Journal of Circulating Biomarkers

OPEN ACCESS
ARTICLE

Short Course in Extracellular Vesicles – The Transition from Tissue to Liquid Biopsies

Meeting Dispatch

Jan Lötvall[1], Johan Skog[2], Alexander V. Vlassov[3], Angel Ayuso Sacido[4], Eva Rohde[5,6], Joanne Gere[7] and Winston Patrick Kuo[8]*

1 Department of Internal Medicine, Gothenburg University, Gothenburg, Sweden
2 Exosome Diagnostics, Cambridge, MA, USA
3 Thermo Fisher Scientific, Austin, TX, USA
4 Thoracic and Brain Tumour Laboratory, CIOCC-IMMA, Fundacion Hospital de Madrid, Madrid, Spain
5 Spinal Cord Injury and Tissue Regeneration Center, Salzburg, Austria
6 Transfusion Medicine Department, Paracelsus Medical University, Salzburg, Austria
7 BioPharma Research Council, Tinton Falls, NJ, USA
8 IES Diagnostics, Inc., Cambridge, MA, USA
*Corresponding author(s) E-mail: winston@iesdiagnostics.com

DOI: 10.5772/60053

Abstract

Extracellular vesicles (EVs), including exosomes and microvesicles, carry a variety of bio-macromolecules, including mRNA, microRNA, other non-coding RNAs, proteins and lipids. EVs have emerged as a promising, minimally invasive (liquid biopsies) and novel source of material for molecular diagnostics, and may provide a surrogate to tissue biopsy-based biomarkers for a variety of diseases. Although EVs can be easily identified and collected from biological fluids using commercial kits, further research and proper validation is needed in order for them to be useful in the clinical setting. Currently, several EV-based research and diagnostic companies have developed research-based kits and are in the process of working with clinical laboratories to develop and validate EV-based assays for a variety of diseases. The successful clinical application of EV-based diagnostic assays will require close collaboration between industry, academia, regulatory agencies and access to patient samples. We expect that international, integrative and interdisciplinary translational research teams, along with the emergence of FDA-approved platforms, will set the framework for EV-based diagnostics. We recognize that the EV field offers new promise for personalized/precision medicine and targeted treatment in a variety of diseases.

A short course was held as a four-session webinar series in September and October 2014, presented by pioneers and experts in the EV domain, covering a broad range of topics from an overview of the field to its applications, and the current state and challenges of the commercialization of EVs for research and an introduction to the clinic. It was concluded with a panel discussion on the regulatory aspects and funding opportunities in this field. A summary of the short course is presented as a meeting dispatch.

Keywords extracellular vesicles, exosomes, short course, liquid biopsies, therapeutics, funding, clinical trials

1. Introduction

EVs are small vesicles, containing diverse "nucleic acids" and protein cargo that are spontaneously secreted by all cells and found in abundance in all human body fluids. Depending upon the cell or tissue of origin, many different roles and functions have been attributed to EVs, for example: the eradication of obsolete molecules, the facilitation of the immune response, antigen presentation, programmed cell death, angiogenesis, inflammation, coagulation, the dissemination of oncogenes from tumour cells and the spread of pathogens such as prions and viruses from one cell to another. Importantly, EVs deliver macromolecular messages that enable cell-to-cell communication and signalling. The short course, *EVs: The Transition from Tissue to Liquid Biopsies* was sponsored by the BioPharma Research Council and was held as a four-session series in October and November 2014. The goal of the short course was to provide, at an introductory level, an exchange between researchers from academia and industry in the characterization of applications of EVs in clinical and translational research and, eventually, clinical practice. The EV topics included an overview of its applications, the commercial aspects, therapeutic hurdles and funding opportunities.

2. Session 1 – September 11, 2014

2.1 Overview of Extracellular Vesicles by Jan Lötvall, MD, PhD

The first session presented by Jan Lötvall, provided an overview of EVs (their function and role in medicine and therapy). Jan started by describing and providing a history of EVs. EVs are membrane vesicles with a lipid bilayer that have surface molecules present on the surface containing cytoplasmic molecules, which can vary in size, with the smaller EVs ranging from 40-150 nm, which have a regulatory function. EVs were first described in 1983 as debris by two groups, Harding et al. and Pan et al., discussing the transferrin receptor sent out from the cells becoming erythrocytes. Earlier studies, in 1967 and 1977, discussed platelet dust and prostasomes, respectively. Jan continued to discuss where EVs are found, what they contain and what can they do in disease. The three major groups of EVs, even though there are many names used in the literature, are: 1) microvesicles budding off the surface of the cells which are released, containing surface receptors, and attaching to another cell (surface-to-surface interaction) or even being taken up by another cell; 2) exosomes, which are smaller EVs, are produced by multivesicular endosomes, contain different types of cargo, and are taken up by another cell (recipient cell); and 3) larger vesicles produced by programmed cell death, the apoptotic bodies. These are important in cell-to-cell communication.

