders saying that any health information “no matter how dire the diagnosis” is always useful, or that the patient is bound to find out anyway and that “it matters little whether that that knowledge is delivered earlier” as a consequence of MCED screening, or that the screening might contribute to developing those very therapies for future patients^[24].

DISCUSSION

As Putcha *et al.* have commented, “[c]ancer sounds like one disease, but is actually many: there are more than 100 different cancers, each with multiple subtypes reflecting different underlying molecular pathophysiologies”^[22]. This means that what is presented as “universal” cancer screening is not so much “screening for cancer, period”, but rather screening for a broad range of disorders with different clinical profiles using one simple test, based on the fact that all cancers happen to shed ctDNA in the circulation. Clearly, this renders the evaluation of MCED screening programs more complex than where concerning screening for one specific form of cancer, as it need not be the case that for each and every type of cancer that the test might detect, the benefits clearly outweigh any possible harms. While the overall benefit-to-harm ratio of MCED screening may well be positive (depending on further evidence from validation studies and pilots), some possible outcomes may represent a moral cost that one would rather avoid.

This is not to say that, in terms of debates about screening ethics, we are entering uncharted territory. Whereas the MCED approach is hailed as a “revolution” in cancer screening, the concept of multi-disorder screening as such is not entirely new, nor is the debate about its ethical implications. For instance, in newborn screening (NBS) for metabolic disorders, the use of tandem mass spectrometry (MS/MS) was introduced in the 1990s, enabling the simultaneous accurate and cost-effective measurement of large numbers of metabolites in dried blood spots on “heelprick” screening cards. As commented in a review, this “has deeply changed the older NBS approach of “one test for one disorder” to “one test for many disorders”, i.e., a multiplex test”^[26]. Current newborn screening programs worldwide use this technology, typically screening for several tens of inherited metabolic diseases^[27]. More recently, we have seen the development of broad scope genomic tests that have significantly changed the field of prenatal screening (based on cfDNA

in maternal blood)^[28]; they are piloted for preconception carrier screening programs^[29] and are expected to further impact the practice of NBS in the years to come^[30].

In each of these contexts, the perspective in the debate about the scope of screening has changed from: “what to test for?” to: “what conditions, if any, to actively exclude from the results of broad scope testing”^[16,31]? For instance, in newborn screening, many commentators would want to hold on to the idea that neonates should not be screened for diseases for which there is no meaningful treatment or prevention, or for diseases that would only manifest later in life and the course of which cannot be altered by preventative measures taken at a young age^[32,33]. This would require using “filters” so as to avoid any unsolicited findings coming available as a result of a comprehensive genetic screening test. However, others have questioned this approach, stressing, e.g., that parents have a right to health information about their children, or that families may benefit from information relevant for future reproductive choices, or also that finding diseases for which there are presently no therapeutic options, would still be useful as it would contribute to research that might lead to developing treatments, in the interest of future children with the same disorder, as well as of society at large^[34,35]. Similarly, while professional societies do not recommend analyzing cfDNA-findings from prenatal screening also for sex chromosome aneuploidies (SCA), pointing to low predictive value, generally mild phenotypes, and uncertain clinical utility^[36], commercial screening offers often do include testing for these abnormalities, with advocates reasoning that it is for the woman to decide if such findings would be relevant for reproductive decision-making. A recent Australian study reports on how this has led to a steep increase in prenatal diagnosis for SCA since the introduction of cfDNA-based prenatal screening^[37].

In our view, it is difficult not to recognize the undercurrent of technology-driven justifications for ever wider screening in these debates, with the fact that we *can* test for something being taken as a sufficient reason as to why we should^[38,39]. As the framing of “universal” cancer screening may well fit in with this “find everything we can” perspective, it is crucial to timely address the arguments relevant to what the scope of the envisaged screening should be. Referring to the above framework, we think the following three criteria from the Andermann list are especially important^[20].

Firstly, “[t]he objectives of screening should be defined at the outset”. What is it that we may want to achieve with MCED screening? Is it accurately detecting cancer signals in the circulation, is it enabling the diagnosis of early-stage cancers that are now often missed, or is it early diagnosis leading to meaningfully changing the clinical outcome in people with different forms of cancer? It seems quite clear that in the context of a public health service, only the latter answer will do. But then, some of the potential outcomes listed in the previous section (such as problems finding the tumor of origin, overdiagnosis, or finding cancer for which no meaningful treatment options are available) should be regarded as at odds with the screening objective and, in so far, problematic. It is important for the screening objective to be “defined at the outset”, in order to enable proper evaluation of ongoing screening programs.

Secondly, “[t]here should be (...) mechanisms to minimize potential risks of screening”. This is especially important in view of the fact that broad scope screening may inevitably yield outcomes at odds with the screening objective. One mechanism of minimizing any resulting harm for MCED screening participants might be the use of filters to avoid such “unsolicited findings”. This may take the form of using specific cut-offs in ctDNA-analysis to limit overdiagnosis, but perhaps also of using tumor of origin information with an eye to avoid finding forms of cancer without meaningful treatment options. The need for specific measures of this type would, of course, need justification in the light of the screening objective. Where such measures are not feasible, the potential for harmful “unsolicited findings” should be included in the weighing of the

overall benefit-to-harm ratio of the screening program. Where screening programs with a risk of such outcomes are offered, proper information and counseling are needed, also in order to protect participants from ending up in a trap that they would rather have avoided.

That brings us, thirdly, to the summarizing ethical requirement that “[t]he overall benefits of screening should outweigh the harm”. In the light of our above discussion (and apart from pandemic-type emergency situations), it would seem necessary to specify this as referring to the benefits and harms “for those participating in the screening”^[17]. This is not to say that screening might not also be beneficial for family members, future patients, or society at large. Indeed, most screening programs are meant to achieve health gains at a population level^[16]. However, it is to say that third party benefits should not be presented as outweighing the harms of specific forms of screening that would not clearly also benefit the participants themselves. This is especially relevant in view of the potentially high research value of the data generated through powerful new broad-scope screening technologies, including MCED^[24]. Without the specification of who is on balance to benefit, there is a risk in these developments of blurring the boundary between screening and research, turning screening participants into research subjects without proper safeguards^[31].

CONCLUSION

The prospect of ctDNA-based early detection of cancer may well change the field of cancer screening as we know it, with potentially large public health and individual benefits. While validation studies of MCED tests are only starting, it is important to pro-actively also consider the ethically relevant aspects of this development. In this connection, it is important to be aware that cancer is not just one disease and that not all findings will therefore have the same clinical utility. As forms of multi-disorder screening have been introduced in other contexts, the ethical analysis may connect to relevant earlier debates about how to deal with specific challenges of optimizing the benefit-to-risk ratio of such forms of screening. As we have argued, the traditional framework for responsible screening still seems to provide useful guidance in this respect.

DECLARATIONS

Authors' contributions

Drafted the paper and wrote the final version: Dondorp W

Commenting and contributing to the text: de Wert G

Availability of data and materials

Not applicable.

Financial support and sponsorship

Not applicable.

Conflicts of interest

Not applicable.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Conference Report

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Rigor and reproducibility: status and challenges for single vesicle analysis

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How to cite this article: Nolan JP, Chiu DT, Welsh JA. Rigor and reproducibility: status and challenges for single vesicle analysis. *Extracell Vesicles Circ Nucleic Acids* 2022;3:244-8. <https://dx.doi.org/10.20517/evcna.2022.28>

Received: 1 Jun 2022 **First Decision:** 11 Jul 2022 **Revised:** 28 Jul 2022 **Accepted:** 10 Aug 2022 **Published:** 23 Aug 2022

Academic Editors: Yoke Peng Loh, Michael W. Pfaffl **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

This report summarises the presentations and activities of the SELECTBIO Workshop on Rigor and Reproducibility in EV Research and Single EV Analysis held in San Diego, USA, in December 2021. The motivation for the session was the recognition that progress in the extracellular vesicle (EV) field is limited by the availability of rigorous and reproducible EV measurement tools. These tools are absolutely required for EVs to evolve from a research lab curiosity to something that will improve our ability to understand, diagnose, treat, and prevent disease. The program focused on guidelines for EV measurement and characterization as laid out in the recent MISEV2018 and MIFlowCyt-EV publications, their implementation in routine practice, and their continued evolution as new EV measurement technologies are introduced. The conclusion of the workshop was that more effort focused on pre-analytical issues and benchmarking of isolation methods is needed to strengthen collaborations and advance more effective biomarkers.

Keywords: Extracellular vesicle, exosomes, flow cytometry, calibration, standardization, reproducibility



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The recent SELECTBIO Conference in San Diego (December 12-14, 2021) included a Workshop on Rigor and Reproducibility in EV Research and Single EV Analysis as part of the Extracellular Vesicles: Technologies and Investigations track. The motivation for the session was the recognition that progress in the extracellular vesicle (EV) field is limited by the availability of rigorous and reproducible EV measurement tools. These tools are absolutely required for EVs to evolve from a research lab curiosity to something that will improve our ability to understand, diagnose, treat, and prevent disease. The program focused on guidelines for EV measurement and characterization as laid out in the recent MISEV2018^[1] and MIFlowCyt-EV^[2] publications, their implementation in routine practice, and their continued evolution as new EV measurement technologies are introduced.

The starting point for discussion was a review of the MISEV2018 reporting guidelines and the MIFlowCyt-EV reporting framework for single EV flow cytometry. Both of these reporting guidelines are intended as recommendations to increase rigor and reproducibility in the EV field, and also act as a tool for editors and reviewers to assess the strengths and weaknesses of manuscripts, proposals, funding applications, and conference presentations. The MISEV guidelines cover, at a high level, all aspects of EV research, from biofluid collection through EV and EV cargo characterization to EV functional analysis^[1]. MIFlowCyt-EV addresses the reporting of essential details of single-EV flow cytometry analysis required for data interpretation and reproducibility, and is an extension of the general MIFlowCyt reporting framework^[3,4]. Key elements of these reporting guidelines include: (1) the use of a calibrated instrument so that measurements can be reported in standardized and biologically meaningful rather than arbitrary units; (2) inclusions of several essential controls to demonstrate the specificity of EV detection and cargo measurement; and (3) comprehensive reporting of methods and results, including data sharing, to enable others to reproduce and extend the work. Workshop attendees were introduced to the significant amount of educational material on these topics available on the website of the EV Flow Cytometry Working Group (<https://www.evflowcytometry.org/>), a group of EV researchers affiliated with ISEV, ISAC, and ISTH that developed and promoted these guidelines^[5,6].

The discussion also covered challenges and opportunities for future advances in single-EV analysis. Rigorous and reproducible measurements of EV number, size and cargo by flow cytometry are possible^[2,7-9] and routine with commercial assay kits^[10-14], but instrument performance generally limits assay sensitivity. Most commercial flow cytometers have been designed to measure lymphocytes, but lab-built flow cytometers with single-molecule sensitivity have been possible for decades^[15-17], and may soon be commercially available. The general lessons for assay development and validation using these single-molecule sensitive instruments still apply, including the need to fully report methodological details, demonstrate the specificity of EV and cargo detection through the appropriate controls, and report data in the appropriate calibrated units. This last point generated additional discussion centered around how to reconcile “top-down” calibration approaches developed for the analysis of cells with the “bottom-up” approach enabled by single-molecule detection.

Flow cytometer fluorescence calibration was initially developed for cell analysis several decades ago, with standards, protocols and software to allow users to readily report particle brightness in standard units (MESF, mean equivalent soluble fluorochromes; ERF, equivalent reference fluorochromes; or ABC, antibodies bound per cell)^[18-21]. Approaches for fluorescence calibration are robust for the measurement of bright particles such as cells, and have been shown to have utility in standardization when extrapolated to the measurement of dim particles such as EVs on suitably sensitive instruments. Along with fluorescence calibration, the development of light scatter calibration methods has shown utility for the standardization

and characterization of small particles^[7,8,17,22,23]. When detecting light from very small numbers of photons (e.g., ~100 fluorochrome molecules), additional factors arise and can affect the uncertainty of these calibrations^[7]. One of these is the natural effect of counting small numbers of anything (including photons, molecules, or particles), where Poisson-type counting statistics introduce a known but stochastic uncertainty into the results. Another has to do with the ways different instruments handle background. Sensitive detection of any analyte is most often limited by background, and definitions of the lower limits of detection (LoD) often reference the variation in the background signal (e.g., LOD is the mean of the background +3 standard deviations), whether molecules or particles are being detected. Most commercial instruments “subtract” the constant background measured by the instrument, but they often perform this with different methods, and cannot remove the variation (or “noise”) in the background, which generally remains. Moreover, beads used for calibration often have their own intrinsic background autofluorescence, the measurement of which will depend on the sensitivity of the instrument. Thus, while the “top-down” calibration approaches developed for cell analysis can be extended into the realm of very dim EVs and the instrument limits of detection, care is required to understand the uncertainties and avoid over-interpretation.

By contrast, single-molecule detection starts with the detection of a single molecule, and a particle that is brighter than a single molecule should be able to be reported as having some number of single molecules (the “bottom-up” approach)^[24]. In practice, for single molecule-based detection approaches, issues of Poisson counting statistics and definition of background also dominate data interpretation, and pose challenges to report values calibrated in biologically meaningful units (e.g., molecules, antibodies, size). First, an inspection of the fluorescence intensity distribution of many detected single molecules generally shows a Log-normal distribution^[25], which demonstrates that the photons detected to form that distribution are produced and detected stochastically, and that there are additional variabilities imposed by the measurement process. This means that intensity distributions of particles bearing one, two, three, or more fluorescent molecules can have significant overlap. Second, often the very sensitive detectors used for single-molecule detection have a limited dynamic range (the difference between the upper and lower LoD), and efforts to measure real biological samples, in which an individual EV may bear from zero to several hundred antibody molecules, can confront a limitation to measure brighter particles. Finally, while mathematical models can deconvolve such distributions post hoc to estimate the numbers of particles bearing zero, one, and two molecules (for example) or account for the effect of detector saturation for brighter particles, the ability to measure, rather than just detect, the estimated number of single molecules on a particle is subject to a number of assumptions and uncertainties.

From this perspective, the convergence of conventional flow cytometry technologies and the “top-down” measurement approach with high sensitivity, single molecule-based detection and the “bottom-up” approaches to measuring the low numbers of cargo molecules on many individual EVs is an exciting challenge in bioanalytical chemistry. As instrument sensitivities improve to strive for single molecule sensitivity, new challenges in high-throughput purification methods may need to be considered in order to remove all unbound label. With existing technologies, titration and dilution are usually sufficient to achieve this due to insensitivity to single molecules. Moreover, as other technologies are applied to measure single EVs, they will be challenged by many of these same issues, including (1) the development of appropriate concentration, size, and molecular cargo standards for single EV measurements; (2) the use of such standards to calibrate instrument response in absolute, rather than relative units, (3) the development of assays that report EV number, size, and molecular cargo in absolute units that can be compared across instruments and between labs; and (4) the sharing of instrument and assay methods and data in a manner that supports replication. These challenges must be solved to develop methods for EV measurement with

the analytical rigor needed to understand their roles in health and disease, and to translate that understanding into useful new clinical diagnostics and therapeutic approaches.

DECLARATIONS

Author's contributions

Conceptualization, writing: Nolan JP, Chiu DT, Welsh JA

Read and approved the manuscript: Nolan JP, Chiu DT, Welsh JA

Availability of data and materials

Not applicable.

Financial support and sponsorship

Work in the authors' labs is funded by NIH 4UH3 CA241687, R01 GM140137, R44 DA044616, R44 GM136165, 4UH3 TR002874. JAW was supported by Intramural Research Program of the National Institutes of Health (NIH), National Cancer Institute, and Center for Cancer Research, NIH ZIA BC011502, NIH ZIA BC011503, and NIH 4UH3TR002881. JAW is an International Society for Advancement of Cytometry (ISAC) Marylou Ingram Scholar 2019-2023.

Conflict of interests

JPN holds equity in Cellarcus Biosciences Inc. DTC has financial interest and is a scientific founder and/or board member of the following companies and their respective affiliates: Micareo, Inc, Lamprogen, Inc, Cellectricon AB, and Fluicell AB.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Conference Report

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A report on ASIC2021: a conference on extracellular vesicle communication mechanisms

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How to cite this article: Russell AE, Sil S, Buch S, Graner MW. A report on ASIC2021: a conference on extracellular vesicle communication mechanisms. *Extracell Vesicles Circ Nucleic Acids* 2022;3:249-63. <https://dx.doi.org/10.20517/evcna.2022.31>

Received: 9 Jun 2022 **Accepted:** 4 Aug 2022 **Published:** 29 Aug 2022

Academic Editor: Yoke Peng Loh **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

The American Society for Intercellular Communication (ASIC) held its first annual meeting, ASIC 2021, on October 21-23, 2021. This meeting brought together researchers from various disciplines and backgrounds to exchange ideas on various means of extracellular communication mechanisms, including tunneling nanotubes, extracellular vesicles (e.g., exosomes, microvesicles, midbody remnants, large vesicles/large oncosomes) and other circulating particles. Due to the ongoing COVID-19 pandemic, the meeting was held in a hybrid format. For those who were able, this afforded them the opportunity to attend in person, while others unable to travel were still able to participate in the meeting remotely via Zoom. Virtual participants were able to ask questions to presenters either verbally or via chat, and those selected for talks could pre-record their presentations and participate in a live Q&A session afterwards.

Although ASIC is a new society and the first meeting was held during the COVID-19 pandemic, the meeting still attracted more than 130 participants and was supported by seven sponsors, including Izon, Particle Metrix, Nanotech, SBI System Biosciences, Nanoview, Purigen Biosystems, Ceres Nano, and *Extracellular Vesicles and Circulating Nucleic Acids* journal. The meeting spanned three days, had 58 talks (38 of which were given in-person), and had one poster session on the 2nd day of the conference.



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Day 1 of meeting: The meeting began on October 21st with opening remarks from Drs. Julie Saugstad and Ursula Sandau.

The first session (Pre-program) was an NIH workshop chaired by Julie Saugstad and Ursula Sandau and focused on funding mechanisms that may be of interest to the meeting attendees. John Satterlee (National Institute on Drug Abuse, National Institutes of Health) began the session by describing various current funding opportunities available through NIDA for all levels (undergraduate through experienced principal investigators), as well as more specific, EV-related R01 and R21 opportunities (e.g., PAR-20-147/PAR-20-148) focusing on extracellular RNA (exRNA) relevant to substance use disorders or HIV. Additionally, Satterlee imparted important advice to the audience about grant writing, including the “10 Commandments for R01 Applications” and encouragement to subscribe to the NIH’s Weekly Funding Opportunities and Notices email.

Next, Jill Morris (National Institute of Neurological Disorders and Stroke, National Institutes of Health) presented numerous funding opportunities in translational research, focusing on the need to bridge the gap between basic and clinical research. For example, the Innovation Grants to Nurture Initial Translational Efforts (IGNITE Program) currently has three opportunities available for translational projects in their early phases (PAR-21-122; PAR-21-123; and PAR-21-124). Through the NIH Blueprint, which provides information about neuroscience research and training opportunities, there are several funding mechanisms to allow for later-stage translational collaborative research projects between academia and industry (PAR-21-163 and PAR-21-233). Additionally, the National Institute of Neurological Disorders and Stroke has a biomarker program with several opportunities for funding at various stages of biomarker discovery including identification and analytical and clinical validation (PAR-19-315; PAR-21-056; PAR-21-057; PAR-21-058; PAR-21-059).

Christine Happel (National Center for Advancing Translational Science, National Institutes of Health) spoke next, providing an overview of the progress the NIH Common Fund Extracellular RNA (exRNA) Communication Program has made since its inception. The main goal of this program is to accelerate research in the field of exRNA biology, and a substantial amount of progress has been made in this area, with numerous landmark publications in *Cell* stemming from these efforts. Stage 1 of this program concluded in 2018 and focused primarily on understanding exRNA biogenesis and function, and clinical uses of exRNAs. Stage 2 aims to understand exRNA carriers and technologies to isolate and characterize single EVs. Data coordination and analysis are at the forefront of both stages and have allowed for the creation of important resources like the exRNA Atlas, which can be found at <https://exRNA.org>. Happel also touched on the similarities between EVs and SARS-CoV-2 and the redeployment of funding opportunities for adapting single-EV isolation technologies for the isolation of SARS-CoV-2 particles through the Rapid Acceleration of Diagnostics (RADx) program.

The final presenter for this session, Elzafir Elsheikh (National Institute of Standards and Technology, National Institutes of Health), discussed the need for EV reference materials and the importance of acknowledging how different isolation and characterization methods yield different results. Each method used to study EVs has its own advantages and limitations and provides its unique data profile of EV sizing and quantification profiles. The utilization of standardized reference materials better allows for comparison across different separation and characterization techniques, the most common of which are dynamic light scattering, nanoparticle tracking analysis, fractionation and light scattering, resistive pulse sensing, and electron microscopy. From several different ATCC cancer cell lines, EVs were isolated and characterized via tangential flow filtration and revealed heterogeneity across the cell lines in terms of EV quantity, size

distributions, proteomic profiling, and vesicle morphology, especially with vesicles visualized via cryoEM. Also noted were the detrimental effects of freeze-thaw cycles on EVs and the need for a large collaborative study to assess reference materials across laboratories, as developing reference materials for EVs is quite a challenge.

Next, Gagan Deep (Wake Forest University) kicked off a pre-program NIH workshop session also chaired by Julie Saugstad and Ursula Sandau, where he discussed recent work examining the effects of a ketogenic diet on mild cognitive impairment. In this work, L1CAM was utilized to pulldown neuron-derived EVs from blood plasma from eleven cognitively normal control patients and nine diagnosed with mild cognitive impairment before and after the implementation of a modified Mediterranean ketogenic diet (MMKD). Post-MMKD, MCI patients had notably lower expression of neuroinflammatory markers associated with neuron-derived EVs (NDE), including amyloid A β 1-42, phospho-Tau, neurofilament light chain, NF- κ B, oxidized proteins, and other neuroinflammatory markers. Further, this work identified that monocarboxylate transporter 2 (MCT2) associated with NDEs may predict responsiveness to MMKD intervention.

Michael Bukrinsky (George Washington University) was the next speaker in this session and discussed recent work from his laboratory focusing on Nef-carrying EVs, which can promote the formation of inflammatory monocytes and macrophages. Many people living with HIV develop other diseases such as HIV-associated neurocognitive disorder (HAND), cardiovascular disease, and other metabolic diseases; chronic inflammation may alter cholesterol synthesis and mediate the development of these disorders in people living with HIV. Despite antiretroviral therapy (ART), Nef is still present on the surface of EVs and has been shown to stimulate cholesterol biosynthesis and increase the abundance of lipid raft-associated TLR4 and TREM1 in macrophages, similar to what is observed when macrophages are exposed to other inflammatory stimuli. These Nef-EVs also drive TNF- α and IGR1R signaling and result in large responses to LPS exposures. Together these effects may result in persistent inflammation even after the use of ART and result in HIV-associated co-morbidities.

The next talk from Anil Prasad (Harvard University) also focused on neuroinflammatory EVs within the context of HIV. As substance use disorders (SUD) and HIV infection appear to have interactive, additive, and synergistic effects on the immune system and neuropathogenesis, this work examined how cocaine use intersects with HAND. The use of cocaine has been shown to exacerbate HAND by inducing inflammation in the central nervous system (CNS), modulating the immune responses, enhancing HIV replication, and altering intracellular trafficking of HIV-containing EVs, preventing lysosomal degradation. Further, cocaine enhances the release of EVs from immune cells infected with HIV, potentially through the downregulation of BST-2 (tetherin). Additionally, these EVs have increased the expression of viral genes, increased infectivity of T-cells, and promoted the release of inflammatory cytokines from human brain microvascular endothelial cells.

Xiaoli Yu (University of Colorado, Anschutz) presented an interesting talk focusing on glioblastoma (GBM) and meningioma (MMA)-derived EVs from blood plasma. These EVs contain high levels of IgG and may mediate complement activation and cascade; in GBM patients, IgG levels are often elevated in plasma and have a unique proteomic profile compared to healthy controls. It was demonstrated that multiple immunoassays could characterize IgG antibodies in plasma and plasma EVs from patients with GBM, MMA, and controls. Higher levels of Fc heavy chain IgG antibodies and IgG1 subclass were shown to be present in GBM plasma and EVs compared to MMA. The IgG antibodies in both GBM plasma and EVs produced complement-dependent cytotoxicity in the neuroblastoma cell line SH-SY5Y used as a neuronal

surrogate. The higher IgG levels in the plasma and EVs in GBM patients and the high capacity of cell killing suggested that GBM IgG antibodies could play an important role in tumor pathogenesis.

