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Extracellular vesicles in veterinary medicine: isolation, characterization and function

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1. Abstract

During the XX century, extracellular vesicles (EVs) have been largely studied in human medicine. EVs are cell-derived nano-shuttles with the ability of transferring cell-material such as proteins, lipids, sugars and nucleic acids to other cells. Because of their membranous structure and carrier ability, EVs are innovative tools which can be applied to different research fields, from physiological to pathological aspects and from the diagnosis of different diseases to their treatment.

Despite EV-research has exponentially increased in the last decades, a lot of information on animal-derived EVs is still lacking. The aim of this work of thesis was to start to fill this gap of knowledge, taking advantage of the multidisciplinary aspect of EVs and studying them in different animal species and for different purposes.

After reviewing the literature on EV-related methodology and the state of art of EVs in veterinary medicine, this thesis preliminary explored EVs in unconventional animal species, describing EVs released in vitro by two cetacean (Tursiops truncates and Ziphius cavirostris) cell lines and a protocol for EV-isolation from Manila clam (Ruditapes philippinarum) hemolymph. The rest of the thesis focused on EVs in canine cancers, comparing the functionality of EVs isolated with two different techniques in vitro from a canine mammary tumor cell line and the expression of microRNA in the plasma of dogs with T-cell lymphoma.

With these studies some new insights on EVs isolated from different animal species were given, showing some of the possible future applications of EV-research, which can fit very well into the concept of One Health, connecting human, animal and environmental health.

2. Riassunto

Nel corso del XX secolo, le vescicole extracellulari (EVs) sono state ampiamente studiate in medicina umana. Le EVs sono nano-shuttle di origine cellulare, con la capacità di trasferire materiale come proteine, lipidi, zuccheri e acidi nucleici ad altre cellule. Per la loro struttura membranosa e la capacità di trasporto, le EVs sono strumenti innovativi che possono essere applicate a diversi campi di ricerca, dalla fisiologia alla patologia e dalla diagnosi delle malattie al loro trattamento.

Nonostante la ricerca sulle EVs sia aumentata esponenzialmente negli ultimi decenni, mancano ancora molte informazioni sulle EVs negli animali. Lo scopo di questo lavoro di tesi è stato quindi quello di iniziare a colmare queste lacune, sfruttando l'aspetto multidisciplinare delle EVs e studiandole in diverse specie animali e per scopi diversi.

Dopo aver esaminato la letteratura sulle metodologie legate allo studio delle EVs e lo stato dell'arte sulle EVs in medicina veterinaria, questa tesi ha esplorato in via preliminare le EVs in specie animali non convenzionali, descrivendo le EVs rilasciate *in vitro* da due linee cellulari di cetacei (*Tursiops truncatus* e *Ziphius cavirostris*) e un protocollo per l'isolamento delle EVs da emolinfa di vongola (*Ruditapes philippinarum*). Il resto della tesi si è concentrato sulle EVs nei tumori canini, confrontando la funzionalità delle EVs isolate con due diverse tecniche *in vitro* da una linea cellulare di tumore mammario di cane e l'espressione dei microRNA nel plasma di cani affetti da linfoma a cellule T.

Con questi studi sono state messe in luce alcune nuove informazioni sulle EVs isolate da diverse specie animali, mostrando alcune delle possibili applicazioni future della ricerca sulle EVs, che possono ben adattarsi al concetto di One Health, collegando salute umana, animale e ambientale.

3. Introduction to Extracellular Vesicles

It is commonly recognized that cells can communicate through the exchange of proteins, RNAs and other small molecules. These molecules can be secreted in the extracellular space and taken up by other cells through transport channels or can be transferred through direct cell-to-cell contact (1). However, during the 80s, our knowledge of cell-communication mechanisms changed, adding a new different kind of "messenger" to the list. These messengers are membranous structures produced by cells, enclosing different molecules (*e. g.* RNA, proteins, lipids, sugars), and released in the extracellular space that are now well known as extracellular vesicles (EVs) (2).

EVs are secreted by all the cells of all organisms and are evolutionary conserved among kingdoms (3). They are surrounded by a double-layered membrane and are very heterogeneous in size and biogenesis. According to their biogenesis, they are mainly classified in exosomes or microvesicles (4). Exosomes are small vesicles (30-100 nm) derived by the inward budding of the endosomal membrane during the maturation of multivesicular endosomes (MVEs), and secreted after the fusion of MVEs with the cell membrane (3). Microvesicles, also called microparticles or ectosomes, are larger in size (50-1000 nm) and originate instead from by the outward budding of the plasma membrane (3,5). Recognizing the origin of EVs is difficult, therefore, nowadays EVs are mainly classified in small-EVs, when their size is smaller than 200 nm, or large-EVs, when larger than 200 nm of diameter (5).

Having the ability of carrying and protecting bioactive compounds, which also represent their cell of origin, a massive increase in EV-research has been observed in the last decades applied to different fields (6). Being involved in cell-to-cell communication and being present in all biological fluids, they are studied for their mediatory role in physiological and pathological processes (Yáñez-Mó et al., 2015). For sure, one of the most studied applications is their use as biomarker to diagnose, stage and predict the outcome of different diseases, especially of cancer (8). But EVs can also be exploited for their therapeutical potential, having immunomodulatory and regenerative effects when coming from specific cell types or biofluids (*e. g.* stem cells, milk) or being innovative engineerable but natural nanoparticles exploitable for instance as target-specific drug carriers (9).

Considering the wide range of applications of EVs, the aim of this work of thesis is to exploit EV versatility for research in veterinary medicine, exploring EV role and features in different animal species and for different purposes, according to the concept of One Health.

3.1 How to isolate extracellular vesicles

EV complexity and heterogeneity lead not only to a wide range of applications, but also to some important issues, mainly related to their isolation. The wide range of sizes (50 – 1000 nm), overlapping to those of other molecules (e. g. lipoproteins, viruses), the complexity and the variability of starting samples (e. g. blood, urine, milk) and the need of both maintaining EV-integrity and functionality and having high purity with low co-isolated molecules, make the isolation of EVs an arduous process. During the last two decades various techniques for EV-isolation have been described in the literature. All the techniques take advantage of the physical or biochemical properties of EVs and differ for processing time, cost, EV-yield and purity (10–12). In order to clarify and standardize methodology for EV analyses, the International Society for Extracellular Vesicles, in 2014 and in 2018, published some guidelines for the EV community known as Minimal Information required to Study EVs (MISEV) and very recently updated in a last version (MISEV 2023, https://www.isev.org/misev) (5,13). These guidelines aim to highlight some critical points of EV-research, shedding light on EV-nomenclature and providing information on the "good practice" for working with EVs (5).

Among the main techniques used to purify EVs, ultracentrifugation (UC) has been considered the gold standard for years (10). UC exploits high centrifugal forces to pellet EVs according to their size and density. Despite being easy to perform and commonly used, UC has got main limitations, related to the co-isolation of other particles (e. g. proteins, lipoproteins), to the possible aggregation of EVs and to EV-losses in the supernatant (12,14). Other techniques allow to overcome these issues but, until now, isolation protocols achieving both high recovery rates and high specificity have not been developed (5). Other techniques applied to isolate EVs and used also in the present thesis are ultrafiltration (UF), size exclusion chromatography (SEC), density gradient centrifugation (DGC), immunocapture and microfluidic based isolation techniques.

UF is a size-based EV-isolation method. Applying pressure to specific filters with pores smaller than EV size, EVs can be strongly concentrated (14). Being considered a recovery method with low specificity, it is mainly used for EV-concentration starting from large volumes (< 10 ml) and can be followed by other EV-purification steps (5). SEC is another size-based purification method. Samples run through porous polymers within specific assembled columns, from which larger particles elute earlier than smaller particles, which are slowed down by entering the polymer pores (10). SEC is considered a medium specificity and medium recovery

rate technique, still co-isolating other non-EV particles but to a lesser extent than other techniques (5,14).

DGC mainly isolates EVs based on their density, combining sucrose or iodixanol density gradients with UC. Here, the main possible co-isolated particles are lipoproteins, which have the same density of EVs, nevertheless the specificity is considered high and the recovery low compared to other isolation methods (14).

Differently, immunocapture exploits the antibody-antigen binding to purify EVs. Plates, beads or chips presenting specific antibodies are used to capture EVs by binding EV-membrane proteins. Because not all EVs express the same membrane markers, immunocapture present the disadvantage of isolating heterogeneous EV-subsets, reducing the recovery compared to other techniques (5,14).

Finally, microfluidic-based strategies have been developed more recently, being the focus of many studies (15). Microfluidic allows EV-isolation based on their physical and biochemical properties simultaneously, increasing the efficiency of traditional purification methods, the purity and reducing EV-losses (11). Moreover, microfluidic is particularly advantageous when processing small volumes (< 1 ml).

To better highlight the potential of EV-isolation through microfluidic strategies in comparison with traditional EV-isolation methods, during my PhD I was involved with some components of my research group in the publication of the following review in collaboration with a team of the department of Physics and Astronomy of the University of Padua working on droplet microfluidic for EV-isolation (15).

3.2 Paper n. 1: "Microfluidic strategies for extracellular vesicle isolation: towards clinical applications"

biosensors

MDPI

Review

Microfluidic Strategies for Extracellular Vesicle Isolation: Towards Clinical Applications

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Abstract: Extracellular vesicles (EVs) are double-layered lipid membrane vesicles released by cells. Currently, EVs are attracting a lot of attention in the biological and medical fields due to their role as natural carriers of proteins, lipids, and nucleic acids. Thus, they can transport useful genomic information from their parental cell through body fluids, promoting cell-to-cell communication even between different organs. Due to their functionality as cargo carriers and their protein expression, they can play an important role as possible diagnostic and prognostic biomarkers in various types of diseases, e.g., cancers, neurodegenerative, and autoimmune diseases. Today, given the invaluable importance of EVs, there are some pivotal challenges to overcome in terms of their isolation. Conventional methods have some limitations: they are influenced by the starting sample, might present low throughput and low purity, and sometimes a lack of reproducibility, being operator dependent. During the past few years, several microfluidic approaches have been proposed to address these issues. In this review, we summarize the most important microfluidic-based devices for EV isolation, highlighting their advantages and disadvantages compared to existing technology, as well as the current state of the art from the perspective of the use of these devices in clinical applications.

Keywords: extracellular vesicles; microfluidics; purification; liquid biopsy; microfabrication; clinics

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1. Introduction

Discoveries in genomics are leading to important outcomes in medicine, improving knowledge of many diseases and leading to the concept of "precision medicine", which is defined as the tailoring of medical treatment to individual characteristics [1]. For example, after a cancer diagnosis, the first approach is often a surgical biopsy to identify the type of tumor by specific marker expression or by genomic analysis [2]. Unfortunately, the latter is an invasive and time-consuming procedure, may not be representative of the entire tumor, and may cause cancer seeding [3]. To face these issues, much attention has been paid to a less invasive procedure called *liquid biopsy*: body fluids (e.g., blood, urine, saliva) are screened for tracers released by cancer tissues, which can provide more rapid and complete information about the original tumor (e.g., type, stage, progression, etc.) and could be used as prognostic and/or diagnostic tools [4]. The most well-known tracers are circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA). The first are cells that are spontaneously released from the cancer tissue and travel in the patient's blood [5]. Similarly, ctDNA are nucleic acid fragments presenting specific tumor mutations that are released from cancer cells and travel in body fluids [6].

Another type of *tracer* that has been discovered in recent decades as potentially useful for liquid biopsy are extracellular vesicles (EVs) [7]. These are double-layered phospholipid membrane structures, released by most cell types, which travel in body fluids carrying various biological molecules of the parental cell (i.e., proteins, lipids, and nucleic acids).

The biogenesis of EVs is mainly related to two pathways: (i) the direct outward budding of the cell membrane and ii) the inward budding of multivesicular bodies that fuse with the cell surface to then be released. In the former case, EVs are known as ectosomes (or microvesicles (MVs), or microparticles), with a size of 100 nm to 1000 nm, and in the latter case, they are known as small extracellular vesicles (sEVs) or exosomes, with a size ranging between 30 and 200 nm [8,9]. Although initially considered cell debris or cell waste, it is now recognized that EVs play a role in cell-to-cell communication, acting as cargo ships between cells by transporting genetic information [10], and therefore participating in a variety of physiological and pathological processes [11]. Therefore, EVs are perfectly suitable for liquid biopsy and are now considered promising diagnostic, predictive, and prognostic biomarkers for many types of diseases. In fact, unlike CTC and cDNA, EVs can provide a variety of information, e.g., either on cardiovascular [12], autoimmune [13], and neurodegenerative [14] diseases, or on various types of cancer [15].

Today, given the invaluable importance of EVs for liquid biopsy, there are some key challenges to overcome regarding their isolation [16]. The current most frequently used approaches, described in Section 2, are based on differential ultracentrifugation (DU), size-exclusion chromatography (SEC), density-gradient separation (DGS), filtration, and immunoaffinity strategies. Additionally, EVs can be collected from various fluids (e.g., cell culture, blood, urine, etc.); thus, the same isolation strategy may present different efficiencies depending on the starting sample [17]. Finally, most of the methods require at least several hours for EV isolation, and thus more rapid isolation protocols are also demanding.

Microfluidic devices have recently been proposed for addressing these issues, as demonstrated by the increasing number of published papers that have appeared over the past ten years on this topic. Figure 1 compares the publications per year obtained using the terms *extracellular vesicles* (or *exosomes*) (Figure 1a) and together with *microfluidics* (Figure 1b) as keywords. Both trends are similarly increasing; however, the ratio between the two numbers (Figure 1b, inset) reports how microfluidics has gained slightly more visibility during the last five years. Notably, considering the low number of articles per year in the microfluidic case, this trend must be monitored in the near future. Microfluidics is commonly defined as the science and technology of systems that manipulate small amounts of fluids (pL and nL ranges), using channels with dimensions that typically range from tens to hundreds of microns [18]. This leads to several advantages, including the development of a portable system for point-of-care analysis, the reduction in sample and reagent volume, down to a million times more than conventional approaches, and the ability to perform parallelized assays that can drastically increase analysis throughput [19]. Given these benefits, it is clear that microfluidics can contribute to simplifying and speeding up the EV isolation process from biofluids, representing a good alternative to conventional protocols. Additionally, microfluidic devices can also be exploited for EV analysis and detection, being embedded within the same microfluidic system or based on other instruments (e.g., a fluorescence microscope). In the latter case, microfluidic devices can be seen as passive tools for EV storage.

In this review, we aim to address the most relevant microfluidic systems devoted to EV isolation, underlining both advantages and disadvantages compared to the conventional existing methods. After reviewing the most common isolation methods, microfluidic approaches are discussed, with particular emphasis on those that seem more promising for future clinical applications. In this context, EVs must be isolated by a microfluidic device and ready for further analysis (see the workflow in Figure 2). Given the variety of microfluidic devices in terms of microfabrication and functionality, they are divided into two main categories: physical and chemical approaches. Whereas the former can be distinguished in active and passive methods, the latter are mainly based on immunocapture on fixed and non-fixed (beads) substrates. In addition, a quantitative analysis of the diffusion of the various microfluidic methods, as well as their capabilities of being used in real clinical studies, is presented. A short description of EV detection methods based on

microfluidics is also introduced; however, for a deeper understanding, a dedicated review can already be found in the literature [20].

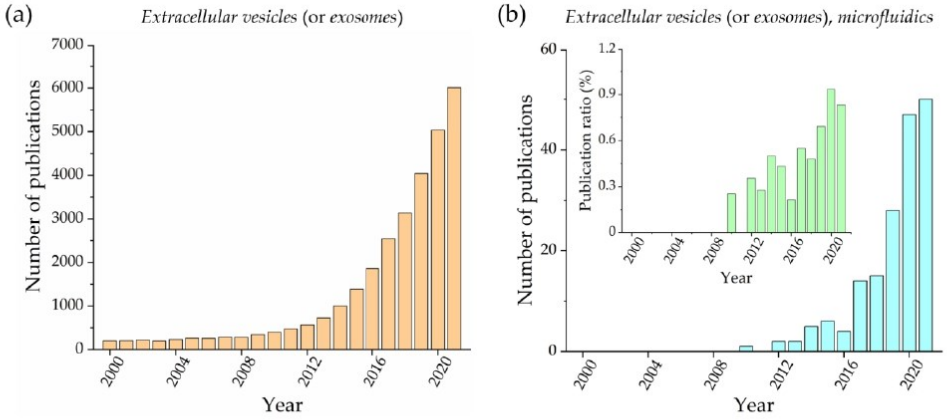

Figure 1. Number of publications per year that contain the keywords: *extracellular vesicles* (or *exosomes*) (**a**) and *extracellular vesicles* (or *exosomes*) and *microfluidics* (**b**). An inset with the ratio between these two numbers is also reported. (Source: Web of Science database, excluding review, meeting abstract, and retracted papers).

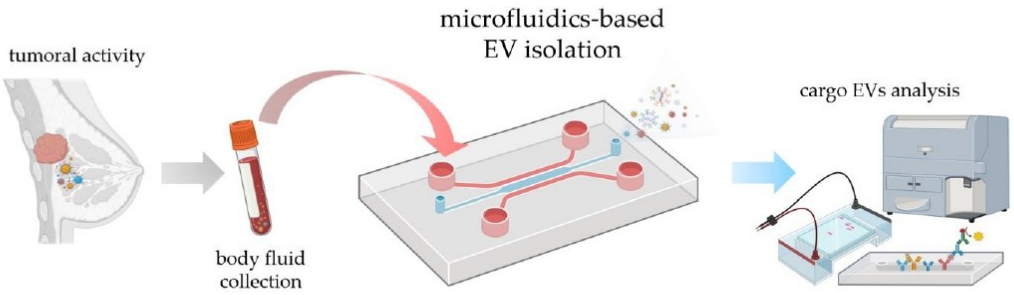

Figure 2. Possible workflow of tumor diagnosis using the microfluidic EV isolation strategy, from sample collection by liquid biopsy to analysis. The elements of the figure are created by BioRender.com.

Importantly, in recent years, different terminology has been used in the literature to classify EVs based on their size or function, since their biogenesis was not easily assessed; however, in 2018, the International Society for Extracellular Vesicles (ISEV) indicated using the word *Extracellular Vesicle* (or EV) as a broad generic term that includes all subtypes of vesicles to avoid confusion in the literature. This recommendation is followed in this review, using the term small extracellular vesicles (sEVs) when the cited articles refer to exosomes or EVs smaller than <200 nm [9].

2. Conventional EV Isolation Strategies

Conventional methods for the isolation and purification of extracellular vesicles can be classified into methods based on the morphological properties (i.e., size, density) and based on their interaction with specific components (solubility, protein reaction). The preferential strategy must be chosen as a function of the initial sample (e.g., cell culture, blood, urine) and the scope of the analysis (e.g., quantity evaluation, diagnosis of specific diseases) [9]. In the following paragraphs, conventional methods will be briefly introduced, reporting their working principle and focusing on their advantages and disadvantages in terms of EV purification. More details on these methods can be found in a specific review [17].

2.1. Differential Ultracentrifugation and Density Gradient Ultracentrifugation

Differential ultracentrifugation (DU) was the first approach used for EV isolation. In general, centrifugation is a label-free method that allows accelerating the natural sedimentation rate of suspended objects that are denser than the surrounding medium [21]. In the case of EV isolation, protocols are typically based on increasing the centrifugal force to progressively remove first cell debris (approximately 1500× g), then large EVs (between 10,000 and 20,000× g), and finally, to collect small EVs (100,000–200,000× g) appearing as a small pellet at the bottom of the centrifugal tube [21]. The limitations of this method are related to the need for expensive equipment (i.e., an ultracentrifuge) and the variable recovery rate (between 5 and 80%), which can often be operator related, preventing comparisons between different studies. In addition, isolated objects are pelleted according to their density, and thus collected materials also contain protein complexes and non-EV nanoparticles (e.g., apoptotic bodies, viruses), leading to an incomplete separation [22]. Additionally, EVs may also cauterize together due to strong centrifugal force. Many different DU protocols can be found in the literature that may puzzle those who are in the research field for the first time. Despite these issues, probably due to its simplicity and recovery rates that can be rather high, DU remains the most widely used approach in research laboratories and is typically combined with other filtration-based EV isolation methods.

Density gradient ultracentrifugation (DGU) is a variation of DU that consists of the addition of specific components (e.g., sucrose) within the suspending medium in order to match the EV density, while allowing the other components to precipitate [23]. Although DGU allows for gaining higher EV purity than DU, some limitations are also reported: the process is time consuming, and molecules with similar EV density (e.g., high-density lipoproteins) can be co-isolated.

2.2. Filtration Methods (Ultrafiltration and Size-Exclusion Chromatography)

Filtration methods are based on the use of a porous membrane to filter objects larger than the porous size [24]. Since EVs typically have sub-micrometric size, membranes with pore sizes between 0.001 µm and 0.01 µm are used combined with ultracentrifugation, in the so-called ultrafiltration (UF) technique; UF allows for faster protocols and better sample quality than DU in terms of purity from protein co-isolation. However, the recovery yield can be biased by the pore size [25].

A highly used filtration-based technique for EV isolation is size exclusion chromatography (SEC), consisting of the elution of EVs in a column composed of packed porous polymeric beads [26,27]. This simple strategy allows for isolating intact EVs from various biological fluids, preserving their biophysical properties, sharp-peaked distribution in size, and high functionality. Thus, among isolation strategies based on the physical properties of EVs, in particular their size, SEC is considered the least invasive in terms of EV integrity [28]. On the other hand, an important disadvantage is the low recovery rate: SEC can be applied to concentrate EV fluids (such as plasma), but in the case of low initial EV concentration (e.g., cell culture media), a pre-concentration step by UF is required. Despite the similarity to microfluidic technologies based on separation by size through pores, SEC relies on the passive motion of particles in a stationary phase, and it is highly time-consuming. Importantly, SEC can also co-isolate other components, such as viruses, protein aggregates, large proteins, and low-density lipoproteins; however, these contaminations are typically less compared to other EV-isolation methods. Today, several commercially available SEC kits can be found specifically designed for EV isolation, depending on the volume and quality of the input sample [29]. It should also be noted that recent studies are trying to improve the capabilities of a standard SEC column, for example, by using a bead size gradient as in particle purification liquid chromatography (PPLC) [30,31]. In any case, despite the good purity of the final sample, SEC requires high costs for disposable filtering columns, in addition to long isolation times.

2.3. Precipitation and Immunoaffinity Methods

Physical–chemical interactions between EVs and solid support are also exploited for their isolation. These approaches are based on EV precipitation [32], adjusting their solubility by chemical compounds, and immunochemistry reactions that exploit the protein present on their surface [33]. EV precipitation can be achieved by properly adjusting the concentration of specific polymers within the starting sample, leading to very simple protocols (e.g., ExoQuick® [34]). However, the final samples are contaminated by the polymer used, which may compromise the downstream analysis. In contrast, immunochemistry reactions are based on the chemical binding between proteins on the EV membrane and specific antibodies, typically grafted onto surfaces or beads. In more detail, some specific tetraspanin molecules are present on most EV membranes (e.g., CD63, CD81, CD9, and others) and are typically used for this purpose [35]. It is noteworthy that the same approach can be applied to isolate a subpopulation of EVs that presents specific membrane proteins associated with a specific EV subtype. The advantages of this approach are its simplicity, the fact that it does not require specific training by the user, and its reproducibility. However, to avoid nonspecific interaction, pre-purification steps are typically required (e.g., differential ultracentrifugation). In addition, isolation kits for specific immunocapturing are usually costly. Another drawback is intrinsic in the approach itself: by selecting the EVs from their surface markers, a subpopulation is always collected, and this could eventually bias the downstream analysis. For this reason, it is preferable to use multimarker antibody cocktails to recover vesicles characterized by different antigens or secreted from heterogeneous cells [36,37].

2.4. Comments

In summary, it is clear that all the conventional approaches listed above have both advantages and disadvantages, as highlighted in Table 1, and the choice between them must be made according to the scope of the study. However, it is important to note that although the chosen approach is the same, the specific parameters for the isolation of EVs are adapted differently from time to time in different laboratories, leading to a lack of standardization and important inconveniencies in comparing the data. On the contrary, microfluidic devices have the potential to overcome some issues, such as the need for expensive facilities and consumables and large sample volumes, which are peculiar to conventional isolation techniques.

Table 1. Summary of the comparison of conventional techniques for EV isolation, based on different isolation strategies. Retention time, quality, and quantity of processed sample and protocol simplicity are evaluated according to the following scale: (–) difficult/very bad, (-) non-trivial/mediocre, (+) easy/good, (++) very easy/excellent.

	Working Principle	Retention Time	Output Sample (Quality and Quantity)	Simplicity
Differential ultracentrifugation	particle size	-	-	++
Density gradient ultracentrifugation	particle density	–	–	++
Ultrafiltration	particle size	–	-	+
Size-exclusion chromatography	particle size	–	-	+
Field-flow fractionation	particle size	-	+	+
Precipitation-based	particle–polymer interaction	-	+	+
Immunoaffinity-based	antigen–antibody binding	-	+	+

3. EV Isolation Methods Based on Microfluidic Devices

Microfluidics is typically applied to bioanalytical protocols by following two different approaches: (i) the miniaturization of existing methods or (ii) the development of new methods that cannot be performed without miniaturization. The first way allows for automating processes while reducing starting volumes, sometimes leading to increased throughput of the analysis and quality of the output sample. In contrast, the second approach tackles the conventional limitations from a completely different angle, leading to results that cannot be compared with existing large-scale methods.

Among the possible ways to classify microfluidic devices, they can be distinguished between "physical" or "chemical" methods according to the nature of forces that regulate the EV isolation process.

3.1. Physical Methods

Physical isolation methods are label-free and exploit physical properties to discriminate vesicles (i.e.,: size or density). They can respond to external physical forces or be based on passive EV collection. In the following, physical approaches are divided into passive or active methods, depending on the presence or absence of driving forces that trigger the physical characteristics of the EVs or of the medium in which they are dispersed.

3.1.1. Passive Approaches

Passive separation methods are label-free isolation strategies that do not require external forces or stimuli. They are intended to enrich EVs by filtering processes through membranes integrated in microfluidic channels or by exploiting hydrodynamic flow properties.

Filtration. A simple method to separate EVs from the initial biological sample based on size requires the use of filtration systems, such as a nanoporous membrane, that allow the passage of vesicles having a dimension smaller than the pore size by exploiting pressure provided by external syringe pumps or by pressure controllers. Inspired by UF and SEC, microfluidic protocols have important advantages in terms of cost, required sample volume, and automation. Filters such as polycarbonate track-etched membranes were integrated within microfluidic devices during the fabrication process by the authors of [38–44]. In this way, the final device is simple to use: it does not require labels or surface treatments, allowing for the processing of very large sample volumes. Nevertheless, the production of such devices can be very complicated because of the strong microfabrication skills required, sometimes preventing mass production. As in the case of conventional filters, these devices can be prone to clogging and are typically disposable; additionally, isolation through filters lacks specificity, except for size. Liang et al. presented a prototyping example, developing a polycarbonate-based double-filtration system to isolate vesicles within a range of 30–200 nm starting from the urine of patients with bladder cancer [38]. An isolation chamber is devoted to collect EVs smaller than the pore size of 200 nm of the first membrane, and particles smaller than 30 nm are trapped through a second filter in a waste chamber (Figure 3a). This method allowed for the isolation of EVs from 8 mL of the pre-centrifuged initial sample in approximately 3.5 h, using a flow rate of 40 µL/min. Further multiple-step filters are arranged in the Exosome Total Isolation Chip (ExoTIC), in which five membranes in a series (pores of 200, 100, 80, 50, and 30 nm) allow for a strict differentiation in the size of vesicles from various types of samples, such as plasma, urine, and lavage [39]. The starting samples have an upper volume limit that can range from 20 mL for cell culture to 500 µL for plasma. Another device called Exodisc takes advantage of centrifugal force with double-step filtering through membranes of different pore sizes [44]. Woo et al. were able to isolate exosome from a 1 mL starting sample of cell culture or urine in 30 min with high purity (95% yield). Sunkara et al. optimized the same platform for processing whole blood, despite using smaller samples (30 µL) and reaching lower recovery rates (exceeding 75%) [45]. Other types of filtering devices rely on cross-flow (tangential-flow) processes, in which the feed flows across the surface of the membrane that acts to concentrate EVs larger than the pore size (30–50 nm). These

devices can pair the cross-flow strategy with conventional (dead-end) filtration [41,42] or with other isolation techniques, such as immunoaffinity-based capture [43], to improve purification efficiency (see also Section 3.2.1).

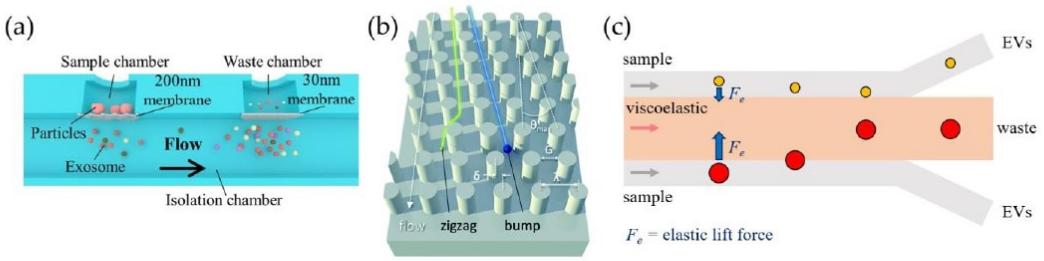

Figure 3. EV isolation methods based on passive approaches. (**a**) Double-membrane system to enrich sEVs (exosomes) of sizes between 30 and 200 nm [38]; (**b**) silicon array of pillars placed inside the chip to induce different zigzag pathways based on particle dimension [46]; (**c**) microfluidic device allowing collection of extracellular vesicles from initial sample mediated by elastic force from viscoelastic fluid.

Inertial force. EV separation can also be performed by exploiting the inertial lift force $\boldsymbol{F_i}$ that particles experience while flowing in a microchannel due to the Poiseuille flow profile [47–49]. In fact, $\boldsymbol{F_i}$ acts to drive the particles orthogonally to the flow direction inside the microchannel in a manner that strongly depends on particle dimension $\boldsymbol{D}$ ($\boldsymbol{F_i} \propto \boldsymbol{D^4}$). Therefore, by properly tuning the channel size and flow rates, it is necessary to focus the particles according to their size [50–55]. A particular configuration consists of spiral channels that strongly favor the lateral migration of particles with different velocities depending on size [56], as used by Tay et al. for rapid isolation from a whole blood sample at 80 µL/min, despite reaching a poor recovery efficiency (20–60%) [57].

Deterministic lateral displacement (DLD). A different approach exploits inertial microfluidics and hydrodynamic interactions of particles with structured channels: particles flowing in microchannels, other than the main force that drives them along the channel itself, also experience lateral forces depending on their size. This effect can be combined with specific and ordered patterns of pillars inside microfluidic channels (see Figure 3b), leading to the so-called deterministic lateral displacement (DLD), which allows for the generation of streamlines that the particles follow depending on the distance of centers $\boldsymbol{\lambda}$ and the gap of the pillars $\boldsymbol{G}$, as well as on the offset angle $\boldsymbol{\theta}$. Their separation can occur whenever the particle size exceeds a critical diameter, which for circular pillars equals $\boldsymbol{D_C = 1.4\, G tan\theta^{0.48}}$, which acts as a cut-off [58]. Specific details about the physical principles of DLD are discussed in devoted articles [59]. DLD has been widely applied for hydrodynamic cell separation depending on the cells' size, and it has also recently been applied to nanometric objects, such as EVs. For example, the pioneering work of Wunsch et al. provided a sharp resolution of particles between 20 and 110 nm separated with a silicon-based pillar array [60]. The production of these pillars, having gaps from 25 to 235 nm, required a sequence of complex micro- and nano-fabrication steps including photolithography followed by reactive-ion etching, then an electron beam process, and finally, deep-UV lithography. Later, Smith et al. integrated 1024 arrays in parallel in another nanoDLD device (Figure 3b) to isolate EVs from serum and plasma, using a similar fabrication approach [46]. This improved design allows for faster isolation processing (up to 900 µL/h), despite an EV recovery of approximately 50%. Other devices, reporting pillar dimensions and gaps of the order of microns, are instead replicated from silicon wafers produced by standard photolithographic and etching techniques, allowing one to reach a high purity of the final sample, but working at low throughput (of the order of µL/h) [61,62].

Viscoelastic force. Most bodily fluids (such as blood, saliva, semen, etc.) exhibit a non-Newtonian behavior when flowing through channels [63]. This viscoelastic property

can be leveraged to separate particles by size by driving a lateral migration owing to the elastic lift forces, without external fields. Specifically, the trajectory of the particles is regulated by the first normal stress difference (N_1), inducing the lateral motion towards the points of minimum shear rate, with relaxation time dependent on medium properties and channel width [64–66]. The resulting elastic lift force F_e depends on the cube of the particle size ($F_e \sim D^3$), and therefore, taking into account a device presenting several outlets (see Figure 3c), larger particles migrate faster to the center line of the channel, whereas smaller EVs are collected at the two sides [67–69]. Unlike DLD-based devices, particles immersed in viscoelastic media can be focused simply by adjusting the rate and width of microchannels, without requiring additional micro- or nanofabricated structures [67,68]. However, to enhance the elastic effect and guarantee good hydrodynamic focusing, specific polymers can be added to the starting samples. As examples, Liu et al. (2017) added a low concentration (0.1 wt%) of a biocompatible polymer to the cell culture medium or serum sample, namely, poly(oxyethylene) (or PEO), to enhance these effects and better control the separation of EVs, achieving high purity and recovery rates greater than 80% and 90%, respectively [70]. Then, in 2019, a similar approach was used to simultaneously separate particles by size and based on membrane protein EVs from breast cancer cell lines and from serum, by using double-stranded λ-DNA molecules in TBE buffer to increase the non-Newtonian effect [71]. In this case, the extracellular vesicles are subjected to the centerline-directed elastic lift force F_e; additionally, larger microvesicles and apoptotic bodies are repelled by the elastic force, competing with the and drag forces F_d ($F_d \propto D$). Asghari et al. exploited oscillatory flows to separate micrometer and sub-micrometer constituents from HEK293T cell lines and was able to focus both λ-DNA strands and vesicles in a sheathless flow [72]. For this purpose, a more complex setup is needed to perform the EV separation, including a pressure-driven chip coupled with an electronic device to actuate valves and generate controlled flow oscillations.