Jan further discussed how the production of EVs is a conserved process, as seen in bacteria, plants and parasites, which is not specific in human cells. A recent paper in *Science* reported bacterial vesicles found in marine ecosystems (the ocean) as they relate to ocean-depth. They demonstrated the transfer of carbon when giving these vesicles to another bacterium to sustain life. In another example, Jan discussed a controversial issue, the presence of rice miRNA in the circulation of Chinese people, where intact miRNA molecules from the rice are present in the EVs, whereby harbouring the rice cargo. Lastly, two abstracts presented at the ISEV meeting discussed the presence of EVs in beer, especially in unfiltered beer, and how they are ingested by humans.

What are the functions of EVs? EVs have several functions, including surface-to-surface interactions (antigen presentation), first described in 1996. Another function is the shuttling of proteins, RNA and lipids between cells. The presence of nucleotides in EVs was described in the late 1990s, considering the packaging of DNA and RNA in apoptotic bodies during apoptosis. In 2006, a study demonstrated that vesicles greater than 100 nm provided evidence that mRNA and protein can be delivered from one cell to another. In 2007, Jan's group showed the presence of mRNA and miRNA in smaller EVs - 40-50 nm - shuttling materials between cells. To prove that the RNA was functional, they took mouse exosomes with mRNA to human mast cells, translating proteins in human cells. They found a number of proteins from exosomes, and a number of proteins that were present as mRNA and not present at proteins in exosomes, suggesting that mouse RNA can be translated to protein in the recipient cell. There was also a presence of functional miRNA.

How do you test whether the RNA is functional? Jan's group exposed exosomes to oxidative stress and placed EVs on top of cells, placing them under stress again – mediating a protective message under stress.

Where do you find extracellular RNA? They are found in all the body fluids, like blood, plasma, saliva, breast milk and urine. The communication of RNA containing exosomes can be made by kissing or may be by breast milk (mother to child communication – uptake by macrophages – immune tolerance and T-regulatory cells).

EVs are present in fetal bovine serum (FBS). In cell culture experiments, FBS is often used to supplement the cell culture medium as a nutrient, but it is important to know that the FBS also contains significant quantities of EVs. Jan's group showed how RNA-containing EVs in FBS can be removed to almost 95% of cases with an 18-hours centrifugation protocol, and this approach strongly reduces the functionality of FBS vesicles in relation to epithelial cell migration.

What can EVs do in medicine? They can promote and attenuate disease. Jan described the presence of bacterial EVs (outer membrane) released from pseudomonas aeruginosa, where his group isolated the EVs from the cell to look to see whether they induce pseudomonas aeruginosa related pathologies/symptoms. In another example, outer membrane EVs from pseudomonas aeruginosa showed strong inflammation (travel through the body quickly by EVs) without any bacteria at all; clearly, EVs are very potent, inducing symptoms. Clearly, all bacterially (both pathogenic and non-pathogenic) released EVs are distributed throughout the body, inducing an inflammatory process. EVs have also been found in the peripheral blood of sepsis patients.

In the cancer field, Jan discussed a few examples of the role of EVs in tumours and cancers. Tumours contain cancer cells, fibroblasts, immune cells and inflammatory cells, but EVs play a role in influencing these cells in the microenvironment. Jan further discusses the role of EVs secreted under hypoxia and how they enhance the invasiveness of prostate cancer cells - cells secreting EVs from the center of the tumour. Another study discussed how EVs from triple-negative breast cancer cells can transfer phenotypic traits. Lastly, Jan discussed a recent study which showed the reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated EVs that actually produce tumours.

Jan stated there are many subsets of EVs. His group showed that apoptotic bodies, microvesicles and exosomes contain fundamentally different RNA profiles, arguing that microvesicles isolated from cell cultures often do not contain considerable amounts of RNA. The rRNA was primarily found in apoptotic bodies, which should be considered when the functionality of RNA in different vesicles is studied.

Exosomes as therapy? Jan discussed a paper published by a group from Tokyo who systemically injected exosomes targeted to EGFR to deliver anti-tumour miRNA to breast cancer cells, suggesting that you can target to tumours partly by functional RNA.

In summary, Jan concluded the following:

1. Exosomes and other EV contain RNA
2. The RNA can be shuttled between cells
3. Mediate biological messages
4. Circulating esRNA can be biomarkers
5. Exosomes and other EVs can deliver therapy
6. Possibilities for treating cancer and inflammatory diseases

In addition, he invited everyone to the upcoming ISEV meeting held in Washington DC that will take place during April 23-26, 2015, at the Bethesda North Marriot Hotel.