Elena Batrakova (University of North Carolina) next presented work focusing on different techniques for loading EVs with drugs, including incubation at room temperature with or without saponin, altering the pH, electroporation, freeze-thaw, sonication, and extrusion. Macrophage-derived EVs were loaded with various cargo which was protected and highly stable, and these vesicles were delivered to the brain in an *in vitro* model. It is thought that the adhesion molecules on macrophage EVs were enabling this. In other murine models, the route of administration is an important consideration when planning drug delivery experiments; in this work, IV, intrathecal, and intranasal administration of EVs all resulted in delivery to the brain. The parental cells from which EVs are derived also influence EV loading and delivery and must also be considered.

The next presenter was Norman Haughey (Johns Hopkins University), whose lab has recently shown that brain-derived EVs communicate inflammatory damage in the periphery. Alzheimer's disease (AD) is thought to induce chronic inflammation of both the central and peripheral immune system, and EVs may participate in the deposition or clearance of A β . In a mouse model of AD, A β was not found to be abundant in plasma or neuronal-derived EVs; however, adoptive transfer of plasma EVs from AD mice into wild-type mice resulted in an acute cytokine response, and these EVs were present in multiple tissues, including the brain. Interestingly, these EVs were enriched with ATPase ATP1A1 and modulated reactive oxygen species levels.

Nicole Noren Hooten (National Institutes of Health/National Institute of Aging) gave the next talk focusing on the HANDLs study which examined differences in life expectancy across various neighborhoods in Baltimore, MD and the use of EVs as potential clinical markers of mortality.

David Greening (Baker Heart and Diabetes Institute, Australia) presented next on a new class of EVs released from colon cancer cells; midbody remnants. These vesicles carry mutated KRAS G12V, activate MAPK signaling, and can be immunoaffinity-purified from plasma. Proteomic profiling also revealed that EVs may be cell signaling regulators. Additionally, the proteomic profile of heart-derived EVs may reflect changes associated with adipose phenotype, and engineered EVs may be useful for delivering cargo specifically to the heart to aid in cardiac repair.

Next, Gurudutt Pendyala (University of Nebraska Medical Center) presented work on sex differences in nicotine addiction. With tobacco use, the primary addictive compound is nicotine and female rats have higher nicotine characteristics and self-administer nicotine more frequently than males. When nicotine enters the brain, it induces dopamine release, altering synaptic activity and brain-derived EVs (bdEVs), as bdEVs from nicotine-addicted females had different proteomic profiles than saline-exposed females. Interestingly, sex-specific bdEV profiles were also observed, which may lend insight into the varying molecular basis of addiction observed between males and females. Using novel technology, Single EV Analyses with Multiplexing (SEAM), his group also validated the identified sex-specific bdEV markers.

Switching gears, Hameeda Sultana (University of Tennessee Knoxville) presented work focusing on arthropod EVs, flaviviruses, and their cargo. Flaviviruses cause diseases like West Nile and Lyme, and are typically transmitted from the bite of an infected insect, like a tick or mosquito. Data presented demonstrated that EVs isolated from tick saliva modulate wound healing and repair by altering the expression of CXCL12 and IL-8 in human skin cells. These EVs alter the immune response in skin cells as

well.

The last research presentation of this session was given by Sowmya Yelamanchili (University of Nebraska Medical Center), who shared recent work investigating the role of EVs in methamphetamine (meth) use disorder. Meth is a highly addictive drug, and relapse is a major problem in recovering addicts; as such, there is a need for therapeutic interventions to prevent relapse. In the brain, meth induces chronic inflammation via microglial activation and causes blebbing from neurons and microglia, which may impact EV biogenesis. To study this, both a Rhesus macaque and a rat self-administration animal model were used. In the frontal cortices of macaques exposed to meth, there was an increase in genes associated with EV biogenesis pathways, specifically endosomal sorting complexes required for transport (ESCRT). Protein expression in these brains suggests there may be an enhanced release of exomeres in meth-exposed brains. EV-associated miR-29a was increased in methamphetamine abuse and may be involved in both drug-seeking behaviors and relapse. Artificial “EV-miR-29a” exposure induced proinflammatory cytokine release from microglia, specifically TNF- α , IL6, and IL β , and resulted in synaptodendritic injury in neurons. Therapeutic interventions, including the administration of anti-inflammatory drugs like Ibuprofen or AV411 in a rat model, resulted in a reduction in inflammation and EV secretion. Additionally, miR-29a was upregulated in the plasma of both macaques and humans addicted to meth, so it may serve as a promising biomarker and warrants further investigation.

The last speaker in the pre-program NIH workshop session was Roger Alexander (Extracellular RNA Communication Consortium; ERCC), who spoke about an online extracellular RNA (exRNA) course from exrna.org.

The official opening of the conference began with the ASIC Meeting Session 1, chaired by Fatah Kashanchi and Meta Kuehn. The first presenter of this session was Nobel Laureate Randy Schekman (University of California, Berkeley), whose presentation focused on selective protein sorting into exosomes with a role in cell differentiation and as a possible tool in genome editing. This study showed the means to deliver Cas9 and a guide RNA (gRNA) enclosed within exosomes, a subclass of small EVs, as a vehicle for efficient and targeted gene editing. Biotinylated Cas9 was expressed in donor HEK cells bound noncovalently to an integral multivesicular body (MVB) membrane protein, CD63-streptavidin. Exosomes formed in the MVBs were thus enriched in Cas9 and a gRNA. These were isolated by buoyant density sedimentation, followed by incubation with reporter cells containing an integrated copy of N-luciferase; exosome delivery of Cas9 and the guide RNA should allow the expression of luciferase. However, exosomes containing a similar level of Cas9 elicited no more than a 50% increase above the basal luciferase expression. The same was true of the conditioned medium containing Cas9-exosomes and even of donor and acceptor cells incubated together, which was separated by a vesicle-permeable membrane in a transwell chamber. Thus, for these EVs, the functional uptake to promote gene expression was not observed as found for those isolated from differentiating neurons. In contrast, donor and acceptor cells cocultured to near confluence showed a 60-fold increase in luciferase expression. Transfer of Cas9 appears to be mediated by open-end membrane nanotubular connections, which is likely dependent on membrane fusion at the point of junction from the donor to the recipient cell. A molecular investigation of the requirements for this transfer may permit the development of an efficient means for targeted delivery of Cas9/gRNA. It also suggests that donor-recipient cell combinations may not be wholly generalized.

The next presentation was from Lance Liotta (George Mason University), who shared work on tumor-derived EVs. The data presented indicated that different EV fractions expressed different proteins; for example, PDL1 was only observed in the 100k \times g pellets. Further, there are different proteins expressed

between *ex vivo* and cell line-derived EVs. Breast cancer-derived lymphatic EVs had very high expression of autophagic markers, specifically mitophagy proteins (e.g., PINK1) and may be transferring these mitophagic proteins to other cells.

Dolores Di Vizio (Cedars-Sinai Medical Center) gave the next presentation focusing on the role of large oncosomes (LOs) in the metastatic spread of cancer to bone marrow. Large oncosomes are a type of EV derived from amoeboid cancer cells ranging from 1-10 μm in diameter. These were separated from highly metastatic amoeboid prostate cancer and breast cancer cells, and were found to induce tumor progression via bone marrow mesenchymal stem cells (BM-MSCs). At the gene level, LOs induced expression of the interferon-gamma and alpha pathways in BM-MSCs. This LO-driven anti-viral response also induced neutrophil recruitment towards BM-MSCs; these neutrophils displayed an immunosuppressive phenotype that could presumably benefit the tumor.

Ryan Flynn (Harvard University) next presented an interesting talk on glycoRNAs, which may bridge the gap between glycobiology and RNA biology. Recent evidence shows that small RNAs can be modified with N-glycans and displayed on the surfaces of cells. Metabolic labeling with azido sugars can be used for detecting biotinylated glycan RNA reporters, and streptavidin can be used to capture these biotinylated glycoRNAs. Many of these are noncoding RNAs, and different RNAs are associated with varying glycan structures.

Yoel Sadovsky (University of Pittsburgh, Magee Women's Research Institute) gave the next presentation on the role of EVs in fetal-maternal communication. Placental-derived EVs from the trophoblast cell layer mediate virus resistance via the C19MC miRNA cluster as they protect against viral replication. The biophysical properties of trophoblast-derived EVs were also assessed and found that they have higher levels of phosphatidylcholines than other types of EVs, and may affect membrane rigidity.

Next, Kevin Morris (Griffith University, Australia) presented work on EV-mediated epigenetic repression of HIV in the context of DNA methylation of the LTR-retrotransposons, and HIV-targeted therapies via zinc finger proteins. An HIV promoter-targeting Zinc Finger protein (ZFP-362) fused to active domains of DNA methyltransferase 3A was developed. Engineered EVs were loaded with RNAs encoding this protein and were found to specifically repress HIV replication in cells while keeping T-cells alive. In NSG mice, these EVs reduced HIV in both the brain and bone marrow.

Meta Kuehn (Duke University) gave the last presentation of the evening, sharing recent insights into the mechanism and functionality of bacterial EVs. Bacteria are ubiquitous, and their EVs may be vehicles for the export of complex products. They can be isolated with similar techniques used to isolate eukaryotic EVs; however, there are many additional variables at play; for example, are the vesicles derived from gram-positive or gram-negative bacteria; are the cargo enveloped or not; are the proteins expressed on the inner and outer membranes of these EVs? All these variables can make for different vesicles. The group has shown that bacterial EVs can interact with plant pathogens and serve a protective function.

Day 2 of meeting: The following morning, the meeting began with a session on EVs and Cancer, and EVs in the CNS, and was chaired by Michael Graner and Ashley Russell.

The first talk of this session was delivered by Phil Stahl (Washington University). This talk focused on the physiological roles of EVs in a microenvironment, such as signaling, seeding, decoys, or exchange, and also recapped his lab's 1983 seminal paper on the loss of transferrin receptors from reticulocytes as they mature

into red blood cells. The EV disposal hypothesis was also presented, which outlines that EVs are “garbage bags” releasing unwanted cellular debris, like protein aggregates, into the extracellular space; however, it is not known whether this is by default or by design in that these vesicles are meant to be discharged, not necessarily as “garbage”. This segued into the signaling hypothesis that EVs mediate intracellular communication, proposed by Raposo in 1996; this sparked a change in mindset regarding the function and purpose of EVs. Some proteins are present in EVs because they participate in the vesicle formation process, whereas others are present because they have other roles, such as cell signaling. Further, Lotvall and Ratajczak showed that EVs can transfer miRNAs and mRNAs, which further supported the hypothesis that EVs participate in cellular communication.

Katia Mallouf (Harvard) presented the next talk, which focused on the use of EVs as tools to monitor neurologic diseases. There is a need for techniques to trace brain cell behavior *in vivo* and monitor the status of cells in the living brain. E-NOMI is a tool designed to trace and pulldown EVs derived from brain cells *in vivo* and *in vitro*, as they can be pulled down with beads due to the FLAG-TAG expressed on them. In this work, engineered human neural progenitor cells were implanted in mice and E-NOMI EVs were pulled out of the mouse blood plasma with anti-FLAG-TAG magnetic beads.

The next presentation was given by Lucia Languino (Thomas Jefferson University) and discussed the use of EVs for cancer therapy as drug delivery molecules containing a specific target receptor, peptide, or drug. For example, integrin $\alpha v \beta 6$ was able to target prostate cancer cells with relative specificity. To make these EVs, cells can be transfected or electroporated with specific siRNAs, as this is much more efficient than electroporating the EVs themselves with siRNAs.

Jeff Franklin (Vanderbilt University) delivered a talk focusing on purification strategies to optimize vesicle yield and parse the heterogeneity. Methodologies discussed were differential centrifugation, Optiprep gradient and direct antibody capture, fluorescent activated vesicle sorting, and size exclusion chromatography (SEC)/FPLC, as well as the use of hollow fiber bioreactors to produce high concentrations of EVs and nanoparticles. Different EV subpopulations from cetuximab-resistant cells were able to promote cetuximab resistance in previously sensitive lines.

Antonio Chiocca (Brigham and Women’s Hospital) continued the discussion on heterogeneity. One of the major reasons cancer therapies fail is the heterogeneity within tumors, as different cells within a single tumor can contain varying mutations. Cancer EVs, and their inherent heterogeneity, further extend this concept with effects on cells of the tumor microenvironment. Within the tumor microenvironment, there are “enablers” that help cancer proliferate; EVs can also be enablers and mediate changes in recipient cells and contribute to tumor immune suppression. This was detailed in the reduced T cell activation when the immune cells were exposed to glioblastoma EVs possessing the PDL1 portion of the PD1/PDL1 immune checkpoint axis.

Bojan Losic (Ichan School of Medicine at Mount Sinai) gave a talk focused on unannotated small RNA clusters associated with circulating EVs in the detection of early-stage liver cancer. Data generated from three independent extracellular RNA (exRNA) cancer datasets from 375 patients, including longitudinal samples, were used for this study. Results showed that exRNA, small RNA clusters (smRCs) were dominated by uncharacterized, unannotated small RNA with a consensus sequence of 20 bp. An unannotated 3-smRC signature was significantly overexpressed in plasma exRNA of patients with hepatocellular carcinoma (HCC). An independent validation in a phase 2 biomarker case-control study revealed 86% sensitivity and 91% specificity for the detection of early HCC from controls at risk. The 3-

smRC signature was independent of alpha-fetoprotein and a composite model yielded an increased AUC of 0.93. These findings lead to the prospect of a minimally invasive, blood-only, operator-independent clinical tool for HCC surveillance, thus highlighting the potential of unannotated smRCs for biomarker research in cancer.

Dilorom Sass (National Cancer Institute) shared work examining EV-associated cytokines in breast cancer, aging, and inflammation. An in-house Luminex assay capable of detecting 35 cytokines probed the surface of EVs from samples of patients with breast cancer. They found intriguing results indicating that EV-IL2 and EV-GM-CSF expression was different between low pain vs. high fatigue in older breast cancer patients, but this trend was not observed in younger patients. These data suggest there may be age-associated differences in cancer-related EV profiles.

The next session on EVs and the CNS began with Aleksander Milosavljevic (Baylor College of Medicine), who presented work focusing on the deconvolution of the exRNA Atlas, which revealed six exRNA cargo types. RNA-binding proteins (RBPs) have over one million distinct exRNA binding sites, and many RBPs appear to carry exRNA in both cell culture supernatants and blood plasma. Interestingly, RBPs may show a preference for specific clusters of RNA, which warrants further investigation.

Julie Saugstad (Oregon Health and Science University) shared recent data outlining the use of miRNAs as biomarkers for AD. This was the first study to obtain CSF from living donors and assess miRNA expression profiles that may be relevant in full-blown AD, and determine if they are also altered during mild cognitive impairment (MCI) in conjunction with APOE4. This work identified five CSF miRNAs that had a downward trend over time for those with MCI, and several miRNAs were also identified as potential candidates from patient plasma. Interestingly, however, these miRNAs were not identified in brain-derived EVs isolated from blood plasma using L1CAM. The miRNAs in CSF EVs were able to distinguish AD from Parkinson's Disease, and pathway analysis showed convincing convergence with neurodegenerative disorders.

Navneet Dogra (Ichan School of Medicine at Mount Sinai) next presented work examining the neuro-secretome. For these experiments, EVs were isolated from brain tissue following mild digestion and fractionation with SEC; the protein and nucleic acid of each fraction were assessed. The first six fractions were found not to contain proteins or nucleic acids, but fractions containing EVs - identified initially by markers ALIX and FLOT1 - have a high abundance of DNA and RNA. In later fractions, enrichment of proteins and nucleic acids is observed once again and is likely non-vesicular. The reproducibility of over 15 brain samples was quite high.

Ursula Sandau's (Oregon Health and Science University) presentation focused on the use of CSF EVs as biomarkers for brain diseases. Proximity of the biofluid to the brain/CNS makes it an attractive biomarker source. However, it is difficult to obtain and has far fewer circulating materials than plasma. Thus, it requires special attention to handling and EV isolation. EVs were isolated from CSF by SEC, using resins of different pore sizes (35 nm and 70 nm). Using various analytical methods, the two different SEC columns differentially separated smaller vs. larger vesicles. Curiously, NCAM1 was not detected in CSF-EVs. RNA profiling showed that some miRNAs reside exclusively in EV or protein fractions, while others can be found in both fraction types.

The last speaker of this session was Tsuneya Ikezu (Mayo Clinic Florida), who presented work focusing on how cell type-specific EVs define disease-related protein networks associated with astrocyte activation in

AD. His group generated human induced pluripotent stem cells (hiPSCs) and differentiated them into neuronal, astrocytic, oligodendrocytic, and microglial cell types. Proteomic profiles of EVs from these differentiated iPSC cells contained cell-type specific markers: excitatory neurons (ATP1A3, NCAM1); astrocytes (LRP1, ITGA6); microglia-like cells (ITGAM, CD300A); and oligodendrocyte-like cells (LAMP2, FTH1). There were also 16 pan-EV marker candidates, including integrins and annexins. Cell type-specific EV proteins could also be found when comparing their data to CSF EV proteomic datasets, which also held true for brain-derived EVs. Correlation networks and pathway analyses identified proteins in each cell subset EVs with co-expression in AD. It was shown that astrocyte-specific EV (ADEV) markers were most significantly associated with AD pathology and cognitive impairment, thereby underscoring the role of ADEVs in AD progression. The hub protein from this module, integrin- β 1 (ITGB1), was elevated in ADEVs purified from total brain-derived EVs and associated with brain A β 42 and tau load in independent cohorts. From this, it was found that astrocytes are likely in an activated state due to IL1 β , and astrocytic AD EVs are enriched in ITGB1. This correlated with A β 42 and phosphoTau, and these EVs enhance neuronal uptake via integrin signaling. Thus, this study provides a featured framework and rich resource for analyses of EV functions in neurodegenerative diseases in a cell type-specific manner.

The next session of the day was EVs, CNS, and Viral Infections, chaired by Ramin Hakami and Leonid Margolis. The first presenter was Jay Debnath (University of California, San Francisco), who presented work on the intersection of secretory autophagy and EVs. Using genetic and molecular tools, the group assessed the proteomic profile of conditioned cell culture media, which revealed numerous proteins, many of which were ribosomal binding proteins (RBPs). Mass spectrometry-based quantitative proteomics found > 200 new putative targets of autophagy-dependent secretion, with co-fractionation of LC3B interactors and EV markers. Interestingly, LC3 processing and lipidation are required for RNA-binding protein secretion. Entire autophagosomes are not being secreted from cells, but various components are. For example, if the endolysosomal pathway is blocked, autophagy cargo receptors are secreted in EVs. If both the lysosomal and Rab27a (EV) pathways are blocked, cargo receptors remain inside the cell, but are not degraded, indicating important roles for both pathways in autophagy and EV release. As lysosomal pathways are often impaired in viral infections and CNS diseases, these results may have important clinical implications.

Next, Shilpa Buch (University of Nebraska Medical Center) presented work focusing on how the HIV-1 protein Tat primes and activates microglial NLRP3 inflammasome leading to synapto-dendritic injury in neurons via exosomes. Even after anti-viral treatment, HIV-1 Tat is still present. Findings from her group showed that HIV-1 Tat can cause the activation of microglial NLRP3 inflammasome, and induce the release of IL1 β , further exacerbating the inflammatory response. These are important contributors to neuroinflammation in HIV-associated neurological disorders (HAND). Microglia-derived EVs (MEVs) were found to carry NLRP3 and IL1 β cargoes, which upon being taken up by neurons resulted in synapto-dendritic injury and increased excitatory currents. Intriguingly, silencing of microglial NLRP3 inhibited the MEV-mediated neuronal damage. The role of NLRP3 in inflammation is well known; however, the role of the same NLRP3 in neuronal damage is an interesting finding and will add to the multifaceted therapeutic potential of NLRP3 blockers in HAND.

The third speaker of this session was Michal Toborek (University of Miami Health System). It is known that aging individuals living with HIV have an accumulation of amyloid in their brains compared to healthy controls. Data from this presentation indicated that endothelium-derived EVs carry A β , and impact the differentiation of neural progenitor cells. HIV infection enhances transendothelial transfer of A across the blood-brain barrier. Endothelial cell-derived EVs can deliver A to neural progenitor cells, induce inflammation, and block their differentiation. Thus, the intersection of aging and EV-driven

neuroinflammation in HIV patients can be viewed as another risk factor for developing HAND.

The next presentation was very topical, focusing on COVID-19 EVs. Navneet Dhillon (University of Kansas Medical Center) presented work focusing on the role of EVs in COVID-19 and vascular injury. EVs were isolated from blood collected from asymptomatic, moderate (not on O₂), moderate (on O₂), and severe COVID-19 patients and found that these EVs are taken up by human pulmonary microvascular endothelial cells. Further, EVs from critically ill COVID-19 patients were more numerous compared to those from other disease states; these EVs had high proinflammatory markers and induced pulmonary microvascular endothelial damage. These studies could implicate blood EVs in vascular damage associated with COVID-19, and those EVs may play roles in “long-haul” disease.

Ross Jacobson (Particle Metrix) wrapped up this session with a presentation on the capabilities of the ZetaView instrument. The instrument takes 11 measurements per sample and provides size, concentration, and zeta potential. Further, vesicles can also be tagged with fluorescent antibodies or stains, and the ZetaView’s fluorescence filters (up to 4 lasers) can provide information on EV subpopulations as well. This work demonstrates the capabilities of combined fluorescence labels and NTA particle recognition which the field has found difficult to obtain.

The third session of the day was EVs, Drug Abuse, and Other Viruses, chaired by Michal Toborek and Sergey Iordanskiy. The first presenter was Christie Fowler (University of California, Irvine), whose presentation focused on nicotine and vaping and their effects on the CNS. Nicotine directly activates choroid plexus cells of the brain; these cells make CSF, and they release and filter CSF-EVs. miR-204 was shown to be upregulated in these cells and their EVs in response to nicotine exposure. When nicotinic receptors were blocked, this phenomenon was inhibited both *in vitro* and *in vivo*. EVs were labeled and injected into a mouse model and observed being trafficked in CSF to the medial habenula cholinergic neurons. Interestingly, THC was also found to upregulate miR-204 expression and affect NCAM1 expression in the brains of male but not female rats.

Presenting for a second time was Ursula Sandau (Oregon Health and Science University). This talk was focused on the effects of methamphetamine on plasma EVs and their miRNA cargo. Meth has been shown to alter Dicer1 and AGO₂ (miRNA processing components) and stimulate EV release from neurons. It is also known that miRNAs are altered in brain regions associated with meth addiction, raising the possibility of EVs as biomarkers in meth addiction. The work involved the Methamphetamine Research Center with a study consisting of over 200 patients’ samples with associated clinical interviews. Plasma EVs were isolated via SEC and subpopulations were measured by vesicle flow cytometry. Platelet EV concentrations were correlated with self-reported depressive measures in patients. Further, several microRNAs were correlated with lifetime exposure to meth, frequency of use, and age of addiction onset, and others were further correlated with anxiety and memory in patients with active meth use.

Next, Guoku Hu (University of Nebraska Medical Center) gave an interesting talk focusing on the effects of morphine use on astrocyte primary cilium development and morphine tolerance. Opioid tolerance is a risk factor in opioid overdose. Via Sonic Hedgehog pathways, morphine was found to induce ciliogenesis and increase primary ciliary length in astrocytes *in vitro*, which is driven by EV release. The working hypothesis is that increased astrocyte EV release induces primary ciliogenesis in neighboring recipient astrocytes, leading to increase opioid tolerance. miRNAs may play a role in this mechanism as knockdown of Dicer resulted in an attenuation of this effect. In mice, the inhibition of primary cilia formation with MLN4924 and inhibition of EV release with GW4896 prevents morphine tolerance. As the opioid epidemic continues,

mechanisms to prevent tolerance, further addiction, and cognitive decline become paramount in our strategies for treatment.

Susmita Sil (University of Nebraska Medical Center) discussed the role of astrocyte-derived extracellular vesicles in morphine-induced synaptic degeneration. It is known that morphine induces amyloidosis in astrocytes, and this is regulated by HIF1A. Astrocytes release EVs with the amyloid cargo, impacting neurons. Her studies demonstrate how morphine can cause activation of the autophagy pathway while blocking the autophagic flux in lysosomes. This resulted in increased EV production from astrocytes carrying autophagy proteins, which upon uptake by neurons, culminated in synaptic degeneration. It was also shown that morphine-mediated dysregulated autophagy and EV biogenesis were regulated by astrocytic NMDA-NR1. This study underscores the role of autophagy cargoes in morphine driven-astrocyte EVs mediated neuronal injury and synaptic alterations. Understanding how morphine hijacks the autophagy machinery to regulate EV release via the astrocytic NMDA-NR1, and how this phenomenon is involved with neurodegeneration is a novel concept, which can set the groundwork for future development of therapeutics for opiate addicts.

