YALE JOURNAL OF BIOLOGY AND MEDICINE 90 (2017), pp.481-491.

MINI-REVIEW

YJBM

Extracellular Vesicles: Evolutionarily Conserved Mediators of Intercellular Communication

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Extracellular vesicles (EV†) are sub-micron circulating vesicles found in all bodily fluids and in all species so far tested. They have also recently been identified in seawater and it has further been shown that they are released from microorganisms and may participate in interspecies communication in the gut. EV are typically composed of a lipid bilayer formed from the plasma membrane and which encases a cargo that can include genetic material, proteins, and lipids. At least two different processes of formation and release have been described in mammalian cells. The exosome population (50 to 150nm size) are produced via a lyso-endosomal pathway, while microvesicles (100 to 1000nm) are formed by budding of the plasma membrane in a calcium dependent process. Both pathways are highly regulated and appear to be conserved amongst different species. EV release has been shown to be upregulated in a number of human chronic diseases including cardiovascular disease, metabolic disorders, obesity, and cancer; evaluation of their presence in veterinary samples may aid diagnosis in the future. This review will provide insight into the formation of EV and their detection in bodily fluids from different veterinary species and how they may provide a novel addition to the veterinary toolkit of the future.

INTRODUCTION

Extracellular vesicles (EV) are a heterogeneous population of cell-derived circulating vesicles that are largely characterized by the presence of a phospholipid bilayer and an internal cargo that can include DNA, mRNA, miRNA, enzymes, growth factors, and lipids. EV have been isolated from plasma, urine, tears, sweat, milk, seminal fluid, cerebrospinal fluid, as well as from plants and microorganisms, and have also been recently identified in seawater. Their cargo can be transferred to distant cells within the body, or to other organisms which may be from the same or different species. Thus, they are increasingly regarded as a novel delivery route, enabling intercellular communication via delivery of genetic material, protein, and lipid mediators. EV are increasingly being targeted

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†Abbreviations: AB, apoptotic bodies; DLS, dynamic light scattering; EM, electron microscopy; EV, extracellular vesicles; FCM, flow cytometry; ISEV, International Society for Extracellular Vesicles; ILV, intraluminal vesicles; MHC, major histocompatibility complex; miRNA, micro RNA; MV, microvesicles; MVB, multivesicular bodies; NTA, nanoparticle tracking analysis; PS, phosphatidylserine; TRPS, tunable resistive pulse sensing.

Keywords: extracellular vesicles, exosomes, equine, bovine, canine, feline

Author Contributions: CL devised and conducted experiments, carried out analysis and wrote the manuscript; DK, EF, EU devised and conducted experiments, carried out analysis; VLF devised experiments and carried out analysis.