Flow fractionation methods. Another possible way to separate microparticles by size by exploiting hydrodynamic forces is provided by asymmetric flow field-flow fractionation (AF4) [73]. This method requires the implementation of thin microchannels (dozens of μm) having one side made of a membrane that allows the generation of a flow perpendicular to the main stream [74]. Thus, the injected sample under laminar flow conditions is subjected to both the cross-flow field and Brownian diffusion. The accumulation of particles is regulated by the competition of these two counteracting forces, which induce large particles to move in proximity to the membrane, and the smaller particles are easily conveyed along the stream. Typically applied for polymer and protein fractionation, AF4 has been used to isolate EVs, being capable of separating two different subpopulations of vesicles by size (60–80 nm and 90–120 nm) from several tumor cell lines [75]. Shin et al. employed a similar fractionation approach, known as EV separation pinched-flow fractionation (PFF) [76], to isolate EVs from apoptotic bodies [77]. Here, a sheath fluid is applied to achieve EVs by focusing within the microchannel.

3.1.2. Active Approaches

Whenever physical forces are applied to fluids that contain suspended particles, they can respond to the stimulus by changing their motion. Active separation exploits applied fields, such as acoustic or electrical ones, without resorting to channel functionalization or patterning, being label-free and contact-free.

Acoustofluidics. Acoustofluidic devices combine the ability of microfluidics to handle small volumes in confined channels with the ability to trigger particle motion with acoustic waves [78–80]. This label-free and contact-free method employs ultrasound waves to induce differential forces on particles according to their size [81]. Particles can be trapped, separated, focused, or transported by regulating the properties of acoustic waves, which can propagate within the bulk material (bulk acoustic waves, BAWs) [82,83] or along the surface of the medium (surface acoustic waves, SAWs) [84]. Importantly, to emit acoustic waves, electrodes or piezoelectric substrates must be included into the microfluidic devices and

properly engineered during their production. In the case of BAWs, the entire piezoelectric material driven by an alternating current (AC) vibrates at the same frequency of the AC signal (100 kHz–10 MHz). In contrast, SAWs are generated by applying an AC signal to interdigitated transducers (IDTs) patterned on a piezoelectric material, which are excited at higher frequencies than BAWs (up to GHz) (Figure 4a). In order to confine small particles such as EVs, high frequency, of the order of dozens of MHz, is generally required, and thus SAW-based devices are employed [85]. As an example, Lee et al. used a $LiNbO_3$ wafer to imprint interdigitated electrodes that can discriminate sEVs and larger microvesicles from red blood cells according to their size; their cutoff value can be set by tuning the acoustic power and flow velocity [86]. Although fabrication is somewhat complex due to the presence of acoustic actuators, acoustofluidic devices can guarantee high isolation efficiency [87–89]. Wu et al. developed a device based on SAWs consisting of two modules to remove larger blood cells and debris, showing separation of EVs with an 82.4% recovery rate and 98.4% purity using flow rates in the order of few µL/min [90]. Other SAW-based platforms have been coupled with commercial acoustic transducers that lead to automated processes [91–93], and others have been implemented together with modules devoted to the detection of sEVs [94–96].

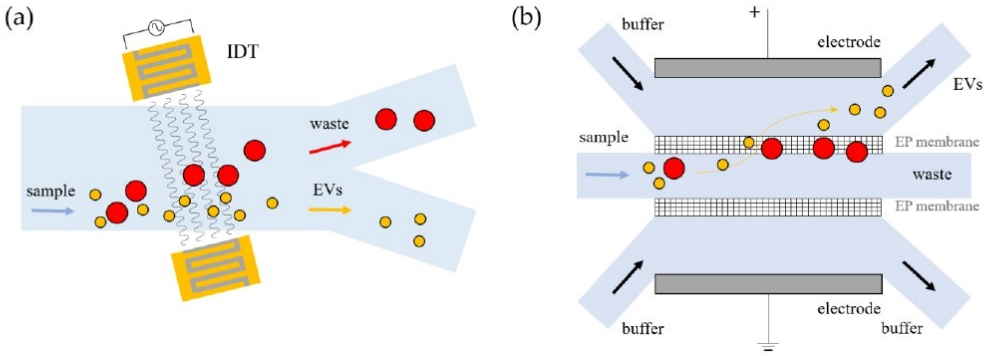

Figure 4. Examples of physical forces to separate vesicles within microfluidic chips. (**a**) Device with interdigitated electrodes generating acoustic waves to separate EVs from the initial sample; (**b**) differentiation of EVs through membranes mediated by electrophoretic (EP) forces.

Electrokinetic force. Electric fields applied to fluids allow for the manipulation of polarizable particles, giving rise to a variety of electrokinetic phenomena: electrophoresis, dielectrophoresis, electroosmosis, etc. These effects provide forces whose magnitude acting on the particles is strictly dependent on their dimension, the dielectric constant, and the charge density of both particles and the surrounding medium. More precisely, the electrophoretic effect (EP) works on monopoles, requiring high forces ($\boldsymbol{F_{EP}} \propto \boldsymbol{E}$) to induce particle manipulation. Dielectrophoresis (DEP) instead allows for controlling the trapping of particles by electric field gradients, to which the DEP force is proportional ($\boldsymbol{F_{DEP}} \propto \nabla|\boldsymbol{E}|^2$) and thus depends on the electrode geometry, rather than the intensity of electric pulses [97]. The latter method happens to be the most widely employed for EV manipulation due to its simplicity with respect to the other electrokinetic phenomena. Ibsen et al. used an alternate current electrokinetic microelectrode to concentrate sEVs from plasma on the edge of the microelectrodes where high field gradients were exerted to process relatively small aliquots (30–50 µL) in less than 30 min, including on-chip fluorescent detection [98]. To improve isolation performance, a device based on dielectrophoretic interactions can be mediated by other types of substrates, such as polystyrene microspheres [99], coupled with automated parts [100], or pneumatically driven components [101]. The latter has been exploited by Davies et al. in a device that takes advantage

of the electrophoretic interaction with a pressure-driven filtration stage of porous polymer monolithic membranes (PPMs), which have variable size pores, to isolate vesicles from 240 µL of whole blood in two hours. In addition to DEP, other examples of electrokinetic phenomena already exploited to trap and concentrate vesicles are electrophoresis [102,103] or electro-osmosis [104,105]. For example, Cho et al. developed a device to enrich plasma EVs by coupling a porous membrane with a dedicated electrode (Figure 4b), in order to remove free proteins and debris subjected to electrophoretic migration through 30 nm pores, with an efficiency of approximately 85% [106].

A recent work by Tayebi et al. combined both dielectrophoretic and acoustophoretic forces to sort extracellular vesicles (<200 nm) and microvesicles (>300 nm) from cell cultures that reached high levels of purity (95%) and recovery (81%) [107]. This kind of virtual DLD (vDLD) permits tuning the balance of the two counteracting forces by adjusting properties of the medium and channel sizes, given a fixed electrode geometry.

3.2. Chemical Methods

Unlike physical methods, approaches based on the chemical affinity between specific antibodies and antigens allow for the recovery of vesicles in a more selective manner. Immunoaffinity-based capture can occur on flat or patterned substrates, as well as on micrometric solid beads or nanoparticles.

3.2.1. Immunocapture on Fix Support

The selective separation of EVs can be achieved by properly engineering the internal microchannel surfaces by adding a specific antibody that can anchor a specific EV membrane protein. The method provides an extremely good specificity and reproducibility and, in the best cases, allows for processing of samples with very high throughput, even of tens of µL/min.

The simplest strategy is to functionalize unstructured channels. However, by using a straight channel with a typical lateral side of dozen to hundreds of µm and considering the typical size of EVs, the binding area available for vesicle capture is relatively low, causing a poor probability of contact. Moreover, the laminar flow prevents the correct mixing of the solution containing EVs, limiting their accessibility to the molecules anchored on the channel walls. This issue has been faced in two ways: i) improving mixing by patterning channels with specific patterns and ii) including micro- and nanostructures within the channel by creating a sort of filter through which the solution is forced to pass. The first method is well known in the microfluidic community, having already been applied to promote chemical reactions or for isolation purposes [108]. In contrast, the second mimics standard filtration methods by integrating specificity, since these 'filters' are coated to capture EVs showing the desired markers [109]. However, as for filtration methods, this approach suffers from clogging and highly complex microfabrication protocols. To increase the surface-to-volume ratio of channel walls, the inner surfaces of microfluidic chips with micro- and nano- structures are also chemically functionalized with antibodies to ensure the EV chemical affinity [110,111]. The most common microstructures are ordered rows of pillars [112–114], herringbone patterns [108,111,115–118], and properly shaped microposts [119,120]. Meanwhile, in the case of nanopatterning, nanorods, nanowires, and more complex 3D structures [121] are typically used. In 2010, a pioneering work by Chen et al. described a way in which to modify PDMS microfluidic channels presenting herringbone grooves with specific surface treatment [122]. The authors flushed inside the chip a solution of 3-mercaptopropyl trimethoxysilane and incubated it with Neutravidin solution before functionalizing it with biotinylated anti-CD63 antibodies, allowing for the isolation of vesicles from 400 µL of serum within one hour. In another work, Chen et al. used an array of ZnO nanowires (Figure 5a) with interconnected macropores to expand the trapping area [109]. The latter approach was validated with small EVs spiked in saline solutions, showing a trapping rate of up to 30 µL/min, and then with both serum and plasma, from which trapped vesicles were detected with horseradish peroxidase (HRP)-

labeled antibody to allow for colorimetric sensing using 3,3′,5,5′-tetramethylbenzidine (TMB). Zhang et al. developed a chip with graphene oxide/polydopamine (GO-PDA) interfaces that provides specific EV absorption from human plasma [119]. The same group then compared a herringbone pattern with solid structures with colloidal silica nanorods (Figure 5b) that showed an improvement in the detection limit of plasma samples at 10 sEVs/µL [123]. Wang et al. fabricated a microfluidic chip structured with a 3D array of ciliated silicon pillars for multiscale filtering of EV-like vesicles or liposomes, mixing filtration properties and immunocapture [112]. Tests with prototyped 83 nm liposomes revealed a retention rate of approximately 60% from a 30 µL of starting volume. This arrangement has been optimized by Qi et al. to improve capture efficiency and preserve EV integrity for drug delivery (Figure 5c) [113]. In this work, the retention rate of sEVs from MDA-MB-231 (breast cancer) cell culture could be increased to 70%, mainly due to the anti-CD63 functionalization of micropillars.

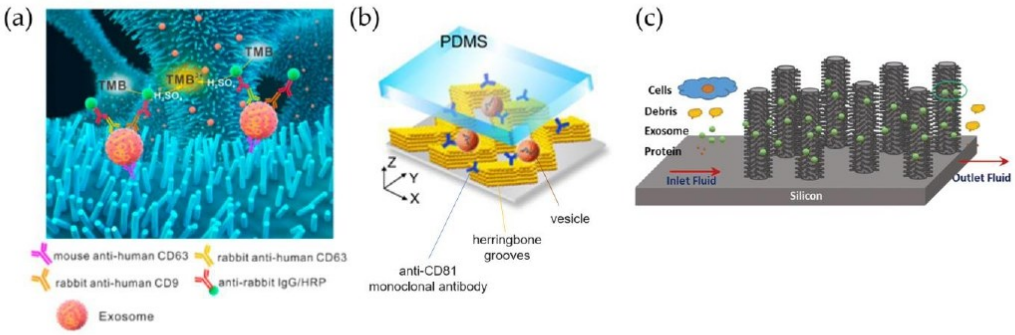

Figure 5. Immunoaffinity capture inside microfluidic devices. (**a**) ZnO nanowires fabricated inside channels for the specific capture of CD63-positive sEVs, modified from [109]; (**b**) colloidal structures arranged in a herringbone configuration inside the microfluidic channel, modified from [123]; (**c**) ciliated silicon nanorods capable of discriminating sEVs from cell debris [113].

3.2.2. Immunocapture on Beads and Nanoparticles

Another strategy to chemically trap EVs requires the use of beads of a size between 0.5 and 20 µm functionalized with the target antibody. In fact, floating beads of micrometer size present a larger surface area. Beads can be directly injected and mixed within the initial sample, enhancing the EV contact probability, without introducing complicated micro- or nano-structures into the microfluidic chip, which typically require costly fabrication approaches and a highly trained operator. Therefore, this technique is one of the most efficient in terms of specificity, but especially for higher recovery rates and analysis sensitivity. On the other hand, the flow rates applied in the microchannel to transport liquids cannot be too low, in order to prevent bead sedimentation, nor too high, to ensure good mixing between vesicles and EVs, even though some works tried to process the sample at a throughput of up to approximately 9 mL/h [124]. There are different possible beads that can vary in terms of material or size. The most commonly used are micrometer-sized commercial immunomagnetic beads that have a paramagnetic core that can be easily handled using external magnets [125–135]. Here, unlike in functionalized channels for immunocapture, an external magnetic force must be applied to manipulate particles and favor isolation. A key aspect in the choice and use of the floating substrate for EV capture is that the time must be sufficient for the substrate to settle in the channel. The sedimentation speed v for a single object dispersed in a viscous fluid can be calculated by the Stokes law: $v = \left(d^2(\rho_d - \rho_f)g\right)/(18\mu_f)$, where d and ρ_d are the diameter and the density of the object, ρ_f and μ_f are the density and the viscosity of the fluid, and g is the gravity acceleration [136]. Thus, considering a polystyrene bead of 1 µm (ρ~1.05 g/cm^3)

and an EV of 200 nm (ρ~1.1 g/cm^3) dispersed in water (μ_f~1 cP), it is possible to notice that the first sediments are more than ten times faster than the second. This occurrence becomes even more critical when using magnetic beads (ρ~1.8 g/cm^3), as their sedimentation speed is two orders of magnitude higher than for EVs. Therefore, the flow rates applied to the liquid within the channel must be fast enough to prevent particle sedimentation, but slow enough to ensure a sufficiently long incubation time for EV capture. Thus, the working range of this type of device is limited.

A highly cited example is given by He et al., who used immunomagnetic beads coated with specific antibodies (e.g., anti-EpCAM) to capture and lyse sEVs inside a unique device to analyze the protein content by chemifluorescent ELISA [125]. Plasma samples of 30 µL volume are processed in about 100 min. Zhao et al. fabricated a device called ExoSearch to enrich sEVs from plasma and measure multiple marker fluorescent signals (Figure 6a) [126].

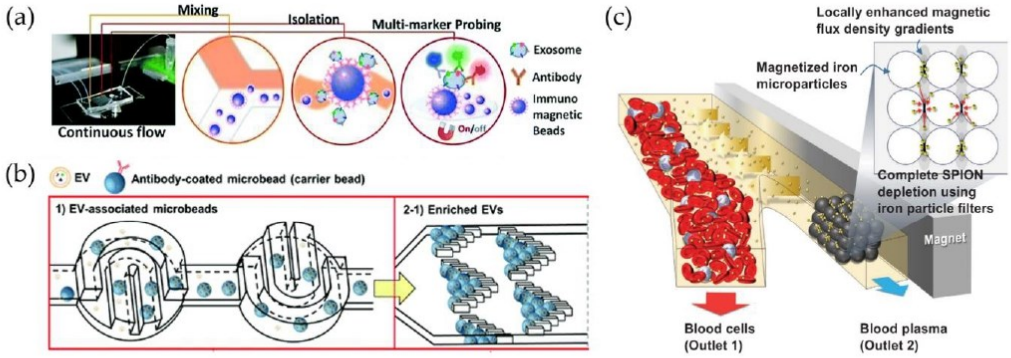

Figure 6. Functionalized beads or nanoparticles used for vesicle capture inside microfluidic devices. (**a**) Immunomagnetic beads coated for the enrichment of vesicles from blood plasma inside ExoSearch chips [126]; (**b**) streptavidin-coated polystyrene beads used as substrate to trap vesicles in herringbone filters and redisperse them [137]; (**c**) microfluidic systems used to flow blood samples and isolate vesicles by means of superparamagnetic nanoparticles (SPIONs), modified from [138].

In addition to magnetic beads, polystyrene beads (PS) can be used for the isolation of EVs [80,99,124,137,139–141] using centrifugation and redispersion instead of magnetic forces. One of the main drawbacks of immunocapturing with beads is the difficulty of breaking the bond with antibodies, preventing EV damage. However, Tayebi et al. used specifically coated PS beads to capture EVs from MCF-7 (a breast cancer cell line) in constrictions along the microfluidic circuit with an aperture of 30 µm to trap a single bead, by exploiting hydrodynamic resistance in channels having different shapes [140]. The trapped EVs were then removed from the beads by rising with a low pH IgG elution buffer (0.1 M glycine-HCl) for 1 min and then waiting 10 min for antibody–antigen dissociation. Finally, neutralization is provided by a solution of pH 7.4 (1 M Tris-HCl). Despite a good purity, this approach limits the amount of EV–bead complexes to the number of trapping sites and the maximum flow rate (50 µL/min). Gwak et al., instead, promoted chaotic stirring of coated PS beads inside horseshoe-shaped mixers, and the work [124], was able to enrich plasma EVs using fish trap-shaped filters (Figure 6b) [137]. Then, using the same elution buffer, different flow rates were tested: under the best conditions, the whole isolation process for the 100 µL sample was completed in 5 min, showing a capture efficiency greater than 97%.

Unlike micrometric beads that are typically larger in size than EVs, an alternative solid substrate is represented by nanoparticles (NPs), which are on the order of a few nanometers, either with magnetic properties (superparamagnetic or ferromagnetic) [134,138,142–147] or simply functionalized with selective markers [148]. In particular, they have a similar or

even smaller size than small EVs, so EVs can be used to capture a single EV on their surface, rather than being encapsulated inside, as used in drug delivery [149]. Notably, these NPs can be an active tool for the capture and manipulation of vesicles, rather than a passive substrate. A pivotal work by Shao et al. showed a strategy for labeling and isolating blood glioblastoma microvesicles, implementing properly functionalized magnetic nanoparticles (core of 7 nm) and using a two-step protein targeting to maximize binding. This approach allowed for better detection of CD63 + vesicles with a micronuclear magnetic resonance (μNMR) system [142]. In another work, Ko et al. exploited magnetic NiFe nanopores (600 nm diameter) to trap EVs labeled with 50 nm coated NPs, allowing for the processing of serum and plasma with flow rates of up to 10 mL/h [144]. Recently, increasing attention has also been paid to superparamagnetic iron oxide nanoparticles (SPIONs) to isolate EVs due to the nanoparticles' reversible magnetic property and easy manipulation, as exploited by Kwon et al. for the purification of blood samples [138]. Here, SPIONs and EVs create a complex cluster that can be isolated by exploiting the magnetic force applied by an external magnet (Figure 6c).

In the following, Table 2 reports a summary of the main EV isolation methods exploiting microfluidic strategies, distinguished between physical and chemical, together with their working principle.

Table 2. Summary of relevant published articles dealing with EV isolation from different microfluidic approaches.

	Methods	Working Principle
Physical: Passive	Filtration [38–45,150,151]	Micro-/nano- filtration process by porous membranes inside chip
	Deterministic lateral displacement [46,60–62]	Particle distribution in size by lateral forces conveyed by ordered array of posts
	Inertial force [50–55,77] Viscoelastic force [67–72]	Imbalance of inertial forces or of shear forces in non-Newtonian viscoelastic fluid
Physical: Active	Acoustofluidics [86–96,107]	Acoustic trapping by ultrasound waves
	Electrokinetic force [98–107,152–154]	Charge separation by electric fields
Chemical: Fixed support	Functionalized fixed support [108–122,130,155–180]	EV capture by specific antibodies on fixed substrate
Chemical: Floating Beads	Magnetic beads [125–135,181–186]	EV capture by specific antibodies on beads for magnetic manipulation
	Polystyrene beads [80,99,124,137,139–141,187]	EV capture by specific antibodies on non-magnetic beads
	Magnetic nanoparticles [93,134,138,142–147,149,188]	EV capture or handling by specific antibodies on magnetic nanoparticles

4. Discussion

4.1. Physical and Chemical Microfluidic Approaches: Pros and Cons

When microfluidic devices are designed to improve the purification and/or isolation of specific analytes from complex starting samples, four main aspects must be considered to balance advantages and disadvantages: throughput of the process, quantity and quality of the output sample, automation capability, and complexity of the microfabrication. In the following section, we discuss the above-presented approaches for EV isolation in microfluidic devices, focusing on these four aspects. A schematic summary of the following discussion is also reported in Table 3.

Table 3. Summary of the comparison of different microfluidic techniques for EV isolation. Each of the four aspects is evaluated according to the following scale: (–) difficult/very bad, (-) non-trivial/mediocre, (+) easy/good, (++) very easy/excellent.

	Throughput	Output Sample (Quality and Quantity)	Possible Automation	Micro-Fabrication Simplicity
Physical: Passive	++	-	+	-
Physical: Active	-	+	+	-
Chemical: Fixed support	+	++	++	+
Chemical: Floating Beads	+	++	–	+

Physical approaches based on filtration, although based on principles similar to those of UF and SEC, allow for the processing of large volumes at high throughput. In fact, microfluidic protocols can be easily automated with the support of external pumps controlled by devoted software [189] to infuse biological samples at high rates throughout filters inside channels. However, the realization of these microfilters represents a critical aspect: as for their correspondent conventional methods, these devices are typically disposable, because they are prone to clogging, and the processing time for complete isolation is quite long compared to that of the other microfluidic techniques.

In contrast, physical approaches based on external stimuli, such as acoustic waves or electric fields, are not directly comparable to existing methods. These contact-free strategies prevent EV damage and preserve the EVs' functional properties [86,106]. In fact, the presented results show good vesicle integrity and a homogeneous size distribution, especially in terms of the acoustic approach [90]. On the other hand, captured EVs have a low purity, because other contaminants that have a similar size and/or density can be isolated together. Then, as in the previous case, the microfabrication requires both an appropriate environment (i.e., a clean room) and trained operators, since the electrodes must be integrated inside microfluidic devices, and high alternate electric fields must be applied and precisely controlled. The latter practice must be executed by equipped laboratories, which include costly facilities, such as photolithographic platforms, metal evaporators, electronic equipment, and related characterization instruments. However, compared with microfluidic filtering methods, cleaning protocols can be considered in the case of SAW or DEP devices, partially reducing the impact of the microfabrication. Most of the aforementioned strategies based on separation by size can be biased by the fact that other membrane-based components also have dimensions similar to those of EVs (e.g., lipoproteins).

Then, approaches rooted in chemical affinity have been integrated within microfluidic devices, with the aim of improving the anchor point by increasing the surface-to-volume ratio, isolation throughput, sample quality output, and process automation [190]. Compared to physical approaches, immunoaffinity provides a highly specific isolation that allows for the distinction between the EV subpopulation [191] and the high purity of the isolated sample. In the fixed support-based approach, molecules are coated on microchannel surfaces that typically present specific micro- and/or nanopatterns to improve the liquid mixing (microstructures) and/or acting as filters (nanostructures). In contrast, the use of micrometric beads as solid floating supports for EV capture allows for using more simple channel geometries, since the beads themselves act as traps that improve the surface-to-volume ratio. However, compared to other microfluidic approaches, the use of micrometric beads leads to difficulties in terms of the complete automation of microfluidic devices, since they are prone to sedimentation and can induce microchannel clogging [144].

Another disadvantage of this approach is the high costs of purchasing commercially available microbead kits devoted to EV capture; therefore, custom and home-made protocols are generally preferable [139]. A completely different perspective is provided using nanoparticles. They present a size comparable to or even smaller than a single EV, leading to an improvement in capture control and preventing damage to the EVs. Additionally, since several characterization methods are now based on optical and spectroscopic techniques (e.g.,: fluorescence microscopy, Raman spectroscopy), nanoparticles can be directly used to improve the signal-to-noise ratio [188]. A possible drawback of the use of NPs is that they require trained operators for their synthesis and, importantly, their stability is strongly influenced by the surrounding buffer, which may affect the EVs as well. Therefore, strong physical-chemical expertise is required to develop a working microfluidic device based on NPs' isolation.

In general, as summarized in Table 3, among the microfluidic approaches analyzed, we considered devices based on the chemical affinity of the fixed support to be the most promising, as they show good throughput and high-quality sample output, supported by simple microfabrication and automation. In this sense, the use of beads requires more precautions due to the possible sedimentation and clogging issues. In contrast, physical approaches suffer from complicated complex microfabrication requiring electrode integration, which, at the current state of the art, is not sufficiently balanced by appropriate throughput or sample quality.

4.2. *Microfluidic Isolation Techniques: Which Is the Most Popular*

To better appreciate how widespread these methods are, Figure 7 reports a statistical representation of all the published articles on EV isolation performed by microfluidic devices.

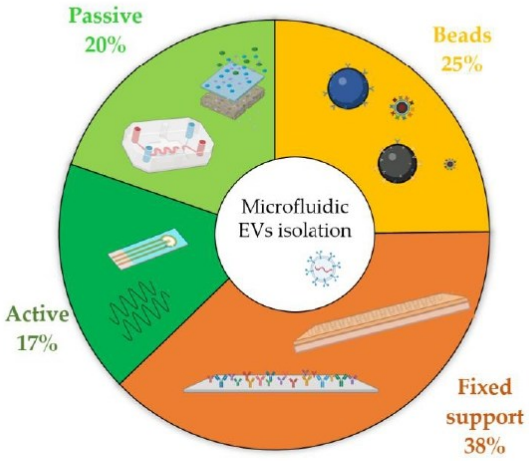

Figure 7. Frequency of the published works from 2000 to present (source: Web of Science database, excluding reviews, meeting abstracts, and retracted papers) related to each of the different isolation techniques that exploit microfluidic devices. Physical approaches are subdivided into active (dark green) and passive (green), whereas chemical immunocapture is separated by fixed supports (orange) and floating beads (yellow).

The pie chart in Figure 7 highlights that the predominance of microfluidic methods makes use of chemical immunocapture, which comprises two-thirds of the total output. This is probably due to the fact that physical approaches leverage concepts similar to conventional ones, such as DU, UF, or SEC, hence carrying similar drawbacks in their usage, and automation has not yet been implemented in the presented proof-of-concept device. Moreover, chemical affinity provides an easy implementation inside microfluidic devices,

especially for flat channels that require a simple functionalization capable of binding to membrane proteins, such as tetraspanins. Additionally, these methods generally perform better than their physical counterparts, mainly in terms of throughput and recovery rate. Among chemical isolation devices, those exploiting fixed substrates are the most commonly used, probably due to the large amount of literature related to the functionalization of microfluidic devices [192], the automatization capability, and the simple microfabrication, despite the fact that the production of certain integrated nano-structures could lead to some difficulties. In contrast, there is no preferred physical approach for EV purification: passive devices are slightly more frequently used, despite the poor quality of final samples, probably because the users do not need specific training to control SAW and DEP, and these devices are more prone to miniaturization. However, these active approaches are relatively newer with respect to their passive counterparts, and therefore, further development is expected in the future to facilitate device production and handling.

4.3. Are Microfluidic Devices for EV Isolation Ready for Clinical Applications

Today, the clinicaltrials.gov database reports more than 350 trials (about 90 already completed) indexed by the keywords *exosomes* and *extracellular vesicles*; among them, only two, which have just started, involve microfluidic systems. The latter can be understood considering the still young character of the EV research field. However, to better analyze the current state of the art from a clinical perspective, we discuss the applicability of the presented microfluidic devices in real diagnostic conditions. In detail, the microfluidic devices devoted to purified and isolated EVs are validated using different starting samples (i.e.,: culture media, blood-derived fluids, urine, etc.), and, in some experiments, EVs are spiked in human fluids. Although we consider all these approaches to be fundamental for the validation of novel technologies, they represent a first proof of concept compared to real clinical assays, since EV isolation may depend on several factors, such as starting samples and EV biogenesis. Therefore, we divided the presented papers based on their validation methods, labeled as: (i) *proof of concept*, if they are limited to cell culture media or spiked EVs in body fluids, and (ii) *clinical sample*, when they directly employ body fluids for EV isolation (e.g., urine, plasma, serum, or whole blood). The result of this classification is reported in Table 4 and Figure 8, together with the isolation approach used.

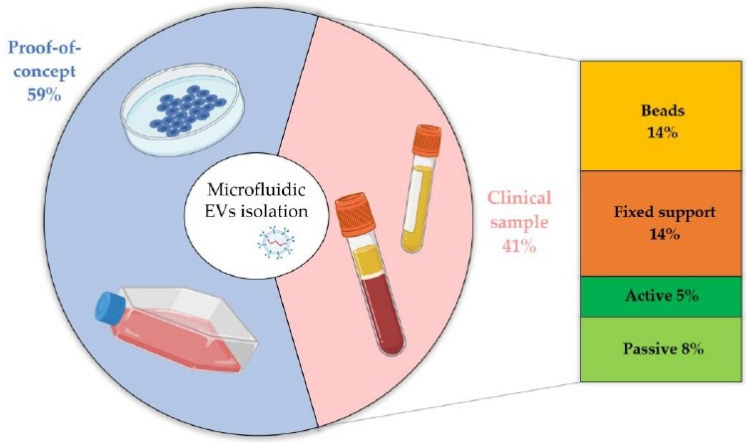

Figure 8. Frequency of published works from 2000 to present (source: Web of Science database, excluding reviews, meeting abstracts, and retracted papers) involving EV isolation from starting biofluids used as a *proof of concept* (cell culture media or EVs spiked in body fluids) or as *clinical samples* (body fluids directly employed) from healthy donors or patients for research studies or diagnostic purposes, further divided according to the isolation technique.

Table 4. Summary of articles published during the years 2000–2022 dealing with EV isolation from microfluidic devices processing samples for research purposes or clinical trials, according to techniques classified in Table 2.

	Proof of Concept		Clinical Sample	
	Starting Sample	**%**	**Starting Sample**	**%**
Physical: Passive	Plasma [52,62] Urine [44,60,95] Cell culture [41–43,50–52,54,55,61,68,71,72,77,88,150,151,193,194]	12	Plasma [39,195] Serum [43,46,71] Blood [45,53,67,69] Urine [38,39,44,46]	8
Physical: Active	Plasma [98,102,103,106,152] Serum [103] Saliva [88] Cell culture [86,89,91,98–100,104,105,107,153,154]	12	Plasma [87,91,94] Blood [90,101] Urine [91,92,95]	5
Chemical: Fixed support	Plasma [87,173] Serum [122,168] Cell culture [109,113,122,155–158,160–162,164–167,172,174–177,179,180,196–200]	24	Plasma [108,114,119,120,123,155,163,171,173,201] Serum [110,117,118,123,130,178,202,203] Blood [170] Urine [115,159,169]	14
Chemical: Floating beads	Plasma [130] Serum [130,183] Cell culture [99,137,139–141,143,147,182,186,188,193,204]	11	Plasma [80,124–127,129,132,135,138,144,185] Serum [131,134,145,147,148,181] Blood [133,142,146] Urine [128]	14
TOTAL		59		41

In Figure 8, it can be observed that 40% of microfluidic devices have been validated using body fluids taken from healthy donors or real patients, representing a good percentage of the total microfluidics-based studies. Among these works, most use immunoaffinity-based devices to capture vesicles, increasing the difference already noted in Figure 7 compared to the use of physical methods. Notably, passive physical methods are disposable and more prone to clogging than their non-microfluidic counterparts, whereas active interactions in real fluids must consider several parameters, such as the fact that viscous biofluids are not as easy to be manipulated as aqueous solution. It is worth noting that most of the works processing *proof-of-concept* samples, such as cell lines or spiked EVs, also include pre-purification steps (e.g., differential ultracentrifugation) before the injection of the biological fluids to favor better isolation of small vesicles after the elimination of heavier debris and cell fragments. In contrast, a device already tested with clinical samples without requiring pre-treatment can be considered ready to use for medical diagnostics, and this kind of validation must be the final goal of future studies.

4.4. Microfluidic Devices for EV Detection and Analysis

Extracellular vesicles collected and purified by the aforementioned approaches can be investigated by several detection techniques. The more conventional ones for EV size estimation require off-chip treatment and characterization by commercial instruments, mostly based on tracking such as nanoparticle tracking analysis (NTA) [205], dynamic light scattering (DLS) [206], or flow cytometry (FC) [207], which reveals information on EV morphology. Indeed, NTA is actually performed in a microfluidic chip for the estimation of size by scattered light based on Brownian particle fluctuations and similarly occurs for DLS; cytometric analysis requires hydrodynamic vesicle focusing that is otherwise achievable by the microfluidic channel.

Microfluidic devices have also been developed for EV detection, mainly based on two approaches: (i) self-standing, in which the device itself works as detector, and (ii) storage, in which the microchannels act as a storing chamber for EVs that are screened by an external microscope or probe. The first approach typically involves the integration of a devoted electrode, as occurs for analysis by field-effect transistors [185], electrochemical sensing [208], zeta potential estimation [102], or lateral flow immunoassay [172].

In contrast, in the second approach, the microfluidic device allows for several types of detection: (i) colorimetric for EV quantification [109], (ii) spectroscopy based on surface-enhanced Raman scattering (SERS) [94], (iii) optical properties by surface plasmon resonance (SPR) [200], (iv) resistive pulse sensing due to EV through nanopores [209], (v) detection by micro-nuclear magnetic resonance (NMR) [142], etc. Furthermore, some microfluidic platforms were engineered to also perform EV content analysis, such as on-chip quantitative PCR [181] or ELISA [44]. More details on detection methods based on microfluidic devices can be found in other specific reviews [20,210].

5. Conclusions and Perspective

This review looks at the most important techniques currently used to isolate EVs using microfluidic devices, analyzing their ability to be applied in clinics. In this perspective, despite the fact that microfluidic technologies have only recently been applied to the isolation of EVs, almost half (40%) of the presented devices use potentially clinical samples for their validation. Therefore, microfluidics seems to represent a promising strategy for medical investigation.