The next presenter was Sergey Iordanskiy (Uniformed Services University), whose presentation focused on how the bystander effect of ionizing radiation was mediated by endogenous retroviruses. It was shown that gamma radiation induced monocytes to differentiate into complex macrophage phenotypes. These macrophages express type I interferons (IFN-I) along with both pro- and anti-inflammatory cytokines via JAK/STAT pathways. These changes correlated with significantly upregulated expression of 622 viral retroelements, particularly from several clades of human endogenous retroviruses (HERVs). This study identified in irradiated macrophages an increased amount of the double-stranded RNA receptors, MDA-5 and TLR3, bound to an equivalent number of copies of sense and antisense chains of viral HML-2 RNA. This binding triggered MAVS-associated signaling pathways, resulting in increased expression of IFN-I and NF- κ B-dependent inflammation-related genes. Knockdown of the HML-2 env gene was accompanied with downregulation of various HERV clades, suggesting, in turn, the dependence of retroviral expression on an interferon signaling that activates transcription of ERV elements whose promoter regions contain interferon regulatory factor- and NF- κ B-binding sites. Silencing of HERV expression led to dramatically reduced expression and secretion of IFN α , proinflammatory and anti-inflammatory modulators in irradiated macrophages. Exposure of non-irradiated reporter THP1 cells with the culture media from HERV-silenced macrophages led to remarkable downregulation of expression of NF- κ B-dependent proinflammatory genes and interferon-stimulated genes. Taken together, these data indicated that radiation stress-induced HERV expression enhances IFN-I and cytokine response, and results in increased levels of proinflammatory modulators released by macrophages, which is a potential inflammation-inducing mechanism in non-irradiated bystander cells.

Pooja Jain (Drexel University College of Medicine) next presented work focusing on the B and T lymphocyte attenuator (BTLA) protein, which can act as both an activator and inhibitor of B and T cell responses in the context of HTLV-1 chronic illness and inflammatory disease. HTLV-1 causes 80 different clinical syndromes and two main diseases, adult T-cell leukemia/lymphoma and HTLV-1-associated myelopathy. HTLV-1 infected cells released high quantities of BTLA, possibly in the form of EVs. In a treatment scenario, anti-viral therapies reduced levels of various immune checkpoint inhibitors in infected cells, including BTLA (seen in this context as a checkpoint inhibitor). There may be a potential role of EVs mediating these diseases as soluble and EV-associated PD-1 was observed in cell culture experiments.

Eva Poveda (Galicia Sur Health Research Institute) was the last presenter for this session and gave a talk focusing on cytokine profiles of EVs in HIV elite controllers a rare subset of people living with HIV that spontaneously control HIV replication without antiretroviral therapy (ART). Despite the rarity of these patients, the group was able to gather samples from 120 subjects, including 20 control subjects. Cytokine profiles (39 cytokines in a multiplexed bead-based assay) were assessed in plasma and EVs of healthy controls, as well as ART-naïve, ART-exposed, persistent elite controllers (control HIV infection over time), and transient elite controllers (control HIV infection, then lose that control). Interestingly, higher levels of IL18 and TNF- α in both plasma and EVs were found in elite controllers vs. ART-exposed patients, and within elite controllers, cytokine profiles can distinguish between persistent and transient control.

After a packed day of both in-person and virtual talks, young investigators participating in the meeting were encouraged to attend a NIH Grant Writing Workshop led by Fatah Kashanchi (George Mason University). During this interactive workshop, Dr. Kashanchi provided information about different types of grants, templates for writing specific aims pages, and walked through an on-the-spot grant proposal crowd-sourced from workshop participants. Also stressed was the importance of preliminary data as grant reviewers need to appreciate the feasibility of the proposed work. This was followed by a poster session.

Day 3 of meeting: The last morning of the conference began with a session on Technology and Treatments, and was chaired by Yuntao Wu and Pooja Jain. The first presenter of this session was David Walt (Harvard University), who focused on new tools for EV isolation, purification, and characterization with the goal of measuring cellular pathologic events based on materials in biofluids. In complex biofluids, it is difficult to separate and differentiate EVs from other particles such as lipoproteins, protein aggregates, *etc.*, so new technologies are needed. Digital ELISAs, such as the single molecule array (SIMOA), use one bead per well with the idea that EVs will bind to beads in a 1:1 ratio. These sensitive measures could combine with optimized SEC to determine parameters for yield or for purity. From this, it was found that L1CAM is soluble and not associated with EVs isolated from CSF or plasma via SEC; however, it was associated with EVs from iPSC-derived neurons.

Emeli Chatterjee (Massachusetts General Hospital and Harvard Medical School) gave a talk focused on an organ-on-chip model to characterize extracellular vesicles as functional biomarkers in cardiorenal syndrome (CRS). Heart failure (HF) can lead to renal injury and vice versa, and CRS is noted for this interface. Like many areas of pathology, EVs in CRS could be involved in organ cross-talk, intercellular communication, biomarkers, and potential therapeutic roles. With the development of a kidney proximal tubule organ-on-a-chip, the group isolated EVs from healthy donors, from patients with HF but no CRS, and from patients with both HF and CRS. Key findings showed that EVs isolated from patients with heart failure (HF) and healthy controls followed by exposure on kidney proximal tubule-chips for 72h led to increased mRNA expression of neutrophil gelatinase-associated lipocalin (NGAL) and IL-18 in human glomerular endothelial cells and proximal tubule epithelial cells following treatment with EVs. The increase was higher in HF patients with Type 1 CRS compared with those without CRS. There was also upregulation of kidney injury molecule 1 (KIM1) mRNA and cystatin C protein, suggesting induction of damage and dysfunction by HF/CRS EVs. This study bridges the gap between *in vitro* and *in vivo* models offering new approaches to identify the role of plasma EVs as potential biomarkers and effectors for CRS.

Next, Ramin Hakami (George Mason University) presented an interesting talk on the development of a novel microfluidic assay for monitoring the effects of live EV exchange in a physiologically relevant 3D environment. Size effects were apparent, as liposomes of 70 and 250 nm could get through the Matrigel (the 250 nm liposomes were less so than the 70 nm ones), but 500 nm fluorescent beads could not cross the

Matrigel. With this platform, cells housed in one chamber of the chip could be transfected to express GFP, and their GFP-containing EVs could traverse through a Matrigel channel to other cells and be taken up by them.

Meredith Chambers (University of North Carolina) gave a talk on the use of direct stochastic optical reconstruction microscopy (dSTORM) for visualizing EVs. This super-resolution microscopy can distinguish between a protein tagged on the surface of an EV and the rest of the EV to allow for vesicular protein localization. Future work aims to optimize three-color 3D dSTORM to allow for the localization of two proteins on a single EV.

Ryan McNamara's (University of North Carolina) talk was presented by Dirk Dittmer. He discussed EV-encased nucleic acids and their use as a scaffold for chemotherapeutic agents for targeted tumor delivery. Kaposi Sarcoma (KS) is a type of cancer that is very prevalent in sub-Saharan Africa and is the most common type of cancer that people living with HIV develop. KS is caused by infection with KS herpes virus (KSHV) and KSHV RNAs promote tumor growth. KSHV-EVs attract endothelial cells; however, if the EVs have been loaded with the chemotherapy drug doxorubicin (DOX), the recruited cells will die. DOX appears to be better contained within EVs than in liposomes (DOXL), which may be due to its interaction with miRNAs in EVs. Interestingly, when DICER is knocked out, over half of the DOX signal in EVs goes away; this is likely due to DOX binding to other small RNAs within EVs other than miRNAs.

Roger Alexander (ERCC) gave a presentation summarizing the April exRNA Data Analysis Workshop (available on YouTube) and provided an update on the exRNA Atlas. There is a major lack of universal reference profiles and a great deal of batch variation, so the ERCC wanted to create a systematic pipeline for researchers to follow. Currently, over 7700 samples from different diseases and sample types are profiled in the exRNA Atlas; small RNA seq data processed through the exRNA toolkit are stored here and researchers are encouraged to submit new data to the Atlas as well.

The final session on EVs, RNA and Therapeutics was chaired by Christie Fowler and Susmita Sil. Alissa Weaver (Vanderbilt University) highlighted the biogenesis of RNA-containing extracellular vesicles at endoplasmic reticulum (ER) membrane contact sites. VAP-A is an integral ER protein that establishes points of intracellular contact between the ER and other organelles. As RNA complexes may be associated with the ER, it was speculated that ER-endosomal/multivesicular body contacts may be sources of RNA loading for EVs. VAP-A knockdown cells exhibited reduced ER-endosome contacts. This study elucidated the number of small RNAs that were altered in VAP-A KD small- and large-EVs compared to control cells. Density gradient fractionation revealed that VAP-A regulated a select subpopulation of small EVs that were enriched with RNA and RBPs. Analysis of small and large EVs for lipid content revealed that VAP-A controlled the levels of ceramide and cholesterol, two lipids involved in EV biogenesis. Furthermore, KD of the VAP-A binding ceramide and cholesterol transporters CERT and ORP1L led to similar defects in EV biogenesis. This study uncovered a novel pathway of EV biogenesis that takes place at the ER membrane contact sites.

Louise Laurent (University of California, San Diego) next presented work on exRNAs as biomarkers for preeclampsia. Preeclampsia is a leading cause of fetal and maternal morbidity worldwide; it manifests in the 2nd trimester, but its origins are thought to exist in the 1st trimester. Thus, early-stage biomarkers could be important to identify patients at risk and establish treatment regimens. Using discovery and verification cohorts from plasma, small RNA sequencing revealed miRNAs that may be associated with preeclampsia, and miRNAs could be clustered based on the patient's status of severe preeclampsia, moderate

preeclampsia, hypertensive controls, and normal controls. Most of the miRNAs originated from the liver, placenta, platelets, and red blood cells, and importantly, the current study did not distinguish between vesicular and non-vesicular RNAs; only total plasma RNA was assessed.

Next, Sophie Anderlind (Penn State Erie, The Behrend College) presented research on the effects of cortisol on iron transport proteins and EV release in placental cells. This work demonstrated that exposure of placental trophoblast cell line, BeWo, to physiologically relevant concentrations of hydrocortisone, resulted in the alterations in the expression of iron transport proteins- Transferrin receptor 1, Ferroportin-1, and DMT-1. Additionally, using size exclusion chromatography, they separated EVs from the conditioned media to assess protein composition following exposure of the cells to hydrocortisone. Future work from their team is aimed at addressing the effects of cortisol exposure on iron transport proteins in EVs, which could influence how iron is delivered during fetal development.

Heather Branscome (ATCC) presented work on retroviral infection of human neurospheres and the use of stem cell EVs to repair cellular damage. In this work, iPS neurospheres were generated from stem cells and infected with HIV-1 as a proof-of-concept platform for studying the effects of antiretroviral therapy on the brain. Interestingly, EVs isolated from the conditioned cell culture media of the stem cells used to create the neurospheres were found to have angiogenic, anti-inflammatory, and antiapoptotic effects.

The last presenter for the day was Botai Xuan (Izon), who presented an overview of Izon's instruments and products, and expressed hope for a standardized workflow in EV isolation.

During the closing ceremony, awards were presented for both poster and oral presentations. Three poster award winners were Sarah Al Sharif, Sebastian Molnar, and Yijun Zhou. Three awards were given for the best talks by junior presenters: Meredith Chambers, Sophie Anderlind and Navneet Dogra.

ASIC would like to thank the Organizing Committee for organizing this meeting, especially given the hybrid format that was necessary to allow for the greatest exchange of scientific ideas. Special thanks are also extended to Gwen Cox for her commitment to ensuring this meeting ran smoothly.

Fatah Kashanchi, George Mason University

Leonid Margolis, National Institute of Child Health and Human Development, National Institutes of Health

Julie Saugstad, Oregon Health & Science University

Meta Kuehn, Duke University

Michael Graner, University of Colorado Anschutz

Janusz Rak, McGill University

DECLARATIONS

Authors' contributions

Organized and wrote the manuscript: Russell AE

Taking notes during the meeting and discussions afterwards: Russell AE, Graner MW

Integrated several sections of the manuscript: Russell AE

Wrote several sections of the manuscript: Sil S, Buch S

Financial support and sponsorship

Russell AE was funded by Penn State Behrend and the Hamot Health Foundation. Graner MW was funded by the National Institutes of Health, grant NIMH 4R33MH118174.

Availability of data and materials

Not applicable.

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.

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Original Article

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Alterations in arthropod and neuronal exosomes reduce virus transmission and replication in recipient cells

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How to cite this article: Fasae KD, Neelakanta G, Sultana H. Alterations in arthropod and neuronal exosomes reduce virus transmission and replication in recipient cells. *Extracell Vesicles Circ Nucleic Acids* 2022;3:264-79. <https://dx.doi.org/10.20517/evcna.2022.30>

Received: 8 Jun 2022 **First Decision:** 15 Jul 2022 **Revised:** 12 Aug 2022 **Accepted:** 23 Aug 2022 **Published:** 31 Aug 2022

Academic Editors: Yoke Peng Loh, Shilpa Buch **Copy Editor:** Tiantian Shi **Production Editor:** Tiantian Shi

Abstract

Aim: Targeting the modes of pathogen shedding/transmission via exosomes or extracellular vesicles has been envisioned as the best approach to control vector-borne diseases. This study is focused on altering exosomes stability to affect the pathogen transmission from infected to naïve recipient cells.

Methods: In this study, neuronal or arthropod exosomes were treated at different temperatures or with different salts or pH conditions to analyze their ability and efficiency in the transmission of tick-borne Langkat virus (LGTV) from infected to naïve recipient cells.

Results: Quantitative real-time PCR (qRT-PCR) and immunoblotting analyses revealed that treatment of neuronal or tick exosomes at warmer temperatures of 37 °C or 23 °C, respectively, or with sulfate salts such as Magnesium or Ammonium sulfates or with highly alkaline pH of 9 or 11.5, dramatically reduced transmission of LGTV via infectious exosomes (human or tick cells-derived) to human neuronal (SH-SY5Y) cells or skin keratinocytes (HaCaT cells), respectively.

Conclusion: Overall, this study suggests that exosome-mediated viral transmission of vector-borne pathogens to the vertebrate host or the viral dissemination and replication within or between the mammalian host can be reduced by altering the ability of exosomes with basic changes in temperatures, salts or pH conditions.



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Keywords: Exosomes, neurons, skin cells, tick, Langat virus, transmission, treatments

INTRODUCTION

Exosomes are membrane-bound small vesicles that have become the highlight of research in infectious diseases^[1-12]. Several viruses perhaps use exosomes [small extracellular vesicles (EVs) of 30-120 nm, with infectious cargo] for transmission of viral RNA/proteins to the naïve recipient host cells^[2-4,13-22]. Our previous studies have shown that arthropod-borne flavivirus full-length RNA genomes and proteins [such as Envelope protein, Non-Structural protein 1 (NS1) and/or polyprotein] are transmitted to the vertebrate host cells via arthropod exosomes^[1,3,4,23]. Flaviviral transmission from the infected vectors (such as ticks and mosquitoes) to the vertebrate host via exosomes could be an important strategy for the dissemination of these vector-borne pathogens^[3,4]. In our previous study, a proof of principle concept illustrates that neuroinvasion of flaviviruses is perhaps mediated via extreme production, release and dissemination of arthropod exosomes through saliva, that fuses with skin barrier cells such as keratinocytes, thus leading to virus dissemination into the peripheral system. High viral loads or viremia in the periphery such as blood, spleen, and liver may breach the blood-brain barrier (BBB) integrity, therefore leading to infection of the CNS^[3,4]. Our novel line of discovery further led us to isolate exosomes from saliva and salivary glands of hard ticks such as *Ixodes scapularis* and *Amblyomma maculatum*^[23,24]. Tick salivary and saliva-derived exosomes showed an immune regulation at the vertebrate skin interface by inhibition of human skin C-X-C motif chemokine ligand (CXCL-12) and induction of Interleukin-8 (IL-8) chemokine^[24]. This immune regulation dramatically delayed cell migration, wound healing and repair process to support continuous and successful blood feeding at the tick bite site^[24]. Detection of exosomes in tick saliva and salivary glands indeed suggested a potential route of pathogen transmission from the infectious vector to the naïve vertebrate host^[3,24]. In both these studies, we have characterized *I. scapularis* tick saliva, salivary gland and ISE6 tick cell-derived exosomes^[3,24]. Given the importance of pathogen transmission via arthropod exosomes to the vertebrate host, it is not surprising to envision that targeting arthropod exosomes could be the best transmission-blocking strategy to interfere and control vector-borne diseases.

Arthropod-borne flaviviruses are transmitted by the bite of an infected vector such as *I. scapularis* ticks which are obligate hematophagous parasites^[3,23,25-31]. The hard ticks, vector/transmit several human pathogens, including tick-borne encephalitis virus (TBEV), Powassan virus (POWV) and Langat virus (LGTV) in the genus flavivirus^[25-31]. There are no known cases of human diseases associated with LGTV, and it does not pose a significant epidemiological threat in comparison to TBEV/POWV^[25-31]. LGTV is used as a model pathogen to study the pathogenesis of deadly pathogens such as TBEV and POWV. During blood feeding, ticks secrete saliva that contains various anti-inflammatory, anti-coagulatory, anti-vasoconstrictory and anti-platelet aggregation factors, which regulate vertebrate host immune responses^[32-39]. Ticks are fed for a prolonged period of time which could be up to 2 weeks. During this lengthy feeding time, ticks must encounter the vertebrate host defense responses to get a complete blood meal, while at the same time also transmitting pathogens that need to evade, survive, replicate, and colonize in the host. At the tick feeding site, there is a triad interaction that involves pathogens, ticks and their vertebrate hosts^[32-39]. Being a key regulator of the triad interactions, the pathogen modulates tick feeding time, efficiency of tick feeding and salivary components/saliva secretion^[1,23,40]. It is noteworthy that ticks co-exist with several pathogens and have been evolving with them for over a period of time. This co-evolution with pathogens perhaps adapts ticks with better strategies to overcome host defense mechanisms by modulating the saliva components with various pharmacologically active molecules and exosomes that could inhibit immune responses at the vertebrate skin interface. These salivary modulations could be pathogen directed and favorable to the vector host in order to co-exist and benefit each other from this

long-term relationship. There are several studies including transcriptomics/proteomics and sialomics, with evidence that tick-transmitted pathogens manipulate the salivary/saliva components to facilitate their transmission, evade host defense reactions and survive in the vertebrate host^[35-39,41-47]. Our findings revealed that tick exosomes are salivary/saliva components and that they do facilitate pathogen transmission to the vertebrate host, suggesting that they are novel means of the dissemination from vector to the vertebrate host^[3,24]. Treatment of arthropod/neuronal exosomes with GW4869 (a pharmacological agent that interferes with the production and release of exosomes) inhibits the viral RNA/proteins and also interferes with the transmission of flaviviruses from exosomes to the naïve recipient host cells^[2-4,25,48,49]. The mechanism(s) of GW4869 affecting viral transmission has not been clearly understood. In this study, we have performed some basic treatments of exosomes at different temperatures with different salts or pH conditions to understand their stability and efficiency in the transmission and replication of tick-borne viruses.

METHODS

Cell lines, cell culture and viral infections. Human neuroblastoma cells (SH-SY5Y; CRL-2266; American Type culture Collection: ATCC), and *Ixodes scapularis* tick embryonic (ISE6; NR12234; BEI Resources) cell lines were purchased from ATCC/BEI and propagated as per the instructions from the supplier. Human SH-SY5Y cells were grown in complete EMEM medium (ATCC recommended) with 10% heat-inactivated Fetal bovine serum (FBS) (SIGMA) at 37 °C and 5% CO₂, respectively. ISE6 cells were cultured and grown in L15B300 medium (SIGMA) at 34 °C and as per the recommendations from Dr. Munderloh^[50,51]. Human skin keratinocytes (HaCaT cells) were obtained from Addexbio Technologies/FisherScientific and grown (as per the instructions from the supplier) in complete Dulbecco's Modified Eagle Medium (DMEM; SIGMA) with 10% heat-inactivated FBS at 37 °C and 5% CO₂. SH-SY5Y or ISE6 cells were plated at densities of 1-2 × 10⁶, and after overnight incubations, cells were infected with Langat virus [LGTV, strain LGT-TP21, 1 MOI (multiplication of infection) for ISE6 cells or 5 MOI for SH-SY5Y cells]. Naïve SH-SY5Y or HaCaT cells were infected via infectious exosomes collected from either SH-SY5Y or tick cells, respectively.

Isolation of exosomes from infected cell culture supernatants and treatment at different temperatures, salts or pH conditions. Exosomes were isolated from the independent batch of LGTV-infected (72 h postinfection, p.i.) human SH-SY5Y or ISE6 tick cell culture supernatants and by differential ultracentrifugation method as previously described^[2-4]. Exosome pellets were resuspended in 300-350 µl of appropriate solutions, depending on the respective treatments and as described in Table 1. Exosome suspensions were aliquoted (as equal volumes) into respective solutions and held overnight at 4 °C (in case of pH or salt treatments) or at respective temperatures (of -80 °C, 4 °C, 12 °C, 23 °C or 37 °C) in 1 × chilled phosphate buffered saline solution (PBS; SIGMA) and as indicated in Table 1. Exosomes collected after pelleting were directly suspended in 1 × chilled PBS (with neutral pH of 7.0 for the temperature group) or in 1 × chilled PBS (tubes were adjusted to achieve respective pH of 1.5, 4, 7, 9 or 11.5). In this study, we used 1 × PBS (SIGMA) with neutral pH of 7, which is adjusted to make either acidic or basic solutions of respective pH. Also, we used 1 × chilled PBS (with neutral pH of 7.0) to prepare 0.1 M (each one) respective salt solutions [of Sodium Chloride, NaCl; Ammonium sulfate, (NH₄)₂SO₄; Sodium Acetate, CH₃COONa or Magnesium Sulfate, MgSO₄]. Each treated group of exosomes from SH-SY5Y or ISE6 tick cells was independently prepared (batch by batch) and processed for independent re-infection on naïve recipient cells (SH-SY5Y or HaCaT cells), respectively. Recipient cells (human SH-SY5Y or HaCaT) that received infectious and treated neuronal or tick exosomes, respectively, were all incubated at 37 °C and 5% CO₂. A detailed schematic representation is shown for experimental procedures and treatments [Supplementary Figure 1].

Infection of naïve SH-SY5Y or HaCaT cells via infectious exosomes treated at different temperatures, salts or pH conditions. For determining the ability and efficiency of infectious exosomes (treated with respective temperatures, salts or pH) to transmit LGTV, we used human SH-SY5Y cells as recipient host cells for neuronal exosomes (prepared from the independent batch of SH-SY5Y cells) or HaCaT cells as recipient host cells for exosomes derived from tick ISE6 cells. Naïve SH-SY5Y or HaCaT recipient cells (at a density of $1-2 \times 10^5$) were seeded per well and as 5-6 replicates per treatment (5 replicates for RNA extractions and 1 sample for protein extraction, respectively). Recipient cells were plated on the same day of exosome isolation, and allowed to adhere while exosomes were independently incubated overnight at respective treatments. Following overnight treatments, exosomes treated with respective conditions were incubated (20 μ L of exosome solution per replicate per treatment/group) on naïve SH-SY5Y or HaCaT cells, respectively. After three days of post-incubation with infectious exosomes, SH-SY5Y or HaCaT recipient cells were lysed in either RLT lysis buffer or modified RIPA lysis buffer for extractions of total RNA or proteins, respectively. A detailed schematic representation is shown for experimental procedures and treatments [Supplementary Figure 1].

Total RNA extractions, cDNA synthesis and qRT-PCR analysis. RNA extractions were performed using Aurum Total RNA Mini kit (Bio-Rad) and by following the manufacturer's instructions. Total RNA from SH-SY5Y or HaCaT cells was eluted in small volumes (50 μ L) of Nuclease free water. RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad). The generated cDNA was used as the template for the amplification and determination of viral loads in the recipient cells. We used previously published primers for the detection of LGTV PrM transcripts^[3,4]. To normalize the amount of template, human beta actin amplicons were quantified using previously published primers^[3,4]. qRT-PCR was performed using iQ-SYBR Green Supermix (Bio-Rad). Standard curves were prepared using 10-fold serial dilutions starting with standards 1 to 6 of known quantities of actin or LGTV fragments. q-PCR reactions were performed using CFX Opus instrument (Bio-Rad) and as previously described^[3,4].

Immunoblotting analysis. To determine the viral loads, 10-20 μ g of total protein from respective cell lysates (either SH-SY5Y or HaCaT cells incubated with neuronal or tick exosomes, treated at different temperatures, salts or pH conditions) were resolved on 12% SDS-PAGE gels. Following gel electrophoresis, Western blotting was performed as described^[3,4]. Blots were blocked with 5% milk buffer and probed with 6E11 anti-Langat monoclonal primary antibody (at 1:1000 dilution; BEI resources) to detect NS1 protein, followed by incubation with mouse HRP-conjugated secondary antibody (at 1:5000 dilution; Boster Biological Technology). Either samples were re-run separately (for SH-SY5Y cells), or blots were re-probed (for HaCaT cells) with anti-CD9 or anti-CD63 monoclonal antibodies, respectively, (Novus Biologicals, LLC), followed by their respective secondary antibodies. Total protein profile gel images were obtained from stain-free gels. Antibody binding was detected with the WesternBright ECL kit. Blots/gels were imaged using the Chemidoc MP imaging system and processed using Image Lab software obtained from the manufacturer (Bio-Rad).