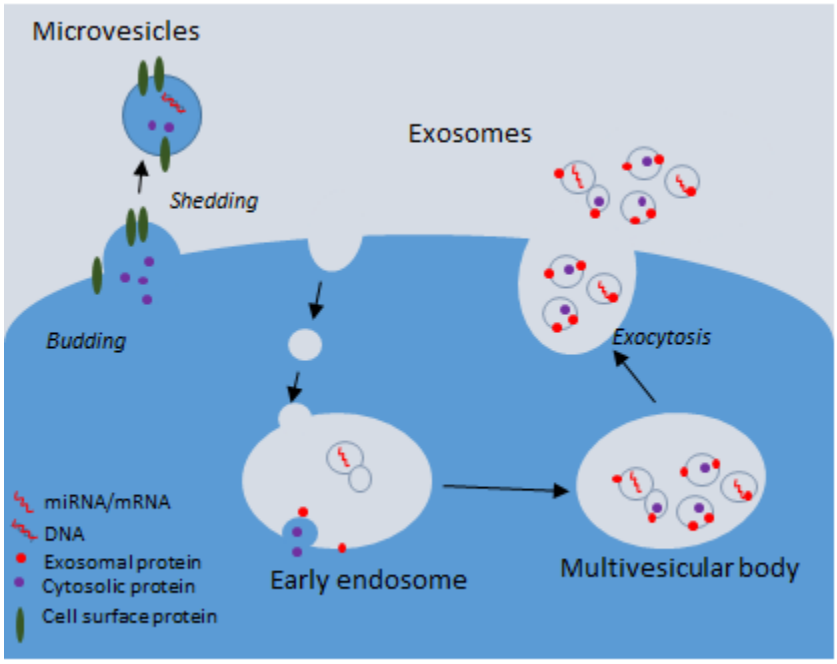

Figure 1. Mechanisms of extracellular vesicle production and release from cells. Extracellular vesicles include exosomes, microvesicles, and apoptotic bodies (not shown on this diagram). **A**. Microvesicles (MVs) are approximately 100nm to 1 µm in size, whilst exosomes are 50-150nm in diameter. MVs are released by membrane budding and are decorated with a number of cell surface molecules specific to the parent cell from which they were released and may also have exposed phosphatidyl serine on their outer membrane. **B.** Exosomes are formed within multivesicular bodies (MVBs) and are released following the fusion of MVB with the plasma membrane. Exosomes have surface markers including CD81, CD63. EV carry cargo including protein, lipids, miRNAs, and mRNAs, which they can deliver to their target cells.

for potential as non-invasive biomarkers in a range of diseases, since samples can be collected with minimal distress to the patient, particularly when measured in bodily fluids such as saliva or urine. A small sample volume is used, they require relatively little processing and often don't need any prior staining. Some veterinary diseases can be hard to diagnose in the early stages due to lack of availability of species-specific diagnostic tools, added to which is the challenge of collecting sufficient biological sample material from some smaller domestic species. The properties of EV make them a particularly attractive proposition for the development of new tests for diagnosis and monitoring.

FORMATION

Extracellular vesicles were first identified several decades ago in a number of different physiological systems; the pro-coagulant platelet derived particles described by [1], later referred to as platelet dust by [2], and the matrix particles identified by [3], were followed by several different studies that identified particles released from adenoma cells, virus like particles released from mammalian cells and prostasomes released in seminal fluid [4]. In the next decade came the first description of the role of multivesicular bodies (MVB) during the formation of exosomes, the smallest of the classes of circulating EV to be described so far [5,6]. In the mid-1990s, interest was once more focused on these populations of circulating vesicles, and the discovery that they contain a range of cargo that includes genetic material such as miRNA has led to renewed attention and a rapid increase in publications [7]. Until recently there has been no standardized nomenclature to categorize the different populations of EV, although the International Society for Extracellular Vesicles (ISEV) has recently published a position paper for the benefit of the growing EV community [8]. EV have been classified both by size and by the mechanism by which they are produced within cells and have been given several different names in the literature. The smallest sized (50-150nm population) are generally known as exosomes, whilst those in the range of 100-1000nm have been variously described as microparticles, ectosomes and more recently described as microvesicles (MV). The third, larger population (> 2000nm) that has been described are apoptotic bodies (AB) and are released during cell death. AB are the most heterogeneous population. For the remainder of this review we will focus on exo-

somes and microvesicles.

Exosomes are produced via a lyso-endosomal pathway. This involves invagination of the plasma membrane to form an intracellular endosome, followed by further inward budding of this within the cytoplasm to form a multivesicular body (MVB) containing multiple intraluminal vesicles (ILV) within. (Figure 1). Exosomes characteristically bear CD63, CD9 and CD81 tetraspanin proteins, which have been shown to be required during assembly of the vesicles [9-12]. The tetraspanins were previously thought to be specific markers of exosomes, enabling characterization after isolation, however, it has recently been shown that they may also be identified in MV. Exosomes may also carry major histocompatibility complex (MHC) class II and heat shock proteins. Tsg101 and Alix have also been identified in multiple studies [11,12].

In contrast, formation of microvesicles occurs directly at the plasma membrane via a calcium-regulated pathway. Less is understood about this process, however, there is a requirement for lipid raft formation where the membrane is pinched or budded off. Enrichment of lipids such as sphingomyelin has been demonstrated in synctiotrophoblast MV in the placenta [13]. Since tetraspanin proteins (CD9, CD63, CD81) have also been identified in MV, it is suggested that they are important for the budding and fusion of the membrane [14]. MV form a more heterogeneous population of circulating vesicles that bear receptors and surface markers from the parental cell. This is likely to be related to their role in intercellular communication and also enables their identification in the laboratory.