The comparison between physical and chemical microfluidic approaches for EV isolation emphasizes a slight preference for chemical methods over their physical counterparts, which becomes much more evident if one considers only the technologies validated with potential clinical samples. This might be the cause for two possible reasons: (i) the device performance itself (higher throughput, better capture efficiency, and simple microfabrication for mass production) or (ii) the general trend of the biomedical community to look for EVs that have specific phenotypes. Actually, we believe that the latter is dominant, even though there is probably a combination of both factors. Although the characterization of EVs was initially based on quantification and size classification, it is now clear that the specific EV subpopulations can provide useful information related to specific diseases [8]. Therefore, we expect that the gap between physical and chemical approaches will increase in the near future. A possible alternative to further improve the efficiency and purity in EV capture might be the implementation of both physical and chemical approaches combined together inside a single device, using passive EV separation from other massive particles, and then a specific immunocapture for distinguishing different subpopulations, providing also high throughput isolation.

In addition to EV isolation, microfluidic devices are also widely employed for EV detection and analysis. Whereas the first devices consisted of simple microchannels that aimed to store EVs for further analysis (as for the NTA), new approaches, which combine microfluidic systems, devoted optical systems, and antibody immunocapturing, point towards the analysis of single vesicles in order to achieve information about their heterogeneity within the same population [211].

In conclusion, we are confident that microfluidics, which is already employed in some gold-standard techniques for EVs analysis (i.e.,: NTA), can bring great support in terms of EV isolation by providing tools capable of performing repeatable and automated protocols, similar to other important biomarkers, such as CTCs and ctDNA. However, microfluidics experts exposed to the field for the first time must also consider biological and medical points of view. Therefore, to be really useful, new technologies must be developed in accordance with the requirements and expectations that, importantly, have progressively changed over the past five years, following the guidelines on the validation of the protocol and the outcomes established by the ISEV community [9].

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3.3 How to characterize extracellular vesicles

After EV-purification, EV presence, purity and specificity should be verified through characterization analysis. Considered the complexity of EVs and the lack of a single technique to count, measure and unequivocally identify all EVs, more than one characterization methods have to be applied (5).

One of the main limitations in EV-research is the impossibility to unequivocally quantify EVs (5). Different methods can be used to estimate EV-concentration but these methods are approximative, lacking precision or being unable to distinguish between EVs and other nanoparticles (5). One of the most used techniques to count EVs is nanoparticle tracking analysis (NTA). NTA detects scattered particles after irradiation with a laser beam. Scattered particles are then recorded by a camera and the NTA software tracking the Brownian motion of particles, measures particle size and concentration (16). Unfortunately, NTA cannot distinguish between EVs and other particles, including in the measurements all the particles in the size range of EVs (e. g. protein aggregates, lipoproteins, salt, small bacteria) (5). Also, Dynamic Light Scattering uses the principle of light scattering to measure the size of particles, but is more reliable when measuring monodispersed particles, which is not the case for heterogenous EV-samples (5,17). EV-size can also be measured after their visualization through electron microscopy (EM). EM is very useful to truly identify EVs after isolation, visualizing their morphology and lipid bilayer, but cannot be applied as a quantification method and size estimation can be biased by procedural steps (e. g. vacuum, freezing) which can affect EV-morphology (18). Measuring EV components, like proteins, lipids or RNA, can also be valuable characterization methods. Despite all these molecules do not belong to EVs only, their quantification can be useful to estimate the quality of the EV-sample. Indeed, the ratio between protein and particle quantification, or protein to lipid quantification, are often used to define the purity of EVs (5).

Flow cytometry (FCM) can be used both to quantify EVs and to assess their phenotype. Despite classical FCM is not a precise method for the determination of quantity and size of small particles, it is a versatile method to characterize EVs by antigen expression, considering the possibility of detecting fluorochrome-conjugated monoclonal antibodies (18). Moreover, high resolution FCM, with improved ability to discern small (< 200 nm) particles have been developed in recent years as a valuable method for EV analyses (17).

Western Blotting (WB) is one of the most recognized methods to confirm EV presence and characterize their phenotype. Like FCM, WB allows to characterize EVs by specific antigen-expression. MISEV guideline suggest that for improved EV-identification, at least both a

membrane and cytosolic marker should be included in the analysis. Moreover, according to the nature of the starting sample, the presence of possible contaminant particles should also be assessed (e. g. lipoproteins for plasma, Golgi cellular proteins to assess cell debris contaminants) (5).

Finally, considered the increased need of precision in identifying EV-origin and cargo, especially for clinical applications of EVs, single EV- and high-throughput analysis platforms are now available and applied in different studies (19,20). These techniques are mainly based on imaging, which can visualize individual EVs (*e. g.* high resolution flow cytometry, super-resolution microscopy), or are droplet-based (19). The latter, load single EVs into droplets where they are barcoded for downstream analysis (21). High-throughput analysis can then be applied to identify EV genomic, transcriptomic, proteomic, lipidomic, and metabolomic profile, leading to the generation of EV-related OMICS data. Importantly, these data are available in various free-to-use web databases (*e. g.* ExoCarta http://www.exocarta.org; Vesiclepedia http://www.microvesicles.org; EVpedia http://evpedia.info), which can be used to improve data analysis (20).

To sum up, considering the heterogeneity of EVs and of the starting samples, and the different applicable isolation techniques, and since there is not a single valid method which can fully identify, measure, and quantify EVs, more characterization techniques should be combined to confirm EV presence and estimate EV quantity, purity, and size.

4 Extracellular vesicles in veterinary medicine

From the moment of their discovery in the ‘80s, research on EVs has increased exponentially in human medicine (2). Except for some early studies preliminarily identifying EVs from animal-derived samples, most of the literature on EVs in veterinary medicine has been published after the beginning of the XXI century (6,22–25). From that moment, the exponential trend of EV-research in human medicine is reflected also in veterinary medicine, as the number of publications is growing year after year (6).

Despite this increasing interest, our knowledge on animal derived-EVs compared to humans’ is still scarce. Moreover, being veterinary medicine so heterogenous, particularly in terms of animal species and different fields of the scientific research (clinical aspects, or infectious diseases and zoonosis studies, animal husbandry and food science), getting lost in so many different studies is easy.

Therefore, to tidy up and show what has been done until now on animal derived-EVs, I and some colleagues working on EVs published the following review on “Extracellular vesicles in veterinary medicine”. The aim of this review is not only to fill the gap of knowledge on this topic, but also to show the issues, the limitations and especially the potential of working with EVs in animals as well as in humans.

4.1 Paper n. 2: "Extracellular vesicles in veterinary medicine"

animals

MDPI

Review

Extracellular Vesicles in Veterinary Medicine

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Simple Summary: Extracellular vesicles (EVs) are small vesicles released by human and animal cells, parasites, microorganisms, and plants. They travel within bodily fluids, transferring the content of their cell of origin to other cells, being both intra- and inter-organism messengers. This EV-mediated method of communication governs many normal functions as well as disease processes. Because of this important role, EVs have been largely studied since 1984, mainly in humans, but more recently also in animals, parasites, and bacteria. In this review, we explore the literature on EVs in animals between 1984 and 2021 and summarize the most important results of approximately 220 scientific papers. Results are presented based on the main topic of research, such as EVs in physiology and pathophysiology, use of EVs as markers to diagnose diseases, or as possible natural transporters of therapies or vaccines. Since working with EVs is challenging, we also address the critical technical points found in the veterinary literature. Finally, we included a brief summary on EVs shed within animal milk, an area of large interest for the multiple applications for human health.

Abstract: Extracellular vesicles (EVs) are cell-derived membrane-bound vesicles involved in many physiological and pathological processes not only in humans but also in all the organisms of the eukaryotic and prokaryotic kingdoms. EV shedding constitutes a fundamental universal mechanism of intra-kingdom and inter-kingdom intercellular communication. A tremendous increase of interest in EVs has therefore grown in the last decades, mainly in humans, but progressively also in animals, parasites, and bacteria. With the present review, we aim to summarize the current status of the EV research on domestic and wild animals, analyzing the content of scientific literature, including approximately 220 papers published between 1984 and 2021. Critical aspects evidenced through the veterinarian EV literature are discussed. Then, specific subsections describe details regarding EVs in physiology and pathophysiology, as biomarkers, and in therapy and vaccines. Further, the wide area of research related to animal milk-derived EVs is also presented in brief. The numerous studies on EVs related to parasites and parasitic diseases are excluded, deserving further specific attention. The literature shows that EVs are becoming increasingly addressed in veterinary studies and standardization in protocols and procedures is mandatory, as in human research, to maximize the knowledge and the possibility to exploit these naturally produced nanoparticles.

Keywords: veterinary pathology; veterinary physiology; biomarkers; therapy

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1. Introduction

Extracellular vesicles (EVs) are heterogenous membrane-bound vesicles released by cells and involved in intercellular communication. Many EV-subtypes are now recognized, classified mainly for their biogenesis or for their size [1]. The outward budding of the plasma membrane produces ectosomes (microvesicles/microparticles), while the fusion

of the plasma membrane with multivesicular bodies, derived from endosomes, originates exosomes [2]. However, since to date biogenesis can only be confirmed through live imaging techniques, EVs are mainly classified according to their size in small EVs (<200 nm) or medium-large EVs (>200 nm) [1]. More recently new EV-subtypes have also been described, such as large (1–10 μm) tumor-derived EVs named oncosomes [3].

EVs are a preserved evolutionary mechanism of communication that mediate the crosstalk between cells within a body, but also between different organisms of the same or even different species [4]. After the release from a donor cell, locally or at distance travelling through body fluids, EVs can interact with a recipient cell through not fully understood mechanisms, including direct contact through ligand–receptor interaction, fusion of EV membrane with the plasma cell membrane and, more often, endocytosis [2,5,6]. EV cargo is complex, made of proteins, lipids, DNA, and small non-coding RNA [2,5]. This complex cargo is responsible for most of the very wide and heterogenous effects of EVs in normal physiological processes as well as in pathological progression [2,5]. Moreover, EVs also have a discarding function, since they often target lysosomes leading to the elimination of molecules (e. g. proteins, lipids) from cells and to the creation of new metabolites [7].

EVs can be isolated from body fluids, cell culture media and from tissues with many techniques (Figure 1) [1]. Differential ultracentrifugation, which exploits increasing centrifugal forces to pellet EVs, has been the gold standard for years, but nowadays, different techniques can be applied and combined to reach the required recovery rate or to purify a specific EV subtype [1,8]. Since different EV isolation methods result in different EV quantities and subtypes, EV presence should always be assessed after purification, characterizing EVs for quantity (e. g. number of particles or protein/lipid content) and for the presence of EV markers and of other co-isolated components [1].

EVs are now investigated for wide potential effects in many research fields, but their study first started in human medicine during the mid of the XX century, when the presence of pelleted particles in high-speed centrifuged blood was first described [9]. During the 1960s and the 1970s, many electron microscopy (EM) studies described the release of vesicle-like structures by cells, but the real starting point of EV research can be dated in 1983, thanks to two publications of the Johnstone and Stahl laboratories [10,11]. These two papers aimed to follow the fate of the transferrin receptor and described through EM the presence of multivesicular bodies in reticulocytes and the release of vesicles upon fusion with the plasma membrane [9–11]. From that moment on, the studies investigating the role and the composition of these vesicles increased exponentially, leading to the tremendous increase of interest during the last two decades. Progressively, since the beginning of the XXI Century, the interest in EVs has grown in many other research areas, including veterinary medicine (VM). With the growing of EV-specific studies and publications on disparate areas of research, it became clear that EVs, with the ability to mediate intercellular transfer functions, play a key role in the physiology and pathophysiology not only in humans but also in other organisms, such as plants, animals, bacteria, and virus, with emerging evidence of EV importance also in inter-organisms' communication [12,13]. A preserved process of EV formation and release across a broad range of species has been demonstrated, strongly suggesting the essential function of EVs in all forms of life [14].

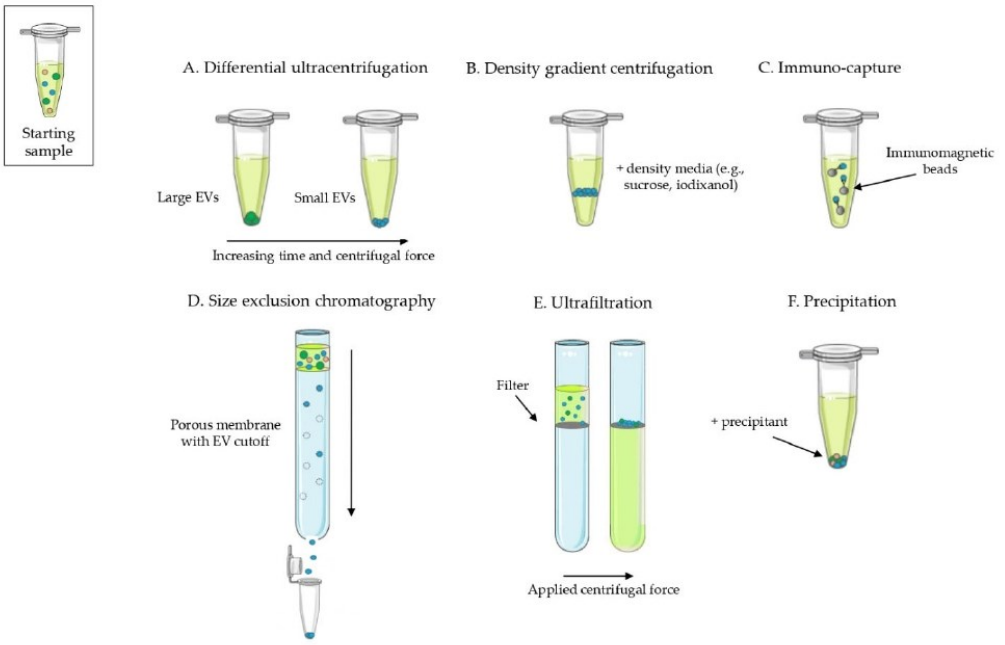
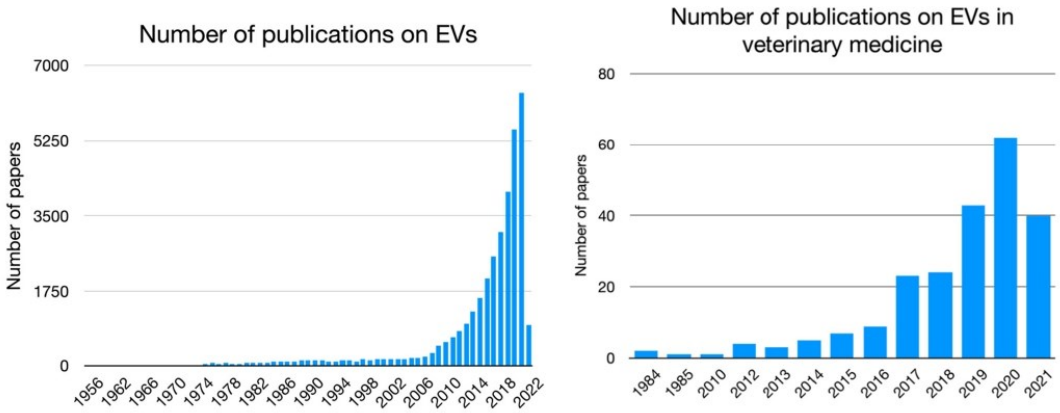


Figure 1. Schematic representation of the most common methods to isolate extracellular vesicles. (**A**) Separation of EVs with differential ultracentrifugation; larger EVs are isolated with lower *g* forces compared to small EVs. (**B**) Separation of EVs according to their density in density gradient centrifugation. (**C**) EVs are capture using specific antibodies against exposed antigens; here immunomagnetic beads bear the antibodies but also plate and chip systems are available. (**D**) Separation of EVs through size exclusion chromatography; while smaller particles are trapped in the porous matrix, larger particles and EVs elute earlier from the column. (**E**) Ultrafiltration allows the concentration of particles larger than the cutoff size of the filter. (**F**) In precipitation, a precipitating agent causes sedimentation of EVs and other particles.

In VM, EV research is a relatively new, but rapidly expanding, field of research, and published research studies on EVs are increasing every year, investigating different roles of EVs in animal physiology, pathology, and their potential applications as diagnostic biomarkers and in therapy. Figure 2a,b show how, despite the delayed and much smaller number of papers, the exponential growth of scientific publications on EVs in VM is similar to the whole EV-related literature (Figure 1a,b). The increase of EV studies in VM mainly reflects worldwide contemporary concerns in the concept of "one health" such as the need for high productivity and welfare in food animal husbandry, control of emerging infectious disease and zoonosis and environmental monitoring [15–17]. Understanding how EVs can act in physiology and pathology of animals could be of great importance in the whole picture of EV research. Moreover, animals and humans show similar physiology and share several diseases, such as cancer, making animals also good spontaneous and inducible models for human research [18,19].



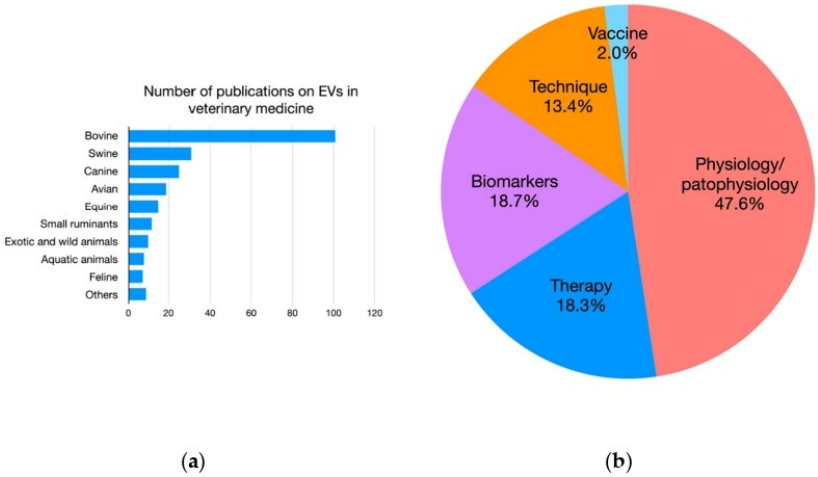
(**a**) (**b**)

Figure 2. (**a**) Bar graph representing the number of papers on extracellular vesicles (EVs) in the literature; (**b**) bar graph representing the number of papers on EVs in veterinary medicine literature.

With the present review paper, the authors intend to summarize the current status of the EV research performed on domestic and wild animals, analyzing the content of scientific literature, including approximately 220 papers published since 1984. Studies on EVs related to parasites and parasitic diseases are excluded. The literature search was conducted on Pubmed database filtering for "other animals" and applying as major keywords: extracellular vesicles, exosomes, microvesicles, and oncosomes. Search within the literature was limited to November 2021.

Most of the EV-related studies in VM have been conducted on pets and farm animals, especially on bovines, pigs, and dogs, but interestingly aquatic organisms and exotic and wild animals are also considered in preliminary EV studies (Figure 3a). The main focus of these studies is related to understanding the role of EVs in physiology and pathophysiology, with fewer but promising works related to the application of EVs as biomarkers or in therapy (Figure 3b). More recently, applications in wild/aquatic animals for environmental monitoring have appeared. Furthermore, a very broad area of interest of EV research in VM regards milk-derived EVs for their role as loadable and natural carriers with therapeutic potentials and as mediators of inter-organisms communication [20].

Small EVs including exosomes are the main targets of these studies (Figure 4), although misnaming is often evident due to lack of coherent isolation protocols, standardized terminology, and classification of EVs. For these reasons, authors will herein use the general term "EVs" when presenting study results. Indeed, the study of EVs is a new research field in VM, with a paramount potential in the "one health" perspective, but it is also a challenge for veterinary scientists as they need to gain more knowledge and expertise on EV isolation and analyses, with specific adaptation of protocols for animals, when needed. With this review, we hope that EV scientists will increase their awareness about the state of the art of EV research in the veterinary field, discovering potential common points and new possible applications of EVs among different species.



(**a**) (**b**)

Figure 3. (**a**) Bar graph representing the number of papers on EVs by non-human species. (**b**) Pie chart representing the percentage of papers on EVs by application.

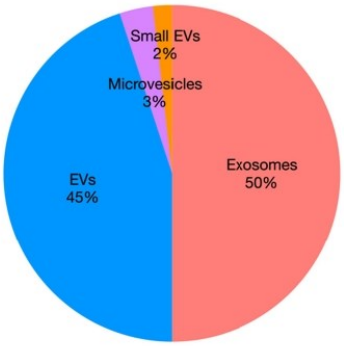


Figure 4. Pie chart representing the percentage of papers by EV subtype.

2. Critical Points of EV Research in Veterinary Medicine

Considering the recent appearance of EVs in VM research, some studies focused on the improvement of EV isolation and on their preliminary characterization from different animal samples.

Despite the first publication on EVs exploiting the use of animal cell culture [21], EV research in the following years proceeding exponentially only in human medicine, leading to the foundation of the International Society of EVs (ISEV) in 2011, to the first ISEV annual meeting in 2012 and to the first consensus guidelines (Minimal Information for Studies of Extracellular Vesicles) in 2014, updated in 2018 (MISEV 2018) [1,22]. Conversely, VM EV research stopped for many years and only in 2012 it started over with the first isolation of EVs from chicken dendritic cells (DCs). Consequently, in the following years, specific studies describing the successful isolation of EVs from canine, feline, and equine cell cultures were published [23–25].

More recently, technical issues have been addressed also referring to MISEV 2018, in which authors compared different purification methods, such as ultracentrifugation (UC), ultrafiltration and precipitation, size exclusion chromatography (SEC) and different starting samples, such as canine serum, feline plasma and urine, and equine uterine fluid [24–28].

However, standardization of protocols and terminology (i.e., MISEV 2018) are still major issues in EV research, and this is even more evident in VM.

The analysis of the reviewed papers reveals that the general main critical points of EV research in VM are the less rigorous selection of EV isolation and characterization techniques as described below.

- Lack of standardization on EV isolation methods, often use of a single method: in the 80% of the reviewed papers a single technique is applied to isolate EVs, with only 20% of papers combining at least two methods. UC is the most commonly used, both as a single or combined technique, followed by commercial EV isolation kits (Figure 5). In addition, limitations of methods and controls are often not properly discussed or included.
- Lack of a constant, proper EV characterization: EVs are characterized in about 85% of the studies, and confirmation of the EV presence with at least two different methods is reported in 64% (Figure 6a). Several methods are used among which standard electron microscopy (EM) (32%), Western blot analysis (WB) (27%), and nanoparticle tracking analysis (18%) are the most commonly used (Figure 6b). However, in 15% of published papers (most of them published 2019–2021), no EV characterization is performed at all.

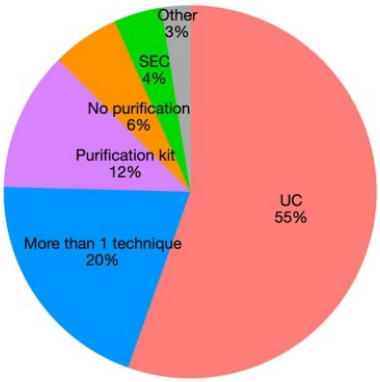
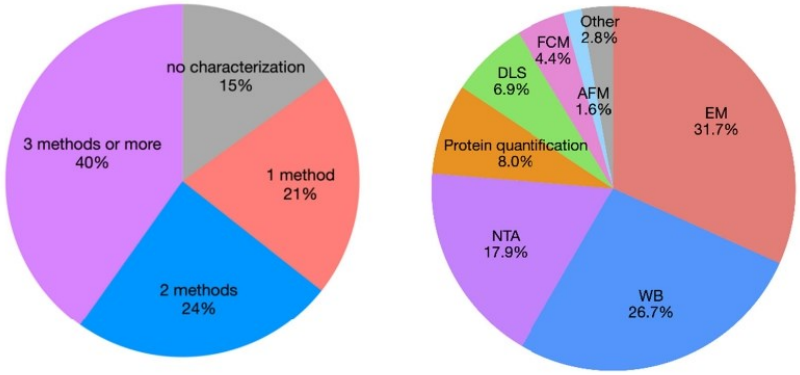


Figure 5. Pie chart representing the number of papers on EVs by purification method.



(**a**) (**b**)

Figure 6. (**a**) Pie chart representing the percentage of papers on EVs by number of applied characterization techniques. (**b**) Pie chart representing the percentage of paper on EVs by characterization technique.

Several other critical aspects have emerged by the analysis of the VM literature related to the still limited number of studies performed on each animal species and the lack of standardization of the VM species-related EV knowledge, such as:

- Lack of a list of specific EV-related or -unrelated markers that can be used for EV characterization in different animal species;
- Lack of tissue- or cell-specific markers to isolate EV subpopulations;
- Lack of reference genes to be applied when EV-associated nucleic acids are investigated;
- Lack of species-specific pre-analytical indications. Often, veterinary scientists try to apply protocols and techniques derived from human (or sometimes lab animals) studies for the analysis of EVs in other animal species. However, there are several factors that can affect EV isolation and analysis, especially when body fluids are analyzed. The starting material (e.g., blood, saliva, urine, milk) can vary in composition depending on the animal species and this can imply differences in methods and protocols to analyze EVs.

The analysis of the VM literature shows that, after milk, reported as the most frequently addressed starting sample investigated for human applications, EVs are isolated from cell culture medium (CCM) in in vitro studies in 42 papers; other works analyzed EVs isolated from other body fluids, with the reproductive fluids (e.g., uterine flushing, follicular fluid, or semen) being the most represented (Figure 7).

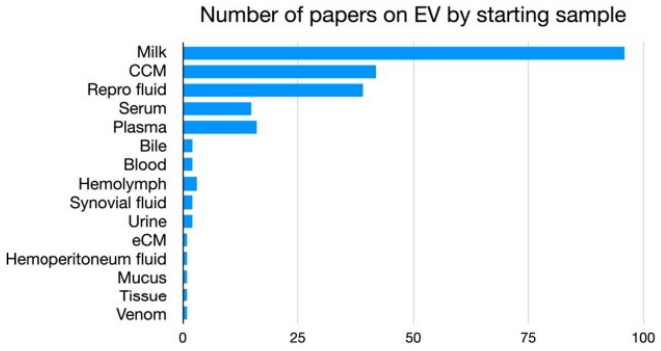


Figure 7. Bar graph representing the number of papers on EVs by starting sample.

Finally, research on EVs in VM is often descriptive, with a lack of functional studies. Functional studies are performed to test one or more specific function/s of the studied EVs and usually are based on in vitro or in vivo analyses in which EVs are uptaken by recipient cells. By the analyses of the selected papers, functional studies are limited to 35% and are mainly based on CCM-derived EVs for therapeutic applications (Figure 8).

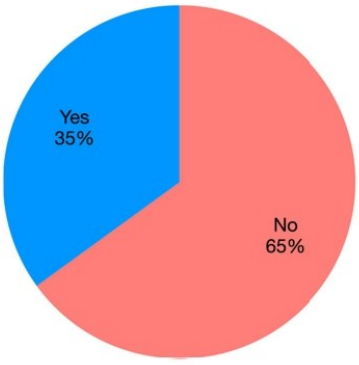


Figure 8. Pie chart representing the percentage of papers using EVs for functional studies.

Based on these considerations, this review presents in the following sections the major findings grouped per topic, selecting papers based on the following inclusion criteria:

- One internationally accepted (MISEV 2018) EV isolation/purification method;
- Minimum of two internationally accepted (MISEV 2018) methods for EV characterization.

Supplementary Table S1 includes the list of all papers that were revised for this work.

3. EVs in Physiology and Pathophysiology

EVs have an important role in intercellular communication both in physiological and pathological processes. Reviewing the VM literature on EVs, it was evident that major attention has been received by the function of EVs in animal immunity and reproduction. The former curiously includes the study of peculiar immune features which characterize animal species living in extreme environmental conditions, such as deep ocean or high altitudes, whereas studies on reproduction refer mainly to large animals, for obvious economic reasons. When it comes to EV roles in pathology, then infectious diseases take the stage since understanding their mechanisms of intra- and inter-species dissemination and of causing cell damage is of paramount importance for their control.

3.1. EVs in Immunity

Considering the rising impact of comparative immunology and the role of EVs in immunity, several animal-specific aspects of the immune system have been investigated with particular attention to the role of post-transcriptionally deiminated proteins contained within EVs [29].

Cartilaginous fishes have peculiar adaptive immune mechanisms and unusual lymphocyte antigen receptors which make them of interest for research in immunotherapeutic fields [30]. Criscitiello and colleagues isolated EVs from plasma of the nurse shark (*Ginglymostoma cirratum*) and assessed the presence of EV-derived deiminated proteins involved in immunity, i.e., hemopexin, a protein which contributes to heme homeostasis and also associated to physiological stress, haptoglobin, an acute phase protein and novel antigen receptor, a particular heavy chain homodimer with high target affinity and selectivity. Some of these EV proteins have been reported to be deiminated in cartilaginous fishes for the first time and further studies may highlight other interesting features of shark immunity [31].

A similar approach to the study of deiminated protein profiles of EVs has been conducted also on teleost fishes. As their mucosal immunity shares features with type I mucosal surfaces of mammals, Magnadottir and co-authors isolated EVs from pooled mucus collected from the dorsal side of farmed cods [32]. They assessed the presence of deiminated cytoskeletal proteins (i.e., elongation factor 1-alpha, fast skeletal muscle alpha-actin, profilin) and found in EV cargo also the complement component C3 and C-reactive proteins, suggesting the role of EVs in natural immunity against pathogens of the aquatic environment [32].

Other species of remarkable interest for comparative pathophysiology are crocodilians. Crocodilians have long life spans, low metabolic rates, strong antibacterial and antiviral abilities and are cancer resistant [33–35]. In alligators, EVs have been studied to analyze their role in the species strong immune resistance. EVs have been isolated from plasma of *Alligator mississippiensis* and their deiminated proteins cargo has been analyzed. Deiminated proteins were found to be involved in metabolism, gene regulation and cancer, providing new insights into the unique physiology of these species [35].

Marine mammals have undergone a range of physiological adaptations to diving and adapted their immune system to the aquatic environment. Research on their immune system may be useful to understand mechanisms such as resistance to cancer, insulin resistance, and adaptations to hypoxia, highly relevant for a number of human pathologies [36–39]. Marine mammals are of research interest also because their environment is changing, due to global warming, pollutant, and anthropogenic presence. These stress factors predispose them to resulting changes in exposure to pathogens and opportunistic

infections with a possible virus-induced immunosuppression and increased bacterial and parasitic infections [40,41].

Therefore, as in humans, EVs could be studied to understand physiological/pathogenic pathways and to be exploited as diagnostic biomarkers to assess marine mammal health status.

In marine mammals, EVs have been isolated from serum of two seal species—gray seal (*Halichoerus gryptus*) and harbor seal (*Phoca vitulina*)—and of five cetaceans—northern minke whale (*Balaenoptera acutorostrata*), fin whale (*Balaenoptera physalus*), humpback whale (*Megaptera novaeangliae*), Cuvier's beaked whale (*Ziphius cavirostris*), and orca (*Orcinus orca*) [42,43]. In these studies, the authors investigated specific EV-associated microRNAs and deiminated protein profiles. Three key miRNAs related to inflammation (miR-21), stress-response (miR-155), and hypoxia and metabolic activity (miR-210) were assessed and species-specific differences in the expression of these miRNAs were found. Deiminated proteins were detected in EVs by WB, indicating that EVs can mediate the transport of critical proteins for immunity and metabolism [42,43]. Additional findings also suggested that EVs could be a better source for the assessment of miRNA expression compared to whole serum, as in all the considered cetacean species the expression of the analyzed miRNA was considerably higher in EVs [43].

Not only marine mammals are exposed to anthropogenic pressures and climate change. Seabirds are the most threatened group of birds worldwide and finding biomarkers to assess their health status would be of paramount importance. EVs have been isolated from plasma of eight Antarctic seabird species: wandering albatross (*Diomedea exulans*), gray-headed albatross (*Thalassarche chrysostoma*), black-browed albatross (*Thalassarche melanophris*), northern giant petrel (*Macroneces halli*), southern giant petrel (*Macronectes giganteus*), white-chinned petrel (*Procellaria aequinoctialis*), brown skua (*Stercorarius antarcticus*), and south polar skua (*Stercorarius maccormicki*). In plasma derived-EVs of three of these species (the wandering albatross, the south polar skua, and the northern giant petrel), deiminated proteins belonging to immune and metabolic pathways were found and may become possible novel indicators of the immunological and physiological status of these animals [44].

3.2. EVs in Reproductive Physiology

Swine, bovine, and chicken are all species of agricultural importance and a better understanding of pregnancy mechanisms to improve outcomes is economically relevant. EVs have been studied mainly in swine and bovine to investigate their role in reproduction and to assess their function to improve reproductive outcome.

In swine, Bidarimath et al. demonstrated that EVs are released by porcine endometrium, chorioallantoic membrane, porcine trophectoderm cells (PTr2) and aortic endothelial cells (PAOEC) and that they contain proteins of signaling pathways relevant for angiogenesis and endometrial vasculature development, which are important processes in early pregnancy stages. In the same study, the potential ability of PTr2 and PAOEC EVs to regulate angiogenesis and the exchange of EVs between these two cell types have been demonstrated through proteomic analysis [45]. Female reproductivity has also been investigated through the analysis of EVs isolated from the follicular fluid (FF) of pigs [46,47]. Matsuno and co-authors evaluated mRNA profiles in EVs isolated from porcine FF compared to mRNAs extracted from mural granulosa cells (MGCs). They found that EVs in FF are mainly secreted by MGCs but also by other types of cells (e.g., tecal cells, oocytes, cells of non-ovarian tissues) and that the mRNAs content was involved in folliculogenesis pathways [47]. Grzesiak et al. identified and quantified FF EVs of small, medium, and large antral follicles of sexually mature gilts to conduct proteomic analysis of their cargo. More EVs were found in medium follicles compared to small and large follicles and proteomic analysis showed the presence of proteins mainly belonging to cytoskeleton or extracellular matrix, suggesting their role in building cell components and in follicular development [48].