Statistical analyses. Statistical significance of differences observed in data sets was analyzed using GraphPad Prism 6 software and Microsoft Excel. The unpaired, two-tailed Student *t*-test was used for all analyses. Error bars represent mean (\pm SEM) values and Standard Error. Also, *P* values < 0.05 were considered significant in all analyses.

RESULTS

Neuronal exosomes infected with LGTV showed the presence of CD9 and HSP70 exosomal markers. We first tested infected neuronal exosomes in this study, since LGTV is very similar to TBEV and POWV

neuroinvasive viruses that cause neuropathogenesis, neuroinflammation, neuronal loss, and encephalitis that ultimately leads to death. Exosomes isolated from human SH-SY5Y cells were processed for detection of exosomal markers such as cell surface glycoprotein, CD9 (a member of the tetraspanins family) and the heat shock protein 70 (HSP70), a component of exosomal lumen. Immunoblotting analysis revealed that both CD9 and HSP70 were detected in LGTV-infected SH-SY5Y total exosomal-protein lysates [Supplementary Figure 2]. It was noted that both CD9 and HSP70 were upregulated in LGTV-infected group in comparison to the respective uninfected control groups [Supplementary Figure 2]. Immunoblotting analysis using LGTV-6E11 antibody revealed detection of viral NS1 in infected groups. Total protein profile gel images serve as loading controls [Supplementary Figure 2].

Neuronal exosomes treated at cold temperatures transmit increased LGTV loads to naïve recipient cells.

Neuronal exosomes derived from LGTV-infected human SH-SY5Y cells, incubated overnight in PBS and at different temperatures (of -80 °C, 4 °C, 12 °C, 23 °C or 37 °C) were allowed to transmit viral loads to naïve human recipient SH-SY5Y cells. After 3-day post-incubations, LGTV loads were determined in recipient cells incubated with infectious exosomes treated at different temperatures [Figure 1A]. qRT-PCR analysis revealed significantly ($P < 0.05$) increased LGTV loads in SH-SY5Y cells incubated with exosomes treated at cold temperatures (of 4 °C or 12 °C) compared to the viral loads noted from cells incubated with exosomes treated at other temperatures (of -80 °C, 23 °C or 37 °C) [Figure 1A]. SH-SY5Y cells incubated with exosomes treated at a higher temperature of 37 °C (which is also the human body temperature) had significantly lower viral loads in comparison to viral loads noted in cells incubated with exosomes treated at 23 °C (room temperature), or 12 °C (cold) or freezing temperature of -80 °C [Figure 1A]. Similar results were obtained with the immunoblotting analysis where LGTV NS1 protein loads were increased in SH-SY5Y cells incubated with exosomes treated at cold temperatures (of 4 °C or 12 °C) when compared to the viral loads noted in cells incubated with exosomes treated at other tested temperatures (of -80 °C, 23 °C or 37 °C) [Figure 1B]. LGTV loads noted in SH-SY5Y cells incubated with exosomes treated at freezing temperature of -80 °C were nearly 2-fold higher or 4-fold enhanced at 4 °C or 12 °C respectively, when compared to viral loads noted in cells incubated with exosomes treated at 23 °C or 37 °C temperatures [Figure 1B]. We also noted the presence of enhanced glycosylated NS1 product in SH-SY5Y cells incubated with exosomes treated at cold temperatures of -80 °C, 4 °C or 12 °C. The NS1 glycosylation product was dramatically lower or faded in cells incubated with exosomes treated at higher temperatures of 23 °C or 37 °C [Figure 1B]. Human CD9 (an exosomal marker protein) loads showed no differences in any samples [Figure 1C]. Total protein profile gel images serve as loading controls [Figure 1B and C].

Neuronal exosome-mediated LGTV transmission is inhibited by both Ammonium or Magnesium Sulfates.

LGTV-infectious exosomes derived from human SH-SY5Y cells, incubated overnight at 4 °C in 1 × chilled PBS (as control) or with PBS enriched 0.1 M salt solutions [of NaCl, (NH₄)₂SO₄, CH₃COONa or MgSO₄], respectively, were incubated on naïve recipient SH-SY5Y cells for 3 days. Treatments with all salt solutions allowed LGTV transmission via infectious exosomes, and replication in recipient cells [Figure 2A]. qRT-PCR analysis revealed that exosomes incubated in PBS, NaCl or CH₃COONa demonstrated significantly high LGTV loads when compared to viral loads observed in cells incubated with exosomes treated with (NH₄)₂SO₄ or MgSO₄ [Figure 2A]. Between these two sulfate treatments, SH-SY5Y cells incubated with exosomes treated with MgSO₄ showed significantly lower viral loads when compared to cells incubated with exosomes treated with (NH₄)₂SO₄ [Figure 2A]. Immunoblotting analysis of these samples also demonstrated lower NS1 protein loads in SH-SY5Y cells incubated with exosomes treated with (NH₄)₂SO₄ or MgSO₄ when compared to the other salt treated groups [Figure 2B]. NS1 protein nearly showed 4-5 folds reduction in SH-SY5Y cells incubated with exosomes that were treated with (NH₄)₂SO₄ or MgSO₄ [Figure 2B]. Human CD9 loads showed no differences between any salt treatments; however, lower

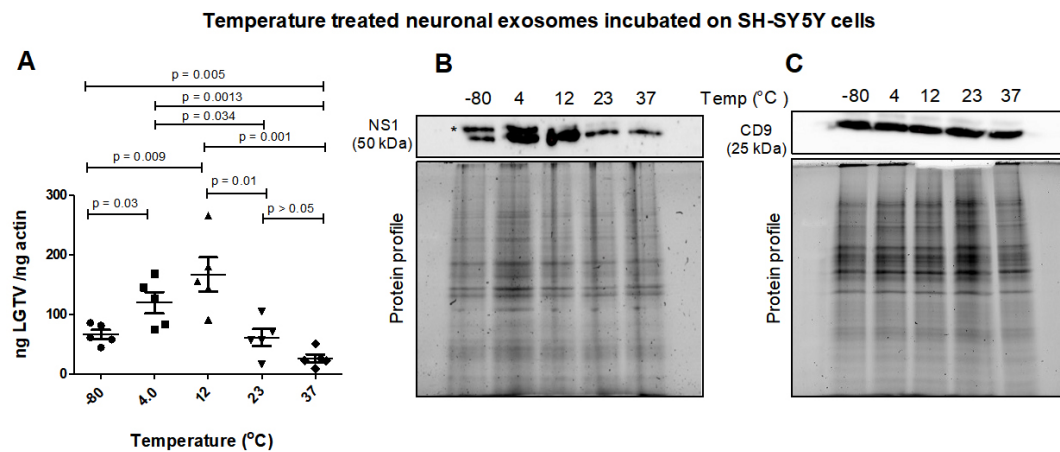


Figure 1. (A) qRT-PCR analysis showing viral burden (PrM transcript levels) from SH-SY5Y recipient cells that were incubated with infectious neuronal exosomes treated at varying temperatures (of -80 °C, 4 °C, 12 °C, 23 °C, or 37 °C) and held overnight in 1 × chilled PBS (of pH 7). All temperatures are represented as degree Celsius. Closed circles, squares, triangles, inverted triangles, or rhombus represents viral loads detected in LGTV-infected SH-SY5Y recipient cells incubated with infectious exosomes treated at indicated temperatures. In panel A, each data point represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to the numbers shown on the graph are from Student's *t*-test. There is no statistical difference between the data observed in -80 °C and 23 °C or 4 °C and 12 °C groups. Immunoblotting analysis is shown for proteins NS1 (B) or CD9 (C) at all tested temperatures. Asterisk (*) indicates glycosylated NS1 protein. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel images in (B) and (C) are shown as loading controls.

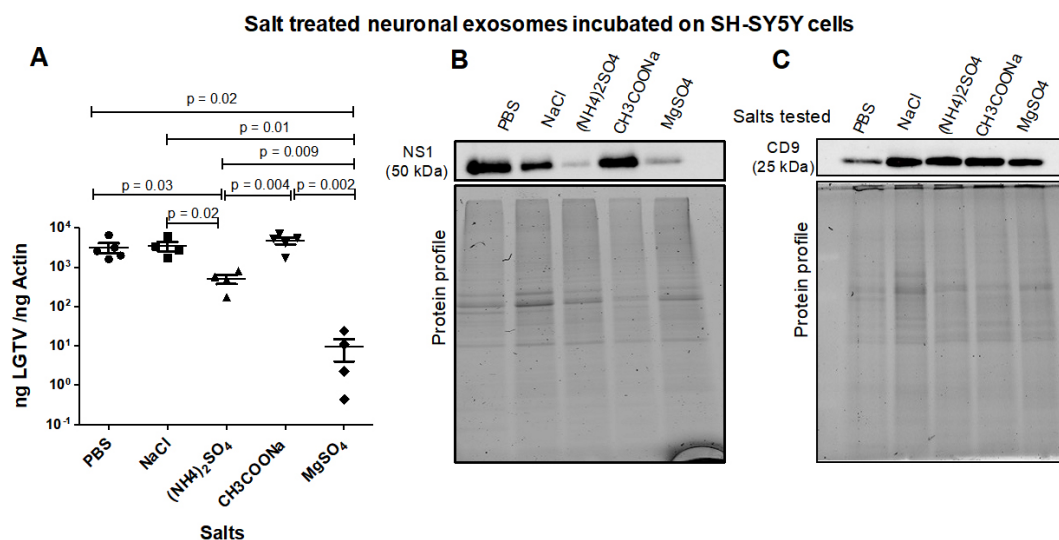


Figure 2. (A) qRT-PCR analysis showing viral loads (PrM transcript levels) from SH-SY5Y recipient cells incubated (for three days) with infectious neuronal exosomes, treated overnight at 4 °C in 1 × chilled PBS (as control) or with PBS enriched 0.1 M salt solutions [of NaCl, (NH₄)₂SO₄, CH₃COONa or MgSO₄], respectively. Closed circles, squares, triangles, inverted triangles or rhombus represents viral loads obtained from LGTV-infected SH-SY5Y recipient cells incubated with infectious exosomes treated with respective salts. In panel A, each data point represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to the numbers shown in the graph are from Student's *t*-test. There is no statistical difference between PBS to NaCl/CH₃COONa, or NaCl to CH₃COONa group. Immunoblotting analysis is shown for proteins NS1 (B) or CD9 (C) and for all tested salts. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel images in (B) and (C) are shown as loading controls.

loads of CD9 in PBS control is perhaps due to loading differences [Figure 2C]. Total protein profile gel

images serve as loading controls [Figure 2B and C].

Neuronal exosome-mediated LGTV transmission is drastically reduced upon treatment at alkaline pH. Neuronal exosomes (derived from LGTV-infected SH-SY5Y host cells) were incubated overnight at 4 °C in PBS solutions with varying pH (of 1.5, 4, 7, 9, or 11.5) and then applied to naïve recipient SH-SY5Y cells for three days post-incubations. It was noted that between the tested pH conditions, both acidic pH (of 1.5 or 4) and the neutral pH of 7-treated infectious exosomes enhanced viral transmission and replication in recipient cells [Figure 3A]. qRT-PCR analysis showed significantly lower LGTV loads in SH-SY5Y cells incubated with exosomes that were treated at alkaline pH of 9 or 11.5 in comparison to the viral loads noted in cells incubated with exosomes that were treated at acidic (of 1.5 or 4) or neutral pH (of 7) [Figure 3A]. Between the two alkaline pH treatments, SH-SY5Y cells incubated with exosomes treated at pH of 11.5 showed significantly lower LGTV loads when compared to viral loads noted in cells incubated with exosomes treated at pH of 9. In contrast, SH-SY5Y cells incubated with exosomes treated at acidic pH of 1.5 showed higher LGTV loads when compared to viral loads noted in cells incubated with exosomes treated at acidic pH of 4 [Figure 3A]. Immunoblotting analysis further confirmed the qRT-PCR data and showed the presence of NS1 in only SH-SY5Y cells incubated with exosomes treated at acidic pH of 1.5 or neutral pH of 7 [Figure 3B]. NS1 protein was not detected in SH-SY5Y cells incubated with exosomes treated at alkaline pH (of 9 or 11.5) or acidic pH of 4 [Figure 3B]. No differences were observed in human CD9 protein levels in all tested samples [Figure 3C] and total protein profile gel images serve as loading controls [Figure 3B and C].

Tick exosomes treated at cold temperatures transmit increased LGTV to human skin cells. LGTV-infected exosomes derived from ISE6 tick cells, held overnight in 1 × chilled PBS solution and at varying temperatures (of -80 °C, 4 °C, 12 °C, 23 °C or 37 °C), were incubated on naïve recipient human skin keratinocytes (HaCaT cells) for three days. LGTV transmission (via infectious tick exosomes) and replication in recipient HaCaT cells were detected in all tested temperatures [Figure 4A]. qRT-PCR analysis showed that HaCaT cells had significantly ($P < 0.05$) increased LGTV loads when incubated with tick exosomes treated at freezing/refrigeration temperatures (of -80 °C, 4 °C or 12 °C) when compared to the viral loads noted in cells incubated with tick exosomes treated at warmer temperatures of 23 °C or 37 °C [Figure 4A]. Immunoblotting analysis also revealed that tick exosomal mediated viral transmission to HaCaT cells showed lower replication of LGTV when recipient cells were incubated with tick exosomes treated at 23 °C [Figure 4B]. However, higher viral loads were noted in recipient HaCaT cells incubated with tick exosomes treated at cold temperatures (of 4 °C or 12 °C) [Figure 4B]. Human CD63 loads were detected in all tested samples and total protein profile gel image serves as the loading control [Figure 4B].

Tick exosome-mediated LGTV transmission is inhibited by Magnesium Sulfate. LGTV-infectious exosomes derived from ISE6 tick cells, held overnight at 4 °C in PBS or PBS enriched 0.1 M salt solutions [of NaCl, $(\text{NH}_4)_2\text{SO}_4$, CH_3COONa or MgSO_4], respectively, were incubated on naïve recipient HaCaT cells for 3 days. All tested salt solutions allowed LGTV transmission and viral replication in HaCaT cells [Figure 5A]. qRT-PCR analysis revealed that HaCaT cells incubated with tick exosomes treated with PBS (control), NaCl or $(\text{NH}_4)_2\text{SO}_4$ demonstrated significantly higher LGTV loads when compared to the viral loads detected in cells incubated with tick exosomes treated with CH_3COONa or MgSO_4 [Figure 5A]. Immunoblotting analysis further supported the qRT-PCR data [Figure 5B]. No differences in CD63 levels were noted in all tested samples [Figure 5B]. Total protein profile gel image serves as the loading control [Figure 5B].

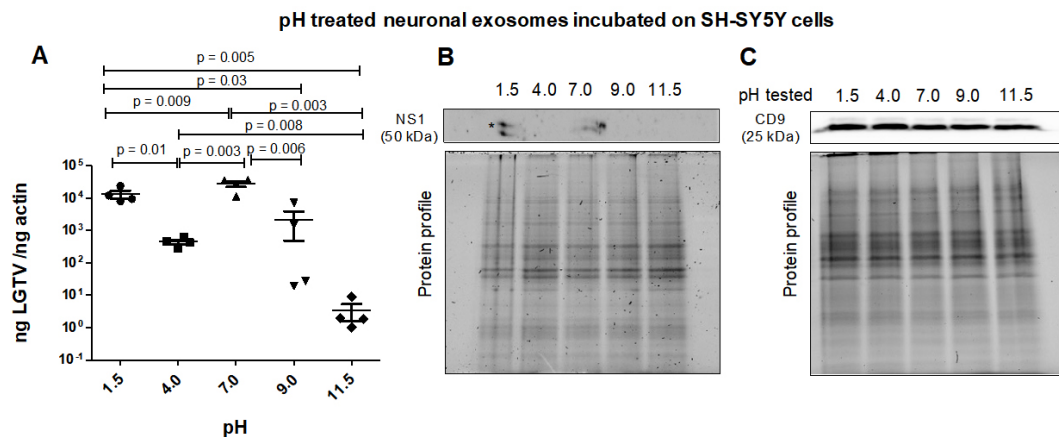


Figure 3. (A) qRT-PCR analysis showing viral loads (PrM transcript levels) from SH-SY5Y recipient cells that were incubated (for three days) with infectious neuronal exosomes, pretreated overnight at 4°C and with different pH conditions (of 1.5, 4, 7, 9 or 11.5), respectively. All pH conditions were set in 1x PBS solution. Closed circles, squares, triangles, inverted triangles or rhombus represents viral loads obtained from LGTV-infected SH-SY5Y recipient cells incubated with infectious exosomes treated at indicated pH. In panel A, each data point represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to the numbers shown in the graph are from Student's *t*-test. There is no statistical difference between groups of pH 4 and 9. Immunoblotting analysis is shown for proteins NS1 (B) or CD9 (C) and for all tested pH conditions. Asterisk (*) indicates glycosylated NS1 protein. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel images in (B) and (C) are shown as loading controls.

Temperature treated tick cell exosomes incubated on human skin (HaCaT) cells

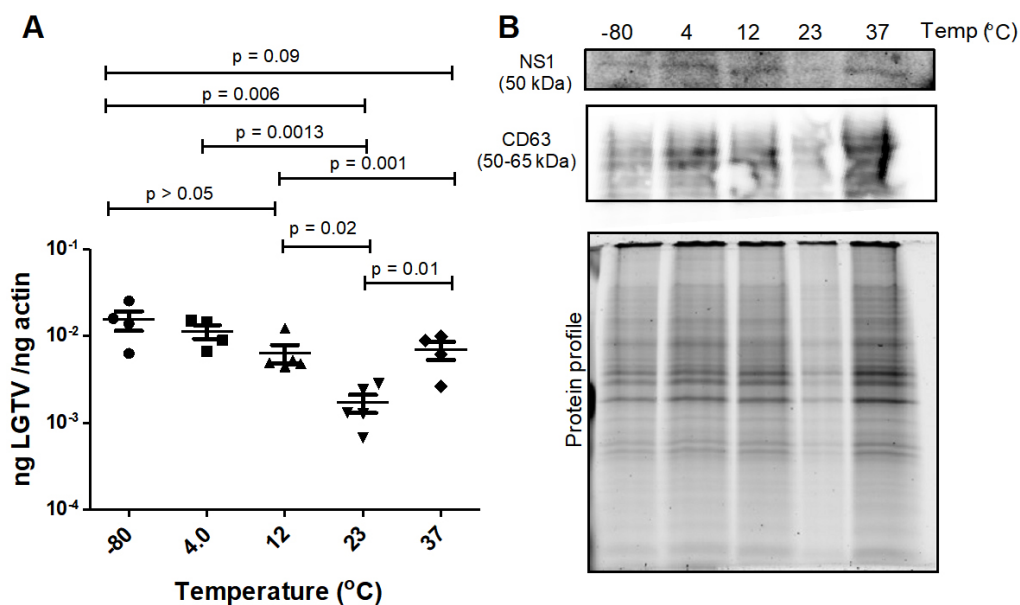


Figure 4. (A) qRT-PCR analysis showing viral loads (PrM transcript levels) from HaCaT recipient cells incubated with infectious tick exosomes treated at varying temperatures (of -80 °C, 4 °C, 12 °C, 23 °C, or 37 °C), and held overnight in 1 × chilled PBS with pH 7. All temperatures are represented as degree Celsius. Closed circles, squares, triangles, inverted triangles or rhombus represents viral loads obtained from LGTV-infected HaCaT cells incubated with infectious tick exosomes treated at indicated temperatures. In panel A, each data point represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to the numbers shown in the graph are from Student's *t*-test. There is no statistical difference between -80 °C and 4 °C, or 4 °C to 12 °C and 37 °C groups. Immunoblotting analysis is shown for proteins NS1 or CD63 (B) at all tested temperatures. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel image in (B) is shown as the loading control.

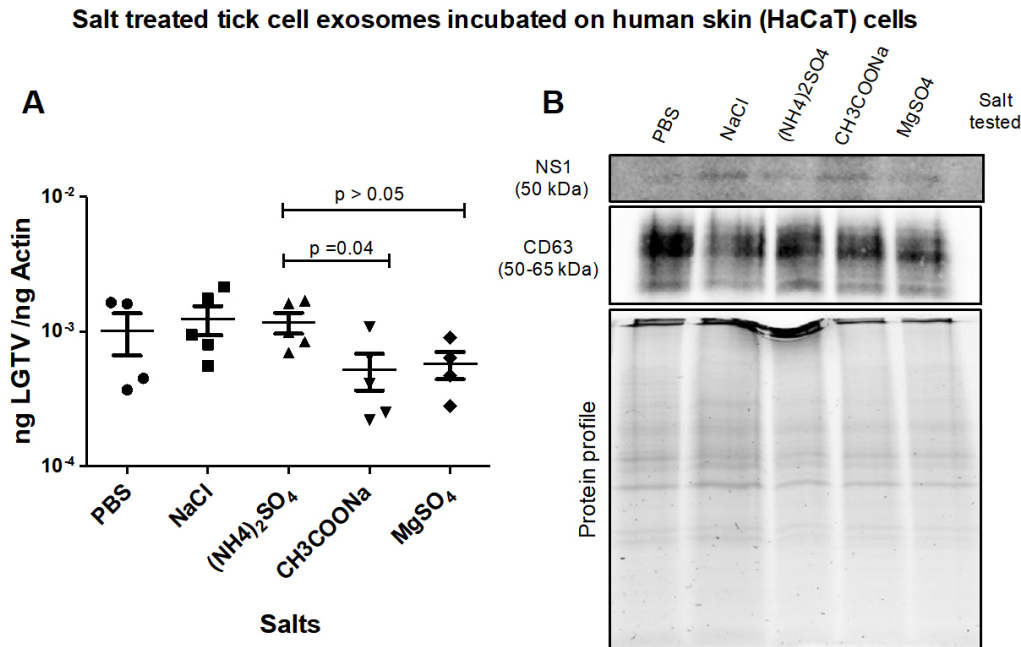


Figure 5. (A) qRT-PCR analysis showing viral loads (PrM transcript levels) from HaCaT recipient cells that were incubated (for three days) with infectious tick exosomes, pretreated overnight at 4 °C in 1 × chilled PBS (as control) or with PBS enriched 0.1 M salt solutions [of NaCl, (NH₄)₂SO₄, CH₃COONa or MgSO₄], respectively. Closed circles, squares, triangles, inverted triangles or rhombus represents viral loads obtained from LGTV-infected HaCaT cells incubated with infectious tick exosomes treated with indicated salts. In panel A, each shape represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to 0.04 are from Student's *t*-test. There is no statistical difference between PBS/NaCl and other tested salts, or CH₃COONa and MgSO₄ groups. Immunoblotting analysis is shown for proteins NS1 or CD63 (B) and for all tested salts. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel image in (B) is shown as the loading control.

Tick exosomes treated at alkaline pH conditions transmit reduced LGTV loads to human skin cells. Infectious tick exosomes (derived from LGTV-infected tick cells) were incubated overnight at 4 °C in PBS solutions with varying pH (of 1.5, 4, 7, 9 or 11.5) and then incubated on naïve recipient HaCaT cells for three days. It was noted that between the tested pH conditions, HaCaT cells incubated with tick exosomes treated at both acidic pH (of 1.5 or 4) had enhanced LGTV loads when compared to the viral loads noted in cells incubated with tick exosomes treated at other tested pH conditions (of 7, 9 or 11.5) [Figure 6A]. qRT-PCR analysis showed significantly ($P < 0.05$) lower LGTV loads in HaCaT cells incubated with exosomes treated at alkaline pH (of 9 or 11.5) in comparison to the viral loads noted in cells incubated with exosomes treated at acidic pH (of 1.5 or 4) [Figure 6A]. Between the two alkaline pH treatments, HaCaT cells incubated with tick exosomes treated at pH 11.5 showed significantly lower LGTV loads when compared to viral loads noted in cells treated with exosomes held at pH 9. In addition, HaCaT cells incubated with exosomes treated at neutral pH (of 7) showed lower LGTV loads when compared to viral loads in cells incubated with exosomes treated at acidic pH (of 1.5 or 4) [Figure 6A]. Immunoblotting analysis showed the presence of NS1 in all tested pH conditions [Figure 6B]. CD63 protein was detected in all analyzed samples and total protein profile gel image serves as the loading control [Figure 6B].

DISCUSSION

Arthropod-borne flaviviruses that are positive-sense single-stranded RNA viruses usually cycle between the vector and natural reservoir host but can accidentally infect human and other vertebrate hosts^[52-55]. Some of the flaviviruses are emerging or re-emerging pathogens that could lead to a large economic burden^[1,23,40].