Phosphatidyl serine (PS) is a phospholipid usually found on the inner leaflet of the plasma membrane. During formation and release from the cell surface the plasma membrane may "flip" as the membrane asymmetry is lost, similar to that seen during apoptosis. This leaves PS exposed on the outer membrane, which may be important for intercellular communication and is also useful during identification.

MEASUREMENT

A range of different methods have been described to measure EV. These can broadly be grouped into optical and non-optical techniques. Electron microscopy (EM) can be considered the gold standard, and the first EV to be described from platelets were observed using this technique [2]. While EM can enable accurate measurement of size and morphological characteristics of different classes of EV within the same fraction, the technique is time consuming, requires specialist expertise which may not be easily available, is not quantitative and cannot be used to further phenotype different vesicle populations (review of methods see [15]). Additionally, the processing required (centrifugation, dehydration, and fixation) alters the size and morphology, producing some artefacts, including the characteristic "cup shape" morphology of exosomes [16,17]. Atomic force microscopy is a newer technique that has also been used to identify EV [15]. This technique does not require chemical fixation [18]. It also enables accurate sizing of vesicles and is advantageous in that it can be used in conjunction with antibody labelling. However, it is also time consuming and as concentration of the sample is required, the technique is not quantitative by nature.

A range of optical methods have been described which include flow cytometry (FCM), which is suitable for measurement of larger (> 200nm) MV, but not usually suitable for smaller exosome detection, although some protocols have been described using latex beads and exosome specific conjugated antibodies [17]. FCM is now accessible in many clinical and research laboratories and so this is probably the most widely reported detection method used to date. One of the benefits of FCM for analysis of MV is that there are a large number of fluorochrome-conjugated antibodies available to enable phenotyping of mixed populations of EV in samples from bodily fluids. Flow cytometers are usually capable of multicolor analysis, so surface marker detection can be coupled with detection of externalized PS using Annexin V reagents, which are available conjugated to many different fluorochromes. This may be important for biomarker detection and especially for veterinary samples where there may be fewer cross-reactive antibodies available to choose from. Standardized guidelines have been published for optimized collection of plasma for later MV detection [19] and there are several protocols to enable differentiation of MV from background noise. Recent advances in FCM technology have enabled direct visualization of MVs using Image-Stream technology [20] and special labelling methods have been established using dedicated flow cytometers to detect individual exosomes. There are some important caveats to detection of MV by FCM, namely that small particles (in older machines this could be anything < 340nm) can scatter light in different ways to the much larger cell populations for which the technique was originally developed. These small particles may also be subject to swarm effect, where several particles below the lower limit of detection are clustered together, and are therefore seen as one larger particle by the laser, which may lead to inaccuracies in the measurements [21]. This can be overcome to some extent by use of pre-calibrated counting and sizing beads as internal controls, which can help with standardization of samples. This could be important to calibrate samples within the same study either on the same machine or for multicenter studies, which may require use of different methodologies [22]. Another key advantage of FCM is that often MV can be stained in