In bovine, Gatien and collaborators analyzed the metabolomic profile of EVs isolated from the oviductal fluid (OF) and identified their metabolite content [49]. They found that the side of ovulation had no effect on EV metabolites concentration, but the reproductive

cycle stage affected maltose, methionine, and glucose-1-phosphate levels in EVs. The pathways involving the analyzed metabolites were related to sucrose, glucose, and lactose metabolism [49]. Interestingly, bovine OF-derived EVs have also been demonstrated to maintain sperm survival and to stimulate processes associated with capacitation, therefore being possibly used to enhance assisted reproductive technologies [50]. OF-derived EVs can not only support sperm but also embryo cells. OF-derived EV supplementation has been demonstrated to increase survival rate of bovine blastocysts after vitrification and warming [51]. Moreover, embryo culture media-derived EVs can positively affect blastocysts, increasing the yield and reducing apoptosis [52]. RNA sequencing of bovine oviductal epithelial cells exposed in vitro to FF-derived Evs showed the presence of differentially expressed genes involved in sperm survival, fertilization, and embryo development [53]. Bovine FF Evs can also promote the synthesis of androstenedione and progesterone in ovarian cortical stromal cells and increase cell proliferation while inhibiting cell apoptosis on the same cells in vitro [54].

High temperatures, and consequent heat stress, are an issue for the reproductive management of dairy and beef cows. An in vitro model of bovine granulosa cells showed that Evs released by cells exposed to heat stress contained differentially expressed miRNAs. Moreover, the supplementation of heat stress-derived Evs to normal cells could induce an adaptive response to heat stress [55].

Few additional species are also included in some studies regarding Evs and reproduction.

In hens, the presence of Evs has been observed in the uterine fluid in vivo, and in sperm storage tubules in vitro [56,57]. Proteins involved in the regulation of sperm function were identified in uterine fluid-derived Evs, suggesting also the possible supportive role of Evs in avian sperm survival [56].

In boar, seminal plasma Evs have been analyzed and their RNA content was associated with immunity pathways which may establish the adequate uterine environment for fertilization and implantation [58].

In pets, a deeper understanding of reproductive mechanisms could be useful for the improvement of assisted reproductive technologies to preserve genetic traits [59]. Feline FF Evs contain proteins involved in follicle and oocyte maturation and can improve oocyte cryopreservation, enhancing their ability to resume meiosis [60]. The effects of feline and canine OF Evs on cryopreserved sperm has also been investigated and their ability to improve acrosome integrity of red wolf and cheetah sperm and red wolf sperm motility has been demonstrated in vitro [61].

3.3. Evs in Pathophysiology of Infectious Diseases

Infectious diseases and zoonoses are some of the main issues in the management of farm animals. Understanding and controlling infective agents is of pivotal importance for animal health and welfare, food safety, and to keep a high productivity, especially in developing countries [15]. In human medicine, many studies showed that human EVs can carry viral elements, being both involved in the spread of infectious diseases and in the induction of an immune response against the infective agent [62–64].

EV-related miRNAs have been proven to be differentially expressed in infected humans, therefore becoming tools to better understand the pathogenic mechanism of a disease or useful diagnostic biomarkers [65].

In VM, some evidence has been demonstrated of the role of EVs as regulators of viral transmission. An example is a study performed on the seminal plasma of cocks infected with subgroup J of Avian Leukosis Virus (ALV-J). Nine EV-related miRNAs were found to be differentially expressed: 3 upregulated and 6 downregulated. Among these miRNAs, the tumor suppressor miR-138-5p was found to be downregulated as in many types of human cancer [66].

Other important viral diseases in farm animals have been preliminarily approached. EVs released by HeLa cells infected with Newcastle Disease Virus (NDV), or by Mardin-Darby bovine kidney cells (MDBK) infected by Caprine parainfluenza virus type 3 (CPIV3),

were demonstrated to carry miRNAs, RNAs, or proteins which enhanced the cytopathic effect of the virus. Furthermore, EVs were also able to suppress interferon (IFN)-beta gene expression in HeLa cells after NDV infection, and to inhibit autophagy during CPIV3 infections, suggesting a significant role of EVs in viral spreading [67,68].

EVs are also involved in long term persistence of some viruses [69,70]. miRNA ecamir-128 which targets CXCL16, the gene involved in the persistence of Equine Arteritis Virus (EAV) infection, was detected in seminal plasma EVs of infected stallions [71].

Understanding the pathogenesis of infectious diseases is an essential step also for their management and prevention. Bovine leukemia virus (BLV) often causes asymptomatic infection and infected animals may remain asymptomatic virus carriers for their entire life [72]. Even if considered a minor route of infection, lactation may transmit BLV particles and BLV-infected somatic cells to calves [73]. Yamada et al. showed that EVs isolated from milk of infected cattle contain BLV structural proteins, but these proteins did not seem to be infectious to cells after in vitro analysis, as proviral DNA was not detected [74]. Montaner-Tarbes and collaborators found the presence of viral proteins in EVs isolated from the serum of pigs infected with African Swine Fever virus. These proteins could be exploited for EV-based vaccines, similarly to what the same authors performed in a trial against Porcine Reproductive and Respiratory Syndrome virus (PRRSV) (see below) [75,76].

3.4. EVs in Environmental Adaptation and Nutrition

Other processes in which animal EVs have been investigated mainly deal with metabolism. In this regard, some animal species have peculiar adaptive mechanisms for living in low oxygen milieu [77]. Among these species, the naked mole-rats are among the most hypoxia-tolerant mammals and this phenomenon is related to their ability to reduce energy (O_2) supply, decreasing their metabolic rate up to 85% [78,79]. Naked mole-rats also have several other adaptive abilities, such as a remarkable longevity and resistance to cancer; they are also the only thermo-conformers mammalian almost completely ectothermic for body temperature regulation [80–82]. These adaptive and immune features make the naked mole-rat an interesting animal model for human diseases and for understanding cancer resistance and longevity pathways. In 2019, Pamenter and collaborators profiled plasma-derived EVs in naked mole-rats to assess the presence of deiminated proteins and stress/hypoxia related miRNAs (e.g., miR-21, miR-155, and miR-210). Interestingly, several deiminated proteins related to glycolysis, Hyopxia-Inducible Factor-1 (HIF-1) pathway, and also targeted miRNAs were all expressed in EVs, indicating their possible role in such an extreme adaptive capability [83].

Camelids are also species of relevant interest for their adaptive strategies [84]. Genomic analysis showed that they have unique adaptive features related to fat and water metabolism, heat stress response, and oxygen transport allowing them to live in extreme environments such as the desert and the high altitudes [84,85]. Because of their adaptive strategies, camelid physiology has been considered useful for human research so that more in depth examinations have recently started involving EVs. In 2020, Criscitiello and colleagues analyzed deiminated proteins in llama (Lama glama) serum and in serum-derived EVs. They found post translational modification in proteins involved in metabolism and immunity and a partially overlapping of deiminated proteins profile between serum and serum-derived EVs, with some proteins identified only in serum and others only in EVs. In llamas, some of these proteins were reported as post-translationally deiminated for the first time, and some of them are known to be involved in human diseases (e.g., dystonin, Xaa-Pro dipeptidase). Further comparative studies may deepen our understanding on the regulation process of these proteins and, consequently, of correlated human diseases [86]. Finally, similarly to sharks, camelids produce small homodimeric heavy chain-only antibodies with variable binding domains that can be highly useful in medicine and biotechnology research applications because of their small size, economic production, specificity, affinity, and stability [30,87,88].

Another curious finding in exotic species is the evidence of EVs in snake venom. Studies on snake venom EVs were conducted to understand their role in the envenomation process and as preliminary analysis to find new future targets for the development of anti-venoms compounds. Snake venom EVs carry proteins which are normally present in snake venom and are functionally active, with fibrinogenolytic and cytotoxic activity [89].

Further, a nutritional role of EVs has been preliminarily investigated in animals. One of the main implications of studying dietary EVs is related to their bioavailability and to the possibility of absorbing their content [90]. A recent study assessed the potential postprandial transfer of bovine colostral EVs and of EV-associated cargo to newborn calves, evaluating the presence of specific colostral proteins and miRNAs in calf circulation. While colostral EV protein markers were detected in calves' post-prandial blood, only a little overlapping of miRNA expression profile was present. These findings may suggest a different uptake according to the localization of EV components, since the investigated proteins were EV membrane-bound whilst miRNAs are usually mainly in the EV lumen [91].

Among avian species, pigeons have the unique ability to produce a nutrient substance which resembles bovine milk. EVs have been therefore isolated from pigeon 'milk' and EV-related miRNA analyses showed that 10 miRNAs are co-expressed in 5 different stages of 'lactation'. They were mainly related to immunity and growth. Moreover, 81 miRNAs were commonly shared with milk of other mammalian species [92].

Finally, an interesting study performed on bovine serum EVs showed that EV cargo can also be influenced by nutrition. The administration of different diets was associated with the presence of differentially expressed miRNAs related to hormone pathways and protein metabolism [93].

4. EVs as Biomarkers

One of the critical aspects of using EVs as biomarkers, as for any other biomarker, is the need for standardization [94,95].

An interesting preliminary study of Narita and collaborators compared three different EV isolation techniques (UC, membrane affinity chromatography, and precipitation) on canine plasma to find reference genes for EV-related miRNAs [96]. The authors found that UC was the most stable method and identified miR-103 as the most reliable miRNA to normalize miRNAs expression [96]. However, many studies suffer technical biases and lack of standardization and details should be carefully assessed. The main findings in VM literature follow.

4.1. EVs as Biomarkers in Reproduction

Hua and collaborators found small RNAs in swine uterine fluid-derived Evs of gilts on days 10, 13, and 18 of pregnancy that were involved in pathways of immunization, endometrial receptivity, embryo development, and implantation. They also highlighted a different expression of some miRNAs during the three phases of pregnancy, whose role as biomarkers to monitor pregnancy could be further studied [97].

Other studies focused on the study of EVs to improve the efficiency of early pregnancy diagnosis in swine. Two miRNAs, miR-92b-3p and miR-15-5p, were found to be upregulated in serum-derived EVs of sows in days of pregnancy 9, 12, and 15, being possible circulating biomarkers to diagnose early pregnancy [98].

In plasma-derived EVs of goats, 5 miRNAs were found exclusively in pregnant animals, being candidates for early pregnancy diagnosis in this species [99].

Embryo-derived EVs could also be useful to indicate cell status and to help select competent blastocysts. In bovine, Mellisho and collaborators demonstrated that bovine blastocysts secrete EVs in the culture media and that the EVs concentration and diameter are different whether embryos are produced by in vitro fertilization or by parthenogenetic activation [100]. Melo-Braez and colleagues also found that miRNA content in EVs released by bovine embryos during their compaction period was different whether embryos were competent and reached blastocyst stage or arrested in the 8–16 cells stage [101].

Finally, the possibility to differentiate EVs according to their origin has been assessed on bovine serum by Raman spectroscopy allowing distinction of placental EVs from mononuclear cell EVs [102]. Raman spectroscopy takes advantage of laser light scattering due to photons interactions to provide information on the biochemical components of a sample [103]. In bovine serum, different clusters, implying a difference in EV cargo composition and origin, were identified. Further, differences in cargo composition were present also in EVs from different pregnancy stages, suggesting that Raman spectroscopy could be a useful tool to monitor placental development [102].

4.2. EVs as Biomarkers in Oncology

Tumor cells produce a high amount of EVs, who take part in every step of tumor progression and whose cargo is related with the stage and the grade of the tumor [104]. These factors highlight the importance of EVs as biomarkers for cancer diagnosis, prognosis, and therapy.

Cancer is one of the main causes of death also in companion animals, which are considered spontaneous models in comparative oncology [105]. Biomarkers are sought for many cancer types also in pets and recently, EVs have become an interesting research target in this field, even if few studies are available. However, standardization of procedures is of remarkable importance in this contest and should be dramatically optimized in VM [106].

MiRNAs are important cancer biomarkers carried by EVs and are largely studied in human medicine for their possible use in liquid biopsy in particular for early tumor diagnosis [107–109]. Studies performed in vitro on canine cell lines provided the first evidence of different EV-related miRNA contents in normal canine mammary epithelial cells versus tumor canine mammary epithelial cells [110]. The benefit of miRNA analysis in tumor diagnosis was also investigated in vivo by Narita and collaborators who found that miR-15b and miR-342-3p derived from plasma EVs could be a potential non-invasive biomarker to differentiate dogs with glioblastoma from dogs with other brain diseases [111].

EV-miRNAs expression can also be exploited for prognostic aims [112,113]. In one clinical trial performed on dogs with multicentric lymphoma, four miRNAs in plasma-derived EVs were found to be differentially expressed between dogs with progressive disease and dogs that reached complete remission [114].

Together with diagnosis and prognosis, the outcome of a chemotherapeutic protocol is another important issue in oncology [115,116]. In vitro studies performed on EVs from canine lymphoma cell lines evidenced the presence of three miRNAs which were differentially expressed between vincristine sensitive and vincristine resistant cell lines [117]. In another study in dogs with long term treatments with doxorubicin, plasma EV-related miRNAs were demonstrated to be efficient in the diagnosis of cardiotoxicity. Indeed, 3 miRNAs were differentially expressed in dogs with cardiotoxicity and miR-502 was upregulated before the third chemotherapeutic dose, being an earlier biomarker than cardiac troponin I (cTnI) [118].

4.3. EVs as Biomarkers for Other Diseases

The use of EV-related miRNAs as biomarkers has been explored also for the diagnosis of other diseases than cancer.

In canine urinary EVs, a pool of 5 miRNAs has been compared in dogs with healthy kidney versus dogs with kidney diseases. miR-26a, miR-10a/b and miR-191 were differentially expressed, suggesting that further studies could assess the role of urine EVs in the diagnosis of kidney dysfunction, as largely demonstrated in humans [119–121].

The first publications on EVs in animals were related to the fate and metabolism of the transferrin receptor (TfR) after endocytosis in cells for iron uptake [10,11,122]. More recently, other researchers focused again on the presence of TfR on EVs membrane, evaluating its role as biomarker for anemia in horses, dogs, and cats [123,124]. In six horses with regenerative anemia, TfR1 expression on serum-derived EVs increased together with the progression of the disease [123]. Another study performed on horses, dogs, and cats focused instead

on the proportion of cytoplasmic domain of TfR (cTfR) and total TfR on serum-derived EV membrane to calculate indirectly the presence of soluble TfR (sTfR), the latter used as biomarker for iron deficiency in humans. No difference between healthy and diseased animals was found in the expression of cTfR, suggesting that quantification of sTfR in animals might be less useful to diagnose iron deficiency during inflammatory disease than in humans [124].

Musculoskeletal and joint diseases are the most common health issue and cause of wastage in the equine industry [125]. However, joint diseases in horses are not only of interest for the veterinary field but also for human medicine, as the horse is considered a good model for human orthopedic research because of the similarities of joints and cartilage [126–128]. Therefore, research on synovial fluid (SF)-derived EVs to unravel both their physio-pathological role and their potential as biomarkers in joint diseases has recently started [129]. Although EV isolation from SF has not been much explored, preliminary research to optimize EV isolation from SF has been performed on horses. SF is a fluid characterized by high viscosity and it has been demonstrated that hyaluronidase pretreatment of SF prior to UC facilitated the recovery of CD44+ EVs [130].

5. EVs in Therapy

Studies regarding the use of EVs in animal therapy are not many and still preliminary.

In pets, only one paper focused on the use of EVs in cancer therapy in vitro. EVs derived from canine M1-polarized macrophages were administered in vitro to canine osteosarcoma and melanoma cell lines. EVs treatment increased the level of pro-inflammatory cytokines and induced apoptosis of tumor cells [131].

Stem cells have attractive biological features which are of interest both for human and veterinary research [132]. Since stem cells have regenerative effects, stem cell-derived EVs are largely studied for therapeutic applications [133].

In a veterinary clinical trial, a horse with a suspensory ligament injury was treated with EVs isolated from adipose-derived stem cells combined with 5-azacytydine and reseveratrol. EV treatment was promising, causing lesion filling, angiogenesis, and tissue elasticity [134].

The effect of mesenchymal stem cells (MSC)-derived EVs has been tested in vitro in some studies performed on equine and canine cells. EVs isolated from equine adipose MSCs and from canine MSCs demonstrated to have anti-inflammatory effects in vitro on recipient endometrial and glial cells, respectively [135,136].

In an in vivo mouse model, canine adipose tissue derived MSC-derived EVs were administered intraperitoneally to mice with dextran sulfate sodium-induced colitis. In mice treated with EVs, inflammation was alleviated, although the injection of TSG-6-depleted EVs reduced this effect. Particularly, the authors confirmed through immunofluorescence on colon samples that TSG-6 in EVs enhanced regulatory T cells and the polarization of macrophages from M1 to M2 [137].

Additionally, a commercial preparation of human plasma-derived EVs was able to increase cell proliferation and collagen deposition in vitro on a canine tenocyte culture. Moreover, EV treatment reduced cellular apoptosis caused by dexamethasone on the same canine cell line [138].

6. EVs as Vaccines

Due to the rise in worldwide population expected in the next decades, a strong increase in global food production is needed, including food of animal origin. In parallel, an enhanced attention for animal welfare issues is likely to increase the expenditure on pet supplies as well as the search for innovative medical approaches. Further, the role of animals in the "one health" perspective and in spreading of novel infections has become more evident. For all these reasons, development of veterinary vaccines is receiving stronger attention and economic investments. At the same time, novel approaches for

vaccine development are required, since classical and live-attenuated vaccines are not totally safe and efficacious [139].

Up to November 2021, three papers on avian species and two papers on swine have been published on the use of EVs as vaccines.

Considering pork industries, one of the main threats is the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) caused by an enveloped RNA virus of the Arterivirus genus, first described in the US in 1987–1988 and referred to as mystery pig disease [140]. Since then, it has become one of the most important swine diseases of the last half-century causing reproductive impairment and pneumonia with no single successful strategy for control [141,142]. As for other diseases, vaccine development has therefore received wide attention. Current available vaccines against PRRSV have a series of limitations such as partial protective immunity, possible reversion to virulence with consequent biosafety problems, inability to induce long lasting and heterologous protection, and high antigenic and genetic differences of strains [76,143]. Interestingly, in 2016, Montaner-Tarbes and collaborators demonstrated that in the serum of animals that had overcome a PRRSV infection there were EVs free of virus but containing viral proteins with immunogenic properties [143]. In 2018, they reported the first trial to immunize pigs with Evs isolated from serum of pigs which had suffered a natural PRRSV infection. They demonstrated that EV-enriched fractions obtained by SEC were free of virus, safe, and with viral peptides capable of eliciting humoral and cellular immune responses with no adverse immune reaction. Moreover, in pigs vaccinated with Evs followed by boosts with synthetic peptides, the authors recorded a specific humoral IgG response which may be useful to differentiate vaccinated from infected animals [76].

The use of EVs as a vaccine was tested also In poultry against coccidiosis. Coccidiosis is a group of parasitic diseases caused in poultry by protozoans of the gender *Eimeria* spp. The infection causes mainly weight loss and poor feed conversion ratio with consequent losses in poultry production [144]. A preliminary trial evaluated the immunization caused by eVs isolated from chicken intestinal dendritic cells (DC) stimulated in vitro with a mixture of *Eimeria* antigens. In vaccinated animals, a T-cell immune response against *Eimeria* was demonstrated [145]. However, isolation of eVs from DCs (or any other cell type) on a large scale for massive vaccinations was considered difficult to practice. Considering serum as a more convenient source of eVs, the protective ability of serum-derived eVs obtained from *E. tenella* infected chickens was tested. A protective immunity was recorded in vaccinated animals, with reduced gut lesions and parasite shedding, increased weight gain, and improved feed efficiency [146]. Moreover, considering the importance of CD80 in antigen presentation, the authors demonstrated that immunity improved when selected CD80+ eVs were administered compared to vaccination with CD80- eVs [146].

The application of eVs for antigen delivery is now very promising in human medicine both for anti-cancer immunotherapy and for conventional prophylactic application against infectious diseases including SARS-CoV-2 [147]. More advances should be made also in VM considering the tremendous role of large-scale vaccination in animals for both animal and public welfare.

Preliminary studies in VM also explored the possible use of eVs, and particularly their miRNAs cargo, as biomarkers to distinguish vaccinated by non-vaccinated animals. Vaccination for Marek's Disease (MD) in chickens, a viral disease causing the development of multiple lymphoid tumors, was associated with an increased expression of tumor suppressor miRNAs in serum eVs. Moreover, in tumor-bearing chickens more EV-related onco-miRNAs were reported, with gg-miR-146a and -21 being good candidates to distinguish non-vaccinated tumor-bearing animals from vaccinated animals [148]. Further studies demonstrated that in serum eVs of chickens vaccinated for MD, mRNA mapping the whole genome of MD virus was present, suggesting the participation of eVs in vaccine immune response [149].

7. EVs from Animal Milk for Human Applications

EVs in human breast milk were first identified in 2007 by Admyre and collaborators who described their immunological potential [150]. In the last 15 years, nearly 100 publications explored eVs in milk from several animal species, mainly focusing on bovine milk, considered an abundant, cost-effective, and biocompatible source of eVs for human medicine research [151]. A non-exhaustive summary is here proposed with regard to the main applications and findings.

Milk is a complex fluid for EV isolation, particularly rich in proteins that may interfere with purification steps. Among these proteins, casein micelles have size and density comparable to eVs, and thus can be co-isolated with milk-derived eVs (MEVs). For this reason, many studies focused on the implementation of MEV purification protocols, which however are not yet properly standardized [152,153]. Preliminary centrifugation steps are used to remove fat globules and casein aggregates and, indeed, centrifugation combined to SEC have been successfully applied for bovine MEVs (bMEVs) purification [154–156]. Recently, it has been demonstrated that adding hydrochloric acid or acetic acid to milk can accelerate casein aggregation and precipitation, facilitating MEV purification and separation from casein, even if a partial degradation of EV-surface proteins has been observed [157–159].

Since it has been demonstrated that MEVs (and their RNA content) can resist digestion, one of the biological relevance of milk lies in the possibility that recipient host cells can uptake viable MEVs and their cargo [160,161]. Although a recent study has demonstrated that many miRNAs in human, bovine, and caprine milk are preserved across species, interspecies MEVs dietary uptake could still result in an exchange of RNA which may regulate host gene expression, despite their low concentration [162–164].

In the last few years scientists have started to investigate the interspecies effects of orally administered bMEVs, primarily on intestinal health and integrity. In vitro studies performed on intestinal crypt epithelial cells exposed to oxidative stress, showed the protective effect of bMEVs pretreatment, with a consequent reduction of reactive oxygen species [165]. In vivo oral administration of bMEVs to mice has demonstrated to have effects on gut microbiota composition and to improve local intestinal immunity, increasing the expression of genes important for mucosal integrity [166]. The positive effect of bMEVs on intestinal integrity has been assessed also in other studies, where they have been tested in models of malnutrition, ulcerative colitis, and necrotizing enterocolitis. In all these studies, bMEV were able to cause a protective or an alleviated effect on treated mice [167–171].

Milk and dairy products contain components associated with bone formation and maintenance, in particular if assumed during childhood and adolescence [172]. Recently, few studies also investigated the role of bMEVs on bone health [173–175]. In vitro studies on human osteoblastic Saos-2 and pre-osteoblastic MC3T3-E1 cells, demonstrated that bMEVs promoted cell proliferation and differentiation [173]. Moreover, in the same study, bMEVs oral administration to rats, enhanced osteogenesis, increasing tibial longitudinal growth and mineral density [173]. Other in vivo studies performed in mice models also showed that oral administration of bMEVs was able to improve bone mineral density in osteoporosis and was osteoprotective and able to reduce osteoclast presence in mice ovariectomized or with diet-induced obesity [174,175].

As well as the positive effects of bMEVs intake, some scientists also investigated the possible negative consequences of dietary assumption. In one study, it was observed that bMEV-associated miR-148 k seems to have a diabetogenic effect, promoting pancreatic β-cell differentiation to a more immature metabolic phenotype. This can affect insulin secretion and cause β-cell apoptosis [176].

The interest in bMEVs is also related to their anticancer properties. At proteomic analysis, Fonseka and collaborators showed that the incubation of bMEVs with neuroblastoma (NBL) cells significantly attenuated proliferation, confirmed by depletion of proteins implicated in proliferation, cell cycle, and Wnt signaling pathway and enrichment in proteins implicated in apoptosis and cellular senescence [177]. Moreover, in the same study,

bMEV administration combined with doxorubicin increased the sensitivity of NBL cells to doxorubicin [177].

In oncology, bMEVs could also be exploited as drug carriers. MEVs are scalable, safe, cost-effective nanocarriers able to deliver a wide range of drugs, small molecules as well as macromolecules [151,178]. Drug-loaded bMEVs can also be engineered on their surface to target specific tumor cells, to improve therapeutic efficacy and reduce toxicity [151]. In an in vitro study, bMEVs were engineered to express hyaluronan (HA) on their surface and loaded with doxorubicin. HA is a specific ligand for CD44, a stem-like cell receptor usually overexpressed on the cell surface of many cancer subtypes. Results demonstrated that HA-bMEVs triggered tumor cell death showing the efficacy for tumor specific drug delivery [179]. bMEVs surface can also be engineered to facilitate their transit. bMEVs coated with polyethylene glycol had enhanced resistance to stomach acid environment and improved permeability to mucin, while delivering functional siRNA [180].

bMEVs may be useful also for dairy herd health management. Cai and collaborators characterized the different expression pattern of miRNAs in bovine bMEV from normal healthy cattle and from milk of cattle affected by mastitis. They found 18 miRNAs involved in immunity, with different expressions between the two groups [181].

Even if most of the studies on MEVs have been performed on bovine milk, there are some exceptions, with studies focusing on milk of other species. Metabolomic and transcriptomic analysis performed on the cargo of MEVs of donkeys and goats showed the presence of metabolites (e.g., arginine, asparagine, glutathione and lysine) and miRNAs with immunomodulatory properties [182,183]. Preliminary studies have been performed on yak milk and in vitro assays demonstrated that yak MEVs can alleviate intestinal inflammation and hypoxia consequences, promoting intestinal cell survival in a rat model [184,185]. Some studies have also been performed on milk not for human consumption, such as porcine milk [186]. Similarly, also the effect of porcine MEVs on the gastro-enteric tract has been evaluated. In particular, MEVs have been demonstrated to be protective against damages caused by the mycotoxin deoxynivalenol. This protective effect has been shown both in in vitro and in vivo studies, where porcine MEVs were able to promote intestinal cell proliferation and to inhibit cell apoptosis in mice [186].

8. Conclusions

The EV research in VM is at an early stage. However, more than 220 papers have been published counting until November 2021, mainly in the last few years, investigating many different areas of EV research in physiology and pathophysiology, as well as potential EV applications as biomarkers or therapeutic tools. All these papers give a glimpse of the enormous potential scientific impact of studying EVs in VM and on the important consequent implications. Furthermore, veterinary researchers are enthusiastic in studying EVs, their diverse roles, and potential applicability in animals, with the main aim of contributing to a better understanding of EVs biology and functions, but sometimes with too little background on EV topic and related technical issues. We strongly believe that a tight collaboration should be encouraged between emerging EV veterinary researchers and specialized EV scientists, in order to decrease technical biases and weaknesses and to get the most from common EV research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12192716/s1, Table S1: list and features of all the references on extracellular vesicles in veterinary medicine.

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5. Extracellular vesicles in unconventional species

As highlighted with the above review, most of the studies on animal derived EVs are mainly addressed to farm animals (6).

Generally, in all disciplines, wild and exotic animals are less studied than farm animals or pets for ethical reasons (especially for *in vivo* studies) and lesser accessibility. However, many wild animals are of interest for human health as comparative models for human diseases but also as relevant reservoir for disease spreading, or as sentinels to monitor environmental health (26–28). Therefore, considering also the concept of One Health, a further aim of my project was to study EVs in aquatic animal species. We preliminary investigated EVs in some unconventional species (*i. e. Tursiops truncatus, Ziphius cavirostris, Ruditapes philippinarum*). Indeed, EVs from these species have been only preliminarily investigated (29,30) but they could be useful tools to gain more information on their pathophysiology and on the environment they live in.

5.1 Cetacean cell derived extracellular vesicles

Marine mammals are species of scientific interest for many reasons. First, they are considered possible good sentinels of the marine environment; being at the top of the trophic chain, they can accumulate elements like pollutants through the diet and these compounds can be stored in tissues and undergo a biomagnification process (31,32). Second, whales particularly, are reported as long living animals which appear to be resistant to cancer, being therefore of extreme interest in comparative oncology and cell-aging studies (33–35).

These interesting features must deal with the challenges of studying wild free-ranging animals, both for ethical reasons and practical sampling difficulties. These issues can be partially overcome using *in vitro* models which, despite being unable of representing completely the *in vivo* complexity, can give useful new insights on scarcely known physio-pathological mechanisms in these species. Unfortunately, *in vitro* studies using cetacean cell lines are still uncommon, due to the difficulties of obtaining fresh well-preserved tissues (36–40).

In addition, EVs could be very interesting tools to gain information on these species, but they have not been widely explored in cetaceans. Only one study described the profile of EVs isolated from the serum of five cetacean species (minke whale, Cuvier's beaked whale, fin whale, humpback whale and orca), investigating the expression of some target EV-associated miRNAs and deiminated proteins (29).

Therefore, to add new information on cetacean derived EVs the following paper on the isolation and characterization of EVs isolated from a bottlenose dolphin and a Cuvier's beaked whale fibroblast cell line was published as part of my PhD. This brief communication is just a preliminary study which aims to describe for the first time EVs released *in vitro* from cetacean cell lines, trying to open the field of EV-research to marine mammals. *In vitro* research combined with EV-study can become an important approach to deepen our knowledge on these fascinating animals, that could be applied to future studies on marine ecotoxicology and comparative pathology.

5.2 Paper n. 3: "Isolation and characterization of cetacean cell-derived extracellular vesicles"

animals

MDPI

Communication

Isolation and Characterization of Cetacean Cell-Derived Extracellular Vesicles

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Simple Summary: Cetaceans are species of scientific interest for many reasons. First, they can be useful to assess environmental health and, second, they have peculiar features which also make them interesting for human comparative pathology. In the last decades, extracellular vesicles have been studied as important carriers in cell-to-cell communication, and many studies in human and veterinary medicine have focused on their role in pathophysiological mechanisms or as biomarker to diagnose diseases. In vitro studies are good models to explore extracellular vesicles. However, cell lines have been poorly used and investigated in these species. For these reasons, here we describe for the first time the isolation of extracellular vesicles from two cetacean cell lines established from bottlenose dolphin and Cuvier's beaked whale. We also compare two different techniques to isolate extracellular vesicles, reporting the difference in the yield and quality of the obtained sample. This preliminary study on extracellular vesicles isolated in vitro aims to be the basis for future research to deepen our understanding on cetacean pathophysiology.

Abstract: Cetaceans are of scientific interest because they are good candidates as environmental bioindicators. However, in vivo research is arduous and in vitro studies represent a rarely used valid alternative. Extracellular vesicles (EVs) are membrane-bound structures playing roles in cell-to-cell communication. Despite being a promising investigative tool in different fields of science, EVs have been poorly studied in cetaceans. To fill this gap, we describe the preliminary characterization of EVs isolated from a bottlenose dolphin and a Cuvier's beaked whale cell line. EVs have been isolated with ultracentrifugation (UC) or size exclusion chromatography (SEC) and characterized with nanoparticle tracking analysis (NTA), Western blotting (WB), and scanning transmission electron microscopy (STEM). UC and SEC allowed the isolation of mainly small EVs (<200 nm). A higher number of particles were isolated through UC compared to SEC from both cell lines. At WB, all EVs expressed the EV-markers CD9 and integrin-β. Only EVs isolated with UC were positive for TSG101. In conclusion, we isolated for the first time EVs from a bottlenose dolphin and a Cuvier's beaked whale cell line using two different techniques. Further studies on cell-derived EVs will be useful to deepen our knowledge on cetacean pathophysiology and health status assessment.

Keywords: extracellular vesicles; ultracentrifugation; size exclusion chromatography; bottlenose dolphin; Cuvier's beaked whale

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1. Introduction

Extracellular vesicles (EVs) are membrane bound nano-vesicles originating from cells and released in the extracellular space [1]. They are heterogeneous in size, ranging from 30 to 1000 nm in diameter, and, as for biogenesis, they originate mainly from endosomes

or from external budding of the plasma membrane [1]. Once in the extracellular space, EVs carry RNA, proteins, lipids, and sugars from their cell of origin through body fluids and reach other cells. Therefore, they are important carriers involved in intercellular communication, having a role in the regulation of both physiological and pathological processes [2]. All these features make EVs a recent outstanding focus in many research fields, but their isolation procedure remains a critical point in all EV studies. Although different techniques and protocols have been developed to isolate EVs from different fluids and from tissues, ultracentrifugation (UC), once considered the gold standard for EV purification, is probably still the most commonly used method [3,4]. UC allows researchers to obtain a high yield of EVs, albeit with the risk of co-isolating other nanoparticles, leading to a low purity of EV sample. Other techniques, such as size exclusion chromatography (SEC) or density gradient ultracentrifugation, allow researchers to collect EVs with higher purity, despite a lower EV yield [4,5].

Most of the studies have investigated EVs in humans for their characterization and classification, to elucidate their role in physiological and pathological processes (e.g., wound healing, tumorigenesis, inflammation), and as diagnostic biomarkers or therapeutical vehicles [1,6,7]. However, EVs have also been isolated and characterized from different animal species, plants, and bacteria [8–10].