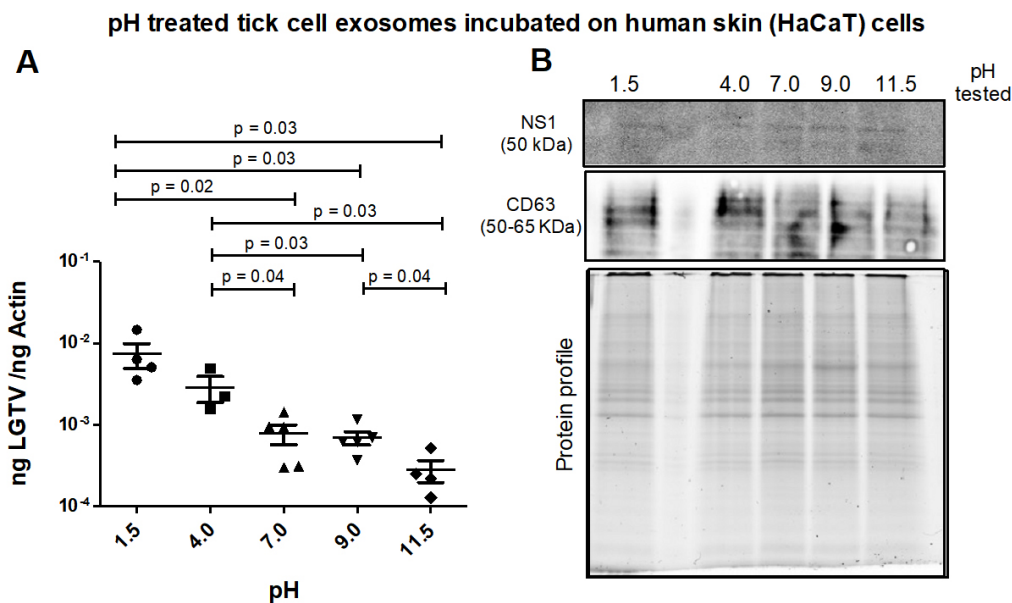


Figure 6. (A) qRT-PCR analysis showing viral loads (PrM transcript levels) from HaCaT recipient cells that were incubated (for 3 days) with infectious tick exosomes, treated overnight at 4 °C and at different pH conditions (of 1.5, 4, 7, 9 or 11.5), respectively. All pH conditions were set in 1x PBS solution. Closed circles, squares, triangles, inverted triangles or rhombus represents viral loads obtained from LGTV-infected HaCaT cells incubated with infectious exosomes treated at indicated pH. In panel A, each data point represents data from one independent culture well in a plate. Each treatment had five independent replicates. The mRNA levels of *prM* gene are normalized to human beta-actin mRNA levels. *P* values less than 0.05 or equal to the numbers shown in the graph are from Student's *t*-test. There is no statistical difference between groups of pH 1.5 and 4, or 7 to 9 and 11.5. Immunoblotting analysis is shown for proteins NS1 or CD63 (B) and for all tested pH conditions. Protein sizes are indicated in kilodaltons (kDa). Protein profile gel image in (B) is shown as the loading control.

Both developed and developing countries are prone to outbreaks of vector-borne flaviviral infections, their related diseases, and deaths^[1,23,40]. Currently, there are no approved vaccines or specific therapeutics to target flaviviral infections. We envision targeting the modes of pathogen shedding or transmission as the best approach to control vector-borne diseases. Our previous studies showed that flaviviruses such as tick-borne LGTV or mosquito-borne dengue (DENV), Zika virus (ZIKV) or West Nile virus (WNV) use arthropod exosomes as modes of transmission from vector to the vertebrate host^[2-4,24,25]. For the first time, our laboratory showed that medically important vectors such as ticks and mosquitoes secrete exosomes in heterogenous populations^[3,4]. Our studies also showed that both tick- and mosquito-borne flaviviruses profusely use arthropod exosomes for transmission from arthropod cells to the human skin keratinocytes or blood endothelial cells^[3,4]. We have detected viral RNA/full-length RNA genomes and viral proteins such as Envelope (E), Non-Structural protein 1 (NS1) and perhaps fully assembled polyprotein in arthropod and neuronal exosomes^[2-4]. We believe that the presence of full-length RNA genomes or polyproteins in exosomes could be sufficient to bring in infection in the vertebrate host, suggesting exosomes as a highly infectious route for viral transmission^[2-4]. We also detected both positive and negative strands of flaviviral RNA that suggest transmission of both assembled and replicative viruses^[3]. Moreover, our extensive studies showed that all tested evaluations, including RNaseA/proteinase K, and treatments with highly cross-reactive/neutralizing antibodies (such as 4G2 or ZV-2/16 Abs), did not interfere with the viral transmission, suggesting the presence of viral material inside the lumen of exosomes^[2-4,25]. These studies suggested that exosomal packed viral RNA genomes and proteins are well secured, highly infectious and replicative in naïve recipient host cells^[2-4,25].

The role of exosomes in infectious viral diseases is highlighted, and several other studies have also contributed to providing evidence(s) that exosomes are important means for the transmission of viruses from infected cells to the naïve recipient cells^[8,56-63]. Our studies showed that GW4869 inhibitor affects flaviviral transmission via arthropod or neuronal exosomes^[2-4,25]. Some studies have shown the effect of temperature and pH on exosomal integrity and functions^[64-68]. Understanding the impact of exosome storage is limited, as it can change the size, number, contents, and function of exosomes. A recent report suggests that -80 °C is the most favorable condition for long-term storage of biofluids and exosomes and 4 °C for short-term storage^[69]. In addition, it has been shown that human saliva-derived exosomes are stable for two months at 4 °C, maintaining their membrane integrity over this long storage period^[70]. Several studies have discussed the inhibitory effects of salts on virus replication^[71-80]. Given the importance of pathogen transmission via exosomes, it is essential to explore the ways we can potentially alter the exosomes stability, efficiency and cargo transport ability from the host to the naïve recipient cells. However, there are no reports showing treatment of exosomes via temperature, pH or salt can dramatically affect viral transmission. We took a simple approach to interfere with the exosomal delivery by treating or affecting them with temperatures, salts or pH conditions. This testing was performed on both neuronal or tick exosomes to understand their stability and efficiency in tick-borne viral transmission.

In this study, different temperatures (of -80 °C, 4 °C, 12 °C, 23 °C, or 37 °C) were selected based on the long-term or short-term storage of exosomes at -80 °C or 4 °C, respectively. Ticks are maintained at 23 °C in laboratory conditions and the human body is at 37 °C; therefore, we chose these temperatures in addition to 12 °C which lies between the selected temperatures. Both acidic (1.5 or 4) or basic pH (9 or 11.5) were selected to address viral replication and transmission. Neutral pH is considered as the control. In the case of different salts, we preferred the highly consumed table salt NaCl and other salts such as MgSO₄, which is taken by several people as dietary supplementation. Sodium acetate (CH₃COONa) is selected as it is a widely used salt across the industrial sectors. In medical situations, sodium acetate is given intravenously as an electrolyte replenishment as it corrects sodium levels in hyponatremic patients. Since ammonium sulfate is used in the purification of proteins including flaviviral proteins, we also considered this salt. We found that LGTV transmission to human SH-SY5Y cells via infectious neuronal exosomes (treated at various temperatures) is increased when incubated with exosomes treated at 4 °C or 12 °C in comparison to exosomes treated at warmer temperatures (of 23 °C or 37 °C) [Figure 1A]. Similar pattern was also noted in human skin keratinocytes that were incubated with tick exosomes treated with same temperatures (as neuronal exosomes), where cold-treated exosomes (treated at -80 °C, 4 °C or 12 °C), resulted in increased viral transmission and replication in naïve recipient skin cells in comparison to exosomes treated at a warmer temperature of 23 °C. This data indicates that at cold temperatures, perhaps exosomes are highly stable and maintain their membrane integrity to secure the inside/luminal contents, including viral RNA/proteins, that could be efficiently transmitted to the naïve recipient host cells. It is noteworthy that neuronal exosomes treated at a higher temperature of 37 °C resulted in less transmission of LGTV to naïve SH-SY5Y cells. However, tick exosomes treated at a higher temperature of 37 °C did not show a lower trend of viral transmission (in comparison to 23 °C) to human skin cells. This observation suggests that tick exosomes, unlike neuronal exosomes, are more stable for prolonged periods, such as tick blood feeding on a vertebrate host that would eventually result in the delivery of infectious exosomes (via saliva secretion), their transport/fusion and transmission of pathogens to human skin cells. In contrast, the neuron-neuron model had very low LGTV loads at 37 °C, thereby suggesting that human body temperature or its slight elevation, such as fever, may control viral transmission between neurons and within the human brain. We also observed a dramatic reduction in NS1 protein loads in naïve SH-SY5Y cells incubated with EVs held overnight at 37 °C. The glycosylated product (visible as a double band) faded at 23 °C or 37 °C in SH-SY5Y cells, suggesting a change in NS1 stability and perhaps polyprotein disassembly at warmer temperatures. The observation of no differences in the CD9 protein levels further suggested a specific change only for viral

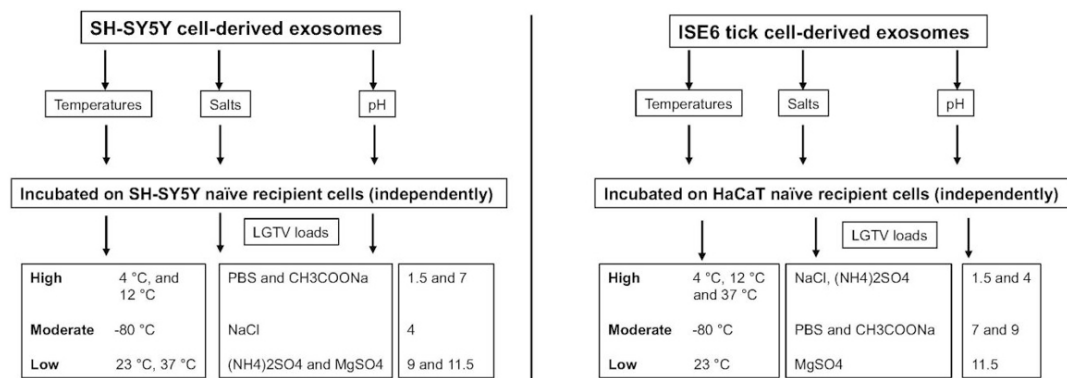


Figure 7. Schematic representation showing the differences with human SH-SY5Y or ISE6 tick cell-derived exosomes in relation to LGTV loads in naïve recipient SH-SY5Y host cells incubated with neuronal exosomes or human skin (HaCaT) cells incubated with tick cell-derived exosomes. Differences are shown as high, moderate or low.

NS1 protein at higher temperatures. We assume that it is a potential time-dependent adverse effect in the host body on the ability of exosomes to either interact or be taken up by their target cells efficiently, or that this could be a time-dependent effect in the host body on exosome stability. Further studies are needed to provide some mechanistic speculations as to why tick exosomes do better than neuronal exosomes at the same physiologically relevant temperature of 37 °C.

Treatment of both neuronal or arthropod exosomes with MgSO₄ showed lower viral transmission to naïve SH-SY5Y or human skin cells, respectively. In contrast to the tick-human skin model, SH-SY5Y cells also showed reduced loads when incubated with neuronal exosomes treated with (NH₄)₂SO₄. This data was further confirmed with reduced NS1 protein loads in the presence of magnesium or ammonium sulfate groups. Also, we did not observe the glycosylated band for NS1 in any of the neuronal samples upon salt treatments, indicating perhaps an influence of salts on the modification of viral protein stability. Taken together, this data suggests that both magnesium/ammonium sulfates affect the transmission of flaviviruses from infectious exosomes. It has been shown that low pH increases the yield of exosome production or isolation from host cells^[81]. Our data on neuronal or tick infectious exosomes treated at low/acidic pH (of 1.5 or 4) show increased transmission in recipient SH-SY5Y or skin cells, correlating with the above findings. Increased viral transmission and replication in naïve recipient cells may be due to increased stability of exosomes at acidic pH (of 1.5 or 4). Additionally, the consistency in reduced viral loads observed in recipient cells incubated with infectious neuronal/tick exosomes (treated at pH of 9 or 11.5) suggested that alkaline pH may inhibit/block exosome-mediated viral transmission to human cells. Our data further boost the reasons for the success of fluid-replacement therapy (with high alkaline fluids in combination with magnesium salts) that are given to dengue/Zika-infected patients with high fevers and thrombocytopenia. In addition, it is interesting to note that treatment of tick exosomes at alkaline pH (both 9 or 11.5) reduces viral transmission and replication in naïve recipient human skin cells. This observation further suggests that humans with alkaline body maintenance/topical application of solutions with high alkaline pH might get fewer tick bites and perhaps protect against the transmission of highly infectious flaviviruses. A summary of the findings from this study is shown [Figure 7]. Overall, our study represents a way to interfere with the transmission of flaviviruses and perhaps other vector-borne pathogens. We believe that this is an important study that could change the way we think about the approaches and strategies to interfere with the modes of pathogen transmission from vector to human and other vertebrate hosts.

DECLARATIONS

Acknowledgements

Authors would like to sincerely acknowledge the useful resource of reagents from BEI. “The following reagent was obtained through BEI Resources, NIAID, NIH: Tick Cell Line ISE6 Derived from *Ixodes scapularis* Embryos, NR-12234.” and “The following reagent was obtained from the Joel M. Dalrymple - Clarence J. Peters USAMRIID Antibody Collection through BEI Resources, NIAID, NIH: Monoclonal Anti-Langat Virus Nonstructural Protein 1 (NS1), Clone 6E11 (produced *in vitro*), NR-40308”.

Author's contributions

Performed cell cultures, exosome preparations/treatments, RNA/Protein extractions, qRT-PCR and immunoblotting analysis: Fasae KD

Supported in immunoblotting, exosome treatments and cell culture: Sultana H

Helped in molecular biology experiments including qRT-PCRs/analysis: Neelakanta G

Collected all required materials and reagents, designed and coordinated the entire study, organized all the data, wrote the manuscript, compiled and supervised overall investigations: Sultana H

Discussed, analyzed, and interpreted the data in several settings, read and edited the manuscript, and approved the submitted version: Fasae FD, Neelakanta G, Sultana H

Availability of data and materials

All the data presented in this study are included in the article and as figures/table, further inquiries can be directed to the corresponding author.

Financial support and sponsorship

Study with tick exosomes/HaCaT cells was supported by funding from National Institute of Allergy and Infectious Diseases (NIAID)/National Institutes of Health (NIH) (Award number R01AI141790 to PI; HS). Work with neuronal exosomes and SH-SY5Y cells is supported by start-up funds from College of Veterinary Medicine at University of Tennessee, Knoxville to HS and GN.

Conflicts of interest

The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Liquid biopsy for monitoring medulloblastoma

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How to cite this article: Eibl RH, Schneemann M. Liquid biopsy for monitoring medulloblastoma. *Extracell Vesicles Circ Nucleic Acids* 2022;3:280-91. <https://dx.doi.org/10.20517/evcna.2022.36>

Received: 31 Jul 2022 **First Decision:** 30 Aug 2022 **Revised:** 31 Aug 2022 **Accepted:** 14 Sep 2022 **Published:** 28 Sep 2022

Academic Editor: Yoke Peng Loh **Copy Editor:** Peng-Juan Wen **Production Editor:** Peng-Juan Wen

Abstract

Despite recent progress in molecular diagnostics defining four distinct medulloblastoma groups, the clinical management of these malignant childhood tumors of the cerebellum remains challenging. After surgical removal of the tumor, both cytotoxic chemotherapy and irradiation can offer additional curative benefits, but they also include a significant risk of long-term damage. Early molecular profiling aims to predict the outcome of such aggressive therapies. This prevents unnecessary damage to patients who may not need it and helps to identify those patients with remaining tumor cells who may benefit from more aggressive treatment with the intent to cure. Monitoring tumor evolution in real time allows personalized precision medicine with an immediate clinical response resulting in a better outcome. Liquid biopsy includes various methodologies already applied in numerous studies and clinical trials for common cancers including brain tumors, but information on medulloblastomas is limited. This review summarizes the recent developments of how liquid biopsy can support or even replace the standard monitoring of medulloblastomas by medical imaging or cytology and discusses what will be needed to make liquid biopsy a new gold standard in diagnosis, therapy, and follow-up of medulloblastomas for the benefit of the patients.

Keywords: Medulloblastoma, monitoring, CSF, cell-free DNA, cfDNA, circulating tumor DNA, ctDNA, biomarker, liquid biopsy, brain tumor



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INTRODUCTION

Medulloblastoma is the most common malignant brain tumor in children, but it can also develop in younger adults. As the father of modern neurosurgery, Harvey Cushing significantly reduced mortality by inventing better neurosurgical procedures a century ago. With Percival Bailey, he also coined the term medulloblastoma for this histological entity of posterior fossa tumors [Table 1]^[3]. Cushing tried local irradiation after surgery, but a major breakthrough with this technique was developed only later; when in 1953 not only the brain but the whole developing CNS including the spinal cord was irradiated to prevent metastatic growth. In the 1980s, cytotoxic chemotherapy was added. Unfortunately, both aggressive chemotherapy and irradiation can lead to severe damage to the CNS as well as, rarely, to secondary tumors^[25]. To avoid over- and undertreatment, it is tempting to identify those patients in advance who may benefit with intent to cure and those who may not need it. Therefore, it was important to realize that medulloblastoma *per se* does not exist, or it does not define such a homogeneous group of tumors as the name suggests. The term medulloblastoma still summarizes morphologically similar but biologically heterogeneous tumors of the cerebellum. The cell of origin remains unclear, but an embryonal origin is supported by single-cell sequencing studies and the peak incidence in early childhood^[9,26-30].

Meanwhile, it became clear that medulloblastomas are genetically distinct from primitive neuroectodermal tumors (PNET), although they share histologically indistinguishable characteristics such as tumor cell morphology, neuroblastic rosettes, and bipotential differentiation with the expression of glial and neuronal markers. A rat tumor model for PNETs developed by Eibl and Wiestler^[8,9], using gene transfer of SV40LT to inactivate tumor suppressor genes including TP53, also triggered the detection of the first TP53 mutations in medulloblastoma biopsies by Eibl three decades ago^[10] [Table 1]. Others were unable at that time to detect such TP53 mutations in tissue biopsies or in xenografts of human medulloblastomas, except in only one cell line^[31]; however, this mutation has been considered a common selection artifact during cell culture. The continued diagnostic and prognostic application of TP53 mutations in medulloblastomas supported further genetic profiling and helped to develop the current tumor classification^[8-10,32-41]: only recently, in 2016^[18] and with an update in 2021^[24], the World Health Organization (WHO) introduced four new diagnostic groups of this childhood brain tumor based solely on molecular genetic features [Table 2].

The correlation between different biological behavior and personalized risk assessment may prevent harmful radiation and chemotherapy when unnecessary or not useful. The first two groups refer to different oncogenic signaling pathways: (1) wingless/Integration-1 (WNT)-activated; and (2) Sonic Hedgehog (SHH)-activated. WNT-activated medulloblastomas show the highest five-year survival and a low prevalence of metastatic disease. SHH-activated medulloblastomas can be further separated into two different subgroups, TP53-mutant or TP53-wildtype. SHH-activated and TP53-mutant occur primarily in older children and have a very poor prognosis, whereas SHH-activated and TP53-wildtype, which are most common in adolescents and young children, have a good prognosis. The other two groups are non-WNT/non-SHH, Group 3 and Group 4, respectively. Group 3 shows an increased prevalence of metastatic disease with the poorest five-year survival, whereas Group 4 has an increased prevalence of metastatic disease with a moderate five-year survival. TP53 mutations in SHH medulloblastomas are associated with poor survival and treatment failures^[18]. Several subgroups have been associated with TP53 and other mutated genes: for WNT-activated, CTNNB1 and APC; for SHH-activated, TP53, PTCH1, SUFU, SMO, MYCN, and GLI2 (methylome); and, for non-WNT/non-SHH, MYC, MYCN, PRDM6, and KDM6A (methylome). Since the WHO classification suggests that the diagnosis from molecular profiling of a tissue biopsy is superior to that of classical histopathology, at least for brain tumors, it is tempting to apply ctDNA-based liquid biopsy for monitoring such mutations in brain tumor patients to avoid repeated and troublesome surgical biopsies. Newer studies successfully used panels of genes.

Table 1. Historical timeframe and developments leading to liquid biopsy of medulloblastomas

Year	Author	Probe	Method	Tumor	Milestone
1868	Ashworth ^[1]	CTC	Microscopy, case report	Skin metastasis of unknown primary tumor, "liquid autopsy"	First report on tumor cells in blood; post mortem; microscopically identical cells in metastatic lesions
1889	Paget ^[2]	CTC	Autopsy	Breast cancer, postulated	Seed and soil "theory of cancer metastasis"
1925	Bailey and Cushing ^[3]	Neurosurgically removed posterior fossa tumors	Histology	Medulloblastoma	Introduced the name medulloblastoma
1948	Mandel and Métais ^[4]	cfDNA	Blood analysis	Not related to cancer, healthy blood donors	First report of (cell-free) nucleic acids in blood
1953	Paterson and Farr ^[5]		Irradiation: 5000 cGy posterior fossa 3500 cGy neuraxis	65% 3-year survival	Irradiation treatment of the whole CNS
1975	Fidler ^[6]	CTC	Experimental metastasis assay	B16 melanoma cell lines	Only a small fraction of intravenously injected tumor cells give rise to metastasis in mouse models
1977	Leon et al. ^[7]	cfDNA	Radioimmunoassay for free DNA in serum	Various cancers	First report on increased DNA levels in some cancer patients; correlation with therapy response
1991	Eibl and Wiestler ^[8,9]	Experimentally induced tumors and derived cell lines	Retrovirus-mediated gene transfer of SV40 LT into neural transplants	PNET	Rat tumor model, histologically identical to human medulloblastomas (neuroblastic rosettes, bipotential differentiation), triggered medulloblastoma research in Germany
1991	Ohgaki, Eibl et al. ^[10]	Primary tumor tissue	SSCP-PCR, direct sequencing	Medulloblastoma	First detection of p53 mutations in primary medulloblastoma tissue by Eibl, supporting Eibl's earlier tumor model of inactivation of p53, also triggered medulloblastoma research
2001	Reya et al. ^[11]	CTC	Applying hematopoietic stem cell knowledge to the heterogeneity of cancer cells, self-renewal	Solid tumors and leukemia, migratory CSC	Cancer stem cell theory (Weissman/Clarke)
2003	Palaña et al. ^[12]	ctDNA	Methylation-specific PCR of MGMT, p16, DAPK, RASSF1A	GBM	Detection of methylated MGMT in serum highly predictive for response to BCNU chemotherapy
2004	Allard et al. ^[13]	CTC	CellSearch™	Prostate, breast, ovarian, CRC, lung, and other cancers	Detection of CTCs in 7.5 mL of blood samples
2004	Cristofanilli et al. ^[14]	CTC	CellSearch™ Amount of CTC	Metastatic breast cancer	Independent predictive marker: reduced PFS and reduced OS
2010	Pantel and Alix-Panabières ^[15]	CTC	Concept of analyzing tumor cells in body fluids	All cancers	Coined the term "liquid biopsy"
2014	Bettegowda et al. ^[16]	ctDNA	Digital PCR, sequencing	14 tumor types	ctDNA detectable for most tumors outside brain
2014	Sullivan et al. ^[17]	CTC	"Negative depletion" CTC-iChip (removing leukocytes from blood)	GBM (usually not metastatic)	Surprising and frequent detection of CTCs in brain tumors
2016	Louis et al. ^[18]	Tissue biopsy	Molecular profile	Medulloblastoma	New WHO classification, introducing four new medulloblastoma groups based on molecular genetics
2016	Donaldson and Park ^[19]	ctDNA	Clinical studies	NSCLC	First FDA ^[20] and EMA approval to use ctDNA for EGFR-targeted therapy
2018	Garzia et al. ^[21]	CTC	Parabiotic xenograft model	Medulloblastoma	Discovery of a hematogenous route of metastasis to leptomeninges by CCL2-CCR2 axis
2018	Cohen et al. ^[22]	ctDNA, plus proteins from blood	CancerSEEK, detecting mutations in 1933 loci of 16 genes; combined with protein tumor markers	8 cancer types	Blood screening test for several common cancers

2020	Lennon et al. ^[23]	ctDNA, protein markers plus PET-CT	Prospective 16 gene locations, 8 tumor proteins, PET-CT	Multi-cancer screening of 10,000 women with no known cancer	Multi-cancer blood testing combined with PET-CT
2021	Louis et al. ^[24]	Tissue biopsy	Molecular profile, incl. methylation profile	Medulloblastoma	Newest WHO classification, four molecular groups further defined by methylome; additional subgroups (4 SHH; 8 non-WNT/non-SHH)

CRC: Colorectal cancer; CSC: cancer stem cell; CTC: circulating tumor cell; ctDNA: circulating tumor DNA; EMA: European Medicines Agency; FDA: US Food and Drug Administration; PCR: polymerase chain reaction; PET-CT: positron emission tomography-computed tomography; SSCP: single-strand conformation polymorphism.