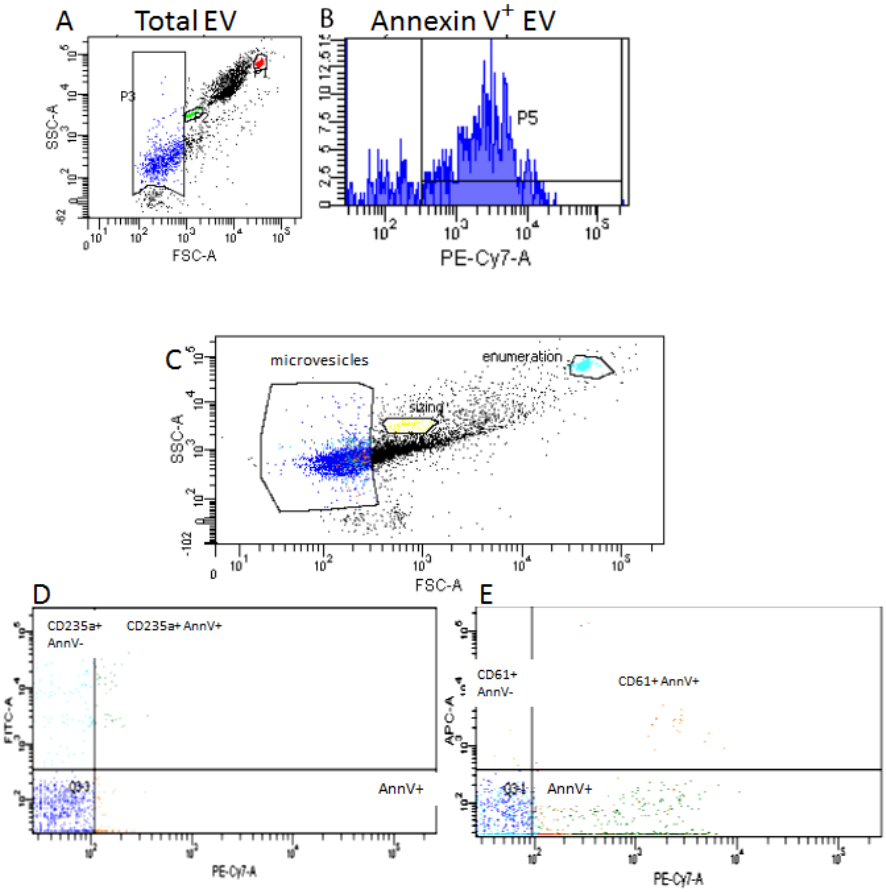

Figure 2. Measurement of microvesicles in plasma using flow cytometry. A and **B.** Measurement of MV in equine plasma. **A.** Gate P1 (red) Commercially available enumeration beads are added to samples to enable accurate quantification of MV number; Gate P2 (green) 1micron latex beads are added to samples to facilitate sizing of MV up to 1000nm diameter; Gate P3 (blue) total MV count in plasma sample. **B.** Gate P5 (blue) shows MV counted in Gate P3 (panel A) that bind to PE-Cy7.7 labelled annexin V (binds to phosphatidyl serine on the surface of MV). **C, D, E** Measurement of MV in feline plasma. C. Gate P1 (light blue) enumeration beads; Gate P2 (yellow) 1micron sizing beads; Gate P3 (blue) total MV count in plasma sample. **D.** Dual florescence analysis of the MV gate for erythrocyte-derived MV. Events in 235+/AnnV+ bind CD235 and Annexin V, indicating erythrocyte cell surface receptors and PS expression. Events in AnnV+ contain nonerythrocyte MV. Events in 235+/AnnV- express erythrocyte cell surface receptors, but do not have PS expression. **E.** Dual florescence analysis of the MV gate for platelet-derived MV. Events in 61+/AnnV+ bind CD61 and Annexin V, indicating platelet cell surface receptors and PS expression. Events in AnnV++ contain nonplatelet MV. Events in 61+/AnnV+ express platelet cell surface receptors, but do not have PS expression. In both **D** and **E** quadrant 3 (Q3) represents debris, machine noise, and MV that did not bind either marker. All analysis was carried out using a Becton Dickinson FACS CANTO II and Diva analysis software (Becton Dickinson, Oxford UK). SCC-A= side scatter; FSC-A= forward scatter; AnnV= Annexin; PE-Cy7-A=Phycoerythrin labelled Annexin V; FITC- fluorescein isothiocyanate labelled anti-CD235a to measure erythrocyte derived MV; APC=Allophycocyanin labelled anti-CD61 to measure platelet-derived MV.

native samples (e.g. whole blood [23]) without purification. This can be important where only small volumes are available, such as those that have been collected and are surplus to requirements for clinical diagnosis.