Among animal-derived EVs, a few preliminary studies have been performed on sea mammal-derived EVs isolated and characterized from the sera of five whales (*Balaenoptera acutorostrata*, *Balaenoptera physalus*, *Megaptera novaeangliae*, *Orcinus orca*, *Ziphius cavirostris*) and of two pinnipeds (*Halichoerus grypus* and *Phoca vitulina*), with the aim of finding possible biomarkers to assess the health status of these species [11,12]. Marine mammals, and specifically cetaceans, are considered good environmental bioindicators because of their top-level position in the trophic chain and due to their unique fat depots which tend to accumulate bio-contaminants [13]. Moreover, they have a long life span and apparently have a low incidence of cancer, making them also of interest for comparative medicine [14]. Further, studies for their conservation are strongly encouraged by national and international legal frameworks, such as the EU Biodiversity Strategy (Habitat Directive, Marine Strategy Framework Directive).

Clearly, in vivo research and sampling from free ranging cetaceans is arduous, and most of the information is obtained through the analysis of stranded animals. Therefore, in vitro research has been recently proposed as an alternative mean to better study their pathophysiological mechanisms [15].

Therefore, considering the still few in vitro studies on cetacean cell lines and the lack of in vitro studies on cetacean-derived EVs, we isolated and characterized for the first time EVs from a bottlenose dolphin (*Tursiops truncatus*) cell line and from a Cuvier's beaked whale (*Ziphius cavirostris*) cell line using two different EV isolation methods; however, the presence of EVs in the plasma of some cetacean species has already been reported [16–22].

2. Materials and Methods

2.1. Cell lines

Two skin-derived fibroblast immortalized cell lines (Sea Sentinels System patent n° 102020000003248; https://www.knowledge-share.eu/en/patent/sea-sentinel-system-for-environmental-studies/ (accessed on 2 October 2023), one derived from a bottlenose dolphin and one from a Cuvier's beaked whale, were cultured in 1× Dulbecco's modified Eagle's medium F12 (DMEM F12) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; PANTM BIOTECH) and 1% penicillin/streptomycin (10 IU/mL and 10 µg/mL respectively; Corning) [16]. Cell lines were regularly tested and confirmed to be mycoplasma-free (Mycoalert Mycoplasma Detection Kit, LONZA, Basel, Switzerland).

2.2. Isolation of Extracellular Vesicles with Ultracentrifugation (UC) and Size Exclusion Chromatography (SEC)

To isolate EVs from each cell line, two p150 petri dishes were seeded with 3×10^6 cells. Then, 24 h before EV isolation, cells were washed twice with PBS and the cell culture medium was replaced with FBS-free (FBSf) medium, in a volume of 25 mL for EV isolation with UC and of 16 mL for EV isolation with SEC.

EVs were isolated from two plates with semi-confluent cells by UC or SEC, as already described [23]. Briefly, the medium from each plate was centrifuged at 300× *g* and at 2000× *g* for 10 min at 4 °C, to remove any cell/cell debris. For EV isolation through UC, the supernatant was transferred to a 39 mL ultracentrifuge tube (Quick-Seal Round Top, Polypropylene, Beckman Coulter, Brea, CA, USA) and ultracentrifuged at 100,000× *g* for 90 min at 4 °C (Optima L-90 K, Beckman Coulter). The supernatant was discarded, and the EV-enriched pellet was resuspended in 100 µL of 0.2 µm double-filtered PBS (dfPBS). To perform EV isolation with SEC, the supernatant was centrifuged with 100 kDa ultrafiltration tubes (Amicon Ultra centrifugal filters, Merck Millipore, Burlington, MA, USA), and the concentrated medium was collected and loaded in the top of qEVoriginal/70 nm columns (IZON Science, Christchurch, New Zealand). SEC was then performed according to manufacturer's instructions and fractions #7, #8, #9, and #10 pooled and centrifuged with a 100 kDa ultrafiltration tube (Amicon Ultra centrifugal filters, Merck Millipore). Concentrated fractions were finally collected and resuspended in 100 µL of dfPBS.

2.3. Nanoparticle Tracking Analysis (NTA)

After EV isolation, EVs obtained with UC or SEC from both cell lines were quantified and evaluated for particle concentration and size distribution using NanoSight NS300 (Malvern). EV samples were progressively diluted in dfPBS until the correct dilution to gain reliable measurements by NTA was reached. For each sample, camera level was set at 12, and three movies of 60 s each were recorded and analyzed using the 3.4 NTA software. For particle quantification, measurements were considered reliable when within the following instrument optimal working ranges: particles per frame from 20 to 120; particle concentration between 10^6 and 10^9 per mL; ratio of valid particles to total particles higher or equal to 1/5. To test differences between the groups, a statistical analysis was performed with ANOVA using GraphPad Prism 8 software. The level of significance was fixed as $p < 0.05$.

2.4. Protein Extraction and Western Blotting (WB) Analysis

For each cell line, cell proteins were extracted from a 15-cm plate with 90% confluent cells using 2 mL of radioimmunoprecipitation assay buffer (RIPA buffer) (Thermo Fisher Scientific) supplemented with protease inhibitor (Pierce Protease Inhibitor Tablets, EDTA-free, Thermo Fisher Scientific) according to the manufacturer's protocol. Proteins from EVs isolated with UC or SEC were resuspended in 30 µL of RIPA buffer supplemented with protease inhibitor immediately after EV isolation.

Cells and EV-derived protein concentrations were calculated using a Pierce BCA protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's protocol.

For WB, 20 µg of proteins extracted from cells or EVs were first denatured at 70 °C for 10 min or at 95 °C for 5 min and then resolved using NuPAGE 4–12% Bis–Tris gel (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane. To block nonspecific binding sites, blots were incubated for 90 min in 5% non-fat dry milk in TBS-T (TBS containing 0.05% Tween-20) at room temperature. Then, blots were incubated at 4 °C overnight with rabbit or mouse primary antibodies against human Integrin-beta 1 (1:5000; GeneTex GTX128839, Irvine, CA, USA), TSG101 (1:1000; GeneTex GTX70255), CD9 (1:200; Bio-Rad MCA694GT, Hercules, CA, USA), and calnexin (1:1000; Cell Signaling #2679, Danvers, MA, USA) diluted in TBS-T containing 1% non-fat dry milk. Then, membranes were incubated with a peroxidase-conjugated secondary antibody (1:3000; anti-Rabbit #32260 or anti-Mouse #32230, Thermo Fisher Scientific) diluted in TBS-T for 1 h at room temperature.

Reactive bands were visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate detection kit (Thermo Fisher Scientific) with the iBright instrument (Thermo Fisher Scientific).

2.5. Scanning Transmission Electron Microscopy

Scanning transmission electron microscopy (STEM) was performed on isolated EVs, resuspended in dfPBS, to analyze their ultrastructural morphology. According to the proper dilution to obtain the best image quality, the samples were adsorbed onto 300 mesh carbon-coated copper grids (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min in a humidified chamber at room temperature. Vesicles on grids were then fixed in 2% glutaraldehyde (Electron Microscopy Sciences) in PBS for 10 min, then briefly rinsed in Milli-Q water and negative stained with 2% phosphotungstic acid brought to pH 7.0 with NaOH. Grids with adhered EVs were examined with a Zeiss GeminiSEM 500 equipped with a scanning transmission electron microscopy (STEM) detector (Zeiss, Germany).

3. Results

After EV purification with UC and SEC, the concentration and size of the isolated particles were measured by NTA. Results are shown in Figure 1 and Table 1.

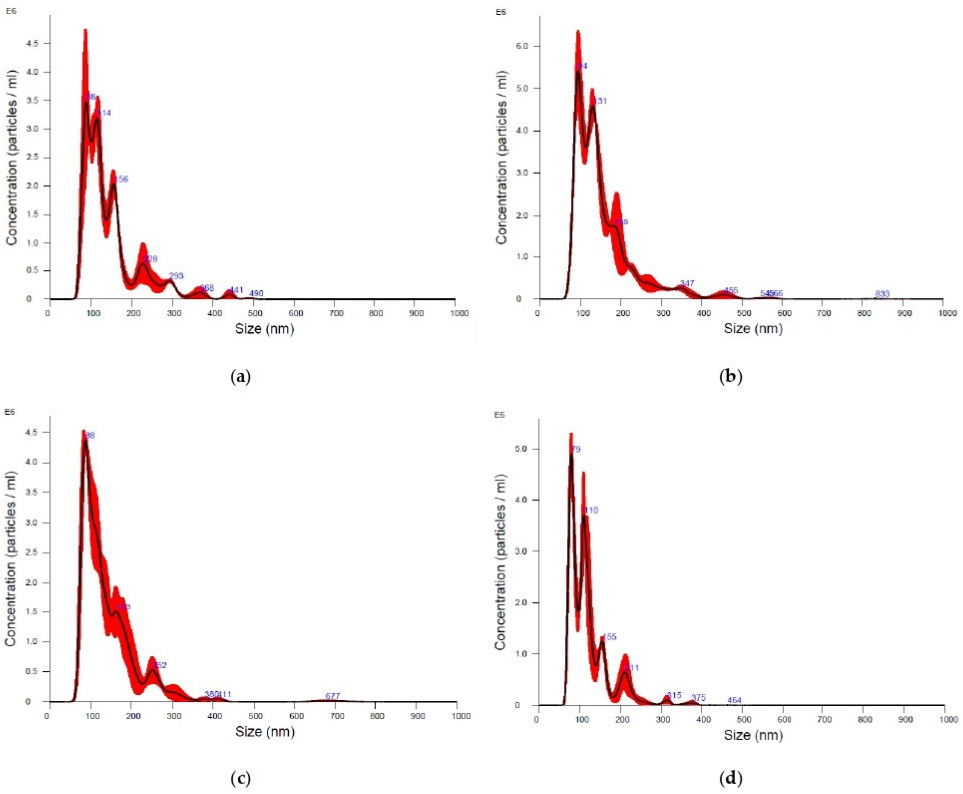


Figure 1. Size distribution of particles analyzed with nanoparticle tracking analysis. (**a**,**b**). Extracellular vesicles isolated with ultracentrifugation from bottlenose dolphin (**a**) and Cuvier's beaked whale (**b**) cells. (**c**,**d**). Extracellular vesicles isolated with size exclusion chromatography from bottlenose dolphin (**c**) and Cuvier's beaked whale (**d**) cells.

Table 1. Nanoparticle tracking analysis performed on extracellular vesicles isolated with ultracentrifugation (EV-UC) or size exclusion chromatography (EV-SEC) from bottlenose dolphin or Cuvier's beaked whale cells.

Sample	Particle Concentration/mL +/− SD *	Particle Mean Size (nm) +/− SD
EV-UC bottlenose dolphin	2.9×10^{11} +/− 2.7×10^{10}	140.8 +/− 3.1
EV-UC Cuvier's beaked whale	4.7×10^{11} +/− 2.8×10^{9}	151.9 +/− 7.3
EV-SEC bottlenose dolphin	2.7×10^{9} +/− 1.9×10^{8}	144.1 +/− 9.9
EV-SEC Cuvier's beaked whale	2×10^{9} +/− 1.4×10^{8}	120.7 +/− 2.9

* SD: standard deviation.

The size distribution of particles after both the UC and SEC isolation procedure showed size ranges within the size of EVs, with the mode size of the diameter ranging from 85 to 106 nm in all samples, demonstrating the isolation of mainly small EVs (Table 1, Figure 1).

NTA showed a similar concentration of particles in EV samples isolated with UC from bottlenose dolphin and Cuvier's beaked whale cells of 2.9×10^{11} +/− 2.7×10^{10} and 4.7×10^{11} +/− 2.8×10^{9}, respectively (Table 1). The particle concentration in EV samples isolated with SEC was lower than UC samples, being 2.7×10^{9} +/− 1.9×10^{8} and 2×10^{9} +/− 1.4×10^{8} for bottlenose dolphin- and Cuvier's beaked whale-derived EVs, respectively ($p < 0.05$) (Table 1).

WB was performed to characterize EVs isolated by UC and SEC from both bottlenose dolphin and Cuvier's beaked whale cells. As a control, proteins extracted from both cell lines were used (Figure 2).

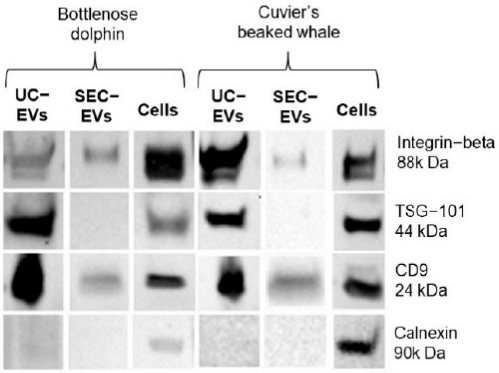
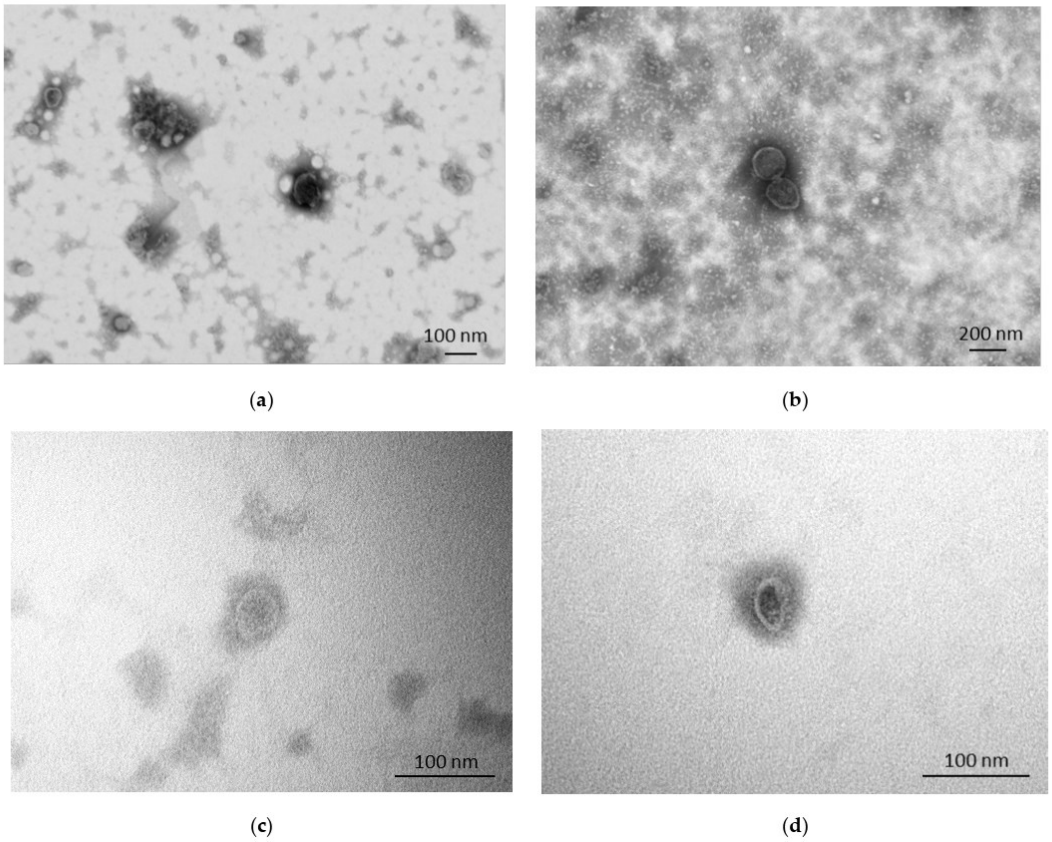


Figure 2. Western blot analysis performed on bottlenose dolphin and Cuvier's beaked whale cells and extracellular vesicles samples. UC-EVs: extracellular vesicles isolated with ultracentrifugation; SEC-EVs: extracellular vesicles isolated with size exclusion chromatography.

CD9 and integrin-beta 1, transmembrane proteins commonly used as EV markers, were detected in EVs isolated with UC and SEC from both cell lines and also in their cellular counterpart. TSG101, a cytosolic EV marker, was detected only in cells and EVs isolated with UC from both cell lines. Calnexin, a marker of the endoplasmic reticulum commonly used as negative control for EVs and as a positive control for cells, was not detected in EVs but was detected in both bottlenose dolphin and Cuvier's beaked whale cells as expected (Figure 2).

STEM further showed in the presence of intact and rounded EVs in all of our samples, as confirmed by the unbroken lipidic bilayer, that appears as a thin white filament enclosing electron dense material (Figure 3). The isolated EVs were mainly small EVs in all samples (<200 nm).



(**a**) (**b**)

(**c**) (**d**)

Figure 3. Scanning transmission electron microscopy performed on bottlenose dolphin and Cuvier's beaked whale extracellular vesicles (EVs). (a) Bottlenose dolphin EVs isolated with ultracentrifugation (UC); (b) Cuvier's beaked whale EVs isolated with UC; (c) bottlenose dolphin EVs isolated with size exclusion chromatography (SEC); (d) Cuvier's beaked whale EVs isolated with SEC.

4. Discussion

The aim of this study was to isolate and characterize EVs from two cetaceans' cell lines using two different isolation techniques. Similar studies have already been performed on other in vitro models [23]. However, considering the novelty of using cetacean cell lines, the fact that EVs released in vitro from cetaceans have never been described, and that EV size, concentration, and marker expression can vary according to the EV source, here we report our detailed protocol and findings. Considering the NTA, WB, and STEM results, we successfully isolated EVs from cell lines derived from bottlenose dolphin and a Cuvier's beaked whale using both different isolation techniques. Indeed, the main differences we recorded in our EV samples were related to the isolation protocol. It is well known that UC allows researchers to isolate more particles than SEC, but it negatively affects the purity of the sample [4,5]. We saw a higher number of particles in EVs isolated with UC from both cell lines, which had a concentration of 10^{11} particles/mL compared to SEC EVs, which had a concentration of 10^{9} particles/mL. These results are partially similar to those present in another study of our research group, focused on the characterization of EVs isolated with UC and SEC from a canine mammary tumor cell line. Despite the fact that the particle

concentration of UC EVs at NTA was similar, if slightly higher (7×10^{11} particles/mL), the SEC EV concentration from canine mammary tumor cells was higher (10^{10} particles/mL) than the concentration SEC EVs from cetaceans (ref). This might be explained by the fact that cancer cells have been demonstrated to generally shed more EVs [23].

In all our samples, particles mainly measured between 50–800 nm in diameter, a size included in the recognized size range of EVs, where EVs smaller than 200 nm of diameter are classified as small EVs and particles larger than 200 nm are classified as large EVs [24]. Considering this classification, we mainly isolated small EVs, accordingly with the used purification procedures.

Considering the protein expression, both EVs isolated with UC or SEC from bottlenose dolphin or Cuvier's beaked whale cells expressed the EV membrane markers CD9 and integrin-beta, and all the EV samples were negative to the negative control calnexin. The absence of this protein in the endoplasmic reticulum in our samples, which was expressed instead in cells, implies the absence of a relevant quantity of cell debris. TSG101, a cytosolic marker of EVs, was only expressed on EVs isolated with UC. It is commonly recognized that UC allows researchers to collect more EVs compared to SEC and, each isolation technique can purify different subtypes of EVs, which might express different EV markers and have different biological functions [25,26]. As such, the absence of expression of TSG101 in our SEC-derived EVs might be explained by a lower quantity of TSG101 compared to UC-EVs, which might be related to the isolation of different EV subtypes with lower TSG101 expression. In relation to our previous study on canine mammary tumor cell-derived EVs, while integrin-beta and TSG101 were not tested as EV markers, CD9 was present in both UC and SEC EVs [23]. Therefore, we demonstrated that all the tested EV markers are also conserved and expressed in cetacean-derived EVs. Finally, through STEM, we clearly showed the presence of intact membranous particles, i.e., EVs, in all our samples.The results herein reported confirmed for the first time the presence of cetacean cell line-derived EVs that could be now considered to study marine mammals' physiology and pathology in vitro, as they have been used for decades in human medicine. Moreover, despite the fact that the presence of EVs in the plasma of some cetacean species has already been reported, they had never been described in the bottlenose dolphin [11,12]. In the study of Magnadòttir and co-authors, EVs were isolated with UC from the plasma of minke whale, fin whale, humpback whale, Cuvier's beaked whale, and orca. Since four different species were included and EVs were isolated from a different biofluid, some aspects, like particle quantification at NTA, cannot be compared with our study. However, the authors interestingly found the presence of other EV markers at WB (CD63 and flotillin-1), also showing the conservation of additional EV markers among different species [12].

Studying EVs in vivo is arduous due to their high complexity and heterogeneity. However, EVs isolated from tissues ex vivo have the advantage of coming from different cell types and, therefore, represent the tissue's in vivo situation. However, until now, we had little information on tissue-derived EVs because of the tissue complexity and possible cell rupture that can contaminate the final EV sample [27]. EVs isolated from body fluids (e.g., blood, urine) have been investigated much more, but they are less representative than those derived from tissues [27]. Single-cell EV isolation and specific RNA sequencing analyses that allow researchers to identify the cell origin of EVs in body fluids are still pioneering and not yet standardized [28]. Therefore, despite their minor complexity and all the limits of in vitro systems, EVs released in vitro can still be useful tools to gain information on specific cell types otherwise difficult to obtain in vivo.

Both the species included in our study are relevant for conservation policies and scientific interests, but they are difficult to investigate in field conditions. Bottlenose dolphins are top predators, living in coastal environments and feeding on commercial species or interacting with fisheries and many other human related activities. For these reasons and because they are also included in Annex II of the Habitat Directive (EU Directive 92/43/CEE) they could be considered good candidates as a sentinel species to monitor both environmental and human health, taking advantage of the bioaccumulation phenomena [29–31].

Additionally, information gained from these species could feed the information on several descriptors of the Marine Strategy Framework Directive (EC/2008/56), including those related to the effects of contaminants (descriptor 8), marine litter (descriptor 10), and underwater noise (descriptor 11). Finally, it should also be noted that several infectious diseases of terrestrial origins and with zoonotic potential have been recently reported in *Tursiops truncatus* caused by bacterial (e.g., *Listeria monocytogenes* and *Salmonella* spp.) or protozoal (e.g., *Toxoplasma gondii* or *Giardia* spp.) agents [32–35]. Also, cetaceans could be relevant hosts for infectious diseases during spill-over events, like those which occurred in the evolution of Cetacean morbillivirus and, more recently, for a highly pathogenic influenza virus [36,37]. On the other hand, Cuvier's beaked whales have developed efficient adaptations to deep waters and a hypoxic environment, and could be considered an interesting animal model for extreme conditions [38–40]. Additionally, they are very sensitive to underwater noise, causing mass strandings, in particular to specific impulsive sounds, such as mid-frequency military sonars [41,42]. It should finally be stressed that cetaceans, in general, also have a very low incidence of cancer despite their long-life span and are frequently exposed to several chemical substances often deemed to be cancerogenic, being, therefore, of interest in comparative oncology studies [43–45].

As described above, the EV isolation techniques included in this study have some limitations mainly related to the lower purity of the EV sample obtained by UC and to the lower yield of the EV isolated by SEC. Both isolation methods are sized-based and apply centrifugal forces with or without a gradient. Additional systems for EV isolation, such as immune-based methods could also be applied for comparison. In future studies, it would also be interesting to assess the functional effect of cetacean cell-derived EVs, investigating cellular uptake and response to external stimuli, which were not included in this preliminary investigation.

5. Conclusions

In this preliminary EV isolation and characterization study, we demonstrated for the first time that bottlenose dolphin and Cuvier's beaked whale cells can release EVs in vitro, like other mammalian cells. We believe that in vitro and EV study can become an additional tool to deepen our knowledge on these threatened and physiologically unique species. Future studies will aim to expand the use of EVs in cetaceans' cell lines to investigate their role as markers after exposure to several natural and artificial conditions, such as underwater pressure, diseases, or chemical substances, also comparing them with results obtained in vitro in other species, including human beings.

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Informed Consent Statement: Not applicable.

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5.3 Isolation and characterization of extracellular vesicles from Manila clam hemolymph

5.3.1 Introduction

During the XIX century, some species of bivalves became very important for the international trade and were introduced in the aquaculture systems of many countries (41). Among these species, Manila clam (*Ruditapes philippinarum*) is considered a conspicuous Italian aquaculture product (42). Being filter feeders widely distributed, easy to distinguish, to collect and to maintain in laboratory conditions, Manila clams can be used as sentinels to monitor environmental pollution (43). Moreover, considering the economic importance of Manila clams as a seafood, new monitoring tools to assess the presence of infectious disease or the effects of climate change, which might damage the production, are needed (41,42,44).

EVs have been proved to be versatile tools, enclosing important information of organisms of any kingdom. Very few studies have been performed on bivalve derived-EVs, most of them mainly focusing on oysters (30,45,46). However, in the few published studies, EV-isolation and characterization procedures are not accurately described or involve purification methods which isolate EVs in low-purity (*e. g.* precipitation kits) (30,45–47).

Considering the few information in the literature on EVs in bivalves, but the high potential that EV research implies in relation to environment and food safety, in my project we studied and optimized a protocol to isolate and characterize EVs from Manila clam hemolymph. Since hemolymph is a complex biofluid, with the same function of blood for mammals but with a different composition, we compared the isolation of EVs from hemolymph diluted in two different buffers: PBS or water with 3.2% of NaCl. While 1x PBS is the most used diluent for EVs from mammalian biofluids, a higher salt concentration might be more suitable when working with hypertonic fluids, such as Manila clam hemolymph. In addition, again due the molecular complexity and density of the hemolymph, both traditional ultracentrifugation (UC) and sucrose density gradient UC were applied for EV isolation.

The following experiments were mainly performed during my abroad period at the Utrecht University, Utrecht (NL), at the department of Biomolecular Health Sciences in the laboratory of prof. Marca Wauben.

5.3.2 Materials and methods

Collection of hemolymph samples

Hemolymph was withdrawn from the posterior abductor muscle of alive commercially available Manila clams (*Ruditapes philippinarum)* using a syringe with a 26G needle. Fresh hemolymph was collected in 4.5 ml aliquots, each derived from a pool of 8 animals. After collection, hemolymph was centrifuged at 800xg to remove hemocytes and then stored at -80°C until EV-isolation.

Isolation of hemolymph-derived EVs

After thawing, each hemolymph aliquot was centrifuged at 2000 × g at 4°C to discharge residual cells and debris. The supernatant was then centrifuged at 10,000 × g at 4°C for 30 minutes in an L90k ultracentrifuge (Beckman Coulter, Brea, California; US) to further eliminate smaller irrelevant debris.

To perform EV-isolation with UC, the 10,000 × g centrifuged supernatant was diluted either in 1:2 in 1x PBS (dfPBS) or milliQ water with 3.2% of NaCl (dfmilliQ), both double filtered (0.2 μm), and transferred to SW40 tubes (Beckman Coulter, Brea, California, US) and ultracentrifuged at 10,000 × g for 90 minutes at 4°C in a SW40 rotor in a Beckman Coulter OptimaL-90k (Beckman Coulter, Brea, California US). Due to technical issues (see Results below) the UC isolates were not analyzed further.

For EV-isolation with top-down sucrose density gradient UC (dgUC), 3.250 mL of the 10,000 × g supernatant (see above) were diluted 1:2 with either dfPBS or dfmilliQ, to reach a final volume of 6.5 mL. The diluted supernatants were loaded on the top of a sucrose density gradient (6 mL), which was made by layering successive sucrose solutions of decreasing density (2.0 M–0.4 M) on top of 2.5 M sucrose in SW40 tubes (Beckman Coulter, Brea, California, US). Samples were then centrifuged at 200,000 × g for 16 h at 4°C in a Beckman Coulter OptimaXE with a SW40 rotor (Beckman Coulter, Brea, California, US). After centrifugation separated density fractions (fr) in the tube were collected and pooled in groups of 3 as follows: higher density-fr 1–3 (mean density: 1.28–1.26 g/mL), fr 4–6 (mean density: 1.24–1.20 g/mL), fr 7–9 (mean density: 1.18–1.13 g/mL), and low-density fr 10–12 (mean density: 1.12–1.08 g/mL). Each pool (about 1.5 ml) was transferred to a SW40 tube (Beckman Coulter, Brea, California, US), diluted with 11 mL of dfPBS and centrifuged again at 100,000 × g for 65 min at 4°C in a Beckman Coulter OptimaL-90K with a SW40 rotor. The final EV-enriched pellets from each dgUC pool [herein as NaCl- or PBS-pools (from hemolymph diluted in dfmilliQ or in dfPBS, respectively) 1-3; 4-6; 7-9; 10-12] were then used for further experimentation, performing new additional dgUC different biological replicates for each assay.

Nanoparticle Tracking Analysis

To assess the size distribution and concentration of EVs, Nanoparticle Tracking Analysis (NTA) was performed in biological triplicates for all dgUC NaCl- and PBS-pools.

Each pellet was resuspended in 100 µl of dfPBS and then in case diluted in the same buffer until reliable measurements were obtained. NTA was performed with NanoSight NS500 (Malvern Panalytical, Malvern, UK) and 3 videos of 60 seconds each were recorded and analyzed using software 3.4, with camera level set at 14 and detection threshold set at 5.

Protein extraction, quantification and staining

The pellet from each NaCl- and PBS-pool was resuspended in 40 µl of radioimmunoprecipitation assay buffer (RIPA buffer) with protease inhibitor and analyzed in technical and biological triplicates for protein concentration with the BCA kit (Thermo Fisher Scientific, Waltham, Massachusetts, US) according to manufacturer's instructions.

The distribution of proteins in each sample was also assessed after gel electrophoresis with sypro-ruby protein staining. Each pellet of NaCl- and PBS-pool was resuspended in 40 µl of sample buffer (125 mM Tris, pH 6.8, 4% SDS, 20% glycerol), heated for 5 min at 95°C, diluted 1:1 with PBS and loaded onto a 12.5% Bis-Tris gel. After gel electrophoresis, the gel was stained with Sypro Protein Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, US) according to manufacturer's instructions. Stained protein bands were visualized with the ChemiDoc MP and Image Lab 5.1 (Bio-Rad, Hercules, California, US).

Negative stain electron microscopy

In order to visualize EVs into the samples, the pellet of each NaCl- or PBS-pool was dissolved in 30 µl of a solution with 25 mM HEPES and 130 mM NaCl and kept on ice for 1 hour. Subsequently, 4 µl of sample was applied to carbon-coated copper grids that were glow discharged (PELCO easiGlow, Ted Pella, Redding, California, US). The sample was allowed to absorb for 30 seconds prior to 2x wash with milliQ water and 3x staining with 2% uranyl acetate solution, with the last washing step lasting for 30 seconds. Grids were allowed to dry in air for 5 min and then imaged on a FEI Talos L120C transmission electron microscope operated at 120 keV, equipped with a CCD camera (Bijvoet Centre for Biomolecular Research, Utrecht University, NL).

Cryo-electron microscopy

To perform cryo-EM, only the pellet of NaCl- or PBS-pool 7-9 was dissolved in 20 µl of a solution with 25 mM HEPES and 130 mM of NaCl solution. 4 µL of sample was loaded onto a Quantifoil 2/1 300 mesh grid (Quantifoil Micro Tools, Großlöbichau, Germany) that was glow discharged (PELCO easiGlow, Ted Pella, Redding, California, US). 1 µL of a BSA-conjugated 10 nm gold beads (Aurion, Wageningen, the Netherlands) suspension was added and the drop was blotted from the back (other side of sample deposition) for 4-6 seconds. Sample was vitrified by plunge freezing in liquid ethane-propane mix (37% ethane) using a manual plunge-freezer (MPI-Martinsried, Planegg, Germany).

Data was collected on a Talos Arctica (Thermo Fisher Scientific, Waltham, Massachusetts, US) transmission electron microscope operated at 200 kV, equipped with a postcolumn energy filter (Gatan) operated in zero-loss imaging mode with a 20-eV energy selecting slit (Bijvoet Centre for Biomolecular Research, Utrecht University, NL). Projection images were collected using a K2 Summit direct electron detector (Gatan, Pleasanton, California, US) in counting mode with dose fractionation, at a magnification of 100,000x (1.359 Å/pix). Target defocus was set at -4 µm and total dose approximately 50 e-/Å.

5.3.3 Results

Isolation of hemolymph-derived EVs

When the hemolymph was preliminarily diluted in dfPBS the formation of whiteish and compact concretions where evident as precipitates after traditional UC, presumably composed of aggregations of salts (Figure 1). Whereas, diluting hemolymph in dfmilliQ with 3% of NaCl did not cause the formation of any precipitates after UC, probably due to the fact that this solution has of the same osmolarity of seawater, which is hypertonic compared to 1x PBS (Figure 1).

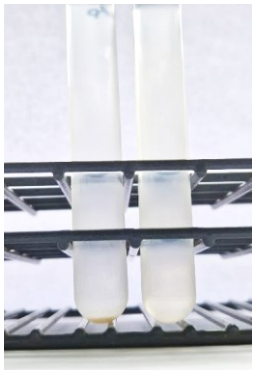

Figure 1. Ultracentrifugation performed on hemolymph samples. On the left, hemolymph diluted with 1:2 with 1x dfPBS; a big pellet of precipitates can be seen at the bottom of the tube. On the right, hemolymph diluted 1:2 with dfmilliQ water with 3.2% of NaCl; no precipitates are present at the bottom of the tube.

Even if without precipitations, performing only traditional UC on hemolymph diluted with water + 3.2% of NaCl did not allow to precisely characterize EVs with NTA, BCA and EM, due to a still high (probably salt) particle concentration in the UC-pellet which interfered with the analysis. Therefore, we decided to perform dgUC to isolate hemolymph derived-EVs, comparing EV-yield and quality when using these two different diluents (dfPBS and dfmilliQ).

Nanoparticle Tracking Analysis

Each NaCl- or PBS-pool isolated with dgUC was analyzed for particle concentration and size distribution at NTA. In both NaCl- and PBS-pools, particles had a wide size range, from 50 to 800 nm of diameter. The mean size of particle diameters ranged from 124.2 to 178.2 nm and the mode ranged from 59.7 to 132.2 nm among NaCl-pools. For PBS-pools, the mean diameter was between 121.6 to 168 nm and the mode between 73.1 and 114.5 nm (Table 1). For both types of buffers, pools 1-3 were those with the highest particle concentration (2.3 and $3.3*10^{11}$ particles/ml, respectively) (Table 1). Particle concentration in both NaCl- and PBS-pools 4-6 and 7-9 was similar (from 1.3 to $1.6*10^{10}$ particles/ml), while the lowest concentration was in NaCl- and PBS-pools 10-12 ($1*10^{9}$ and $6.1*10^{8}$ particles/ml respectively) (Table 1).