Table 2. Molecular classification of four groups of medulloblastomas according to the WHO

Medulloblastoma, molecularly defined	Pathway
Group 1	WNT-activated
Group 2	SHH-activated and TP53-wildtype SHH-activated and TP53-mutant
Group 3	(non-WNT/non-SHH)
Group 4	(non-WNT/non-SHH)

SHH: Sonic hedgehog; WHO: world health organization; WNT: wingless/integration-1.

For brain tumors including medulloblastomas, CSF offers another chance to find ctDNA with a higher sensitivity than plasma or serum^[42-46]. ctDNA from CSF represents the genomic mutations better than plasma, and CSF shows an increased sensitivity for putative actionable mutations and CNA (copy number aberrations; EGFR, PTEN, ESR1, IDH1, ERBB2, and FGFR2)^[47]. This improves prognostic evaluation, therapy decisions, and monitoring of treatment, e.g. irradiation, chemotherapy, and future immune therapies.

It is reasonable to apply this molecular expertise from classical tissue biopsy to liquid biopsy in order to improve the monitoring of tumor evolution and response to treatment, as well as to avoid elaborate surgical biopsies with a higher risk of neurological or infectious complications. Tumor-derived, cell-free nucleic acids (cfDNA/RNA) and extracellular vesicles (EV) can be found outside of the original tumor in body fluids, such as blood, cerebrospinal fluid (CSF), peritumoral cysts, and urine. Surprisingly, even intact circulating tumor cells (CTC) can be found in blood and CSF [Figure 1]. Analysis of cell-free nucleic acids allows improved and personalized monitoring of patients to adapt rapidly to new therapy decisions without the higher risk of neurosurgical tissue biopsies. Here, we provide an overview of recent developments without an emphasis on technological methods and details, but with the clinical application potential, as well as current limitations and challenges in how future standards need to be developed in order to improve the clinical management of medulloblastomas within the next few years.

LIQUID BIOPSY

Within the past two decades, different methodologies have evolved that can be summarized as liquid biopsy [Figure 1]^[50,51]. In principle, tumor-derived material, including whole cells or parts thereof such as nucleic acids and extracellular vesicles (EV), can be detected at locations quite distant from the original tumor or its metastases. This can often be achieved with easy and less risky access than a classical surgical tissue biopsy. Whereas blood tends to be the biofluid of choice for many other cancers, CSF appears to be more suitable for brain tumors. This is partly due to the blood-brain barrier (BBB), preventing cells from entering the bloodstream. CSF also offers less background in terms of leukocytes or cell-free DNA compared to blood, resulting in a better signal-to-noise ratio for many analytical methods. Many medulloblastoma patients

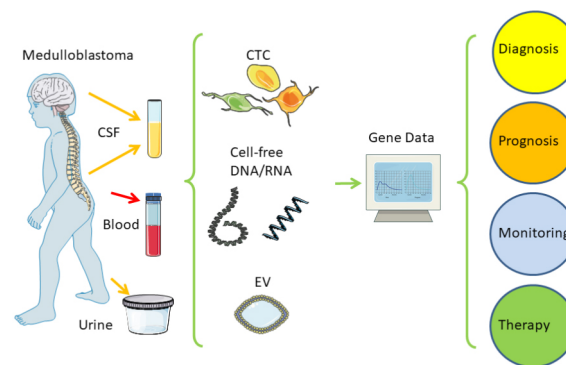


Figure 1. Liquid biopsy of medulloblastomas. Distant to the cerebellum, body fluids such as blood, CSF, or urine can be taken at low risk and then analyzed for relevant genetic information from the childhood brain tumor to support clinical decision making. CSF: Cerebrospinal fluid; CTC: circulating tumor cell; EV: extracellular vesicle. Created/modified with SMART^[48,49].

develop hydrocephalus which is commonly drained before tumor removal. This can also be an easy source for obtaining CSF, in addition to standard lumbar puncture for follow-ups. The historical timeframe for the development of liquid biopsy and medulloblastoma research is shown in Table 1.

ctDNA

ctDNA is a varying part from much less than 1% to 10% of the total cell-free DNA. Individual changes in ctDNA amounts often correlate with tumor development [Figure 2]^[50]. An increase of ctDNA can point to metastatic progression, whereas a reduction of ctDNA indicates a treatment response. No reduction of ctDNA after treatment indicates a lack of response. A later increase after an initial decrease indicates resistance development. More diagnostic and prognostic information comes from sequence analysis, which can be targeted or non-targeted to detect mutations or epigenetic signatures of methylation in the tumor.

CTCs, EVs, miRNA, circRNA and other biomarkers

In 2004, the detection of CTCs with CellSearch was approved for clinical use to detect and count the number of CTCs per blood sample. This detection system uses an epithelial marker to select carcinoma cells, but this marker is not present in brain tumors. Other methods or modifications need to be used and further developed to reach the required sensitivity on brain tumors. Since CTCs are extremely rare, it was surprising to detect CTCs in the blood of glioblastoma patients^[17,52-57], an aggressive brain tumor usually found in adults. In 2018, Garzia and colleagues were able to detect CTCs in the blood of medulloblastoma patients^[21]. Those patient-derived CTCs were able to spread in a xenograft model via the blood to form leptomeningeal metastases, thus questioning the general assumptions of medulloblastomas spreading only, or preferentially, via the CSF. A chemokine highly expressed on medulloblastoma cells, CCL2, was identified with its receptor CCR2 to drive this leptomeningeal homing. Similar potential mechanisms of organ-specific metastasis involving chemokines and their receptors, thus mimicking lymphocyte homing, have been investigated, including at the single-molecule level, with atomic force microscopy^[58-65], but not yet with medulloblastoma cells.

Tumor and normal cells can release small, extracellular vesicles (EVs), which protect their included proteins and different sorts of nucleic acids. Recently, EVs from blood, urine, or tissue samples have sparked great interest in liquid biopsy. EVs collected from CSF appear to be superior due to the reduced number of EVs from leukocytes compared to blood^[66]. EVs from urine can also be used to obtain EV-encapsulated marker candidates with a nanowire scaffold, even from non-urollogic cancers^[67].

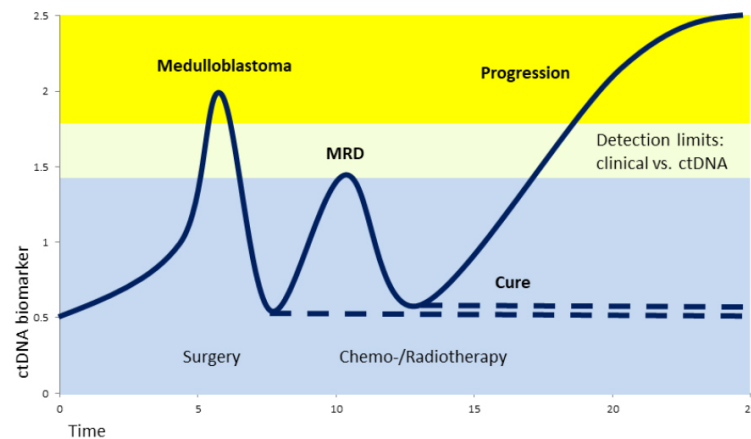


Figure 2. Scheme of ctDNA biomarker level during medulloblastoma development, therapy, and progression. Sequential analysis of CSF supports diagnosis and early detection of minimal residual disease (MRD) as well as clinical decisions for the best benefit of the patient. CSF: Cerebrospinal fluid.

MicroRNAs (miRNA, miR) are 20-24 nucleotides long, non-coding RNA molecules with regulatory and stabilizing effects of translating mRNA. miRNAs seem to play a role in tumor biology, angiogenesis, and immunology. Although their functions are not fully understood, they can serve as markers or potential therapeutic targets in glioblastomas^[68]. In a diagnostic model, urinary miRNA detection was able to confirm different CNS tumors, including neuroblastoma, with high sensitivity and specificity^[69].

Circular RNAs (circRNAs) consist of a closed loop without polyadenylation signal. They appear to be more stable than miRNAs and seem to be similarly involved in gene regulation, although their functions are not well understood. Many circRNAs may just serve as a sponge for miRNAs, thus inactivating the function of a specific miRNA. Due to their stability, they may serve as candidate markers for disease, e.g. circ_463 for medulloblastomas^[70].

Proteomic analyses from blood, CSF, or urine may also reveal protein-based biomarkers for screening and monitoring of medulloblastomas in the future. Recently, bioinformatics allowed discriminating medulloblastoma patients from healthy individuals by analyzing a combination of potential protein biomarkers in urine samples^[71].

Many studies have been developed by academic collaborations leading to publications that offer significant access to data for reproducibility, metanalysis, and data mining. Large clinical studies usually have major industrial findings and may have more restrictions for sharing data, but they may also eventually allow at least partial access to data. As in many developing fields, there is a demand for data to satisfy the FAIR principles of “findable, accessible, interoperable, and reusable data”^[72], which may help to compare different studies.

CLINICAL STUDIES

Few studies on medulloblastomas have been developed recently in the field of liquid biopsy with varying levels of success, but they basically confirm CSF as currently the most suitable source to analyze ctDNA, followed by blood and urine [Table 3]. Unfortunately, the challenges of repeatedly isolating sufficient amounts of ctDNA even from CSF appear to be high, thus reducing the sensitivity and applicability for many medulloblastoma patients. Furthermore, the low number of mutations in medulloblastomas poses a source for artifacts and needs further evaluation. More clinical studies are needed to establish suitable

Table 3. Studies using ctDNA or ctRNA from CSF for screening or monitoring medulloblastomas

Year	Author	Tumor	Method	Findings
2020	Escudero et al. ^[73]	MB	WES, CNVs	ctDNA from CSF sufficient for diagnosis of MB-subgroups, risk stratification and monitoring (proof of concept study)
2020	Li et al. ^[74]	Pediatric MB	Whole genome methylation sequencing	High specificity and sensitivity to monitor treatment response of epigenetic signatures in ctDNA from CSF, potential diagnostic and prognostic value
2021	Liu et al. ^[75]	MB	WGS	ctDNA from serial CSF samples as prospective marker for MRD, in half of the patients before radiographic progression
2021	Sun et al. ^[76]	Pediatric MB	Deep sequencing/NGS, ctDNA in CSF	More alterations detectable in ctDNA from CSF than from primary tumor, superior monitoring technique when ctDNA is detected from CSF
2022	Lee et al. ^[70]	MB	RT-PCR sequencing	Circular RNA circ_463 as a candidate biomarker
2022	Pagès et al. ^[77]	Pediatric CNS tumors, incl. MB	ULP-WGS, deep sequencing of specific mutations and fusions	ctDNA is detectable better in CSF than blood, not in urine. Molecular profiling is feasible for a small subset of high-grade tumors (incl. MB). Liquid biopsy remains a major challenge for such tumors with low clonal aberrations
2019-2024	NCT03936465 ^[78] ongoing Phase I study, 66 patients	Pediatric cancer, incl. brain tumors	ctDNA	Clinical toxicity study; ctDNA markers in blood and CSF planned as a response to treatment

CNV: Copy number variation; CSF: cerebrospinal fluid; MB: medulloblastoma; MRD: minimal residual disease; ULP-WGS: ultra-low-pass whole-genome sequencing; WES: whole exome sequencing.

standards. Currently, ctDNA as a routine marker for tumor monitoring appears to be useful mainly for subsets of medulloblastomas, i.e. progressed high-grade tumors, those with a close connection to the CSF, or for larger children or adult patients with easier access to sufficient amounts of CSF and blood.

As a proof-of-concept study, Escudero and colleagues showed that ctDNA from CSF can provide valuable information about diagnosis and prognosis^[73]: the genomic alterations represent and characterize the heterogeneity of the tumor and allow the identification of medulloblastoma subgroups and subtyping with risk stratification. In all cases, the CSF was negative from the cytologic analysis, i.e. no intact tumor cells were detectable. Prior to the analysis of ctDNA, somatic mutations from matched tumors were detected with WES and then validated for ddPCR. Before surgery, CSF-ctDNA was detectable in 77% of patients, but not in the plasma, except in 1 of 13 patients. This sensitivity demonstrates the feasibility and superiority of CSF-ctDNA above both cytology from CSF and ctDNA from plasma. CSF-ctDNA monitoring can identify the minimal residual disease (MRD) and genomic tumor evolution.

Since oncogenic mutations in medulloblastomas appear to be much less frequent than those in most other tumors, Li and colleagues used a different approach for tumor monitoring of serial CSF samples^[74]. Epigenetic changes were reliably detected by whole-genome methylation sequencing (WGMS). DNA methylation as well as hydroxymethylation profiles from CSF matched the signatures from the original tumors in the same patients, thus allowing ctDNA to be used in monitoring treatment response and tumor recurrence. High sensitivity to detect MRD was shown in serial samples after treatment, even when the cytology was negative. The high specificity and sensitivity of these epigenetic signatures from CSF samples may be used for diagnostics and prognosis.

In a prospective trial, Liu and colleagues confirmed the clinical utility of CSF-ctDNA with 476 serial samples from 123 children with medulloblastoma^[75]. Low-coverage whole genome sequencing allowed the detection of 54% of localized and 85% of metastatic disease cases at baseline. Response to therapy is shown by a reduction of ctDNA, whereas persistent detection after therapy points to a higher risk of progression. ctDNA as a surrogate marker for MRD can detect tumor progression earlier than MRI or CSF cytology.

Primary tumors located adjacent to the CSF reservoir allowed Sun and colleagues to isolate and investigate ctDNA from 15 out of 58 patients with medulloblastoma^[76]. Alterations between primary tumor and CSF-ctDNA are shared, but more alterations were detected in the CSF-ctDNA, which may reflect the evolution of the tumor as well as the heterogeneity of the primary tumor. Undetectable ctDNA was associated with complete remission after surgery, but it was also found in tumors with no direct access to the CSF. Gene panels with 500 and 952 genes were used to analyze and compare tissue DNA with ctDNA obtained from CSF and plasma. Mutations detected in CSF were: KMT2D (32.0%), KMT2C (28.0%), SMARCA4 (24.0%), BCOR (20.0%), TP53 (12.0%), PTCH1 (8%), EP300 (8%), NF1 (8%), SETD2 (8%), MED12 (8%), SPEN (8%), CTNNB1 (4%), CREBBP (4%), PIK3CA (4%), LRP1B (4%), and FBXW7 (4%)^[76]. Mutations detected in plasma were attributed to possible damage to the blood-brain barrier facilitating the entry of ctDNA into the bloodstream. CSF-ctDNA can predict disease progression and can detect more mutations than matched tissue. This may help in diagnosis, monitoring, and targeted therapy.

Lee and colleagues analyzed the CSF from medulloblastoma patients and identified metabolites, lipids, and cancer-specific RNAs for hypoxia, as well as cancer-specific RNAs^[70]. Although subgrouping was challenging and not the primary goal, the study was able to reveal a group of omics signatures to separate cancer from normal CSF. A novel circular RNA, circ_463, as a sensitive biomarker for recurrence should be validated in further clinical studies.

Pages and colleagues confirmed a major challenge of very low ctDNA levels in 258 pediatric CNS tumors of 13 different tumor types, mostly low-grade gliomas ($n = 102$), but also including almost 10% of embryonal tumors including medulloblastomas ($n = 27$)^[77]. Harvesting ctDNA from CSF allowed the detection of CNAs in 20% and sequencing alterations in 30% of the samples, whereas plasma reached only detection sensitivities of 1.3% and 2.7%, respectively. Urine samples were all negative. Therefore, molecular profiling of ctDNA appears to be feasible for only a small subset of primary CNS tumors in children, such as medulloblastomas and other high-grade tumors. The low number of clonal aberrations in most medulloblastomas poses a challenge for the clinical application of sequencing methods.

Despite over 300 “medulloblastoma” studies listed on the clinicaltrials.gov webpage, only one includes “ctDNA” as a monitoring marker in blood and CSF. Therefore, most clinical studies on medulloblastomas focus on standard MRI and CSF cytology monitoring for measuring progression-free survival (PFS) and overall survival (OS).

CONCLUSION

MRD-guided clinical decisions are now the standard of care for pediatric leukemia, sparing toxic therapy if not needed and identifying poor patient responses for more aggressive treatments. To achieve similar success in medulloblastoma management, sensitive and specific markers for the detection of MRD in medulloblastoma patients need to be confirmed. Recent progress in diagnostics and subtyping of medulloblastomas with risk stratification based on genetic profiling of primary tumor samples can reproducibly be applied to ctDNA as long as sufficient amounts can be isolated from body fluids of the patient. Gene alterations found in ctDNA such as TP53, PTCH1, MYCN, GLI2, SUFU, and 17p loss represent the original tumor and allow a less invasive molecular diagnosis and prognosis. Currently, CSF is the preferred source of ctDNA and appears to be much more sensitive than blood or urine. Varying but promising results can be obtained for early molecular diagnosis, even before the removal of the tumor. For some but not all patients, sequential analysis of ctDNA also allows close monitoring of tumor development after treatment. Compared to standard clinical monitoring by MRI and CSF cytology, ctDNA can detect MRD and relapse up to several months earlier, which opens a window for a better outcome. Early risk

stratification should help the clinician to identify those patients who may benefit from aggressive chemo- and radiotherapy with the intent to cure as well as other patients who may not need it to protect them from unnecessary long-term damage including direct neurologic damage and secondary tumors. Ideally, CSF should be acquired shortly before and four weeks after the resection, followed by regular controls connected to clinical events. The clinical use of ctDNA analysis in medulloblastoma management can offer clear advantages over standard monitoring by MRI and cytology of CSF. The challenges of sensitivity to low amounts of ctDNA and artifacts are well defined and may be overcome with only small steps in technology improvement, including new devices and methods, and then providing the next gold standard. Fast and high-quality processing of CSF may improve the ctDNA quality and should be validated in larger clinical studies. In contrast, the potential use of CTC detection in medulloblastomas currently appears to be a much bigger challenge and most likely only restricted to highly specialized academic environments. Due to the low mutation rate in medulloblastomas, epigenetic markers, as well as specific circRNAs, should be included as markers for MRD in addition to classical mutation profiles. Ongoing progress in analytical methods, including proteomics and the potential role of EVs, as well as the reduction of artifacts, may offer new chances to establish liquid biopsy not only from smaller samples of CSF but also from blood or urine. After a century of milestones in neurosurgery, irradiation, and chemotherapy, the new molecular classification of medulloblastomas will progress with ctDNA from CSF as a promising biomarker for early diagnosis and better monitoring for improved clinical management of these childhood brain tumors.

DECLARATIONS

Acknowledgments

We thank Stuart Fraser for helpful comments on the manuscript.

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis, data acquisition and interpretation, as well as provided administrative, technical, and material support: Eibl RH, Schneemann M

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Review

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Extracellular vesicles in reproduction and pregnancy

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How to cite this article: Smith TI, Russell AE. Extracellular vesicles in reproduction and pregnancy. *Extracell Vesicles Circ Nucleic Acids* 2022;3:292-317. <https://dx.doi.org/10.20517/evcna.2022.27>

Received: 27 May 2022 **First Decision:** 21 Jul 2022 **Revised:** 30 Aug 2022 **Accepted:** 14 Sep 2022 **Published:** 30 Sep 2022

Academic Editors: Yoke Peng Loh, Carlos Salomon **Copy Editor:** Peng-Juan Wen **Production Editor:** Peng-Juan Wen

Abstract

Extracellular vesicles (EVs) are small, lipid-bound packages that are secreted by all cell types and have been implicated in many diseases, such as cancer and neurodegenerative disorders. Though limited, an exciting new area of EV research focuses on their role in the reproductive system and pregnancy. In males, EVs have been implicated in sperm production and maturation. In females, EVs play a vital role in maintaining reproductive organ homeostasis and pregnancy, including the regulation of folliculogenesis, ovulation, and embryo implantation. During the development and maintenance of a pregnancy, the placenta is the main form of communication between the mother and the developing fetus. To support the developing fetus, the placenta will act as numerous vital organs until birth, and release EVs into the maternal and fetal bloodstream. EVs play an important role in cell-to-cell communication and may mediate the pathophysiology of pregnancy-related disorders such as preeclampsia, gestational diabetes mellitus, preterm birth, and intrauterine growth restriction, and potentially serve as noninvasive biomarkers for these conditions. In addition, EVs may also mediate processes involved in both male and female infertility. Together, the EVs secreted by both the male and female reproductive tracts work to promote reproductive fertility and play vital roles in mediating maternal-fetal crosstalk and pregnancy maintenance.

Keywords: Extracellular vesicles, reproduction, pregnancy, preeclampsia, gestational diabetes mellitus, preterm birth, intrauterine growth restriction, infertility, maternal-fetal crosstalk, spermatogenesis



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INTRODUCTION

Extracellular vesicles (EVs) are small, lipid bound packages [Figure 1] that are secreted by all cell types and are thought to play a role in both normal, homeostatic mechanisms, and several diseases including cancer^[1], autoimmune disorders^[2], and neurodegenerative disorders^[3,4]. These particles are naturally released as a form of intercellular communication^[5] and can be found in all biological fluids, including saliva, urine, cerebral spinal fluid, and intravascular fluids. They function to carry bioactive molecules like RNA and DNA^[6-8], amino acids, lipids, and metabolites throughout the body^[9], and have been shown to influence various regulatory mechanisms such as skin cell development^[10], immune system activation^[11-13], and antitumor responses^[14].

Intercellular communication via EVs can contribute to both physiological and pathological changes in target cells^[15] as they play a major role in homeostasis and various diseases, such as cancer and neurodegenerative disorders^[16,17]. Cancer is characterized by cells that divide uncontrollably and develop the ability to destroy normal body tissues. EVs have been involved in metastatic seeding which occurs when secondary tumors develop in tissues other than the tissue the cancer originated in^[18]. EVs have also been implicated in several neurodegenerative diseases such as Parkinson's disease, prion disease and Alzheimer's disease^[16,19]. In these cases, EVs contain pathological cargo and contribute to the spread of pathology from their cells of origin, into the extracellular environment^[15,20,21] and to both neighboring and distal cells. For example, EVs injected into the tail vein of mice were eventually observed in the lungs, spleen, bone marrow, and liver^[22], indicating that EVs originating in one location can influence cells in other areas of the organism.

INVOLVEMENT IN THE REPRODUCTIVE SYSTEM

Part of maintaining homeostasis includes the maintenance of the reproductive organs, however, the role of EVs within this system is still under intense investigation.

Extracellular vesicles in the male reproductive system

The male reproductive system consists of the testes, the epididymis, accessory glands, and the penis^[23] [Figure 2A]. Sperm are produced within the testes and begin to mature as they move through the epididymis where they are stored. Prior to ejaculation, sperm move from the epididymis to the vas deferens, which join with the seminal vesicles and prostate gland. Together with the bulbourethral glands, the seminal vesicles and prostate produce the seminal fluid that sperm mix with to produce semen. EVs have been shown to be associated with sperm development and maturation at various locations within the male reproductive tract.

In the testes

Sperm are continuously produced by spermatogonial stem cells (SSC) within the testes' seminiferous tubules^[24]. The stem cell niche plays a key role in SSC proliferation and EVs released from developing sperm cells can suppress the proliferation rates of these SSCs, potentially serving as negative regulators of spermatogenesis^[25]. Additionally, Sertoli cells within the seminiferous tubules have also been reported to potentially release EVs, which may impact spermatogenesis as well^[26]. Testicular EVs [Figure 2B] have also been shown to be taken up by developing sperm cells at various points in spermatogenesis, and by epithelial cells within the seminiferous tubules, likely serving as important intercellular communicators^[27]. The majority of studies examining male reproductive tract EVs however, focus on those produced in the epididymis or within seminal fluid.

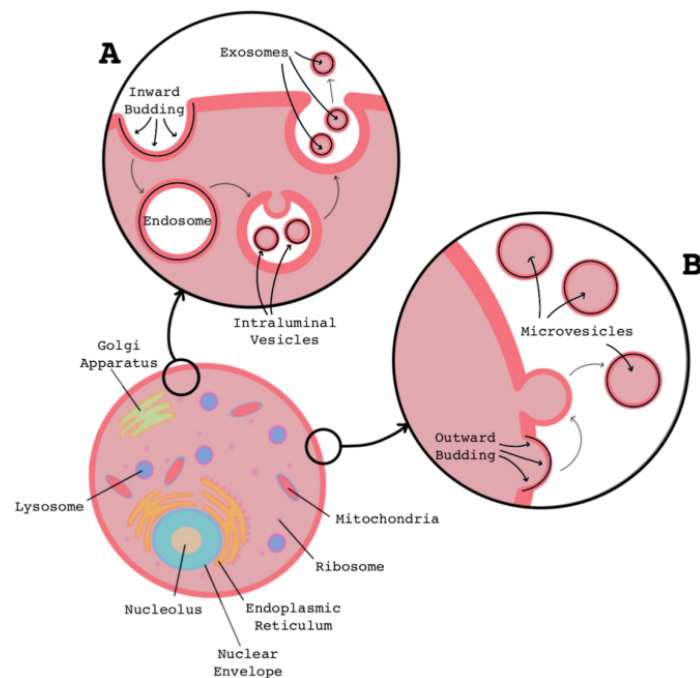


Figure 1. Common mechanisms of extracellular vesicle biogenesis. Formation of exosomes (A) begins with the inward budding of the cell's plasma membrane to create an endosome. The plasma membrane of the endosome then invaginates creating intraluminal vesicles. The endosomal plasma membrane can then fuse with the cell's plasma membrane and release the intraluminal vesicles into the extracellular space as exosomes. Microvesicles (B) are formed as the cell's plasma membrane blebs outwards and sheds, releasing vesicles into the extracellular space.