A range of additional optical techniques have been developed and optimized for measurement of the smaller exosome population (50 to 150 nm). These include dynamic light scattering (DLS), tunable resistive pulse sensing (TRPS), and nanoparticle tracking analysis (NTA) [15]. All these methods have a much lower limit of detection (below 50nm size) and so enable quantification of exosomes. They are also much more efficient in terms of both time and cost per sample than either EM or atomic force microscopy. DLS should be approached with particular caution. Although a very time efficient method, the technique has been optimized for use with

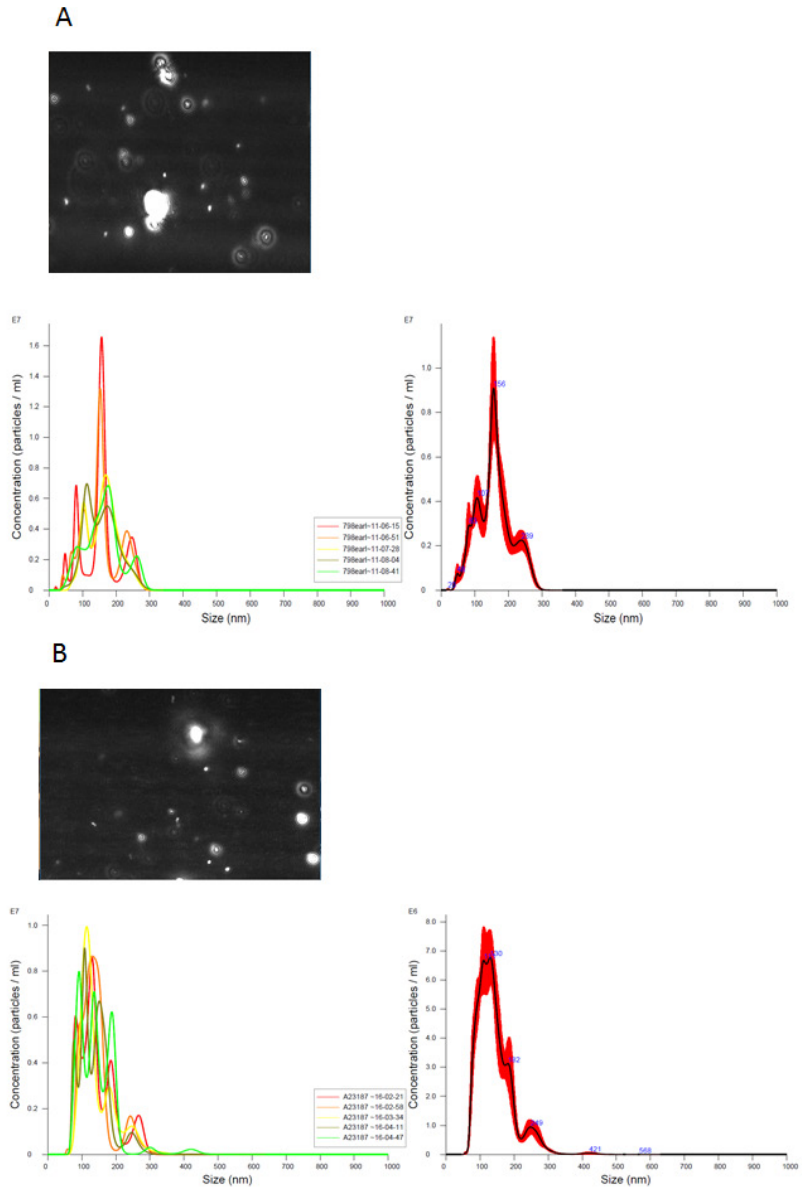

Figure 3. Measurement of exosomes using nanoparticle tracking analysis. A. measurement of exosomes purified from raw cow's milk, exosomes were isolated using a modification of the method in (Thery 2006 for methods please see Appendix A: supplemental methods); **B**. measurement of exosomes in supernatant from dispersed cells collected from zebrafish embryos at 96h post fertilization. **A** and **B**, left panel, Brownian motion of individual particles is recorded for 60 seconds; middle panel, overlaid histograms of the concentration of particles at different sizes taken from multiple measurements; right panel, smoothed histogram from middle panel showing average concentration and size. Nanosight LM10 instrument (Malvern Instruments Ltd, Malvern, UK) was used for all analysis.