Table 1. Nanoparticle tracking analysis performed on sucrose density gradient ultracentrifugation (dgUC) pooled fractions of hemolymph diluted with dfmilliQ water (NaCl-pools) or with PBS (PBS-pools).

dgUC pools	Mean concentration particles/ml +/ SD	Mean size (nm) +/- SD	Mode size (nm) +/- SD
NaCl-pools 1-3	$2,312*10^{11}$ +/- $2,35*10^{10}$	124.2 +/- 9.2	59.7 +/- 5.6
NaCl-pools 4-6	$1,59*10^{10}$ +/- $2,52*10^{9}$	178.2 +/- 1	128.6 + /- 6.1
NaCl-pools 7-9	$1,29*10^{10}$ +/- $2,74*10^{8}$	149.6 +/- 1,9	120.2 +/- 8.4
NaCl-pools 10-12	$1,06*10^{9}$+/- $2,76*10^{7}$	163.4 +/- 2.3	132.2 +/- 6
PBS-pools 1-3	$3,34*10^{11}$ +/- $1,36*10^{10}$	121.6 +/- 3.8	73.1 +/- 2
PBS-pools 4-6	$1,13*10^{10}$ +/- $1,23*10^{9}$	168.9 +/- 1.8	107.9 +/- 6.1
PBS-pools 7-9	$1,64*10^{10}$ +/- $2,08*10^{9}$	145.8 +/- 2	111 +/- 5.4

PBS-pools 10-12	$6{,}09*10^{8}$+/- $2{,}74*10^{7}$	134 +/- 2.5	114.5 +/- 4.7

* *SD = standard deviation.*

Protein quantification and staining

BCA assay performed on all the dgUC NaCl- and PBS-pools, showed a higher protein concentration in all the NaCl-pools compared to the PBS-pools. In particular, three PBS-pools (4-6; 7-9 and 10-12) could not be reliably measured because protein concentration was under the kit detection range (Figure 2A). The highest protein concentration was in NaCl-pool 1-3 (mean = 0.58 +/- 0.02 µg/µl), while in the other NaCl-pools protein concentration resulted similar (mean protein concentration: fr 4-6 = 0.10 +/- 0004 µg/µl; fr 7-9 = 0.15 +/- 0.003 µg/µl; fr 10-12 = 0.08 +/- 0.02 µg/µl) (Figure 2A). Sypro Ruby protein staining showed the presence of proteins of different molecular weight in all samples (Figure 2B). More protein bands were visible in NaCl- than in PBS-pools.

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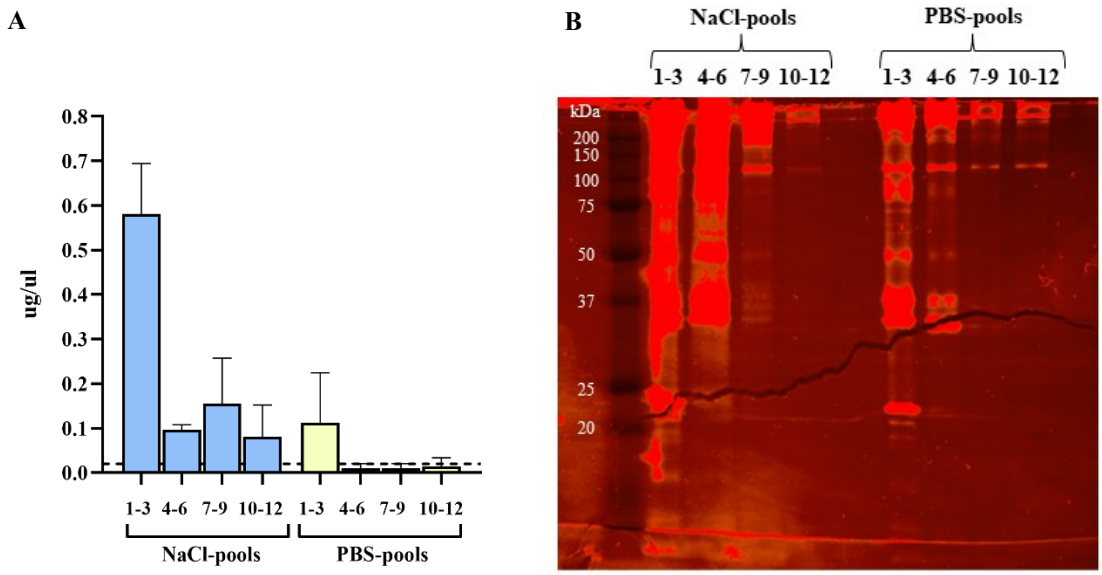


Figure 2. A. Protein quantification performed on sucrose density gradient ultracentrifugation pooled fractions derived from hemolymph diluted in dfmilliQ (NaCl-pools) or in PBS (PBS-pools). B. Protein gel staining with Sypro-ruby on NaCl-pools and PBS-pools. On the left, the molecular weight in kDa of the ladder.

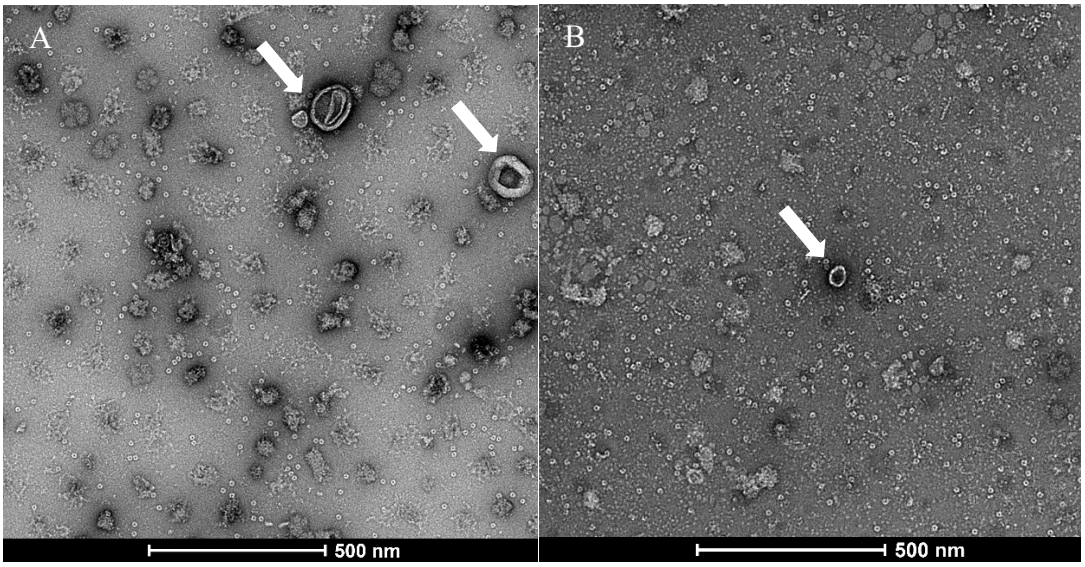
Electron microscopy

Negative stain EM was performed on all the NaCl- and PBS-pools. Membrane-bound vesicular structures presenting the typical cup-shaped and consistent with EVs, were detected in all NaCl- and PBS-pools (Figures 3A-F). EV-size was mainly referable to that of small EVs, being smaller than 200 nm. Most EVs with least contaminant co-isolated particles in the background (most likely proteins, seen as white or grey smaller granular or fibrillar material) were detected in both NaCl- PBS-pools 7-9 (Figures 3E; 3F). Lot of similar vesicular structures consistent with EVs were present also in pools 4-6, but with a higher background material (Figures 3C; 3D). Comparing the two buffers, the NaCl-pools 4-6 and 7-9 seemed to contain a more abundant number of EVs than the same PBS-pools, with the latter instead presenting some collapsed amorphous vesicular structures (presumably consistent with collapsed EVs) which were not present in the corresponding NaCl-pools (Figures 3C-F).

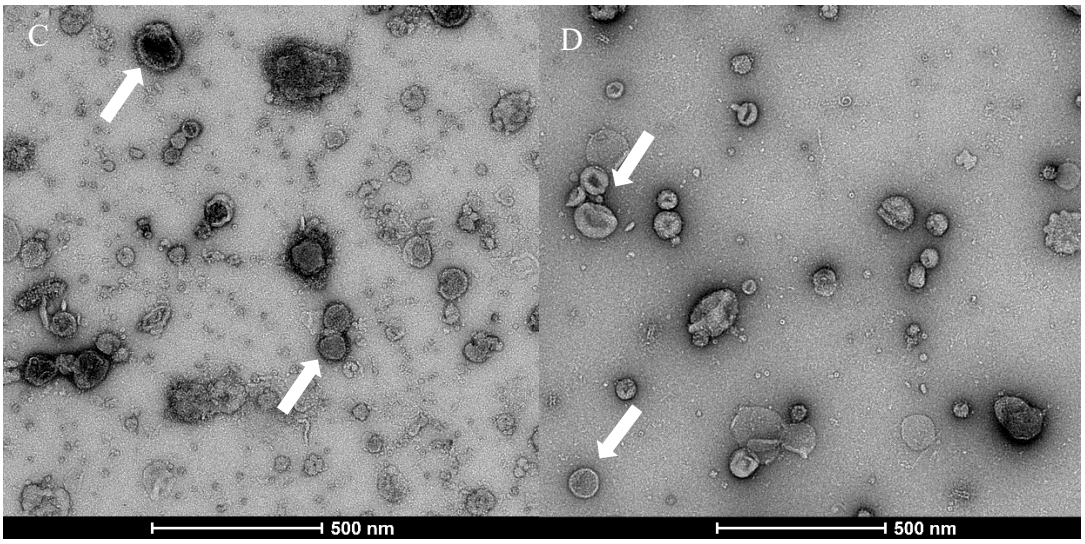
Few EVs were detected in NaCl and PBS-pooled-fr 1-3, where most of the isolated material was of non-vescicular (EV) nature (proteins and salts, the latter seen as darker spots especially in PBS-pooled-fr 1-3), and in NaCl and PBS-pooled-fr 10-12, where almost no particles or vescicles were present (Figures 3A-B; 3G-H).

NaCl-pools **PBS-pools**

1-3



4-6



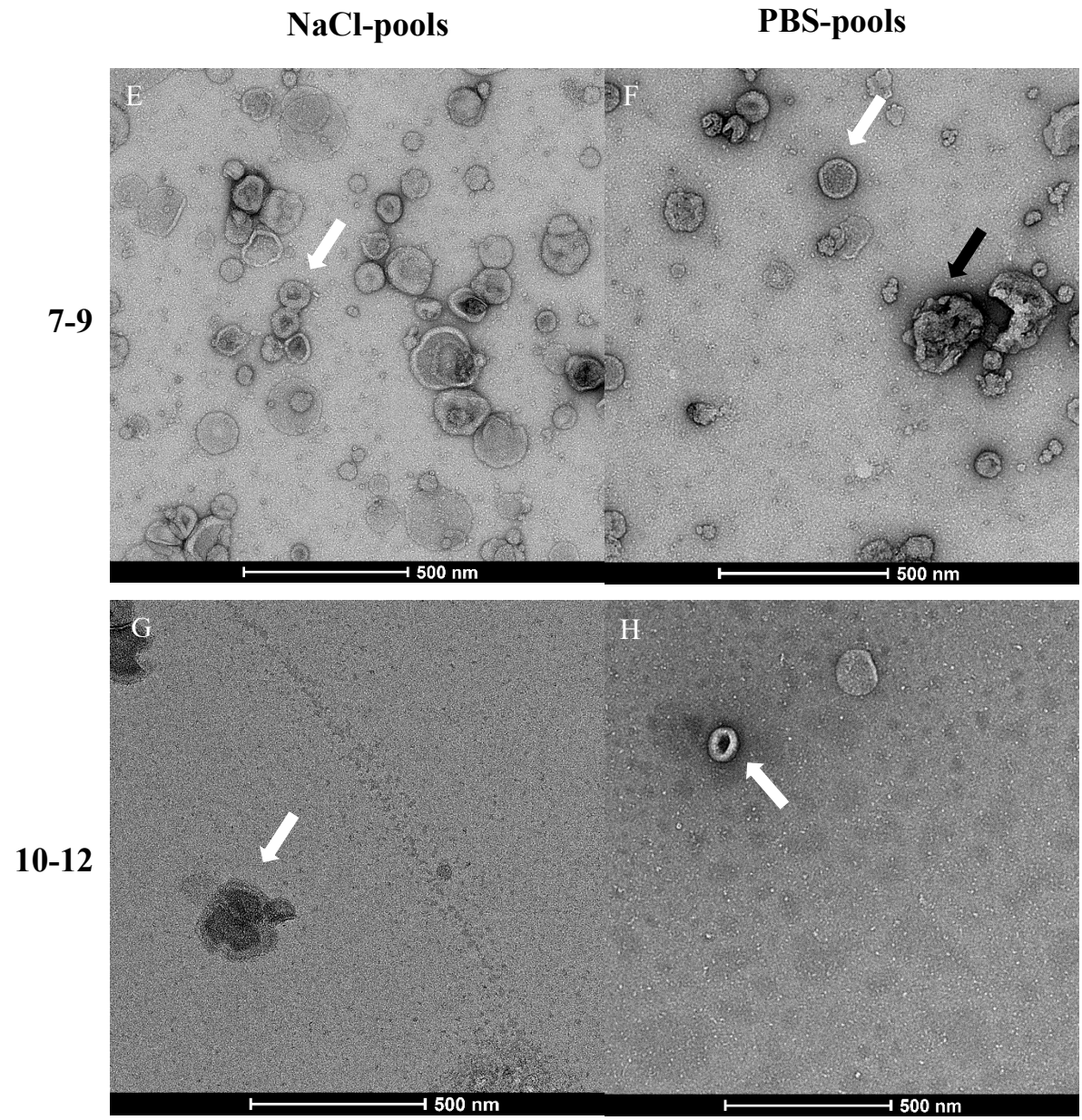


Figure 3. Negative staining electron microscopy performed on density gradient pooled fractions derived from hemolymph diluted in dfmilliQ (NaCl-pools) or in PBS (PBS-pools). White arrows point vesicular structures consistent with extracellular vesicles (EVs). A. NaCl-pools 1-3; B. PBS-pools 1-3; few EVs are surrounded by abundant amorphous structures in the background (probably proteins) (A-B); C. NaCl-pools 4-6; D. PBS-pools 4-6; groups of EVs are present. In the background many white and grey granular and fibrillar structures consistent with proteinaceous material are present (C-D). E. NaCl-pools 7-9; F. PBS-pools 7-9; an increased number of EVs can be seen, with few white granular structures (proteins) in the background (E-F); the black arrow points a huge amorphous structure, possibly consistent with collapsed EVs (F). G. NaCl-pools 10-12; H. PBS-pools 10-12; presence of scarce EVs and background material (G-H).

Since most of vesicles consistent with EVs were present in dgUC pools 7-9, NaCl- and PBS-pools 7-9 were selected for cryo-EM (Figure 4A-B).

Cryo-EM confirmed the presence of vesicular structured surrounded by a lipid bilayer, consistent with EVs. As for negative staining, Cryo-EM confirmed the presence of mainly small EVs (< 200 nm) in both NaCl- and PBS-pools 7-9 (Figures 4A-D). In both samples, EVs appeared with heterogenous shapes, with an intact lipid bilayer and with small granular dark particles, probably proteins, coronating their surface (Figures 4A-D). The main difference between NaCl- and PBS-pools 7-9 was the presence of diffused crystalline structures in PBS-pool 7-9, probably related to the presence of salt precipitates which made the image acquisition more difficult and that were not clearly detected at the negative staining (Figure 4D).

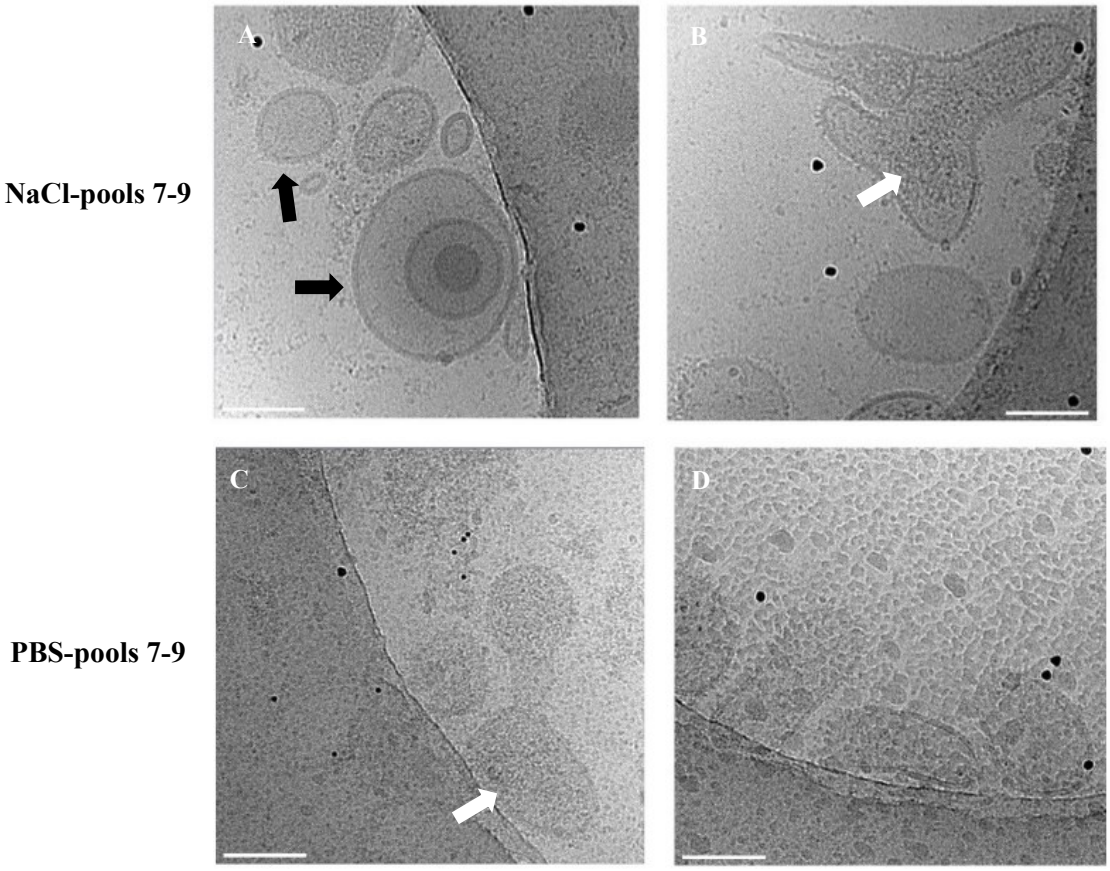


Figure 4. Cryo-electron microscopy performed on dgUC pools 7-9 derived from hemolymph diluted in dfmilliQ (NaCl-pools) or in PBS (PBS-pools). A-B. NaCl-pool 7-9. A. roundish EVs heterogenous in size surrounded by an intact lipid bilayer (black arrows); B. polymorphous EV coronated with protein on the surface (dark granular small material – white arrow).; C-D. PBS-pool 7-9; C. EVs coronated by proteins on the surface (white arrow); D. Diffuse crystalline material (probably salt precipitates) covering the sample; the scale bar is set at 100 nm.

5.3.4 Discussion

In this study, we report a tailored protocol for EV-isolation from Manila clam hemolymph.

A high salt component evident as precipitated concretion or more dissolved salt particles was present in the UC-pellet of hemolymph diluted with either dfmilliQ or dfPBS. Therefore, to better separate EVs from salts which affect EV-analysis, we chose to perform sucrose dgUC to isolate EVs. dgUC is recognized as a high specificity EV-purification method, allowing to separate the particles of a sample according to their density (14). Moreover, to test whether the salts precipitates caused by dfPBS could dissolve in the sucrose density gradients or cause further issues in the EV-isolation and analysis procedures, we still included the dfPBS ad a buffer in comparison with the hypertonic dfmilliQ.

Protocols for EV isolation with sucrose dgUC are standardized for mammal biofluids and the density gradient fractions containing EVs are known in advance (14,48,49). However, this is not the case for hemolymph which has never been tested by this method. Therefore, to precisely assess EV-distribution in dgUC fractions, we pooled in groups of three all the sucrose density gradient obtained fractions and characterized all of them for EV-presence.

According to the literature where the same sucrose dgUC isolation protocol was performed on a complex and dense biofluid such as bovine and human breast milk, pools 7-9 have been demonstrated at cryo-EM, EM negative staining and Western Blotting as the fractions containing most EVs with least co-isolated contaminants (49,50). Pools 4-6 can also present many EVs, but less than pools 7-9 (49).

In our study, NTA and BCA were performed to quantify particle and protein concentration, respectively, on all NaCl- and PBS-pools. However, NTA can only detect nanoparticles by Brownian motion analysis, with no precise indication of their nature and origin (i.e. EVs versus other particles), while BCA only quantifies proteins. Therefore, EV presence in the different dgUC pools was then also assessed with EM. Coherent to the literature, EM negative staining showed that most of vesicles consistent with EVs with least background contaminants were present in NaCl- and PBS-pools 7-9, followed by NaCl- and PBS-pools 4-6 which still presented many EVs, but to a lesser extent (49).

Considering the comparison between dfPBS and dfmilliQ with 3.2% of NaCl as diluents, we observed mild differences in the dgUC pools affecting some EV-analysis.

Cryo-EM was performed on NaCl and PBS-pools 7-9, to highlight possible differences in EV quality related to the two different diluents. Intact small EVs were present in both samples but, in PBS-pools 7-9 salt precipitates were spread in the background, partially covering EVs. In the literature, there is no accurate description of the characterization of EVs isolated from hypertonic and complex biofluids like hemolymph. However, in relation to our samples, we might suppose that salt precipitates detected at cryo-EM might have affected also other analysis,

explaining the discrepancy between the NTA and the BCA data. While particle concentration at NTA was similar among the corresponding NaCl and PBS-pools, protein concentration at BCA was much higher in all the NaCl-pools. We might speculate that this difference could be due to the presence of salt aggregates in the PBS-pools, possibly measured by the NTA (5,16).

To better assess the nature of the components of dgUC pools more information on Manila clam hemolymph is needed. In the literature, some studies report its composition mainly in relation to disease or stress conditions (*e. g.* bacterial or viral infections) or focus on the analysis of specific components, but large datasets describing the detailed biochemical properties are still lacking (e. g. nitric oxide, cycloxigenase-2, hemocyte description) (51–54).

To our knowledge, few studies have analyzed hemolymph derived EVs in bivalves, and none of them investigated Manila clam EVs. Most studies, focused indeed on different species of oysters (*Crassostrea gigas, Crassostrea virginica, Pinctada fucata*) or on other bivalves (*Mytilus edulis, Mya arenaria, Ensis leei*) (30,45–47). In these studies, EV-isolation from hemolymph was mainly obtained with precipitation kits which do not guarantee a good purity of the EV-samples (45–47). In the study of Bowden and coauthors, EVs were isolated instead with ultracentrifugation (twice at 100,000 x g for 1 h) from hemolymph of four different bivalve species (*Mytilus edulis, Mya arenaria, Ensis leei, Crasosstrea virginica)* and were correctly characterized through NTA, EM and Western Blotting (WB) (30). However, EM pictures show a high background in all samples and do not clearly highlighted EV presence. Moreover, WB showed a positivity for the EV-marker CD63, however the detected band was at a different molecular weight than that reported for the same protein in the literature and declared from the antibody producing company (70 kDa band detected in bivalve EVs versus 28 kDa; see: https://www.abcam.com/products/primary-antibodies/cd63-antibody-late-endosome-marker-ab216130.html) (30).

In our study it was not possible to obtain WB characterization of the isolated EVs since there was lack of cross-reaction between Manila clam proteins and some commercial antibodies that we tested with several WB protocols (data are not shown). The applied antibodies were raised against human antigens normally associated to EVs (CD81, HSP70, HSP90, TSG101) and were not able detect a positive signal from the extracted proteins obtained from the NaCl- and PBS-pools. Therefore, further analysis on Manila clam protein sequences, still scarcely characterized, should be performed to find good markers for EV-characterization.

To conclude, here we report a protocol for EV-isolation from Manila clam hemolymph. Diluting hemolymph in hypertonic solution compared to 1x PBS can avoid the formation of salt aggregates which can affect some EV-analysis. Considered the scarce knowledge on Manila

clam EVs, further studies should be performed to better characterize hemolymph derived-EVs, paying attention to protein composition and cargo. Being Manila clams a seafood of interest for human health, for the international trade and for environmental research, (55,56), further studies on hemolymph derived-EVs could be performed to investigate their application as monitoring tools for animal and environmental health assessment.

6. Extracellular vesicles in cancer

The role of EVs in the carcinogenic process is one of the most studied functions of EVs, especially in human medicine (57). Indeed, EVs have been shown to be involved in all the main processes of carcinogenesis, including cancer progression, invasiveness, metastasis and drug resistance (55,58,59)

Cancer EVs have been demonstrated to modulate the tumor microenvironment, transferring oncogenic proteins and nucleic acids to the stromal cells which will then support tumor growth, invasion, and metastasis (60). EVs can also be secreted by different cells of the tumor microenvironment, improving cancer progression, for example transferring angiogenic factors to endothelial cells (60,61). Moreover, despite EVs contain tumor antigens capable of priming an anti-tumor immune response, there is strong evidence that this anti-tumor immune response can also be suppressed by tumor cells through EVs, by reducing the activity of immune cells or inducing their apoptosis (59,60). Cancer EVs can also mediate drug resistance by directly exporting or sequestering cytotoxic drugs, reducing their concentration at target sites or conferring resistance to drug-sensitive cancer cells (62,63).

Besides their role in the regulation of cancer-related pathways, EVs have also been studied because of their possible application as cancer biomarkers (8,57,64). Since EVs are stable in various types of body fluids and reflect the current state of their parental cell, targeting EV-cargo allows to obtain crucial information about the health status of an organism. Moreover, EV sampling is minimally invasive when performed by certain body fluids, so EVs and their cargo can be used as clinical diagnostic, prognostic, and predictive cancer biomarkers (58).

EVs carry different types of molecules which could be targeted as biomarkers, such as proteins, lipids, mRNAs and lncRNAs. However, miRNAs are for sure among the molecules investigated the most as cancer biomarkers, both in human and veterinary medicine (58,63). miRNAs normally regulate molecular RNA networks but are often dysregulated in cancer, supporting both its progression and suppression (58). They can be detected as free circulating molecules in the blood, but also as associated to EVs, where they seem to be more resistant to RNase activity. This makes EV-associated miRNAs good candidates as cancer biomarkers (58). However, despite several trials assessing the application of EVs for human cancer diagnosis, staging and response to therapy have been performed, fine standardization procedures are still needed to truly use EVs as biomarkers in the clinical routine (58,65).

6.1 Functionality of cancer-derived extracellular vesicles

In human medicine, several studies have demonstrated the ability of EVs of transferring their cargo to other cells, of the same or of a different cell-type, in an autocrine or paracrine manner or also through distant cell signaling (66). This horizontal transfer of molecules can have different implications in cancer, and tumor derived EVs can spread an acquired phenotype, promoting for example cell proliferation, metastasis, drug resistance or inhibiting apoptosis (67,68).

Considering the complexity of studying EV-related pathways *in vivo* and the need to reduce animal trials, *in vitro* studies are still the first choice to study EV-uptake and trafficking and to evaluate the function of tumoral EVs (69). Despite *in vitro* studies on cancer-derived EVs are frequent in human medicine, they are much rarer in veterinary medicine. Until now, few studies have been published on *in vitro* cancer derived EVs, and even fewer evaluated EV-functionality in veterinary medicine (70,71).

The most common neoplasia in both women and female dogs is mammary tumor (72). For human breast cancer, many *in vitro* studies showed how both cancer derived EVs and EVs released by the tumor associated microenvironment can functionally promote tumor survival, spreading and metastasis by activating specific cell-signaling pathways (73–75). In dogs, despite mammary tumor is the most common neoplasia, only two studies investigated the role of cancer derived EVs, one focusing on the characterization of EVs from *in vitro* mammary tumor cell lines and the other one on the analysis of EV-associated miRNAs derived from five different canine mammary tumor cell lines (71,76).

Therefore, an additional part of my PhD project was to highlight possible autocrine effects on cell proliferation, invasiveness and migration of canine mammary tumor cell line derived EVs isolated and administered to the same cell line *in vitro*. Additionally, considering the importance of the EV purification protocol which might select specific EV-subpopulations in different conditions of purity, the EV-profile and functionality of EVs isolated with two different methods, UC and SEC, was also compared. The following publication was the result of these studies.

6.2 Paper n. 4: "Characterization and function of extracellular vesicles in a canine mammary tumor cell line: ultracentrifugation versus size exclusion chromatography"

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ORIGINAL ARTICLE

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Characterization and function of extracellular vesicles in a canine mammary tumour cell line: Ultracentrifugation versus size exclusion chromatography

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Abstract

Extracellular vesicles (EVs) are cell-derived membrane-bound vesicles involved in many biological processes such as tumour progression. For years, ultracentrifugation (UC) has been considered the gold standard for EV isolation but limited purity and integrity allowed the diffusion of alternative techniques. In this study, EVs were isolated from a canine mammary tumour cell line using UC and size exclusion chromatography (SEC) and analysed for size and concentration by nanoparticle tracking analysis (NTA) and for protein expression by western blot (WB). EV autocrine effect on cell proliferation, migration and invasiveness was then evaluated in vitro. In all samples, particles were in the EV size range (50–1000 nm), with a higher concentration in UC than in SEC samples (10^{11} and 10^{10} particles/ml respectively), and expressed EV markers (Alix, CD9). Functional assays did not show statistically significant difference among conditions, but EV treatment slightly increased cell proliferation and invasiveness and treatment with SEC-isolated EVs slightly enhanced cell migration compared to UC-isolated EVs. In conclusion, the main differences between the two isolation techniques are the quantity of the final EV-product and slight differences on EV functionality, which should be further explored to better highlight the real autocrine effect of tumoral EVs.

KEYWORDS
CIPp, dog, extracellular vesicles, mammary tumour cell line, size exclusion chromatography, ultracentrifugation

1 | INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous family of membrane bound vesicles originating from endosomes or cellular plasmatic membrane.[1] EVs are involved in physiological and pathological processes, being used by cells to communicate and to modify the behaviour of target cells through autocrine or paracrine interactions, exchanging molecules such as proteins, lipids, sugars and nucleic acids.[1–4] EVs have been classified according to their size and biogenesis as small "exosomes" (30–100 nm), originating within endosomes, and larger plasma membrane-derived "ectosomes" (microparticles/microvesicles) (100–1000 nm).[2,3] Since biogenesis is not easily definable, the use of size ranges has been recommended, identifying small (<100 nm exosomes and 100–200 nm microvesicles) and medium-large EVs (>200 nm).[5,6] More recently, larger (1–10 μm) tumour-derived EVs–named oncosomes–and new specific EV subtypes have also been described.[7]

The role of EVs in tumorigenesis, tumour prognosis and therapy has been the focus of many studies in human medicine in the last years.[2,8,9] Tumour-derived EVs can target and transfer their cargo to different cells within and outside the tumour.[3,10] The uptake of tumoral EVs by neoplastic cells has consequences on many tumour-associated pathways, stimulating angiogenesis, regulating immune response and transferring drug resistant phenotypes.[3,8,11] EV cargo can also promote cancer cell migration, invasiveness, and metastasis and EVs isolated from malignant cells and transferred to less malignant cells were demonstrated to increase the migration of the recipients in vitro.[12]

When studying EVs, the isolation procedure represents a critical step. EVs can be isolated through different methods. In the past years, ultracentrifugation (UC) was considered the gold standard for EV purification and concentration, but this technique presents major limitations,[5,13] such as the co-deposition of non-EV components, the formation of EV/protein aggregates and the damage of EVs during centrifugation.[14] For these reasons, the popularity of other isolation techniques has increased, and different methods can be combined to gain higher specificity.[5] An alternative isolation method to UC is size exclusion chromatography (SEC). SEC should reduce the co-precipitation of contaminants, preserve EV integrity and avoid EV-aggregation more efficiently than UC.[10,14]

In veterinary medicine, few studies investigated EVs isolated from different species, mainly focusing on identification, characterization and preliminary cargo description.[15–18] However, tumour-derived EVs have not been widely explored yet in veterinary medicine. Studies on circulating EVs demonstrated the higher concentration of EVs in the blood of tumour-bearing dogs compared to healthy dogs[19,20] and investigated EV-related RNAs to find possible diagnostic and prognostic biomarkers.[18,21,22] In vitro research focused on preliminary isolation and characterization of EVs from tumoral cell culture medium (CCM) and on the analysis of their RNA content to elucidate tumour biological behaviour.[22–25] To our knowledge, no study has explored the role of canine mammary tumour-derived EVs in tumour progression and aggressiveness performing in vitro functional assays.

In this study, we compared the isolation of EVs with UC and SEC from a canine mammary tumour cell line (CIPp) and evaluated EV autocrine effects in vitro on cell proliferation, migration and invasiveness. A comparison between the presence or absence of fetal bovine serum (FBS) in CCM was included and assessed for particle concentration and effects on cell proliferation. Our findings showed that UC allows the isolation of more particles consistent with EVs than SEC; however, SEC-isolated EVs manifested a slightly higher autocrine effect.

2 | MATERIALS AND METHODS

2.1 | Cell line validation statement and culture conditions

Canine primary mammary carcinoma CIPp cell line was established by Uyama et al.[26] and kindly provided by Prof. R. De Maria (University of Turin, Italy). The cell line has been regularly tested and confirmed to be mycoplasma-free. Cells were cultured in Rosewell Park Memorial Institute (RPMI 1640) (Thermo Fisher Scientific) supplemented with 10% FBS (PANTM BIOTECH) and 1% penicillin/streptomycin (Corning).