3. Session 2 – September 18, 2014

3.1 Clinical Diagnostic Applications of Extracellular Vesicles by Johan Skog, PhD

The second session, by Johan Skog, discussed the clinical diagnostic applications of EVs, including sample collection and processing approaches, and platform applications, including exosome RNA mutation analysis and expression profiling. Johan started off by describing an overview of EVs. Various imaging technologies like electron microscopy (EM) and scanning EM can be used to characterize EVs, and it has been shown that EVs are heterogeneous in nature and that they vary in size (30 nm to greater than 1,000 nm). Cryo-EM show that EVs are a double-lipid membrane, but interestingly EVs are single-lipid bi-layers and one can see vesicles within vesicles. The contents of the EVs are interesting - they contain mRNA, miRNA, non-coding RNA and a variety of proteins – a good platform for biomarkers. The package of EVs is about 10 kb of RNA, not the entire transcriptome. You will find one or a few transcripts per vesicle – carrying different messages.

Johan discussed the extraction of nucleic acids from biofluid such as plasma. Using the exosome platform enables the sub-fractionation of RNA from different cellular processes by way of total exosome extraction (ultracentrifugation, filter purification, etc.) and affinity purification (magnetic beads, microfluidics, etc.). In using whole-plasma extraction, there is a problem in that there is no selectivity from the sub-fractions (RNA is acquired from all kinds of components) and there is a limit in the efficiency of the RNA extraction with higher volumes due to RNase activity. Johan showed that exosomal isolation demonstrates the good quality/yield of intact RNA and 18S and 28S ribosomal RNA peaks compared to direct extraction.

Johan stated that the pre-processing of one's samples is a critical step, like avoiding cell carry-over. He emphasized that the need for standardized SOPs for the collection and pre-processing of your samples is important. When isolating nucleic acid isolation, you need to know whether you are extracting DNA or RNA, since small amounts of RNA are easily be biased by DNA. It is important to have controls in your extraction method. Johan uses the ExoRNA isolation method because it is scalable. Most blood collection tubes are compatible with extraction methods, but have different biases. Johan emphasized using controls/standards and the same collection protocol for all patients. Exosome RNA is stable once isolated, even under freeze-thaw cycles. Johan mentioned that there are multiple sources of RNA in biofluids and that exosome RNA is not the same as the direct precipitation of RNA. By conducting the lysis on the filter using the exoRNeasy kit, one can capture the RNA, even the flow-through (miRNA), directly. High volumes of samples are needed for high-sensitivity applications, like looking for oncogenes and tumour mutations.

Exosome RNA profiling can be used to monitor treatment responses - Johan found unique expression changes in the responder group when compared to the non-responder group and when validated by single-plex qPCR validation.

Johan concluded there are benefits in measuring tumour mutations in biofluids because there is less risk for the patient than invasive biopsy, it is cheaper than surgery, and it can obtain multiple samples over time to track changes longitudinally (although there are still challenges in isolations and in detecting rare tumour transcripts).

4. Session 3 – September 25, 2014

4.1 Commercialization Aspects of Extracellular Vesicles by Alexander "Sasha" Vlassov, PhD

The third session on the commercialization aspects of EVs was presented by Alexander "Sasha" Vlassov. His session discussed how the spectrum of current scientific interest in exosomes is wide, ranging from studying their functions and pathways, to utilizing them in the development of diagnostics and therapeutics. For example, *Exosome Diagnostics* developed a biofluid-based molecular diagnostic test for use in personalized medicine. The company's proprietary exosome technology makes use of this natural enrichment to achieve high sensitivity and specificity for rare gene transcripts, as well as the expression of genes responsible for cancers and other diseases. *Caris Life Sciences* developed Carisome – an innovative, proprietary and versatile testing technology that has the potential to reveal critical information about disease at its earliest stages, from a simple blood test. Currently being developed for a number of types of cancers, including prostate, breast, lung and colorectal cancers, the technology has the potential to provide diagnostic, prognostic and theranostic information for patients. *Exosome Sciences* developed exosome-based solutions to improve the identification and monitoring of acute and chronic conditions. Candidate products are focused on diagnostic advancements in the fields of oncology, infectious disease and brain injury. *Exosomics Sienna* performs applied research and R&D activities in the field of exosome-associated biomarkers, with a specific focus on the development and validation of proprietary immunometric multiplex assays for non-invasive cancer diagnostics and monitoring and point-of-care devices for cancer screening.

In speaking about therapeutic applications, Sasha mentioned how exosomes have tremendous potential as vesicles for the *in vivo* delivery of various therapeutic cargos. For instance, one group reported the successful use of exosomes for the delivery of short interfering RNA (siRNA) to the brain in mice. Targeting was achieved by engineering dendritic cells to express Lamp2b, a membrane protein found in exosomes, fused to the neuron-specific RVG peptide3. Loaded exosomes were purified from conditioned culture media of these cells with synthetic siRNA using electroporation and intravenously injected using RVG-p3-tagged exosomes that delivered siRNA specifically to neurons, microglia and oligodendrocytes in the brain, resulting in a specific gene's knockdown.