monodispersive particles, i.e., particles of one particular size in a homogenous population, whereas there are usually a range of vesicles types and sizes present in biological fluids (so called polydispersive samples). It is then important to develop an accurate mathematical model because larger particles scatter light more effectively so any measurements are skewed towards smaller numbers of large particles (for review [21]). The major limitation with NTA and TRPS (although this may change in the next few years) is that they lack the multiple lasers seen in most flow cytometers, so they are not suitable for phenotyping using multicolor analysis with a range of antibodies. Additionally, multiple purification steps may be required for some of the instruments to ensure that only exosomes are counted. Confirmatory techniques such as western blotting and immunodetection for characteristic

exosome proteins may be required as they are not able to reliably distinguish between protein aggregates and exosomes [7]. For veterinary samples this may require the optimization of cross-reactive antibodies.

COMPARATIVE BIOLOGY OF EV

Release of EV appears to be an evolutionarily conserved process and therefore we hypothesized some time ago that they could be detected in bodily fluids from veterinary and non-mammalian laboratory species and that they might be useful as non-invasive biomarkers for a range of diseases of veterinary importance. One key difficulty with development of any new diagnostic or research test for use on veterinary samples is the lack of available antibodies, meaning that analyses that do not necessarily require species-specific reagents can be invaluable. We have now identified MV in equine and feline plasma, as well as from bovine milk (Figure 2; for methods please see Appendix A: supplemental methods), from zebrafish embryos (5dpf) and insect cell cultures by flow cytometry and from human and rodent counterparts (data not shown and [24-26]). In addition, we have identified exosomes from bovine milk and zebrafish embryos (5dpf) by NTA (Figure 3; for methods please see Appendix A: supplemental methods). Using a candidate based approach we have identified cross-reactive antibodies to feline antigens to identify platelet (CD61) and erythrocyte (CD235a) MV in plasma (Figure 2). A number of other groups have also identified equine and canine extracellular vesicles, both from plasma and from tissue culture medium using similar techniques to those outlined above [27-30].

RELEVANCE TO DISEASE PROCESSES AS BIOMARKERS AND THE ADVANTAGES FOR VETERINARY SPECIES OVER OTHER BIOMARKERS

One important aspect that has become clear as the field has grown is that EV have great potential as biomarkers for a range of different diseases, and this may be translatable to common diseases of veterinary species. They have been identified in plasma from patients with a range of different cancers [31] as well as cardiovascular disease including valve disease and heart failure [32,33] and metabolic disorders including obesity, insulin resistance, and type II diabetes [7,34]. Some of the methodology outlined above enables measurement without pre-staining with antibodies (NTA, TRPS) and a number of sophisticated protocols have been developed enabling isolation of genetic material, in particular miRNA, from different populations of EV. These techniques are gradually becoming more affordable and less time consuming/higher throughput. At the same time, the feline, canine, equine, and bovine genomes are becoming better annotated and more is being understood about the role of different miRNAs in regulation of gene expression. Circulating miRNA has been identified in canine plasma, serum and urinary exosomes [35,36], feline plasma [37,38], equine serum and ovarian follicular fluid [39,40], and bovine plasma and milk [41,42]. Similar to findings in humans and rodent models, circulating miRNA in different bodily fluids reflects different disease or physiological states in veterinary species. Thus, the ability to collect this important information regarding the cargo of EV will enable development of new biomarker assays in the clinic and give further insight into the role of EV in different (patho) physiological processes in veterinary species.

In summary, the study of extracellular vesicles is providing us with new insights into how cells communicate both within the individual and also between organisms of the same or different species. Understanding more about their biology enables the generation of new tools and techniques to evaluate their cargo and function and gives us exciting opportunities to develop novel assays for clinical use. This is particularly important for veterinary diseases where simple and robust diagnostic tests are often lacking in the clinic. Development of assays to measure EV from veterinary species will enable high quality care and enhanced welfare for our patients.