2.2 | Isolation of EVs with UC and SEC

To isolate EVs, two p150 petri dishes were seeded with 1.6×10^6 cells each. CCM was replaced 24 or 48 h before EV isolation with FBS-free (FBSf) medium or with 5% EV-depleted FBS (EV-dFBS) medium respectively, in a volume of 25 ml for UC and 16 ml for SEC. Growth medium from plates processed in the same way but without cells, unconditioned medium (UCM), was included in all experiments as negative control.

EV-dFBS was prepared by overnight (16 h) ultracentrifugation at 100 000 g at 4°C. The pellet was discarded and the supernatant (EV-dFBS) was sterile filtered using a 0.2 μm filter (Sartorius Stedim Biotech).

EVs were isolated by UC and SEC (50 ml and 32 ml of medium respectively) from two plates with 90% confluent cells. The medium was first centrifuged at 300 g for 10 min at 4°C to remove any cell/cell debris. The supernatant was then centrifuged at 2000 g for 10 min at 4°C to remove additional debris. For EV isolation through UC (herein UC EV) the supernatant was transferred to a clean ultracentrifuge tube (Beckman Coulter) and ultracentrifuged at 100 000 g for 90 min at 4°C. The supernatant was discarded and the EV-enriched pellet resuspended in 100 μl of double filtered (0.2 μm) PBS (dfPBS). For EV isolation with SEC (herein SEC EV), the supernatant was transferred into a 100 kDa ultrafiltration tube (Merck Millipore) and centrifuged at 5000 g for 30 min at 4°C. All the material that did not pass through the filter was loaded onto qEVoriginal columns (Izon Science) and SEC was performed according to manufacturer's instructions. Yielded fractions #7, #8, and #9 were pooled and centrifuged with a 100 kDa ultrafiltration tube (Merck Millipore) at 4000 g for 20 min at 4°C. The remaining material that did not passed through the filter was collected and resuspended in 100 μl of dfPBS.

All functional studies were performed in biological triplicates. For western blot (WB) and nanoparticle tracking analysis (NTA), also SEC EV fractions from #10 to #18 were collected. UC and SEC were similarly performed on UCM, so that the herein called UC UCM and SEC UCM (pooled SEC UCM fractions #7, #8, and #9) were used as negative control in each experiment.

2.3 | Nanoparticle tracking analysis

After EV purification, samples of UC EV, UC UCM, SEC EV and SEC UCM from fFBS and 5% EV-dFBS media were quantified and evaluated for concentration and size distribution using NanoSight NS300 (Malvem). For SEC EV fractions from FBSf medium, fractions from #7 to #18 were pooled and analysed. Resuspended (100 μl dfPBS) samples were kept on ice for 1 h and then progressively diluted in dfPBS until reliable measurements were obtained by NTA. To assess background particles in the original media, unprocessed samples (herein unprocessed media) of FBSf RPMI and 5% EV-dFBS RPMI without

cells were also measured by NTA. Three movies of 60 s each were recorded for each sample and analysed using the 3.4 NTA software with camera level set at 12. For particle quantification, reliable values were those within instrument optimal working ranges: particles per frame from 20 to 120; particles concentration between 10^6 and 10^9 per ml; total particles to valid particles ratio higher or equal to 1/5. In addition, for size measurements we also reported D90, D50 and D10, which represent the size point below which 90%, 50% and 10% of the particles, respectively, is included.

2.4 | Protein extraction and western blotting analysis

Cell proteins were extracted from 90% confluent cells on a 15-cm plate using 2 ml of RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitor according to manufacturer's protocol. Proteins from UC EV and SEC EV from FBSf media and from UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media were extracted resuspending them in 60 or 20 μl of RIPA buffer supplemented with protease inhibitor after UC and SEC, respectively.

Cells and EV-derived protein concentrations were calculated using Pierce BCA protein Assay Kit (Thermo Fisher Scientific), according to manufacturer's protocol.

For WB, 20 μg of proteins from cells/EVs were used for samples, which were in BCA assay quantification range. Instead, 21 μl were used for samples with protein concentration below detection range. Samples were first denaturated at 70°C for 10 min or at 95°C for 5 min, then were resolved using NuPAGE 4%–12% Bis-Tris gel (Thermo Fisher Scientific) and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked for 90 min in 5% non-fat dry milk in TBS-T (TBS containing 0.05% Tween-20) at room temperature. Blots were then incubated at 4°C overnight with rabbit or mouse primary antibodies against human Alix (1:200; Santa Cruz sc-5358), CD9 (1:200; Bio-Rad MCA694GT) and Calnexin (1:1000; Cell Signalling #2679). Then, membranes were incubated with a peroxidase-conjugate secondary antibody (1:3000; anti-Rabbit #32260 or anti-Mouse #32230, Thermo Fisher Scientific) for 1 h at room temperature. All antibodies were diluted in TBS-T containing 1% non-fat dry milk. Reactive bands were visualized using a chemoluminescent detection kit (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific) with the iBright instrument (Thermo Fisher Scientific).

2.5 | Cell proliferation assay

A 5000 cells per well were seeded in 100 μl of FBSf medium in a 96 well plate. Within 1 h 10 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media) were added to each well. After 24 or 48 h, 20 μl of CellTiter 96® Aqueous One Solution cell proliferation assay (MTS, Promega) was added to each well and after 1 h of incubation at 37°C, absorbance was measured with a spectrophotometer (Packard Instrument, Meriden) at 490 nm.

2.6 | Cell migration assays

Cell migration was studied using wound healing assays and transwell migration assays. For the wound healing assay, 2×10^5 cells per well were seeded on a 6-well plate in 10% FBS medium. When cells reached confluency, cells were washed with PBS and the medium replaced with FBSf medium and 100 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. After overnight incubation (14 h), the cell monolayer in each well was scratched twice vertically using a 1 ml sterile pipette tip with a distance of approx. 1 cm between the two scratches. Cells were photographed at 10x using an inverted microscope (Olympus IX50) after 0, 4 and 8 h in three fixed points per scratch (6 fixed point per well). The width of the gap was calculated in each fixed point using ImageJ (https://imagej.nih.gov/ij/), considering 10 measurements per fixed point. To measure migration over time, the mean of measurements after 4 and 8 h were subtracted to measurements at time 0.

In the transwell migration assay, 1.5×10^4 cells were seeded in 100 μl of FBSf medium on a 8-μm pore membrane insert of a transwell 24-well plate (Corning) and 15 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. In the lower chamber 400 μl of RPMI with 10% FBS were added. After 6 h, the migrated cells on the lower surface of the transwell membrane were stained with 0.5% crystal violet and visualized using a digital microscope (DMD108, Leica). Pictures were taken at 5× on 5 different fields per condition and ImageJ was used to count the migrated cells.

2.7 | Transwell invasion assay

Cell invasion ability after EV treatment was detected using a 8-μm pore membrane transwell 24-well plate (Corning). Each transwell membrane was pre-coated with 50 μl of 1% Matrigel (BD Biosciences) diluted in FBSf medium and dried at 37°C overnight. A 1.8×10^4 cells were seeded in the upper chamber of each well with 100 μl of FBSf medium and 18 μl of dfPBS resuspended samples (UC EV, SEC EV, UC UCM or SEC UCM from FBSf medium) were added to each well. In the lower chamber, 600 μl of RPMI with 10% FBS were added. After 24 h, membranes were stained with 0.5% crystal violet and cells on the lower surface were visualized using a digital microscope (DMD108, Leica). Pictures were taken at 5× on 5 different fields per condition and ImageJ was used to count the migrated cells.

2.8 | Statistical analysis

Statistical analyses were performed with GraphPad Prism 8 software. Differences between two groups were tested with the two-tailed unpaired Student's t-test when data were normally distributed or the Mann–Whitney test when data were not normally distributed. Differences between more than two groups were tested with ANOVA

TABLE 1 Particle size recorded at nanoparticle tracking analysis

Sample type			Size mean (nm) ± *SD*	Size mode (nm) ± *SD*	D90 (nm) ± *SD*	D50 (nm) ± *SD*	D10 (nm) ± *SD*
Unprocessed medium		FBSf	Unreliable	–	–	–	–
		EV-dFBS	129.7 ± 4.9	97.9 ± 7.9	198.8 ± 9.6	115.4 ± 4.7	83.8 ± 1.3
UCM	UC	FBSf	Unreliable	–	–	–	–
		EV-dFBS	142.1 ± 4.5	118.1 ± 8.1	222.1 ± 6.0	121.8 ± 0.6	87.6 ± 2.7
	SEC 7/8/9	FBSf	Unreliable	–	–	–	–
		EV-dFBS	141.5 ± 4.6	105.1 ± 2.6	211.1 ± 8.5	126.9 ± 4.8	88.5 ± 2.9
EV	UC	FBSf	180.0 ± 2.0	138.2 ± 2.3	283.4 ± 11.8	154.9 ± 3.2	109.0 ± 1.5
		EV-dFBS	188.2 ± 4.8	141.7 ± 7.5	295.2 ± 10.9	162.4 ± 6.3	119.1 ± 3.3
	SEC 7/8/9	FBSf	139.1 ± 1.0	101.2 ± 2.5	201.4 ± 4.7	127.7 ± 4.4	97.1 ± 1.7
		EV-dFBS	169.1 ± 1.6	117.6 ± 4.7	254.1 ± 10.5	150.3 ± 1.5	109.9 ± 3.0
	SEC 10/11/12	FBSf	140.0 ± 3.6	99.0 ± 3.3	207.9 ± 17.5	121.6 ± 1.3	90.5 ± 1.7
	SEC 13/14/15	FBSf	Unreliable	–	–	–	–
	SEC 16/17/18	FBSf	Unreliable	–	–	–	–

Note: D90, D50, D10 represent the size point below which 90%, 50% and 10% of the particles is included.
Abbreviations: EV, extracellular vesicles; EV-dFBS, 5% EV-depleted FBS medium; FBSf, FBS-free medium; SD, standard deviation; SEC #/#/#, fractions of size exclusion chromatography; UC, ultracentrifugation; UCM, unconditioned medium.

when data were normally distributed and Kruskal-Wallis when data were not normally distributed. Level of significance was set at $p < .05$.

3 | RESULTS

3.1 | EV size and concentration

After EV purification with SEC and UC, concentration and size of isolated particles were measured by NTA. Results are shown in Table 1 and Figure 1.

Measurements were not reliable in UCM samples from FBSf medium and SEC EV fractions 13/14/15 and 16/17/18 because of the low particle concentration (Table 1). The size distribution of particles from all other conditions showed instead size ranges within the size range considered for EVs (mode and mean of measurements ranging from 94 to 187 nm) (see Table 1). D90, D50 and D10 of the evaluated samples ranged from 195–289 nm, 115–164 and 84–116 nm, respectively, indicating the purification of mainly small EVs (Table 1).

In UC UCM and in SEC UCM fractions 7/8/9 from 5% EV-dFBS a concentration of 10^{10} and of 10^{9} particles/ml respectively, was observed (Figure 1).

Nanoparticle tracking analysis (NTA) showed similar concentration of particles in UC EV from FBSf medium and from 5% EV-dFBS medium ($7.7 \times 10^{11} \pm 1.8 \times 10^{10}$ and $4.2 \times 10^{11} \pm 6.2 \times 10^{10}$ particles/ml respectively). Particle concentration of UC EV samples from both medium was higher of one order of magnitude than particle concentration in SEC EV fractions 7/8/9 from 5% EV-dFBS or FBSf medium which resulted similar ($7.9 \times 10^{10} \pm 4.1 \times 10^{9}$ and $6.2 \times 10^{10} \pm 3.2 \times 10^{9}$ respectively) (Figure 1). These two latter samples had more particles than SEC EV fractions 10/11/12 from FBSf ($9.1 \times 10^{9} \pm 5.1 \times 10^{8}$ particles/ml) (Figure 1).

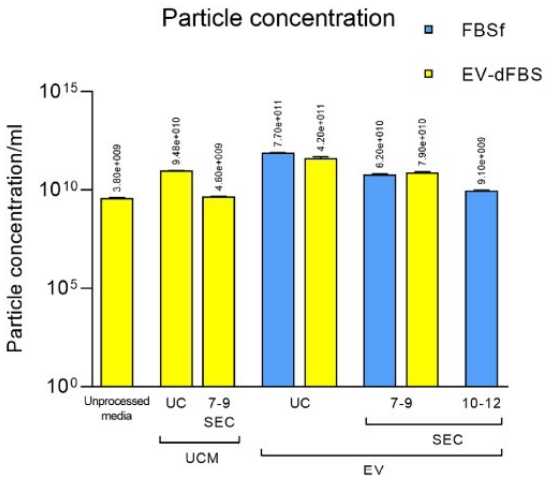

FIGURE 1 Particle concentration/ml recorded at nanoparticle tracking analysis (NTA); UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; #-# SEC, fractions of size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium. Samples with unreliable measurements are not included.

3.2 | EV characterization

WB was performed to characterize EVs isolated by UC and SEC.

Alix, a cytosolic marker of EVs was detected in UC EV and in SEC EV fractions 7/8/9, where the majority of EVs should elute according to manufacturer. Alix was also detected in SEC EV fractions 10/11/12, suggesting EV presence in later fractions (Figure 2). One of

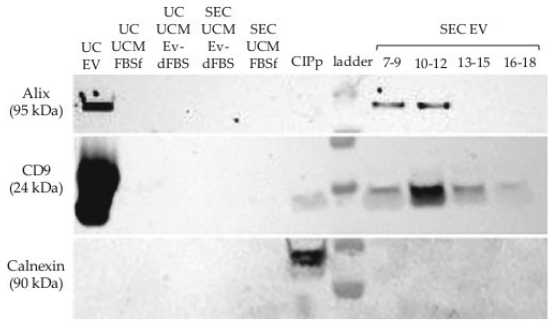

FIGURE 2 Western Blot analysis for EV markers on cells and on EV/UCM samples. UC EV, extracellular vesicles purified with ultracentrifugation; UC UCM, unconditioned medium from ultracentrifugation; SEC UCM, unconditioned medium from size exclusion chromatography; SEC EV #-#, pooled fractions of extracellular vesicles purified with size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium.

the most widely accepted tetraspanin marker for EVs, CD9, was detected in UC EV and in SEC EV fractions from 7 to 18, also suggesting EV presence in late SEC fractions (Figure 2). Calnexin, a marker of the endoplasmic reticulum commonly used as negative control for EVs and positive control for cells, was not detected in EVs but as expected, was detected in cells (Figure 2).

In negative controls (UC UCM and SEC UCM from both FBSf and 5% EV-dFBS media) no EV markers were detected.

3.3 | EV effect on proliferation, migration and invasion

To evaluate EV function, we performed cell proliferation, migration and invasion assays.

First, we evaluated the proliferation of CIPp treated for 24 or 48 h with SEC EV, UC EV, SEC UCM and UC UCM from both FBSf medium and 5% EV-dFBS medium. We did not evidence any

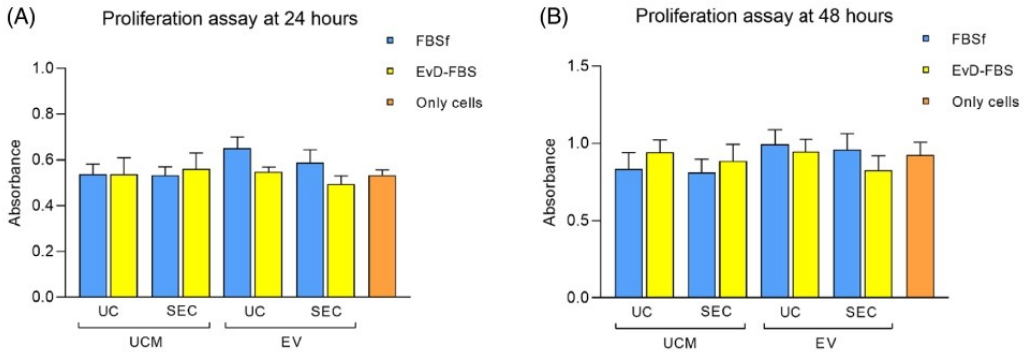

FIGURE 3 (**A**) Proliferation assay performed with MTS assay showing cells absorbance level at 24 h after EV treatment and (**B**) at 48 h after EV treatment. On the ordinate axis, absorbance value is proportional to the number of living cells. UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography; FBSf, FBS-free medium; EV-dFBS, 5% EV-depleted FBS medium. The error bar represents the SE of the mean.

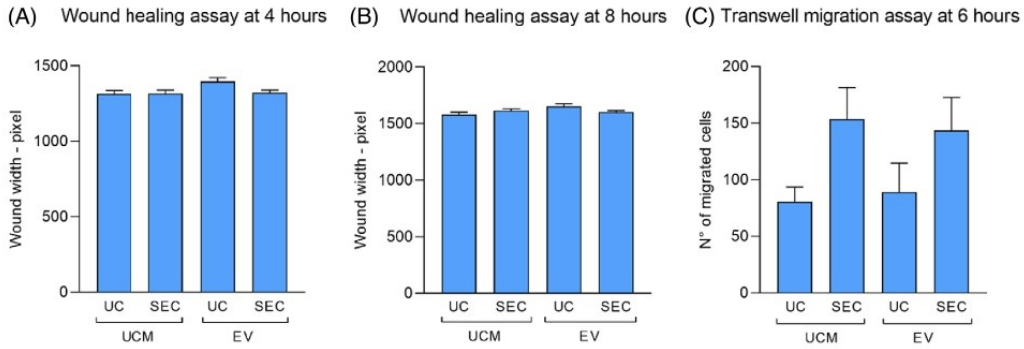

FIGURE 4 (**A**) Wound healing assay showing the reduction of wound width in pixels after 4 h from EV treatment; (**B**) and after 8 h from EV treatment; (**C**) Transwell migration assay showing the number of migrated cells after 6 h from EV treatment. UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography. The error bar represents the SE of the mean.

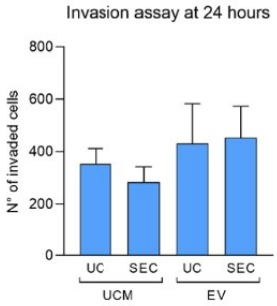

FIGURE 5 Transwell invasion assay results showing the number of invaded cells after 24 h from EV treatment; UCM, unconditioned medium; EV, extracellular vesicles; UC, ultracentrifugation; SEC, fractions 7/8/9 of size exclusion chromatography. The error bar represents the SE of the mean.

significant difference in cell proliferation among conditions after 24 and 48 h, except for a slight increase of cell proliferation of CIPp treated with UC EV and with SEC EV compared to their corresponding controls (UC UCM and SEC UCM, respectively) (Figure 3A,B). This increase was only evident when treating with EVs from FBSf medium and not from 5% EV-dFBS medium.

Secondly, to evaluate the ability of EVs to influence cell migration, we performed wound healing and transwell migration assays considering only FBSf medium samples. In the wound healing assay, cell migration was similar among conditions, both 4 and 8 h after scratch (Figure 4A,B). In the transwell migration assay, 6 h after EV treatment, we evidenced an increase in cell migration of cells treated with SEC EV compared to those treated with UC EV (Figure 4C) but with no statistical significance. However, EV treatments did not differ significantly from their relative controls (SEC UCM and UC UCM respectively).

Finally, to evaluate the effect of EVs on cell invasiveness, we performed a transwell invasion assay considering only FBSf medium samples. We did not find any statistically significant difference in cell invasiveness 24 h after treatment (Figure 5). However, cells treated with SEC EV and UC EV migrated more compared to their corresponding controls (SEC UCM and UC UCM).

4 | DISCUSSION

The aim of this study was to compare two EV isolation techniques, SEC and UC, on a canine mammary tumour cell line to evaluate the autocrine effect of EVs on cell proliferation, migration and invasiveness, including an evaluation of the presence or absence of FBS within the CCM.

We were able to isolate EVs from CIPp cell line using both UC and SEC.

Considering the technical aspects, the main difference between the two EV purification methods was the quantity of the isolated particles measured by NTA, that was higher with UC compared to SEC. It is now recognized that UC allows to collect a greater quantity of EVs compared to SEC, although EVs tend to aggregates and to co-precipitate with extra-EV proteins.[5,14] The presence of co-precipitated contaminants and aggregates can influence NTA measurements, with the impossibility to precisely discern EVs from non-EV particles or EV complexes.[5] Therefore, NTA concentration measurements can be influenced by particles present in FBS and in culture media before cell growth.[27]

In our study, while in unprocessed media and in UCM from FBSf medium, particle concentration was low (unreliable), in unprocessed media and UCM from 5% EV-dFBS medium, particles were within detection range (10^8/ml in unprocessed media, 10^9/ml in SEC UCM and 10^{10}/ml in UC UCM), meaning that the addition of 5% EV-dFBS increased particle concentration. However, since no EV marker was detected in UCM samples at WB, these particles could be FBS-derived EVs with a quantity of proteins below WB detection threshold, proteins/protein aggregates or other media/FBS components (e.g., amino acids, vitamins, salts, minerals), detected as particles at NTA. Additionally, FBS-related particles were no longer detectable after cell growth, since NTA revealed the same order of magnitude of particle concentration in SEC and UC EV from FBSf or 5% EV-dFBS medium. This may indicate that FBS-related particles were consumed by cells, being media components or that in EV samples from 5% EV-dFBS medium, EV concentration was lower compared to EV samples from FBSf medium, where there were no FBS-derived particles. A previous study performed on N2a neuroblastoma cells, showed trough NTA that cells cultured in serum free conditions shed more EVs than cells cultured with EV-depleted serum.[28] In our study, the absence of difference in particle concentration between EV from FBSf and 5% EV-dFBS medium may also be due to different cellular behaviour (N2a vs. CIPp), different culture conditions or by the impossibility to distinguish EV from non-EV particles.[5,29] Anyhow, based on this data and as already mentioned in other studies,[5,27] removal of FBS from CCM is strongly suggested to avoid any biases when working with CCM-derived EVs.

According to WB results, we found EVs distributed in SEC fractions from 7 to 18. The SEC columns producer company indicates SEC fractions #7/8/9 as those where the majority of EVs elute with the least contaminant proteins. EVs elute also in later fractions but with more contaminant proteins. Even if mainly performed on plasma, other studies have evidenced the presence of EVs through WB and NTA in SEC fractions beyond those suggested to collect when isolating EVs by manufacturers.[30–32]

Considering the functional studies, we did not detect any statistically significant biological effect of EVs nor any remarkable functional difference between UC EV and SEC EV. A slight increase in proliferation of cells treated with UC or SEC EV compared to controls, in cell migration when treating with SEC EV compared to UC EV, and in cell invasiveness when treating with SEC EV compared to controls were found.

The poor measured effect of EVs may be related or to technical aspects, or to the lack of a real biological autocrine effect of the studied EVs.

Considering the technical aspects, UC EV might be damaged and less functional,[14,33] and this might have contributed to the slightly higher effect of SEC EV, despite the lower concentration, in the transwell migration assay, as already demonstrated in other studies.[34–36] UC EV might also be associated with non-EV proteins,[5,14] which could influence cell growth. In a study performed on EVs isolated from starved Mesenchymal Stromal Cells, the authors found a deleterious effect of UC EVs on treated cells, which, after a comparison with EVs and proteins isolated with SEC, was referred to the presence of UC co-isolated particles and not to EVs themselves.[37]

Co-isolated particles might have also influenced the proliferation assays, where we evidenced a slight increase in cell proliferation only when treating cells with EVs isolated from FBSf medium compared to controls, but not when treating cells with EVs from 5% EV-dFBS. This latter lack of effect may be related to FBS co-isolated proteins, which could have interfered with EVs, or to starving which might have changed EV composition or quantity.[5,28,29]

Another technical issue in EV functional studies is the EV/recipient cells treatment ratio, for which standardization and overlapping with in vivo situation is arduous.[38] In our assays, EV concentration might have been too low or different from physiological concentration. In functional studies, EVs have been demonstrated to have dose-dependent effects on cell proliferation and migration, especially in regenerative medicine.[39–41] Hence, in further studies, EV dose could be changed to define dose-dependent effects in vitro and enhance broader qualitative differences. In stem cell research, EV effect on cell proliferation has been reported to be also time dependent, with incubation being usually longer than 24 h.[40,42] Unfortunately, in our study longer incubation was not possible because of the fast doubling time of CIPp cell line (24.6 h).[26]

Generally, considering the investigated biological effects of EVs, the literature suggests an enhancing effect of tumour EVs on cell proliferation, migration and invasiveness.[4] However, most studies deal with heterologous EVs, treating recipient cells with EVs isolated from different donor cells.[43–45] Studies on the functional effects of autologous EVs are fewer and some of them report a lack of clear effects. Menck and co-authors reported that the administration of autologous microvesicles to breast cancer cell lines enhanced cell invasion but had no effects on cell proliferation.[46] Moreover, in the same study, microvesicles enhanced cell invasiveness more than exosomes, suggesting the different properties of different EV subpopulations.[46] In our study, using UC at 100 000 × g, we mainly isolated small EVs, apparently mainly microvesicles (100–200 nm size range), and fewer large vesicles (>200 nm).[14] In another study, exosomes isolated from glioma associated-human mesenchymal stem cells, enhanced glioma stem-like cells (GSC) proliferation, but the administration of autologous exosomes to GSC had no effect on cell proliferation.[44] An additional work highlighted the ability of tumoral EVs to enhance cell migration in recipient cells according to the metastatic potential of the donor cells.[47] The authors compared two human breast cancer cell lines, the metastatic/invasive MDA-MB-231 and the non-metastatic/non-invasive MCF7, treated with autologous or heterologous EVs isolated from the same cell lines. EVs isolated from MDA-MB-231 cells had greater autocrine and paracrine migratory potential compared to MCF7-derived EVs.[47] In our study, the lack of significant pro-proliferative, migratory and invasive effects of CIPp-derived EVs may also be related to a low metastatic/invasive profile of the donor cells.[26]

To conclude, we purified EVs from a canine mammary tumour cell line with two different techniques: UC and SEC. UC allowed the isolation of a greater number of particles compared to SEC but SEC EV apparently showed a slightly higher effect on proliferation, migration and invasion. Further analyses are needed to better elucidate the role of EVs and their autocrine effect, taking into consideration their quantity, heterogeneity, purity, integrity and the cells of origin.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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6.3 Extracellular vesicles as cancer biomarkers

One additional application of EVs in cancer is their possible use as cancer biomarkers in liquid biopsy. Ideally, the liquid biopsy is a low invasive and cost-effective method to early diagnose cancer and monitor its progression or recurrence, detecting representative molecules in the circulation without performing tissue biopsies (Zhou et al., 2021;Yu et al., 2021). Liquid biopsy can target circulating tumor DNA, circulating tumor cells or circulating EVs (identifying tumor-derived EVs is still challenging!) and their cargo with a particular emphasis on EV-associated miRNAs (77). Despite we are still far from using EVs as biomarkers in the clinical routine, about 50% of the clinical trials in human medicine focuses on using EVs as biomarkers for different diseases and, more than the 70% of these trials apply EVs as cancer biomarkers (65).

Despite improvements in liquid biopsy research would allow to find early, non-invasive and non-expansive biomarkers, until now results are very scarce in veterinary medicine and only few studies have investigated the use of EV-associated miRNAs as possible canine cancer biomarkers (76,79,80).

Particularly, among canine tumors, lymphoma is still a significant challenge for veterinary oncologists. Lymphoma is one of the most common tumors in dogs and is mainly categorized in B-cell lymphoma, T-cell lymphoma or non-B/non-T cell lymphoma. The T-cell phenotype is less common the B-type but more rapid, more aggressive and can more often develop drug resistance (81).

Therefore, in collaboration with the ANICURA Veterinary Institute of Novara (Granozzo con Monticello (NO), Italy) and Dr. Chiara Leo, in my project I also investigated the expression of EV-associated and free circulating miRNAs in dogs with T-cell lymphoma compared to healthy dogs. The EV-associated miRNAs that we found as dysregulated in our study could be used for further studies on larger samples of patients to better investigate their application as diagnostic, prognostic or predictive biomarkers in canine T-cell lymphoma. The following paper summarizing the results has been recently submitted to PLOS ONE.

6.4 Paper n.5: "Free circulating versus extracellular vesicle-associated microRNA expression in canine T-cell lymphoma"

Canine T-cell lymphoma: free circulating versus extracellular vesicle-associated microRNA expression

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Abstract

Canine lymphoma (cL) is one of the most frequent cancers in dogs. The T-cell lymphoma (TcL) is not the most common phenotype but presents an aggressive behavior. MicroRNAs (miRNAs), are small, single-stranded, non-coding RNA molecules which can circulate freely in blood or be associated with extracellular vesicles (EVs). The dysregulation of certain miRNAs has been identified in numerous types of human cancers and they have been largely investigated as possible tumors biomarkers in human medicine, while research in veterinary oncology is still scarce. The aim of this study was to compare the expression patterns of free circulating and EV-associated miRNAs in dogs with T-cell lymhoma (TcL) and healthy dogs. Eight dogs with TcL were selected as the lymphoma group (LG) and eight dogs were included as controls (Ctrl). Plasma samples were collected at the time of the diagnosis and EVs isolated with ultracentrifugation. miRNAs were extracted from both the circulating EVs and the plasma supernatant, obtaining EV-associated and free-miRNAs. Quantitative real-time PCR was performed to analyze the expression of 88 target miRNAs. Nine and seven differentially expressed miRNAs between LG and Ctrl were detected in EV-associated and free-miRNAs, respectively. Among EV-associated and free-miRNAs, only has-miR-222-3p was overexpressed in both conditions. All the differentially expressed miRNAs detected in this study, have been already described as dysregulated in other human or canine cancers. The EV-associated miRNAs, which appear to be more stable and better conserved than free-miRNAs, could be investigated in further larger studies to better assess their use as possible biomarkers for TcL.

Introduction

Canine lymphoma (cL) is among the most frequently diagnosed cancers in dogs and represents the most managed neoplasia in veterinary oncology [1]. Most of the cases are B-cell lymphoma (70%), while the others can be T-cell lymphoma (TcL) or non-B/non-T cell lymphoma [1]. Generally, cL is routinely diagnosed by cytologic examination of affected tissues but other diagnostic investigations may be pursued for

immunophenotyping, grading and clinical staging, mainly to predict the biological behavior [2]. Multi-agent chemotherapy represents a gold standard treatment for cL and remission is often achieved, despite TcL can develop more commonly drug resistance [2]. MicroRNAs (miRNAs) are small non-coding RNA molecules of approximately 22 nucleotides in length that participate in post-transcriptional gene regulation. Having this role, they are involved in the regulation of different biological processes, including carcinogenesis being either oncogenes or tumor suppressors [3]. Many studies have demonstrated that the expression of many oncogenic miRNAs is dysregulated in tumors, enhancing proliferative signaling, evasion from growth suppressors, cell death resistance, invasiveness, and angiogenesis [3]. Many mechanisms are involved in this dysregulation and include amplification or deletion of miRNA genes, abnormal transcriptional control of miRNAs, dysregulated epigenetic changes, and defects in the miRNA biogenesis machinery [3]. Given that miRNAs exist not only in cells and tissues but also across various body fluids, coupled with their significant stability, they offer a vast resource of minimally invasive biomarkers [2]. Moreover, miRNAs can circulate in the blood freely or associated with extracellular vesicles (EVs). Extracellular vesicles (EVs) are heterogenous membrane-bound vesicles released by cells [4]. EVs, which transport molecules like proteins, sugars, lipids, and nucleic acids, play a crucial role in cell-to-cell communication [5]. EVs are mainly divided into exosomes and microvesicles, according to their biogenesis [5, 6]. However, other cell-derived particles can be categorized as EVs, including for example apoptotic bodies, prostasomes, and oncosomes. Considering the heterogeneity of EVs and the difficulties in recognizing their origin, they are usually also classified according to their size in small-EVs (< 200 nm) or large-EVs (> 200 nm) [5, 6]. Among many other functions, EVs play a role in promoting the carcinogenic process, inducing angiogenesis, tumor dissemination, immune escape, metastasis, and drug resistance [5, 7, 8]. EVs are present in all body fluids (e. g. blood, saliva, urine, bronchoalveolar fluid, breast milk, semen) and being stable for long time and are easy to sample, being suitable for sequential collection [8]. EV-associated RNAs have been reported as possible tumor biomarkers for cancer diagnosis or for monitoring cancer progression in many studies in human medicine [8]. However, even though there have been some studies in veterinary medicine, knowledge remains limited regarding the expression of both free circulating and EV-associated miRNAs in dogs with lymphoma [2, 9, 10]. Considering the need of non-invasive biomarkers in veterinary oncology and the paucity of information regarding miRNA expression in canine T-cell lymphoma (TcL), the aim of this study was to identify expression patterns of specific circulating miRNAs in dogs with TcL, investigating both free circulating miRNAs and EV-associated miRNAs as possible diagnostic biomarkers.

Materials and methods

Case recruitment

This was a prospective double-arms study. Eight dogs were included as controls (Ctrl) and eight dogs diagnosed with TcL were selected according to eligible criteria for the lymphoma group (LG). All LG and some Ctrl samples were provided by Anicura Istituto Veterinario Novara (Novara, NO, Italy). The remaining Ctrl samples were provided by the Veterinary teaching hospital of the University of Padua (Legnaro, PD, Italy). All lymphoma patients were treated at the AniCura Istituto Veterinario Novara with chemotherapy protocols that required systematic hematology controls. Blood samples were obtained at the time of diagnosis (T0), during the clinical diagnostic routine procedures and leftovers were used for the study. Blood samples from Ctrl were obtained during elective surgery for sterilization and leftovers were kept for the study.