A completely different therapeutic approach was proposed by *Aethlon Medical*. A few years ago, it was reported that tumour-secreted exosomes actually suppress the immune response to the cancer. Aethlon Medical developed the Hemopurifier® medical device to selectively remove the tumour-secreted exosomes from the circulatory system. Their postulate is that this will restore the immune system of the cancer patients. The technology uses a large format flow-through canister that can work in an apheresis mode. Proprietary lectins attached to the canister-bed matrix act as unique affinity-capture moieties for exosomes, targeting mannose residues on their surface.

Taking into account that the number of applications and commercial opportunities is rapidly increasing, exosomes are very small and complex entities - there is a growing need for quick and easy methods for both the isolation of exosomes and the analysis of the containing cargo. Life Technologies (now part of Thermo Fisher Scientific) developed a complete exosome workflow solution: (i) the fast and efficient recovery of exosomes from serum, plasma, urine, cerebrospinal fluid, amniotic fluid, ascitic fluid, milk, saliva and cell media using total exosome isolation reagents; (ii) the extraction of their "cargo" with total exosome RNA and a protein isolation kit; (iii) the characterization of exosomal RNA content using the Ion Torrent Proton sequencing and qRT-PCR with TaqMan® assays. A number of additional kits and reagents were released recently, allowing the versatile and advanced analysis of exosomes and EVs (www.lifetechnologies.com/exosomes). For example, Dynabeads with conjugated anti-CD63, CD81, CD9, EpCAM antibodies enable the isolation of exosomal subpopulations.

The continuous development of next-generation tools is crucial in furthering our understanding of exosomes and expanding the range of their practical applications. Moreover, based on the recent reports, this goes beyond mammalian systems to plants, bacteria and - possibly - other kingdoms, where EVs have many undiscovered roles and many exciting practical uses including, for example, food and cosmetic industries.

5. Session 4 Part 1– October 2, 2014

5.1 Extracellular Vesicles: Therapeutic Hurdles by Eva Rohde, MD

Part one of the fourth session was a presentation on the therapeutic hurdles in the EV ecosystem by Eva Rohde. Eva expressed the increasing interest in the putative therapeutic potency of human cell-derived EVs. EV-based therapeutics are biologicals that may be categorized as 'high risk medicinal products' due to currently unknown mecha-

nisms of action (MoAs). She stressed the challenges in EV characterization and the design of preclinical studies as well as aspects of pharmaceutical engineering which have to be addressed to overcome the hurdles on the way to clinical application. A lack of safety data as well as particular knowledge about the nature of the therapeutic target or further biomedical uncertainties (as, for example, the most representative animal models for EV-based therapies) may setback the translational process. Unsurprisingly, data from clinical trials (CTs) testing EV-based therapeutics are scarce. From this point of view, we are not yet perfectly prepared for the clinical testing of EV-based therapeutics. Nevertheless, study protocols evaluating the therapeutic potency of EVs will emerge and broad clinical testing will hopefully start in the foreseeable future. To accelerate the developmental process bringing EV-based therapies into clinics, a close collaboration between researchers and public health authorities is urgently required.

6. Session 4 Part 2– October 2, 2014

6.1 Funding opportunities for Extracellular Vesicle Research by Angel Ayuso Sacido, PhD

The second part of the session was a discussion on the funding opportunities for EV research by Angel Ayuso Sacido. Angel primarily focused on the funding opportunities within the European Union based on his experience. He provided an overview of the structure of the European Parliament, the Council and the Commission. The European Union is based on a 10-year milestone process. The previous programme, entitled the "Lisbon Strategy", from 2000-2010, was based on growth and jobs, whereas the Horizon 2020 is very broad, whereby 3% of the EU's GDP is focused on research and development. Angel suggested three cores - industrial leadership, societal challenges and excellent science - in which EV research could fall under.

Angel also mentioned partnership opportunities, "Looking for Partners" using a matchmaking tool, "Fit for Health" and "Funding for Networks" by "EraNets", a networking mechanism of national and regional programmes, and "COST", the European Cooperation in Science and Technology programme.

7. Conclusion

Based on the session presentations, it was evident that the field of EV research is quickly evolving and that it continues to advance in all facets of science. Overall, the short course fulfilled its goal of providing a balanced forum of relevant content from researchers from both academia and industry. The webinar presentations are available on the BioPharma Research Council website (http://www.biopharmaresearchcouncil.org/webinar-short-course-in-the-exosome).