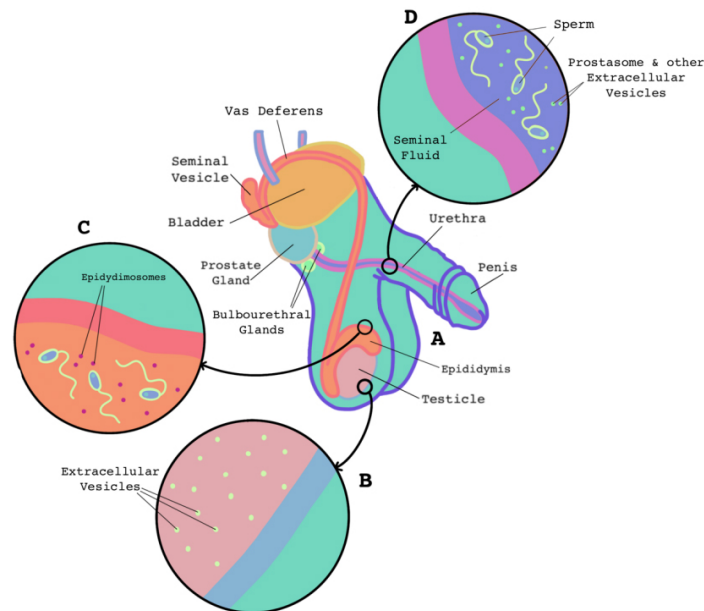


Figure 2. Anatomical structure and location of EVs in the male reproductive system. The primary components of the male reproductive tract (A) consist of the testes where sperm are made, the epididymis where sperm mature, the vas deferens which transports sperm to the seminal vesicles and prostate gland which produce seminal plasma, the urethra, and the penis. EVs have been identified in the testes (B) and may influence sperm production. EVs found in the epididymis (C) are referred to as epididymosomes. EVs in seminal fluid are produced by the seminal vesicles and prostate and are mixed with sperm as it enters the urethra (D). EVs: Extracellular vesicles.

In the epididymis

Sperm are produced in the testes and as they move through the epididymis, they mature and acquire the ability to fertilize an ovum. EVs located in the epididymis, also known as epididymosomes [Figure 2C], were first observed in hamsters^[28] and have now been found in other mammals including mice^[29,30], rats^[31], bulls^[32], rams^[33], and humans^[34]. The protein cargo found in epididymosomes typically includes various enzymes, as well as adhesion, structural, and trafficking molecules and can act as a vehicle for modulating the molecular structure of sperm^[28,34-37]. More specifically, epididymosomes carry proteins including, but not limited to, sorbitol dehydrogenase, hexokinase 1, acrosin, and zona pellucida binding protein 1 and 2 that interact with receptors located on the head of the sperm and mediate processes associated with maturation, protection, and the acrosome reaction^[38,39].

Interestingly, epididymosomal cargo varies depending on which segment of the epididymis they are isolated from^[35,36,40]. Microarray data suggest that miRNAs are differentially expressed in cauda versus caput epididymosomes, but a large majority of these miRNAs are expressed at similar levels in the epididymal epithelial cells from which they are derived^[40]. Conversely, small RNA sequencing data reveal that epididymosomes isolated from the cauda epididymal segment have a more complex miRNA profile relative to caput epididymosomes, and these miRNA profiles differ from those observed in the epididymal epithelial cells from which they are derived^[36]. These disparate findings may be attributed to a number of methodological differences in sample processing, the techniques used for vesicle and RNA isolations, and how miRNAs are profiled (microarray vs. sequencing). Species differences may also impact epididymosomal miRNA profiles.

As sperm travel through the segments of the epididymis, their miRNA signature changes, and they acquire various capabilities such as ample motility and the ability to fertilize the female ovum^[41,42]. Sperm are not able to perform de novo synthesis of proteins or RNAs, so many of these changes can be attributed to the uptake of epididymosomes^[40,43-46].

Arrdc4 is important for sperm cells to develop fertilization capacity within the epididymis^[47]. *Arrdc4* is a member of the α -arrestin protein family and plays a role in EV biogenesis^[48]. When compared to *Arrdc4*^{+/+} cells, *Arrdc4*^{-/-} epididymal epithelial cells exhibited significantly reduced epididymosome production and interestingly, *Arrdc4* knockout mice produce sperm with impaired functionality^[47]. Impaired sperm functionality could be rescued by incubating *Arrdc4*^{-/-} sperm with wild type epididymosomes, indicating that *Arrdc4* is critical for both normal epididymosome biogenesis and sperm maturation^[47].

In addition to facilitating sperm maturation, epididymosomes also appear to be the main source of non-coding RNAs found in sperm, and these RNAs can alter the epigenetic inheritance patterns of offspring^[49-53]. Epididymosomes may also play an important role in quality control of sperm cells as well by transferring epididymal sperm binding protein 1 (ELSPBP1) to dead sperm cells, possibly to protect viable sperm in the vicinity from dead or dying sperm^[54].

In the seminal vesicles and prostate

Prior to ejaculation, sperm travels up the vas deferens and mixes with the seminal plasma produced by the seminal vesicles and prostate gland to create semen [Figure 2D]. Seminal plasma consists of sugars, salts, amino acids, and other compounds necessary to maintain sperm viability. EVs have also been observed in seminal fluid since the late 1960's, however their main physiological function is still not yet fully understood^[55]. Commonly, seminal fluid EVs are referred to as prostasomes, which refers to a specific type of EV released from prostate epithelial cells; however, EVs in seminal fluid are highly heterogeneous and are

likely derived from a number of cell types within the male reproductive tract^[56-59].

Seminal fluid EVs are thought to play a number of important roles in sperm functionality including forward sperm motility, capacitation, acrosomal reaction, and membrane stabilization^[60-68]. Interestingly, prostasomes have been shown to inhibit spontaneous capacitation and acrosomal reactions by decreasing spermatozoa tyrosine phosphorylation^[65], and only after capacitation has been initiated do they fuse to sperm^[64]. Indeed, it seems that seminal fluid EVs only interact with sperm after they have been introduced to the female reproductive tract^[69]. Proteomic analysis of prostasomes revealed over 139 proteins, including prostate-specific antigen and prostatic acid phosphatase^[70]. One third of the identified proteins were categorized as enzymes suggesting prostatic cargo may have the capacity to influence a cell's metabolic state, while the remaining proteins function primarily as transport and structural proteins, GTP proteins, chaperones, and signal transduction proteins^[70].

Semen is highly enriched in EVs containing a variety of small RNAs, including miRNA, tRNA, Y RNAs, and fragments of mRNAs^[71]. In humans, the most abundant miRNAs were from the let-7 family, followed by miR-148a, miR-375, and miR-22; these miRNAs all have validated immune-related mRNA targets, suggesting that they may modulate the immune function of semen in the female reproductive tract^[71]. In boar semen, miR-21-5p, miR-148a-3p, miR-10a-5p, miR-10b, miR-200b, and the let-7 family are the most abundant EV-associated miRNAs^[72]. Interestingly, EV-associated miR-21-5p has been found to potentially reduce sperm fertility, and along with miR-148a and the let-7 miRNAs, may impact immune function^[72].

Extracellular vesicles in the female reproductive system

The female reproductive system consists of the ovaries, fallopian tubes, uterus, and vagina [Figure 3A]. Within the ovary, eggs are surrounded by follicular fluid. During ovulation, an egg is released from the ovary and brought into the fallopian tube, filled with oviductal fluid. The egg then travels down the fallopian tube to the uterus where, if fertilized, it will become implanted into the uterus and begin to develop into an embryo.

The role of EVs in the female reproductive system is heavily understudied but is beginning to gain traction, especially in livestock like cows, horses, and sheep. In humans, EVs have been detected in follicular fluid, oviductal fluid, the intrauterine environment, and in the vagina [Figure 3].

In the ovaries

Follicular fluid (FF) is the liquid content that surrounds the developing oocyte, the cell in an ovary that has the potential to form an ovum [Figure 3B]. Within the ovary, mural granulosa cells line the follicle and communicate with the cumulus-oocyte complex (COC; specialized granulosa cells that surround and support the oocyte). Prior to ovulation, the COC expands, allowing for the oocyte to undergo complete meiotic maturation and likely occurs due to communication between granulosa cells and the COC^[73]. EVs within the FF (FF-EVs) have been shown to induce COC expansion and this mechanism may be mediated by miRNAs within these EVs^[74,75]. Indeed, FF-EVs have been shown to be taken up by granulosa cells, which further supports the notion that these EVs impact follicle growth and development^[76]. Human derived FF-EVs were previously shown to contain miRNAs that target genes associated with the inhibition of follicular maturation and the resumption of meiosis, including miR-132, miR-212, and miR-214^[77]. Conversely, another group demonstrated that FF-EVs arrest meiotic maturation of oocytes through the CNP-NPR2 signaling pathway^[78]. These differences in EV mediated effects may be attributed to changes in their molecular cargo at differing times in the menstrual cycle. Indeed, several studies identified significant changes in EV cargo at different phases of the menstrual cycle, suggesting that FF-EVs and their cargo are

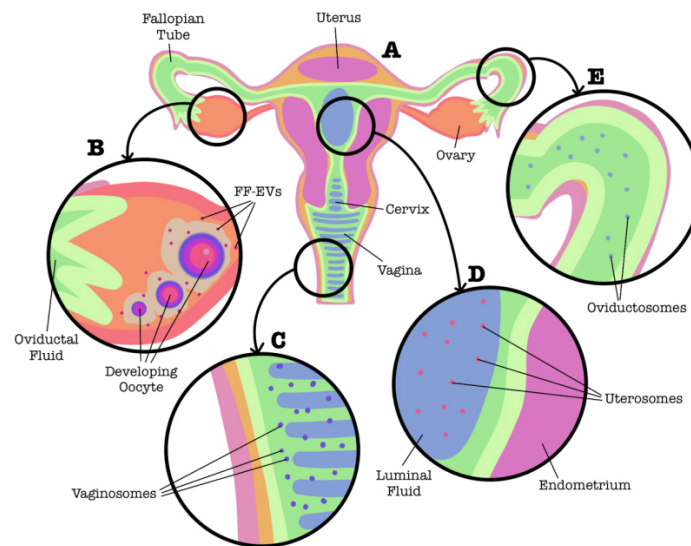


Figure 3. Involvement of EVs in the female reproductive system (A). In female organisms of many species, secretions from the epithelial lining of the ovary (B), cervix and vagina (C), endometrium (D), and fallopian tubes (E) contain EVs. EVs: Extracellular vesicles.

dynamic players in folliculogenesis^[79-81].

The FF is a rich source of EVs and through proteomic profiling, hundreds of proteins have been identified^[76,82,83]. Many of these proteins are associated with protein and RNA folding, molecular transport, and signal transduction, and likely play important roles in oocyte competence and follicular homeostasis^[82]. Small RNA sequencing of extracellular RNA isolated from FF reveals the presence of numerous types of noncoding RNAs, including miRNA, snRNA, snoRNA, and tRNA^[84,85]. Separation of EVs from the FF supernatant reveal significant overlap in expression of several miRNAs that target genes involved in reproduction, cell proliferation, and immune system development^[84]. The mRNA profiles contained within FF-EVs have also been sequenced, and many of the identified sequences code for proteins involved in metabolic pathways, DNA-protein interactions, and transcriptional regulation^[85,86].

Proper signaling of the transforming growth factor- β (TGF- β) pathway is required for follicular development and oocyte competence^[87]. FF-EVs from horses have been shown to contain ACVR1 (a member of the TGF- β superfamily) mRNA and protein, as well as miRNAs that regulate expression of ACVR1, including miR-27b, miR-372, and miR-382^[76,88]. Porcine and bovine FF-EVs were also found to contain miRNAs that target genes involved in the TGF- β signaling pathway^[89,90]. These data further support the notion that FF-EVs participate in mediating follicular development and homeostasis.

FF-EVs have also been shown to be taken up by and alter the transcriptome of epithelial cells that line the fallopian tubes, leading to the expression of genes that would assist in increasing the likelihood of fertilization and embryo development^[91].

In the fallopian tubes

The fallopian tubes, or oviducts, are filled with fluid produced by secretions from epithelial cells that line the oviducts and the transudation from the blood [Figure 3E]. This oviductal fluid also contains EVs which are referred to as oviductosomes^[92].

Oviductosomes have primarily been studied within the context of fertilization as they have been shown to transfer key proteins to sperm within the female reproductive tract (discussed later in "Extracellular vesicles and fertilization" below). Proteomic analysis of feline oviductosomes revealed over 1000 proteins, many of which are involved in metabolism and cellular organization^[93]. The bovine oviductosome proteome was less diverse with slightly over 300 proteins identified, however gene ontology analysis revealed the majority of these proteins were also involved in metabolism and localization^[94].

The protein and RNA cargo of bovine oviductosomes has been shown to fluctuate throughout the course of the estrous cycle^[95]. Similar findings were observed for murine oviductosome miRNA and metabolomic profiles as well^[96,97]. These data suggest that oviductosome composition is under hormonal regulation.

In the uterus

Extracellular vesicles found in the luminal fluid of the uterus [Figure 3D] are commonly referred to as uterosomes^[98]. The protein and RNA cargos of these vesicles differ from the endometrium lining, and change during pregnancy^[99,100]. Expectedly, the estrous cycle also affects the release and cargo composition of uterosomes as well^[100-102]. Uterosome associated proteins released from endometrial epithelial cells appear to be involved in key embryo-implantation mechanisms, indicating that these vesicles play important roles in early pregnancy^[102]. Aside from being released from endometrial cells, it has also been shown that uterosomes can be taken up by endometrial epithelial cells and significantly alter their transcriptome^[103].

In the vagina

Very recently, EVs present in the vaginal canal have begun to be examined [Figure 3C]. Termed vaginosomes, these vesicles released from the vaginal lining have been shown to modulate sperm capacitation and acrosome reaction in mice, similar to EVs found in other female biofluids^[104]. When assessing the miRNA profiles of vaginal fluid however, the majority of miRNAs identified were non-vesicular^[105]. Interestingly, there is some evidence that suggests extracellular RNAs found in the vagina can protect against HIV-1 infection, specifically miR-186-5p^[105]. Although not released directly from the vaginal epithelium, there is evidence to suggest that bacterial-derived EVs found in the vaginal compartment also provide protection against HIV-1 infection^[106,107].

ROLES OF EXTRACELLULAR VESICLES IN NORMAL PREGNANCY

Extracellular vesicles and fertilization

When an egg is released, it is transported to the fallopian tube where it may be fertilized. As discussed previously, the fallopian tubes contain oviductal fluid and oviductosomes, which have been shown to play a number of different roles in mediating fertilization, including sperm capacitation, the acrosome reaction, gamete maturation, and embryo quality.

Sperm capacitation, also known as sperm activation, occurs in the female reproductive tract and initiates the signaling pathways necessary for the sperm to penetrate the multiple layers of the female egg, including alterations to the sperms metabolism, membrane structure and permeability, and pH^[108,109]. In the first step of fertilization, the sperm's plasma membrane binds to the outer layer of the oocyte; the zona pellucida. After this binding event, the acrosome reaction occurs, in which the head of the sperm releases hydrolytic enzymes to soften the zona pellucida, allowing for further entry into the oocyte. Lastly, the cortical reaction occurs when the female egg acknowledges the presence of the male sperm and releases cortical granules to harden the zona pellucida to prevent polyspermy, and the sperm's genetic information is deposited inside of the oocyte.

During sperm capacitation, sperm cells acquire plasma membrane Ca^{2+} -ATPase 4a (PMCA4a) and PMCA1 from oviductosomes, uterosomes, and vagisomes^[92,104,110]. This mechanism plays an essential role in fertilization as post-testicular acquisition of these calcium pumps is paramount for sperm motility and the acrosome reaction^[92]. Oviductosomes and uterosomes have also been shown to transfer tyrosine phosphorylated proteins to sperm, which may also modulate capacitation as this is a key signaling event that occurs in capacitation^[98,110].

In addition to proteins, miR-143-3p, miR-22-3p, and miR-34c-5p were also shown to be transferred from oviductosomes to sperm cells, and were localized to sperm heads^[96]. Most interestingly, miR-34c was localized near the centrosome where it has previously been shown to play a key role in the first cleavage division of the fertilized egg^[111].

Incubation of sperm with oviductosomes improved sperm viability and motility in a dose and time-dependent fashion, including sperm from frozen semen which holds great promise for improving *in vitro* fertilization (IVF) outcomes^[93,112]. Aside from being taken up by sperm, oviductosomes have also been shown to be taken up by oocytes and participate in the regulation of monospermy, potentially by delivering the oviduct-specific protein OVGP1, which has previously been shown to help prevent polyspermy, a key issue with IVF^[112-114]. Additionally, uterosomes have been shown to transfer Sperm Adhesion Molecule 1 (SPAM1) to sperm, which is a crucial protein involved in the fertilization process^[30]. Uterosome EVs taken up by sperm cells have been shown to stimulate capacitation as well^[115].

IVF requires the addition of sperm to an extracted oocyte, however the oocyte needs to fully mature prior to fertilization. Several extracellular miRNAs isolated from the FF of oocytes retrieved from women undergoing IVF were identified to be indicative of high fertilization potential; specifically miR-202-5p, miR-206, miR-16-1-3p, and miR-1244^[116]. MiR-92a and miR-130b were found to be significantly upregulated in FF-EVs derived from oocytes that failed to be fertilized^[117]. Incubation of retrieved oocytes with FF-EVs and/or oviductosomes has been shown to improve oocyte maturation and embryonic development^[85,118,119]. Interestingly, the method by which FF-EVs or oviductosomes are isolated from follicular or oviductal fluid impacts IVF-embryonic competence, with vesicles isolated via OptiPrep™ density gradient ultracentrifugation yielding higher quality blastocysts than those isolated via size exclusion chromatography^[118].

Embryo implantation and maternal-fetal crosstalk

After fertilization, the conceptus trophectoderm releases EVs into the uterine fluid and these vesicles likely mediate some communication between the endometrium lining and the fertilized egg prior to implantation^[99,120,121]. Interestingly, embryonic stem cells within the inner cell mass of the embryo also release EVs and promote implantation of the blastocyst^[122]. Seminal fluid EVs present within the female reproductive tract may also play a role in mediating endometrial decidualization and promoting prolactin secretion, both of which are necessary for embryo implantation^[123].

The endometrium releases uterosomes which are taken up by the embryo prior to implantation, and notably, EV-associated miR-30d increases the adhesion of the embryo to the endometrium^[124]. Hormonal signaling also influences the cargo of uterosomes and can impact the adhesive capacity of embryos through focal adhesion kinase (FAK) signaling^[102]. MiR-100-5p was identified in EVs isolated from endometrial cells and has also been shown to activate FAK signaling and promote embryo implantation^[125]. Other miRNAs identified in the conditioned cell culture media of endometrial cells include miR-200c, miR-17, and miR-106a, all of which are involved in pathways associated with embryo implantation^[121]. The bidirectional

communication of the endometrial and trophoblast cells is mediated in part by EVs, allowing for the transfer of important cargo to facilitate embryo implantation, such as angiogenic and proliferation factors^[126].

The number and size of EVs released from IVF embryos may be indicative of embryo quality as recent evidence suggests that lower quality embryos release more EVs relative to higher quality embryos, and these EVs are slightly smaller in diameter on average^[127-129]. Further, the small RNA cargo of blastocyst derived EVs may also be indicative of quality and viability as some evidence suggests there may be specific miRNA profiles that are up- or down-regulated in EVs released from viable/non-viable embryos^[130].

Co-culturing IVF embryos creates a microenvironment that relies on paracrine communication and results in improved embryonic development compared to embryos cultured independently. This phenomenon may be due in part to EVs released from the embryos that contain the pluripotency genes *Nanog*, *Klf4*, *Oct4*, *Sox2*, and *c-Myc*, which improve the developmental competence of co-cultured, neighboring embryos^[131]. Together these data may help improve IVF outcomes by creating optimal culturing environments and improving identification of the most promising embryos.

Placental derived extracellular vesicles

As the embryo makes itself at home in the uterine lining, it must continue to communicate with the surrounding environment to ensure that the maternal immune system does not reject it, as this could result in spontaneous abortion. Early on in pregnancy, maternal immune tolerance during implantation is typically mediated by regulatory T cells which function to inhibit inflammatory responses and allow for implantation of the developing embryo.

The placenta is an organ that develops during pregnancy and serves as the main form of communication between the mother and the growing fetus [Figure 4]. The placenta attaches to the mother's uterus between weeks 10 and 12 in humans, through remodeling of the uterine wall spiral arteries, mediated by extravillous cytotrophoblasts. The placenta sustains the fetus for several months, serving as the main transporter of oxygen and nutrients for the developing fetus^[132].

As the placenta begins to develop, this transient organ works to modulate the maternal immune response to ensure that the maternal immune system does not reject the developing fetus; this also allows the placenta to interface and communicate with the maternal circulatory system^[133]. Some of this communication is thought to be mediated by EVs released from the placenta^[134,135]. Indeed, EVs released from cytotrophoblasts have been shown to be involved in the migration of extravillous trophoblasts into the decidua of the uterus^[136].

Placental derived EVs are typically thought to express placental alkaline phosphatase (PLAP) and have been shown to increase in maternal circulation throughout pregnancy, beginning in the first trimester^[137]. Placental derived EVs have been shown to express GLA-G5, B7-H1, and B7-H3, which are immunomodulatory proteins that can modulate T cell responses and may be implicated in maternal-fetal tolerance^[138]. Additionally, placental derived EVs have been shown to express Fas ligand (FasL) and induce FasL-mediated apoptosis in T cells, which also assists in maternal-fetal tolerance^[139,140]. Placental derived EVs also carry ligands for the natural killer cell receptor, NKG2D, including UL-16 binding proteins (ULBPs), and MHC class 1 chain-related (MIC) proteins A and B^[141]. These proteins participate in the fetal immune escape by reducing natural killer cell cytotoxicity^[141].

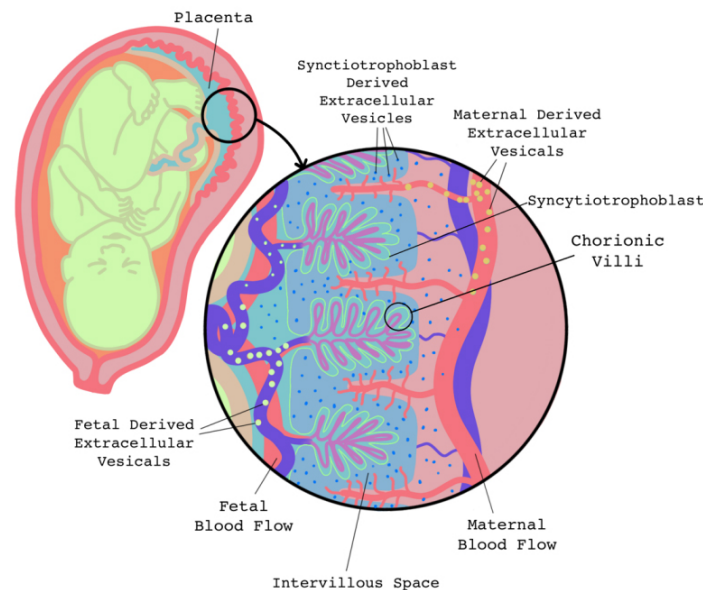


Figure 4. Maternal-fetal communication through EVs. The placenta acts as a central hub throughout the duration of the pregnancy. During this time, the fetus, placenta, and maternal cells release EVs that facilitate communication between the maternal environment and the fetus. EVs: Extracellular vesicles.

As the placenta develops, it invades the uterine decidua and induces reorganization of the uterine spiral arteries. Recent evidence suggests that placental expression of miR-18a, miR-518b, and miR-376c promote trophoblast invasion of the decidua, whereas miR-34a suppresses invasion^[142-145]. Although some of these miRNAs have been observed in other tissues, miRNA profiling of placental tissue revealed a cluster of primate-specific miRNAs, chromosome 19 microRNA cluster (C19MC), which is one of the largest miRNA gene clusters found in humans and is predominately expressed in placental tissues^[146]. Interestingly, C19MC miRNAs are of the most abundant miRNAs identified in placental derived EVs both *in vitro* and *in vivo*^[147,148].