Approval by an ethics committee was therefore not required for this study. Inclusion criteria for the Ctrl included: no clinical, hematology or biochemistry abnormalities. Inclusion criteria for LG included a cytological or histological diagnosis of lymphoma, no previous treatments with chemotherapy or steroids, and absence of other comorbidities. The presence or suspicion of leukemic lymphoma was considered exclusion criteria. Informed consent was obtained from all owners. The diagnosis, grading, and staging of lymphoma were performed according to the Kiel updated classification (Lennert K, Feller CA: Histologie des Lymphomes Malins Non Hodgkiniens Selon la Classification de Kiel Actualise´. Doin, Paris, France, 1991). For each case, clinical examination, complete blood count (CBC) and biochemistry profile, chest radiographs, abdominal ultrasound (US) and cytology of lymph nodes, liver (with one exception) and spleen were performed according to standard diagnostic procedures. To confirm the T-cell origin of lymphomas, the immunophenotyping was assessed by flow cytometry and the grading was determined by the size of cells (majority of small-sized cells or majority of medium- and large sized cells) and mitotic index [11]. The cL phenotypes were determined by flow cytometry (CyFlow Space, Sysmex Europe GmBH, Norderstedt, Germany) and the data analyzed with the FlowMax software (Sysmex Europe GmBH, Norderstedt, Germany). For flow cytometry, lymph node samples were collected in Eppendorff containing 500 microliters of autologous serum and analyzed within 24 hours from collection. Erythrocyte lysis was performed using erythrocyte lysis buffer containing 8% ammonium chloride if deemed necessary due to hemodilution. The percentages of dead and live cells were assessed using propidium iodide (PI) to evaluate samples' preservation before the staining with antibodies. A panel of antibodies that included CD45 and CD44 (pan-leukocyte), CD3 and CD5 (T lymphocytes), CD4 (T-helper lymphocytes), CD8 (T-cytotoxic lymphocytes), CD21 (B lymphocytes), CD34 (blast cells) was used. For each sample, 50 µL of cell suspension were added to the tubes containing different combinations of antibodiesS1 Table.

Isolation and characterization of extracellular vesicles from plasma and microRNA extraction

Four ml of venous blood were collected by each dog and transferred to an EDTA tube. Upon collection, each sample was centrifugated at 4°C at 2000 x g for 15 min to obtain the plasma fraction. The plasma was stored at -80°C until isolation of EVs. EVs were isolated from plasma of Ctrl and LG dogs with ultracentrifugation (UC). Each sample was first centrifuged at 4°C (2000 x g for 10 min) to remove large debris. The supernatant was then transferred to a new tube and centrifuged twice at 3850 x g. The final supernatant (about 1 ml) was diluted in phosphate-buffered saline (PBS) 0,2 µm double- filtered (dfPBS) to reach a final volume of 4 ml, transferred to an ultracentrifuge tube (Ultra-Clear Open top, Beckman Coulter, Brea, CA, US) and ultracentrifuged in a swinging bucket rotor (SW55ti, Beckman Coulter, Brea, CA, US) at 100.000 x g for 90 min at 4°C (Beckam Coulter Optima L-90K, Beckman Coulter, Brea, CA, US). After UC, the obtained pellets containing EVs (EV-pellet) were lysed and nucleic acids extracted using miRNAeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions to finally obtain EV-associated miRNAs. To extract free circulating miRNAs (free-miRNAs), miRNaeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions on UC-supernatant. Because of the low volume of plasma samples, two more Ctrl samples, not included in the following miRNA analysis, were used as techinical controls to assess EV-presence after UC. After performing EV-isolation as described above, one of these two samples was used to perform Nanoparticle Tracking Analysis (NTA) and one for Western Blotting (WB).

Nanoparticle tracking analysis

NTA was performed to assess the size distribution and concentration of EVs. After UC, the EV-pellet was resuspended in 600 µl of dfPBS and then diluted 1:100 in the same buffer. NTA was performed with NanoSight NS300 (Malvern Panalytical, Malvern, United Kingdom) and 3 videos of 60 seconds each were recorded and analyzed using software 3.4, with camera level set at 14 and detection threshold at 5. Values considered reliable were those included in the following ranges: particles per frame 20 to 120; particles concentration: 10^6 to 10^9 /ml; total particle to valid particles ratio $\geq$1/5.

Western blotting

WB was performed to verify the presence of EV-markers in the EV-enriched pellet. After UC, proteins from EV-enriched pellet were extracted using 20 µl of RIPA buffer (Thermo Fisher Scientific, Waltham, MA, US) supplemented with proteinase inhibitor. First, protein concentration was quantified by Pierce BCA protein Assay Kit buffer (Thermo Fisher Scientific, Waltham, MA, US), according to manufacturer's instructions. 20 µg of proteins were denatured for 10 min at 70°C, resolved by electrophoresis gel using NuPAGE 4%-12% Bis Tris gel (Thermo Fisher Scientific, Waltham, MA, US) and transferred on to a nitrocellulose membrane. Non-specific binding sites were blocked by a 90 min incubation at room temperature with a TBS-T solution (Tris-buffered saline with 0,05% of Tween-20) supplemented with 5% of skimmed milk powder. Blots were incubated overnight in TBS-T supplemented with 1% of skimmed milk with primary anti-human antibodies against TSG101 (cytosolic protein, dilution 1:1000, GTX70255, GeneTex, Irvine, CA, US) and integrin-beta (a membrane protein, dilution 1:5000, GTX128839, GeneTex, Irvine, CA, US) at 4°C. After overnight incubation, membranes were incubated at room temperature for 1 h with a peroxidase-conjugated secondary antibody (dilution 1:3000, anti-Rabbit #32260 or anti-Mouse #32230, Thermo Fisher Scientific, Waltham, MA, US). Finally, bands resulting from antigen-antibody binding were visualized using a chemiluminescent detection kit (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, US) with the iBright instrument (Thermo Fisher Scientific, Waltham, MA, US).

Reverse transcription and quantitative real-time PCR

The extracted RNA from each sample (8 LG subjects and 8 ctrl subjects) was reverse transcribed to complementary DNA (cDNA), using the miRCURY LNA RT Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To each RNA, 0.5 µl of spike-in UniSp6 RNA was added as control for monitoring successful reverse transcription and to be used as inter-plate calibrator on qPCR. The obtained solution was incubated at 42°C for 60 min and then at 95°C for 5 min. Finally, qPCR was performed immediately, or cDNA samples were stocked at -20°C for up to 5 weeks. Quantitative RT-PCR was performed for each sample using a 96-well plate miRCURY LNA miRNA Focus PCR Panel (Qiagen, YAFD-201Z, Qiagen, Hilden, Germany) and miRCURY LNA SYBR® Green PCR Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. These plates are designed to target 84 different miRNAs and 4 reference genes (U6 snRNA, 5S rRNA, RNU5G, RNU1A1) while the remaining wells are for positive controls and interplate calibrators (i.e IPC UniSp3) S2 Table. The reaction mix was prepared by adding 495 µl of Nuclease-free water, 510 µl of 2x miRCURY SYBR® Green PCR Master Mix, 5.1 µl of ROX and 10 µl of cDNA. For each target/assay the qPCR reaction was carried out in a final volume of 10 µl. The amplification protocol consisted of an initial step of 2 min at 95°C, followed by 40 cycles of 10 s at 95°C and 60 s at 56°C. All experiments were carried out in a Thermo ABI

7500 (Applied Biosystems, Waltham, MA, US).

Statistical analysis

The obtained cycle threshold values (Ct) were analyzed using the GeneGlobe miRNA PCR Array Data Analysis (www.geneglobe.qiagen.com/analyze). To verify the efficiency of reverse transcription and qPCR reactions, the spike ins cel-miR-39-3p and Unisp6 were used as internal amplification controls. A first control based on Ct value was applied on both target and reference miRNAs before normalization. Ct values above 36 were assigned the undetermined status, as the transcript is considered "undetectable". miRNAs with Ct between 33 and 36 are considered "detectable but not quantifiable," and miRNAs with Ct below 33 are considered "quantifiable" and thus usable for subsequent statistical analysis. A first inter-plate normalization was performed based on the IPC UniSp3. The software calculated the calibrator factor (CF), obtained from the difference between the Ct of the IPC of each sample (IPC plate) and the mean of the IPC plate values of all samples (IPC overall). For each sample, the Ct value of all genes was corrected (CtC) based on the CF value. The CtC values of each miRNA were then normalized based on the NormFinder method. Relative quantification was determined using the $\Delta\Delta$Ct method. Fold change (FC) was calculated using $2^{-(\Delta\Delta CT)}$ to assess for differences in miRNA expression between the control and lymphoma group: FC greater than 1 were indicative of overexpression, while FC less than 1 represented under expression. To compare the difference between the mean expression of each miRNA between the two groups, the Student's t-test was performed and FC greater than 1.5 and p values (p) less than 0.05 were considered statistically significant.

Results

Patient characteristics

Sixteen dogs were enrolled in the study, eight dogs in the LG and 8 dogs in the Ctrl. The LG included four females and four males with a median age of 7,75 years (4-12) and belonging to various breeds (mixed, Boxer, Labrador retriever, Weimaraner, Bernese Mountain dog). The most common form of lymphoma was the multicentric one (five dogs), with one patient having nasal involvement as well. The other lymphoma presentations were mediastinal (one dog), hepatosplenic (one dog), cardiac (one dog) and in the jejunal lymph node (one dog) Table 1.

Table 1. Characteristics of dogs in the lymphoma group.

ID	Gender	Age	Breed	Grade	Stage	Substage	Localization	Hypercalcemia
1	M	12	LR	HG	3	b	Multicentric	No
2	M	8	B	HG	5	b	Mediastinal	Yes
3	F	5	W	HG	4	b	Multicentric	No
4	F	4	M	HG	ND	b	Digiunal lymph node	Yes
5	M	6	B	HG	5	b	Hepatosplenic and cardiac	No
6	M	11	M	LG	5	a	Nasal and multicentric	No
7	F	6	BMD	HG	4	ND	Multicentric	No
8	F	10	M	HG	4	b	Multicentric	No

ID=assigned identity number; M=male; F=female; LR=Labrador Retriever; B=Boxer; W=Weimaraner; M=Mixed breed; BMD=Bernese Mountain dog; HG=high grade; LG=low grade; ND= not determined

The cytological and flow-cytometric evaluation confirmed seven dogs (87,5%) presenting high-grade lymphoma, and one dog with low-grade lymphoma (ID 6). One

dog had stage 3 lymphoma, three dogs had stage 4, and three dogs presented a stage 5 lymphoma. Six dogs (75%) were classified with substage b Table 1.

Extracellular vesicle characterization

To confirm EV-size and concentration, NTA was performed on one EV-enriched pellet after UC Fig 1A. The particle size distribution showed ranges within those of EVs, mainly between 80 and 600 nm, with mean and mode having values of 130.4±3.1 and 111.3±12.3 nm respectively, meaning that mainly small-EVs were isolated. Particle concentration was $2.53\times10^{1}2\pm2.54\times10^{1}1$ particles/ml.

Fig 1. Extracellular vesicle characterization results. A: Size distribution recorded at nanoparticle tracking analysis of plasma-derived extracellular vesicles isolated with ultracentrifugation. B: Western Blotting performed on plasma-derived extracellular vesicles (EVs) isolated with ultracentrifugation. The sample was positive to TSG101, a cytosolic marker of EVs, and to integrin-beta, a membrane marker.

WB was performed on one sample to confirm the presence of EV markers in the EV-enriched pellet. The EV-enriched pellet resulted positive to two typical markers of EVs, TSG101, a cytosolic protein, and integrin-beta, a membrane protein Fig 1B.

Quantification of extracellular vesicle-associated and free-circulating miRNAs

For each dog included into the study miRNA analyses were carried out both on EV-associated (EVs-LG and EVs-Ctrl) and free-miRNA (Free-miRNA-LG and Free-miRNA-Ctrl) samples. The free-miRNA sample ID 6 Table 1 (6-Free-miRNA-LG) showed CtC values for all target miRNAs much higher than all other samples, with a consequent low percentage of quantifiable miRNAs ($\leq$30%). For these reasons, this sample was excluded from statistical analyses. For all the other samples, the analysis of the amplification profiles identified a variable percentage of undetectable miRNAs, generally higher for free-miRNAs (missing data ranging from 7.29% to 20.83% for EV-associated miRNAs and from 12.50% to 46.88% for free-miRNAs) Table 2.

Among the EV-associated miRNAs and the free miRNAs, 5 miRNAs with the best stability score, were selected by the software as reference miRNAs for each of the two miRNA-sources S3 Table. The coefficient of variation CV%, of the 5 reference miRNAs was calculated as the ratio of the standard deviation of each miRNA to its mean CtC among all samples, multiplied by 100. An optimal CV value is considered having value lower than 15%. In this study the calculated CV% for the reference miRNAs showed a value between 3-7% S3 Table.

Differential miRNAs expression between the two groups

A total of 9 EV-associated miRNAs were found to be differentially expressed in LG compared to Ctrl. One additional miRNA (miR-103a-3p) had a p-value close to significance (p-value = 0.0506) Table 3.

A total of 7 free miRNAs were found to be differentially expressed in LG compared to Ctrl Table 4.

In LG, only one miRNA (miR-222-3p) was found as overexpressed both in EV-associated and free-miRNAs Table 3 4. As shown in the volcano plot, among the differentially expressed EV-associated miRNAs in LG, 8 were statistically significantly overexpressed, one was significantly under-expressed, and one was overexpressed but marginally significant Fig 2A.

Table 2. Percentage (%) of missing data at the analysis of the amplification profile for each sample. Each sample is described by the ID number followed by the miRNA source and the group of patients.

Sample	% of missing data
$1.EVs - LG$	13.54%
$2.EVs - LG$	11.46%
$3.EVs - LG$	11.46%
$4.EVs - LG$	13.54%
$5.EVs - LG$	11.46%
$6.EVs - LG$	15.63%
$7.EVs - LG$	7.29%
$8.EVs - LG$	17.71%
$9.EVs - Ctrl$	12.50%
$10.EVs - Ctrl$	18.75%
$11.EVs - Ctrl$	20.83%
$12.EVs - Ctrl$	14.58%
$13.EVs - Ctrl$	18.75%
$14.EVs - Ctrl$	14.58%
$15.EVs - Ctrl$	17.71%
$16.EVs - Ctrl$	15.63%
$1.Free - miRNA - LG$	33.33%
$2.Free - miRNA - LG$	34.38%
$3.Free - miRNA - LG$	12.50%
$4.Free - miRNA - LG$	29.17%
$5.Free - miRNA - LG$	41.67%
$7.Free - miRNA - LG$	34.38%
$8.Free - miRNA - LG$	22.92%
$9.Free - miRNA - Ctrl$	27.08%
$10.Free - miRNA - Ctrl$	46.88%
$11.Free - miRNA - Ctrl$	42.71%
$12.Free - miRNA - Ctrl$	37.50%
$13.Free - miRNA - Ctrl$	27.08%
$14.Free - miRNA - Ctrl$	43.75%
$15.Free - miRNA - Ctrl$	42.71%
$16.Free - miRNA - Ctrl$	40.62%

EVs=plasma derived extracellular vesicles; free miRNAs=plasma free-circulating miRNAs; LG=canine T-cell lymphoma group; Ctrl=control group

Fig 2. Volcano plot of differentially expressed genes between the lymphoma and the control group. A: Extracellular vesicle-associated miRNAs; B: free circulating miRNAs. Group 1 = lymphoma group

Among the differentially expressed free miRNAs, 6 miRNAs were statistically significantly overexpressed, and one was significantly under-expressed in the LG Fig 2B. Finally, a principal component analysis (PCA) on differentially expressed genes in EV-associated miRNA samples, showed a separation between LG and Ctrl Fig 3A.

Fig 3. PCA of differentially expressed genes between the lymphoma and the control group. A: Extracellular vesicle-associated miRNAs; B: free circulating miRNAs. cL = lymphoma group; Ctrl = control group

PCA performed on differentially expressed genes of the free-miRNA samples didn't

Table 3. Extracellular vesicle-associated miRNAs significantly differentially expressed between the lymphoma and the control group.

$miRNA$	Fold Change	P-value
$hsa-miR-103a-3p$	0.67	0.009344
$cfa-miR-191$	1.71	0.000227
$hsa-miR-192-5p$	1.99	0.046515
$hsa-miR-222-3p$	2.88	0.002108
$rno-miR-223-3p$	2.18	0.034584
$bta-miR-27a-3p$	1.73	0.047376
$hsa-miR-30b-5p$	1.89	0.000372
$cfa-miR-30d$	2.05	0.032016
$hsa-miR-378a-3p$	2.92	0.022901
$hsa-miR-130a-3p$	3.35	0.050624

Table 4. Free circulating miRNAs significantly differentially expressed between the lymphoma and the control group.

$miRNA$	Fold Change	P-value
$hsa-miR-106b-5p$	1.60	0.017385
$hsa-miR-146a-5p$	3.62	0.038298
$gga-miR-18a-5p$	2.35	0.013136
$hsa-miR-21-5p$	3.23	0.002350
$hsa-miR-222-3p$	2.50	0.030518
$hsa-miR-93-5p$	1.62	0.021672
$hsa-let-7b-5p$	-1.98	0.005933

show instead any separation between LG and Ctrl Fig 3B.

Discussion

Canine TcL continues to pose significant challenges for veterinary oncologists, with a particularly rapid and aggressive behavior and no curative approaches developed in the past 25 years despite numerous attempts [1,12]. Similar to other pet tumors, early detection remains elusive, and the focus on biomarker research is seen as a beacon of hope for identifying lymphomas at an early stage, or to provide prognostic value. In this context, in vivo studies are crucial, as in vitro research on cancer cells fails to identify the early stages of the disease and does not accurately replicate the complexity of in vivo conditions [13,14]. MiRNAs have gained popularity in veterinary and human medicine as tumor minimally invasive biomarkers, given their stable presence not only in cells and tissues but also across various body fluids [2,3]. This preliminary study aimed to identify the expression patterns of circulating miRNAs in a small group of canine T-cell lymphoma dogs, and compare it to healthy dogs, investigating the expression of both free circulating miRNAs and EV-associated miRNAs. EV-characterization performed on two technical controls, showed that EV-isolation with UC allowed the purification of particles that were similar in terms of concentration and size distribution to those of other studies in which mainly small-EVs were isolated using UC from canine plasma [15,16]. Moreover, the isolated particles were positive to two typical EV-markers at WB, a cytosolic (TSG101) and a membrane marker (integrin-beta) [6]. After EV characterization, we conducted qPCR and data analysis on both EV-associated and free circulating miRNAs. As general considerations, more undetermined Ct values were detected in the free circulating miRNA samples compared to the EV-associated ones,

meaning that less miRNAs were overall detected in the free-miRNA samples. Furthermore, there was a marginally higher number of differentially expressed miRNAs in EV-associated compared to free-miRNAs when comparing LG and Ctr (9 vs 7). Among the differentially expressed miRNAs, only one was in common between EV-associated and free-miRNA samples (miR-222-3p). This lack of correspondence between the differentially expressed miRNAs that were either EV-associated or free in the plasma might be due to the fact that EV-associated miRNAs constitute only a small portion of the total plasma miRNAs and their expression can differ from the free circulating ones [17, 18]. Moreover, EV-associated miRNAs are more stable and better shielded from RNAse activity, which could account for the higher amount of determined Ct values observed in EV-associated miRNAs in our study [19, 20]. Consistently with our results, a recent review underscored that among numerous studies examining miRNA expression in human prostate cancer patients, either as total circulating miRNAs (including both EV-associated and free-miRNAs) or EV-associated miRNAs, only miR-21 was identified as overexpressed in both miRNA sources across different patient groups [21]. Since usually studies analyze only EV-associated miRNAs or the total circulating miRNAs, to our knowledge, our work is the only one comparing the EV-associated miRNAs and the free-circulating miRNAs extracted from the EV-depleted plasma, in the same group of patients [21]. While literature on cancer both circulating and EV-associated miRNAs is abundant in human medicine, fewer studies have been performed in veterinary medicine [2, 9, 10, 22–24]. Garnica and coauthors investigated the relationship between serum EV-associated miRNAs and the response to therapy in dogs with multicentric lymphoma (MCL). Among the investigated miRNAs, the authors found mir-222 being more expressed in the group that achieved complete response, and mir-93 being overexpressed in the group showing progressive disease [9]. In our study, miR-222 was found to be overexpressed in both EV-associated and free-miRNAs in the LG. On the other hand, miR-93 showed overexpression in free-miRNAs, but not in the EV-associated ones. The discrepancy in EV-associated miRNA profiles between our study and that of Garnica and colleagues might stem from several factors: i) their specific focus on MCL involving both B and T cells, ii) their exclusive examination of small-EVs, and iii) their analysis of serum-derived EVs as opposed to plasma-EVs, matrixes which have been shown to possess different EV profiles [9]. Circulating miRNAs in lymphoma bearing dogs were also examined in another study, which focused on the analysis of total circulating serum miRNAs, encompassing but not differentiating between free- and EV-associated miRNAs [10]. In this study, the only miRNA that was differentially expressed and consistent with our findings was miR-let-7b, downregulated in cL cases in both studies and differentially expressed as free-miRNA in our analysis. Once again, different results could be attributed again to different case selection criteria (exclusively TcL versus various types of lymphomas) and the distinction between analyzing EV-associated miRNAs and total circulating serum miRNAs [10]. Lastly, also miR-18a, which was overexpressed among the free-miRNAs in our study, has been reported as overexpressed in dogs with cancer, specifically in the serum of dogs with mammary carcinoma [25]. According to the literature, all miRNAs identified as differentially expressed in both free- and EV-associated samples in this work, have been previously noted for their overexpression or downregulation in human tumors and appear to play an important role in both tumor oncogenesis and oncosuppression [26–37]. Indeed, some of the miRNAs that we found differentially expressed in TcL have been reported as markers in human lymphoma. miR-21 and miR191 have been found to be overexpressed in the serum of patients with advanced stages of diffuse large B-cell in lymphoma (DLBCL), and miR21 has also been reported being overexpressed in plasma-EVs of patients with Hodgkin lymphoma [38, 39]. miR-30d, that was found overexpressed in EV-associated miRNAs,

was at contrary downregulated among the total circulating serum miRNAs of human patients with chronic lymphocytic leukemia [40]. Interestingly, miR-let-7b and miR-18a, previously identified as dysregulated in canine lymphoma and canine mammary carcinoma respectively, have also been observed as dysregulated in the total circulating serum miRNAs of human patients suffering from DLBCL. Notably, miR-let-7b has been linked with more advanced stages of this disease [10, 24, 41]. The other miRNAs found as differentially expressed in our study, which have not been reported in canine malignancies or human lymphoma so far, have been investigated in other types of human cancers, and some of them have been detected as associated to circulating EVs. miR-146 and miR-223, that we found as overexpressed in free- and EV-associated miRNAs respectively, were underexpressed in serum-EVs of human patients with laryngeal squamous cell carcinoma [42]. miR-223 has been found overexpressed and downregulated in patients with colorectal and epithelial ovarian cancer respectively when EV-associated [43, 44]. In colorectal cancer patients, also miR-192 and miR-27a have been found to be differentially expressed when EV-associated and miR-30b, overexpressed among EV-associated miRNAs in our study, was found underexpressed in plasma EVs of women with breast cancer recurrence [43, 45, 46]. Lastly, miR-103a and miR-130a, which were respectively downregulated and overexpressed when EV-associated in our study, have been found overexpressed in the total circulating serum miRNAs of human patients with lung cancer and urinary bladder cancer, respectively [47, 48]. miR-106, that we have found overexpressed among free-miRNAs, has been considered a potential circulating diagnostic biomarker for hepatocellular carcinoma and a prognostic biomarker for gastric cancer [49]. While the findings of this study align to some extent with prior research in human and canine oncology, suggesting a subset of miRNAs as potential early diagnostic biomarkers for TcL, we have to acknowledge some limitations of this study. A limited number of patients was included due to difficulties in obtaining blood samples from a homogenous subset of subjects. Additionally, to exclude patients that had undergone different treatment protocols, samples were only gathered at the time of diagnosis. Consequently, to achieve more substantial results, further investigations should involve a larger, uniform group of individuals including additional samples collected at different timing post-diagnosis. These investigations could focus on the analysis of the herein found differentially expressed miRNAs, evaluating their efficacy not only as diagnostic, but also as prognostic and predictive biomarkers. This would involve adding follow-up data from larger sample groups. Moreover, although UC has always been considered the gold standard method for EV isolation and is a very well-established technique that we an excellent margin of certainty allows isolation of EVs, we could analyze EV concentration, size and characterization only on two samples used as procedural controls. Further, there are other techniques that, according to the literature, allow the collection of purer samples being less operator-dependent [6, 50, 51]. Therefore, future studies could also consider using and comparing different EV-isolation techniques possibly applying them to the whole subset of analyzed samples.

Conclusion

This preliminary prospective double-arms study compares for the first time the expression of EV-associated and free-circulating miRNAs in the plasma of dogs with TcL and healthy control dogs. Nine EV-associated and seven free circulating miRNAs were found differentially expressed between LG and Ctrl, with hsa-miR-222-3p being overexpressed in both EV-associated and free circulating miRNAs. Several miRNAs identified as differentially expressed in this study have previously been recognized as potential markers in TcL, making them promising candidates for more in-depth analysis.

The choice of using EV-associated miRNAs as biomarkers over free circulating miRNAs would have several advantages, as EV-content is stable and protected from degradation by circulating RNAse. The miRNAs found in this study could be used in further studies with a larger number of patients and additional follow-up and therapeutic information to investigate their possible use also as prognostic and predictive biomarkers. These preliminary results add new insights into EV-associated miRNAs within the field of veterinary medicine, enhancing our understanding of their expression in TcL. Our expectations are to contribute to the development of quicker, more effective, and innovative diagnostic and therapeutic tools.

Supporting information

S1 Table. Antibodies combinations used to determine canine lymphoma phenotypes In brackets the dilution used. Abbreviations. FITC: fluorescein isothiocyanate, PE: phycoerythrin, APC: allophycocyanin, CD: cluster of differentiation.

S2 Table. Layout of the PCR 96-well plate miRCURY LNA miRNA Focus PCR Panel (Qiagen, YAFD-201Z) used to perform quantitative real time PCR.

S3 Table. Reference miRNAs calculated for the extracellular vesicle-associated miRNAs (EVs) and free circulating miRNAs (free-miRNAs) groups and corresponding mean cycle threshold (Ct), standard deviation and coefficient of variation (CV%).

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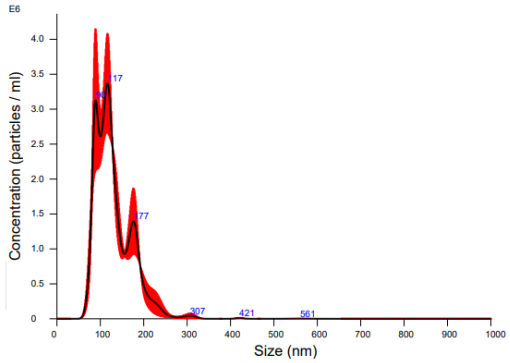
A



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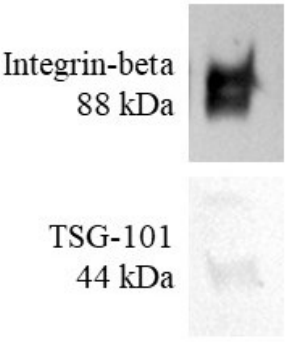

Figure 1

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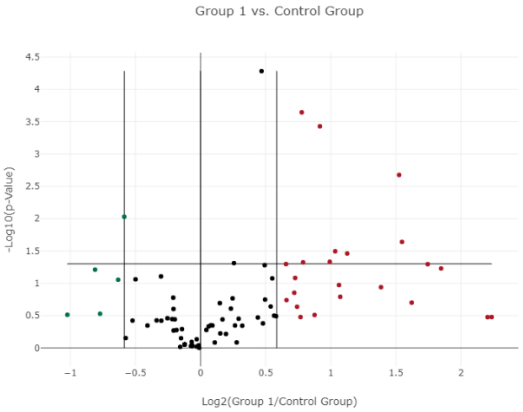

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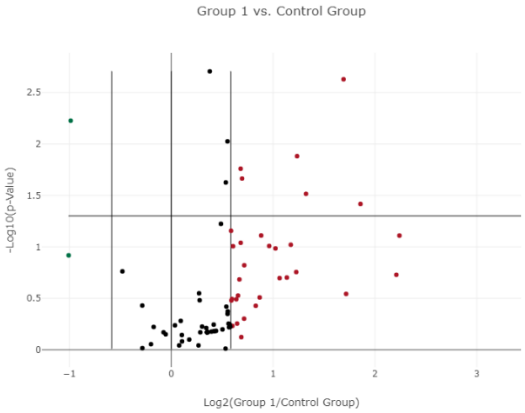

Figure 2

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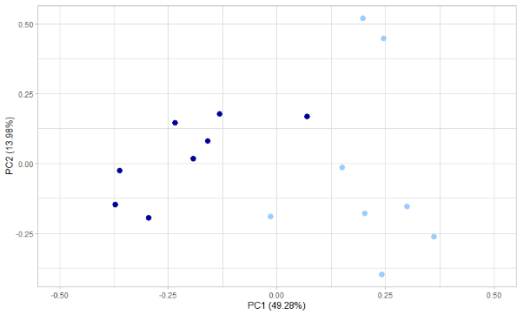

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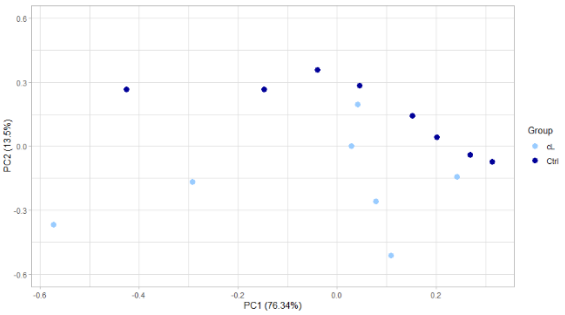

Figure 3

7. General conclusions

EV-research has a wide range of different applications, from their use in pathophysiology studies, to their practical application as diagnostic biomarkers or as therapeutics.

In veterinary medicine, despite the increasing number of publications, still little is known about animal-derived EVs.

In this PhD thesis, different applications of EVs, isolated from different animal species and biofluids were explored to gain more information and expertise on these powerful messengers also in animals.

The added value of EV-research is that all these different applications can fit very well into the concept of One Health, since EVs can be used to assess the health of humans and the relation between animals, humans and the environment, having the ability to travel and connect different kingdoms (82). To better explore this interconnector role of EVs, we need for sure more information on animal derived-EVs, on the possible mechanisms of release in biofluids and in the environment and on their role in intra and inter-species communication.

With the purpose to follow this thread in the future, I hope this research work lain another brick on the wall, adding new information on animal derived EVs and showing the potential and the versatility of this new research tool.

8. Side projects and related papers

Besides from the here above reported papers and research works, during my PhD I could participate to a side project in collaboration with another group. My contribution was mainly based on my expertise on EVs and on the NTA technology applied in the study. Here is the published paper.

- Mecocci S, De Paolis L, Zoccola R, Fruscione F, De Ciucis CG, Chiaradia E, **Moccia V**, Tognoloni A, Pascucci L, Zoppi S, Zappulli V, Chillemi G, Goria M, Cappelli K, Razzuoli E. Antimicrobial and Immunomodulatory Potential of Cow Colostrum Extracellular Vesicles (ColosEVs) in an Intestinal In Vitro Model. Biomedicines. 2022; 10(12):3264

I also participated to other research works not involving EVs. The list of these works is reported below followed by a short description of my contribution in each study.

- Crescio M I, Ru G, Aresu L, Bozzetta E, Cancedda M G, Capello K, Castagnaro M, Carnio A, Cocumelli C, Uberti B D, Eleni C, Foiani G, Fonti N, Gibelli L R, Maniscalco L, Manuali E, **Moccia V**, Paciello O, Petrella A, Petrini A, Poli A, Puleio R, Razzuoli E, Scaramozzino P, Varello K, Vascellari M, Zappulli V, Ferrari A, On Behalf Of NilovTorrigiani F, Moccia V, Brunetti B, Millanta F, Valdivia G, Pena L, Cavicchioli L, Zappulli V. The Italian Network of Laboratories for Veterinary Oncology (NILOV) 2.0: Improving Knowledge on Canine Tumours. Veterinary Sciences. 2022;9(8), 394.
  Contribution: canine tumors data collection from the register of the pathology diagnostic service of the Comparative Biomedicine and Food Science dept. of the University of Padua.

- Torrigiani F, **Moccia V**, Brunetti B, Millanta F, Valdivia G, Pena L, Cavicchioli L, Zappulli V. Mammary Fibroadenoma in Cats: A Matter of Classification. Veterinary Sciences. 2022;9(6), 253.
  Contribution: laboratory support (for immunohistochemistry); writing, editing and reviewing of the original draft.

- Rensi N, Sammarco A, **Moccia V**, Calore A, Torrigiani F, Prosperi D, Rizzuto MA, Bellini M, De Maria R, Bonsembiante F, Ferro S, Zanetti R, Zappulli V, Cavicchioli L. Evaluation of TFR-1 Expression in Feline Mammary Cancer and In Vitro Antitumor Efficacy Study of Doxorubicin-Loaded H-Ferritin Nanocages. Cancers (Basel). 2021; 10, 13(6):1248
  Contribution: *in vitro* assays (cell proliferation assays); Western Blotting analysis

- Zappulli V, Ferro S, Bonsembiante F, Brocca G, Calore A, Cavicchioli L, Centelleghe C, Corazzola G, De Vreese S, Gelain ME, Mazzariol S, **Moccia V**, Rensi N, Sammarco A, Torrigiani F, Verin R, Castagnaro M. Pathology of coronavirus infections: a review of lesions in animals in the one-health perspective. Animals. 2020; 10, 2377
  Contribution: writing of a chapter ("Coronavirus in pets").

Finally, I published also another study as first author. The aim of the following study was to histologically evaluate the organ and to describe and score the histological distribution of AA-amyloid deposits in nine shelter domestic shorthair cats with confirmed systemic AA-amyloidosis.

- **Moccia V**, Vogt A. C., Ricagno S., Callegari C., Vogel M., Zini E., Ferro S. Histological evaluation of the distribution of systemic AA-amyloidosis in nine domestic shorthair cats. PLoS One. 2023; 18(11):e0293892
  Contribution: histological description and scoring of amyloid deposits; writing of the original draft.

9. Bibliography

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