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Appendix A: **Supplemental Methods**

Measurement of EV in plasma

All blood collection was approved by The Royal Veterinary College Ethical Review Board. Venous blood was collected into EDTA tubes and removed to the laboratory for processing as soon as possible. Plasma was centrifuged twice at 2,500 xg for 15 min and the supernatant was decanted and stored at -80°C until use. 5µL plasma was diluted with 45µL Annexin V buffer (eBioscience, Thermo Fisher, Dartford UK) together with 2µL Cy7.7-labelled Annexin V (eBioscience). For feline samples 2µL fluorescently conjugated cross-reactive antibodies against cell surface markers were added (anti human CD235-FITC, anti human CD61-APC, both from eBioscience). Samples were incubated for 15 min on ice before addition of 450 µL Annexin V buffer. 1.1µm latex sizing beads (Sigma, Poole, UK) and 10µL Flowcount Fluorospheres (Beckman Coulter, Hemel Hempstead UK) were added before analysis on a Becton Dickinson FACS CANTO II and Diva analysis software (Becton Dickinson, Oxford UK). Please see Figure 2A for gating strategy. Briefly, events that were 200-1000nm were gated and then analyzed for Annexin V (PS) staining and presence of CD235a (Figure 2d) or CD61 (Figure 2e). Numbers of events in each region were calculated based on the numbers of events counted in an "enumeration gate" based on the number of Flowcount beads calibrated by the manufacturer and according to the equation

Number of gated events of interest per microliter =

$$\frac{\textbf{20 x \#gated MV events x dilution factor of sample}}{\textbf{\# enumeration beads counted (in gate)}}$$

Isolation of exosomes from cow's milk

Milk was obtained from Holstein Friesian cows housed at Bolton's Park Farm (The Royal Veterinary College, Potters Bar, UK). The samples were transported back to the laboratory. 20µL of EDTA was added before centrifugation at 1500×g for 5 minutes. This centrifugation step skimmed the milk, resulting in a layer of cream on the top which was removed. A protocol was optimized for the isolation of exosomes from fresh bovine milk. Fourteen ml of the milk was transferred into a 15ml tube for centrifugation at 2000×g at 4°C for 10 minutes (Thermo Scientific Haraeus™ Multifuge™ X3 FR). Any residual cream forming on the top was removed and a white pellet (possibly formed of proteins and fats) was observed at the bottom of the tube. The supernatant was removed and transferred into Beckman Polyallomer tubes**.** The samples were centrifuged at 17,000×g for 15 minutes at 4 °C in Beckman-Coulter Optima™ L-80 ultracentrifuge (Sw-40-Ti rotor). Acceleration throughout the ultracentrifugation steps was set high, whilst deceleration was set to low. A solid, white pellet was observed on the bottom of the tube. The supernatant was carefully removed and transferred into a clean tube. This ultracentrifugation step was repeated three times until the liquid appeared clear. 10 ml of the supernatant from the final centrifugation step was layered onto a 30% sucrose (Sigma, UK; dissolved in 1xPBS) cushion. These samples were then centrifuged at 100,000×g for 70 minutes at 4°C. A cloudy floating pellet formed over a gelatinous yellow/white pellet on the bottom of the tube. This floating pellet was expected to contain the exosomes and was transferred into a clean ultracentrifugation tube. The tube was filled up with ice-cold 1x PBS and centrifuged again at 100,000×g for 60 minutes at 4°C to purify the exosomes. The resulting floating pellet was

removed and filtered with a 0.22µm filter. The samples were diluted 1:5000 in 1x PBS for analysis by Nanosight LM-10 (Malvern, Malvern UK).

Preparation of EV from Zebrafish embryos

Five zebrafish embryos at 96h post fertilization were placed into an Eppendorf tube with 100µL 1x PBS and homogenized using a single use mini homogenizer (Thermo Fisher). The homogenate was centrifuged at 3,000 xg 5 min 4°C to pellet cellular material and the supernatant was transferred to a clean tube for analysis by Nanosight LM-10 (Malvern, Malvern UK).