MiRNAs within the C19MC have been shown to play important roles in pregnancy and these miRNAs have been observed in circulating EVs of pregnant women^[147,149]. For example, miR-517a/c expressed in villous trophoblasts helps to mediate the endothelial to mesenchymal transition these cells must undergo to properly invade the decidua^[150]. EV-associated miR-519c was shown to be a placental-specific immune regulator and may have anti-inflammatory properties^[151]. Trophoblast cells confer resistance to maternal viruses via expression of miRNAs in the C19MC, specifically miR-517-3p, miR-516b-5p, and miR-512-3p, and this resistance can be transferred to nonplacental cells via EVs^[152]. These miRNAs are detectable in maternal blood EVs as early as two weeks after implantation of IVF embryos^[153]. In addition to the C19MC cluster, the miR-371-3 cluster (also present on chromosome 19) and the C14MC (present on chromosome 14) are also predominately expressed by the placenta and detectable during pregnancy^[154].

Understanding the biodistribution of EVs during pregnancy would better elucidate their specific roles in various tissues across time. For example, recent evidence suggests that placental-derived EVs traffic to the maternal lung and liver, and specifically interact with immune cells in these organs through surface integrins^[155]. Interestingly, when assessing distribution of large *vs.* small placental EVs, macro-vesicles were found to localize to the lungs exclusively, while micro-vesicles were found in the lung, liver, and kidneys^[156]. Similarly, human placental EVs injected into a pregnant mouse model were found to localize to the kidney, lungs, and liver, and after a 30-min exposure period, the acetylcholine-mediated vasodilatory response of

the murine mesenteric arteries was increased; however, after 24 h this effect was reversed^[156]. Through use of a transgenic mouse line, it was shown that just over a third (35%) of maternal plasma EVs are of fetal origin and are also localized to the maternal uterine environment^[157]. Further, injection of bioengineered EVs into pregnant mice showed trafficking of EVs to fetal cells as well, suggesting that maternal EVs can cross the placenta and influence the fetus^[157].

ROLES OF EXTRACELLULAR VESICLES IN PREGNANCY COMPLICATIONS

Much of the research focused on placental derived EVs focuses on how they may be used as diagnostic tools or biomarkers for various pregnancy complications as many women throughout the United States experience complications with pregnancy every day^[158]. Usually, these complications are health problems that occur during pregnancy and can impact either the health of the mother, the baby, or both. Some of these issues can be present before the pregnancy begins but are exacerbated by it, like hypertension or anemia^[159]. Others, such as preeclampsia and gestational diabetes, arise after the woman becomes pregnant. [Table 1](#) describes the cellular origin and potential function of these EVs in preeclampsia, gestational diabetes mellitus, preterm birth, and fetal growth restriction.

Extracellular vesicles and preeclampsia

Preeclampsia is a hypertensive pregnancy disorder distinguished by the development of high blood pressure and proteinuria at around twenty weeks of gestation, as well as poor placentation and endothelial dysfunction^[184,185]. There are several risk factors that may contribute to the development of preeclampsia, such as the mother's age, weight, and current health status^[186].

Numerous groups have demonstrated that the concentrations of circulating EVs are significantly higher in pregnant women versus nonpregnant women, and even more so in preeclamptic and eclamptic women relative to normotensive pregnant women^[137,187-189]. Although the etiology of preeclampsia is not yet fully understood, it is thought that the process of forming new blood vessels to supply the placenta is compromised, and this may be mediated, in part, by EVs [[Figure 5](#)]. Syncytin-2, a protein that facilitates embryo implantation and trophoblast cell fusion, is expressed at lower levels in EVs isolated from preeclamptic women relative to their normotensive counterparts^[160,190]. Cytotrophoblast cells are believed to release EVs that induce extravillous trophoblast cell invasion of the decidua; however hypoxia can impair this process and result in insufficient arterial remodeling^[136]. Placental hypoxia also induces the release of high mobility group box-1 protein from trophoblast cells, which then stimulates the release of endothelial cell derived EVs^[163].

Interestingly, the majority of EVs identified in the circulation of preeclamptic women were found to be of endothelial origin and may contribute to the procoagulant phenotype seen in preeclampsia^[163]. Activated platelets, signified by externalized phosphatidylserine (PS) contribute to coagulation, and activated platelet-derived EVs have recently been shown to express increased levels of procoagulant tissue factor (CD142) and PS in preeclamptic patients, relative to healthy controls^[165,166]. Placental-derived EVs from preeclamptic women have also been shown to activate platelets, further perpetuating the procoagulant effect seen in preeclampsia^[164]. Additionally, plasminogen activator inhibitor-1 (PAI-1) is overexpressed on EVs isolated from preeclamptic placentas, providing additional support for the notion that EVs contribute to coagulation in preeclampsia^[161].

In addition to the procoagulant effects observed in circulating EVs of preeclamptic women are the anti-angiogenic properties of these EVs^[191,192]. Indeed, EVs obtained from placental perfusions exhibited increased levels of anti-angiogenic proteins (i.e. Eng and PAI-2), which may impact placental

Table 1. Cellular origin and potential function of EVs involved in the pathogenesis of various pregnancy related diseases

Pregnancy condition	Cellular origin	Potential function	Citation
Preeclampsia	Placenta	Reduced expression of syncytin-2, which alters syncytiotrophoblast formation	[160]
	Placenta	Alter fibrinolytic and angiogenic processes	[161]
	Placenta	Activation of peripheral blood mononuclear cells and induce proinflammatory response	[162]
	Trophoblast cells	Stimulate EV release from endothelial cells	[163]
	Syncytiotrophoblasts	Activate platelets, promote coagulation	[164]
	Endothelial cells	Express high-mobility group box 1 and promote coagulation and neutrophil activation	[163]
	Platelets	Promote coagulation	[165,166]
	Podocytes	May be involved or indicative of renal injury	[167]
	N/A	Alter sodium reabsorption in the kidney	[168]
Gestational diabetes mellitus	Placenta	Induce inflammation from endothelial cells	[169,170]
		Mediate skeletal muscle insulin sensitivity	[171]
		Alter metabolic pathways associated with GDM	[172]
	N/A	Mediate glucose intolerance during pregnancy	[173]
		Insulin secretion and regulation; Glucose transport	[173,174]
Preterm birth	Placenta	Biomarkers of placental function	[175]
	Amnion epithelial cells	Increase inflammation in maternal uterine cells	[176]
		Carry HMGB1; Induce labor	[177]
	Group B <i>Streptococcus</i>	Induce labor	[178]
Fetal growth restriction	N/A	Biomarkers of preterm labor	[179-181]
	Placenta	Mediate maternal immune tolerance to the fetus	[140,182]
	Umbilical cord blood	Reduce angiogenic properties of human umbilical vein endothelial cells	[183]

EVs: Extracellular vesicles; GDM: gestational diabetes mellitus.

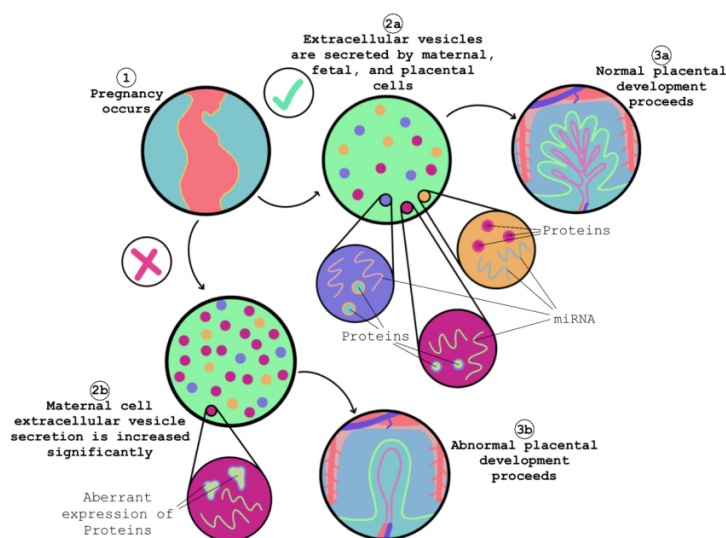


Figure 5. EVs released by various cell types during pregnancy may contribute to the etiology and pathophysiology of preeclampsia by transferring various RNAs and proteins that negatively impact placental development and implantation. EVs: Extracellular vesicles.

circulation^[161]. EVs in preeclampsia may also impact immune cell function and further modulate the etiology and pathophysiology of preeclampsia^[193].

A recent meta-analysis examining the miRNA profiles of preeclamptic women indicated significant elevation of miR-16, miR-20b, miR-23a, miR-29b, miR-155, and miR-210 compared to controls^[194]. Not surprisingly, many of these miRNAs are involved in pathways that regulate trophoblast proliferation and invasion, as well as angiogenesis^[194]. When assessing EV cargo specifically, seven miRNAs (miR-153, miR-222-3p, miR-224-5p, miR-325, miR-342-3p, miR-532-5p, and miR-653-5p) were found to be significantly upregulated in preeclamptic women^[195]. These miRNAs may contribute to the pathogenesis of preeclampsia, but further investigation is warranted.

Early diagnosis of preeclampsia is of utmost importance to ensure the health and safety of both the mother and fetus. EVs in urine, blood plasma, and gingival crevicular fluid (GCF), which is an inflammatory periodontal exudate utilized for diagnosing periodontal disease^[196], are now being explored as noninvasive biomarkers for preeclampsia. GCF EVs from preeclamptic patients had significantly higher expression of the anti-angiogenic protein, soluble fms-like tyrosine kinase-1 (sFlt-1), and lower levels of the pro-angiogenic protein, placental growth factor (PlGF) compared to controls^[197]. Notably, these GCF EVs were also positive for PLAP, indicating that they were indeed placental derived^[197].

Urine is easily attainable and routinely collected during prenatal visits to assess protein and sugar levels as indicators for preeclampsia and gestational diabetes, respectively. As such, there is interest in using urinary EVs to detect and diagnose preeclampsia, which may also indicate renal injury associated with preeclampsia^[198]. Urine from preeclamptic women has been previously shown to contain both podocytes (specialized epithelial cells that cover the outer surfaces of glomerular capillaries) and the podocyte specific proteins PARD-3 and PARD-6, which support the hypothesis that preeclampsia is characterized by podocyte loss and injury^[199,200]. The number of EVs of podocyte origin isolated from the urine of preeclamptic women was significantly higher than in normotensive pregnant women^[167]. Further, preeclamptic urinary EVs were also shown to have altered expression of the NaCl2-k co-transporter 2, Na-Cl co-transporter, and the epithelial sodium channel which may increase sodium reabsorption in the kidney and perpetuate hypertension in preeclampsia^[168].

Extracellular vesicles and gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is associated with inadequate cell functionality and marked insulin resistance and is a form of hyperglycemia first detected during pregnancy^[201]. GDM appears to result from the same physiological abnormalities that characterize diabetes mellitus outside of pregnancy^[202]. In general, hyperglycemia results from an insulin supply that is too low to maintain blood glucose regulation^[203]. During pregnancy, hyperglycemia caused by GDM can be associated with preterm delivery, low birth weight, and even clinical neonatal hypoglycemia^[204]. EVs may participate in the pathophysiology of this disorder and also hold the potential to serve as noninvasive biomarkers^[205].

Recent evidence is conflicting on EV profiles in pregnant women. Some studies suggest that the total number of circulating EVs does not change between women diagnosed with GDM and healthy controls^[206], while others report significantly more EVs in GDM plasma relative to controls^[169]. These differences may be due, in part, to EV isolation and characterization techniques as Franzago *et al.*,^[206] profiled EVs from whole blood through flow cytometry, while Salomon *et al.*^[169] first separated EVs from blood plasma using a density gradient. Interestingly, Franzago and colleagues' work demonstrates no significant differences in the number of total circulating EVs, but did find that the proportion of adipocyte-derived EVs is significantly higher in controls, relative to GDM patients^[206]. Placental-derived EVs were shown to be at significantly higher concentrations in plasma obtained from GDM patients, relative to healthy controls^[169,173]. These EVs were also shown to induce proinflammatory cytokine release from endothelial cells, which is in line with the

hypothesis that GDM is associated with chronic, low-grade inflammation^[169,170]. Circulating GDM EVs infused into a nonpregnant mouse model also conferred insulin resistance, providing further evidence that EVs play a key role in the pathophysiology of GDM^[173].

miRNA profiles of serum-derived EVs reveal significant differences in control versus GDM patients, and many of the dysregulated miRNAs are associated with insulin regulation, glucose transport, and trophoblast proliferation^[174]. EVs isolated from chorionic villi explants from GDM and healthy controls revealed distinct miRNA profiles, and many of the altered miRNAs target carbohydrate metabolism and cellular migration pathways^[171]. Urinary EVs also reveal differential expression of miRNA profiles in GDM patients, specifically downregulation of several C19CM miRNAs during the third trimester; these miRNAs target genes associated with insulin resistance and pro-inflammatory responses^[172].

Extracellular vesicles and preterm birth

Babies born prior to 37 weeks gestation are considered preterm, and the mechanisms that initiate the transition of a resting uterus to a laboring one, known as parturition, are not fully understood. Increased inflammatory signals at the feto-maternal interface (FMI) may play a role. Amnion epithelial cells undergoing oxidative stress release EVs that significantly upregulate inflammatory marker expression from maternal uterine cells^[176]. The inflammatory signaling marker, high mobility group box 1 protein (HMGB1) is elevated in the amniotic fluid, and umbilical cord and maternal blood in preterm births, and has recently been shown to be released from senescent amnion epithelial cells at the FMI in EVs^[177]. Further, EVs expressing HMGB1 injected into pregnant mice induced preterm labor, indicating a key role in this EV-mediated inflammatory signaling pathway involved in the induction of preterm labor^[177]. As gestation progresses, expression of EV-associated inflammatory mediators significantly increases^[207]. Pregnant mice (E15) injected with EVs isolated from late-gestation plasma (E18), but not early-gestation (E9), showed increased expression of inflammatory mediators in their reproductive tracts and delivered preterm^[207].

Inflammation-induced preterm labor can also be mediated by infections, as vaginal infection with Group B *Streptococcus* (GBS) has previously been shown to induce preterm labor. Interestingly this phenomenon may be mediated by EVs released from the bacteria themselves^[178]. A recent study found that EVs engineered to express IB, an NF- κ B inhibitor, could be used to suppress infection-induced preterm labor by reducing the fetal inflammatory response^[208].

To better understand the role of circulating EVs in preterm labor and investigate their potential as diagnostic biomarkers, EVs isolated from the blood plasma of pregnant women have been assessed for their lipid, protein, and miRNA content. Lipidomics revealed a panel of five EV-associated lipids that were found to be predictive of preterm birth^[179], proteomics identified 96 differentially expressed proteins in placental-derived circulating EVs^[175], while RNA sequencing identified over 150 significantly altered miRNAs in circulating plasma EVs^[180,181]. Interestingly, a number of differentially expressed miRNAs were associated with placental development^[181]. Together, these data support the need for further mechanistic studies and validation of the use of EV profiling for the prediction of preterm labor.

Extracellular vesicles and intrauterine growth restriction

Intrauterine growth restriction (IUGR) occurs when a fetus' growth is less than expected for their gestational age^[209]. IUGR occurs in a subset of pregnancies and usually characterized by placental insufficiency, and sometimes linked to preeclampsia^[210]. As EVs have been implicated in the growth, implantation, and angiogenic properties of the placenta (discussed in "ROLES OF EXTRACELLULAR VESICLES IN NORMAL PREGNANCY"), it is not surprising that EVs may also play a role in IUGR. In a cohort of 30 pregnant women, 20 of which were either small for gestational age or experienced

IUGR, fewer placental-derived EVs were found in maternal plasma from IUGR pregnancies compared to normal birthweight pregnancies^[211]. An important aspect of normal pregnancy is maternal immune tolerance to the fetus, and this may be mediated, in part, due to placental derived EVs expressing FasL and other immunomodulatory molecules^[140,212]. In IUGR pregnancies, circulating EVs from maternal plasma express lower levels of FasL relative to those from normal pregnancies, and which may result in less maternal immune tolerance of the developing fetus and impaired fetal growth^[182].

Another study observed the downregulation of several C19MC cluster miRNAs in EVs isolated from the blood plasma of women during their first trimester who would then go on to develop IUGR^[213]. Further, in a porcine model of IUGR, significant differences in EV-associated miRNAs associated with angiogenesis, specifically decreased expression of miR-150, were observed and may contribute to abnormal placentation in IUGR pregnancies^[183]. Similar to IUGR, studies on small for gestational age pregnancies also show altered expression in EV-associated miRNAs, with increased expression of miR-20b-5p, miR-942-5p, miR-324-3p, miR-223-5p, and miR-127-3p^[214]. The role of EVs in mediating the pathophysiology of IUGR is just beginning to be elucidated and warrants further mechanistic studies.

Extracellular vesicles and infertility

Currently, infertility is defined as one year of unwanted non-conception with unprotected intercourse in the fertile phase of the menstrual cycle^[215]. After six cycles of attempted conception, about 50% of the couples trying to conceive will do so spontaneously in the next six cycles and the remaining would be considered as having slightly reduced fertility^[215]. After twelve unsuccessful cycles, 50% of these couples will conceive in the next 36 months (about 3 years) while the rest are nearly completely infertile; by forty-eight cycles of attempted conception, couples are equivalent to sterile^[215].

In cases with a more positive prognosis, most couples are encouraged to wait for any assisted reproductive treatment because their probability of conceiving with or without treatment is very much the same^[216,217]. Most of the time, self-monitoring of the reproductive system is recommended and may be all that is necessary to improve the chances of conception^[218,219]. However, cases with a more negative prognosis, such as tubal pathology or severe male infertility, immediate assisted reproductive treatment should be discussed because it would increase the chances of conception rather than with self-monitoring^[220].

Male infertility

Male infertility is typically characterized by low sperm count (oligozoospermia), low sperm motility (asthenozoospermia), both low sperm counts and motility (oligoasthenozoospermia), or no sperm in the semen at all (azoospermia). Extracellular RNAs have been posited to play a role in male infertility and recent evidence suggests that alterations in their expression may be indicative of the different types of infertility and potentially be involved in the underlying mechanisms associated with male infertility^[221].

Relative to normozoospermic fertile individuals, patients suffering from oligozoospermia had significantly reduced expression of miR-34b in both semen and testicular biopsies^[222-224]. Further, microarray data from human semen EVs indicate that miR-21 and miR-148a are underexpressed in men with oligoasthenozoospermia relative to control patient samples^[225]. These data are in line with small RNA sequencing data from both human and boar semen EVs in which these miRNAs are overexpressed in control samples^[71,72].

Proteomic analysis of seminal EVs identified significant differences in expression patterns between asthenozoospermia and normospermia semen samples, and notably decreased expression of ADAM7 and

TRPV6, which modulate sperm motility^[226].

Azoospermia is typically categorized as obstructive or nonobstructive; nonobstructive implies that the testes suffer from decreased sperm production. Patients with nonobstructive azoospermia can undergo sperm retrieval procedures, but this is successful in just over half of cases and runs the risk of severe complications^[227,228]. Seminal EV long-noncoding RNAs may be indicative of promising candidates for sperm retrieval by revealing whether viable sperm are present in the testes^[229]. Similarly, seminal EV miR-31-5p may be predictive of azoospermia, and miR-539-5p and miR-941 may indicate whether residual sperm is present in the testes or not^[230]. New approaches to treat nonobstructive azoospermia are emerging, utilizing EVs derived from mesenchymal stem cells or amniotic fluid, in hopes of restoring spermatogenesis^[231,232].

Female infertility

Although much progress has been made in treating infertility, women with polycystic ovarian syndrome (PCOS), intrauterine adhesions (IUA), or premature ovarian insufficiency (POI) are still struggling to conceive^[233,234]. EVs may participate in the pathogenesis of these disorders and may also be utilized as therapeutic agents, specifically mesenchymal stem cell derived EVs (MSC-EVs) as they exhibit higher biological stability and lower immunogenicity than traditional MSC cell-based therapy^[235].

PCOS is the most common cause of female infertility and is characterized by high levels of androgens, which inhibit normal oocyte development and release. Expression of several miRNAs in the FF of PCOS patients, including miR-132 and miR-320, were significantly lower than in controls; these miRNAs target genes associated with steroidogenesis which is typically impacted in PCOS^[84]. Proteomic profiling of FF-EVs from PCOS patients revealed enrichment of S100 calcium-binding protein A9, which disrupts steroidogenesis and activates the NF-κB signaling pathway, therefore inducing inflammation^[236].

Endometriosis is another common cause of sub-fertility characterized by endometrial tissue developing outside of the uterus. Recently, uterosomes have begun to be investigated for use as biomarkers for endometriosis and patients diagnosed with endometriosis have significantly more circulating EVs relative to control patients^[237,238]. Vaginal EVs may also serve as non-invasive, early biomarkers of endometriosis as evidence from nonhuman primates indicates that there are fewer EVs in cervicovaginal fluids from a rhesus macaque diagnosed with endometriosis relative to healthy controls^[239]. Unique to patients diagnosed with endometriosis, plasma EVs contain miR-30d-5p, miR-16-5p, and miR-27a-3p, all of which have previously been associated with endometriosis^[240]. Further, endometriosis EVs have also been shown to express unique long noncoding RNA (lncRNA) and proteomic profiles that may transfer inflammatory and angiogenic factors to endothelial epithelial cells^[240,241].

EVs isolated from cultured endometrial stromal cells biopsied from patients with endometriosis had reduced expression of miR-214, which targets the fibrotic markers connective tissue growth factor (CTGF) and collagen α^[242]. Unregulated expression of CTGF and collagen may be involved in the development of endometrial tissue outside of the uterus. Additionally, another group found that EVs isolated from endometriosis patients affected immune and angiogenic factors within the uterine microenvironment^[240]. EVs derived from primary endometrial cells cultured from a mouse model of endometriosis were shown to be taken up by macrophages both *in vitro* and *in vivo*, attenuate their phagocytic capacity, and induce them to polarize into the M2 phenotype^[243]. These data suggest that endometriosis-EVs alter the immune microenvironment of the uterus and may contribute to the pathophysiology of the disease.

Recent studies have shown that MSC-EVs induce angiogenesis in the ovaries of mouse models of chemically induced POI^[244,245]. Increased ovarian angiogenesis may rescue ovarian function and be an avenue of exploration for treating POI^[244]. Indeed, rescue of fertility by MSC-EVs has recently been demonstrated, with little to no adverse effects on offspring born to previously infertile mice^[245]. Aside from angiogenesis, MSC-EVs may also activate the Hippo pathway, which is a key mechanism that mediates folliculogenesis and ovarian function^[246].

CONCLUSION

The role of EVs in reproductive health and pregnancy is an important area of study that is under intense investigation. EVs are key mediators in sperm production and maturation and have been found in every major compartment of the male reproductive system (i.e. the testes, epididymis, seminal vesicles, prostate, and ejaculate). Similarly, in the female reproductive tract, EVs in the ovaries, fallopian tubes, uterus, and vagina have been shown to play significant roles in oocyte development, maturation, and release during the menstrual cycle. Further, EVs from both the male and female reproductive tracts are critical for fertilization and initial development of the embryo, and shortly after fertilization, the embryo itself begins to release EVs that participate in important maternal-fetal crosstalk to ensure proper uterine implantation. As development proceeds, the placenta also begins to release EVs; these EVs are highly abundant in the maternal circulation and likely assist in the development of maternal immune tolerance to the fetus.

In pregnancy related disorders such as preeclampsia, GDM, preterm birth, and fetal growth restriction, EVs have been implicated as direct participants in disease pathophysiology and are being investigated for their use as non-invasive early diagnostic biomarkers. Early identification of pregnant individuals at risk for the development of these complications could facilitate the implementation of life-saving measures to decrease both maternal and fetal mortality. Recent evidence also suggests that the ability to even get pregnant in the first place may rely heavily upon proper EV-mediated signaling pathways. As EVs have been implicated in sex cell development and maturation, it is not surprising that infertility related conditions are characterized by aberrant expression of various proteins and RNAs associated with reproductive tract EVs.

Understanding the pathophysiology of various reproductive and pregnancy related diseases and conditions will undoubtedly include the investigation of the role EVs play in these conditions. EVs are key functional players in ensuring optimal reproductive health, as well as the initiation and maintenance of successful pregnancies.

DECLARATIONS

Authors' contributions

Made substantial contributions to design of the manuscript and performed writing, illustration of figures, and editing: Smith TI

Made significant contributions to the conceptualization and overall design of the manuscript, and performed writing, critical reviewing, and editing, as well as funding acquisition: Russell AE

Availability of data and materials

Not applicable.

Financial support and sponsorship

This work was supported by Penn State Behrend and The Hamot Health Foundation.

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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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.

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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.

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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.

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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.

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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

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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. <i>N Engl J Med</i> 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. <i>Hypertension</i> 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. <i>J Urol</i> 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
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Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and biliary 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. <i>Proc Natl Acad Sci U S A</i> . Forthcoming 2002.

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2.4 Manuscript Format

2.4.1 File Format

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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.

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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;

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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;

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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.

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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

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