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Advances in Extracellular Vesicle Research Over the Past Decade: Source and Isolation Method are Connected with Cargo and Function

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The evolution of extracellular vesicle (EV) research has introduced nanotechnology into biomedical cell communication science while recognizing what is formerly considered cell "dust" as constituting an entirely new universe of cell signaling particles. To display the global EV research landscape, a systematic review of 20 364 original research articles selected from all 40 684 EV-related records identified in PubMed 2013–2022 is performed. Machine-learning is used to categorize the high-dimensional data and further dissected significant associations between EV source, isolation method, cargo, and function. Unexpected correlations between these four categories indicate prevalent experimental strategies based on cargo connectivity with function of interest being associated with certain EV sources or isolation strategies. Conceptually relevant association of size-based EV isolation with protein cargo and uptake function will guide strategic conclusions enhancing future EV research and product development. Based on this study, an open-source database is built to facilitate further analysis with conventional or AI tools to identify additional causative associations of interest.

1. Introduction

Extracellular vesicles (EVs) are a heterogeneous group of membrane-enclosed cell-derived vesicles involved in cell-to-cell communication. Most prominent representatives under the umbrella-term EV are nanometer-sized endosome-derived exosomes and outer cell membrane-derived ectosomes as well as oncosomes, microvesicles, and apoptotic bodies that measure up to microns in diameter. Additional members of the growing EV universe are migrasomes and elongated neutrophil-derived structures, among others, as defined by their biogenesis.[1] Recent research further identified large (3500–4000 nm) exopheres as cell-derived information carriers (Box 1). The growing family of biological nanoparticles lacking bilayer membrane or lumen was recently reviewed elsewhere and was not covered by our analysis.[2]

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Documented functions of EVs range from cell signaling in health and disease,[3] tumor niche formation,[4] and immune response modulation,[1] to disposal of cellular material for maintenance of cell homeostasis.[2] The heterogeneity of the numerous types of EVs and nonvesicular extracellular particles is a major challenge in their analysis and toward understanding the biological role of the multiple individual biological nanoparticle entities.

Starting with the early observation of a high-velocity (31 000 x *g*) centrifugation-depletable thromboplastic factor by Chargaff and West in 1946[5] and the first description as "platelet dust" with "coagulant activity" by Peter Wolf in 1967,[6] EV studies have only recently expanded exponentially.[7] It is meanwhile apparent that EVs are not just biological waste, expelled by virtually every cell, but play an important role in cell biology.[8] Over the past decades, EV research has changed our understanding of cell-to-cell and cell-to-matrix communication.[3] A canonical view distinguished contact-dependent from contact-independent mode of cell signaling, the latter being considered previously as purely soluble factor-mediated.[9] The advent of EV science introduced a third dimension into our understanding of cell-based communication, with cell-derived biologically active EVs realizing cell membrane- and/or EV cargo-related signaling over distance.[3,10–12] EVs can transfer a rich cargo, inside and at their surface, comprising a plethora of proteins, lipids, sugars, small molecules and nucleic acid species, including regulatory small RNAs, for distant action.[1,13,14] EVs may also allow targeted delivery of cargo across biological barriers, functioning as a drug delivery platform.[15,16]

The vast gain of knowledge in the EV field, particularly over the past decade, makes it difficult to recognize relationships between the various increasingly complex isolation and analysis methodologies that may considerably vary depending on additional parameters such as storage conditions.[17,18] Particularly the broad range of methods used for EV isolation and subsequent characterization of quantity, identity, cargo and function introduce a high level of uncertainty due to their mutual interdependence. It is well established that different isolation methods enrich for diverse types of vesicles comprising highly variable protein cargo.[19] Most recently, hydrodynamic radius-based isolation methods like tangential flow filtration and size-exclusion chromatography, partly preserving EV-associated proteins, enabled discovery of a functional EV corona that was otherwise artificially removed by depleting proteins from EV isolates.[20–25] Due to their surface-to-volume ratio, EVs < 180 nm in diameter can carry more cargo on their surface than in their interior.[26] Generally, size-based isolation leads to EV nanoparticle preparations of diverse density, while density-based isolation results in diversely sized nanoparticles of comparable density.[27] Ultracentrifugation with its high forces has been used extensively for depleting the nanoparticle corona.[28] Precipitation methods can lead to EV aggregation and reduced functionality.[29–31]

Still, many "known unknowns" regarding EV biogenesis and mode of action,[32] in part related to methodological limitations combined with lack of standardization in EV analysis,[33] hampered more rigorous mechanistic research and clinical translation. We therefore performed a systematic text mining analysis of the entire EV research literature in PubMed over the past decade to create an up-to-date global display of connectivities between various EV sources, isolation methods, cargo, and function. We furthermore identified significant associations deviating from expected quantitative outcome, indicative of superior experimental strategies to be tested rigorously. The very interesting commonalities and differences between viruses and EVs would be an interesting target for another study using the tools developed our current analysis but were out of focus in the current study.

2. Rising Number and Heterogeneity of EV Studies

Our PubMed search identified 40 684 records related to EVs for the period 2013–2022, by February 8, 2023, leaping from 1277 records in 2013 to 9151 records in 2022 (Figures S1 and S2A, Supporting Information). To assess the coverage of our search, we compared the overlap with publications submitted to the EV-TRACK database[34] and found that 98.7% of EV-TRACK publications (n = 1693/1715 by January 31, 2023) were recovered. To restrict our analysis to primary research articles, we used a sequential approach. First, we selected PMIDs flagged by PubMed as "research articles" (n = 28 496). To exclude publications inadmissibly flagged as research articles, we next applied an artificial intelligence approach (random forest algorithm; Figure S3 in the Supporting Information) using the abstracts as input to classify "research articles" versus "others" and found 564 publications out of the 28 496 that were identified as nonprimary research (Figure S1, Supporting Information). Finally, based on the availability of download links and access, we retrieved 22 519 open access (OA) and non-OA publications, consistently comprising around 70% OA publications over the observation period (Figure S2AB, Supporting Information). After identifying publications containing material and methods and/or results sections, 20 364 studies, considered original research articles, were retained for the text mining and meta-analysis.

To display the multidimensional dataset, we next used dimensionality reduction by uniform manifold approximation and projection (UMAP).[35] We defined EV source, isolation, cargo and function as four categories for collecting more detailed information. Within these categories, we selected the 18 most common sources, as well as six of each of the most common isolation methods, cargo types, and function parameters, out of 157 parameters analyzed totally. We assigned binary values (0 and 1) to each parameter, indicating absence (0) or presence (1) in each publication, respectively. We investigated the interaction between the parameters ("variables") within the four categories to contextualize connectivities. The resulting multidimensional dataset was used as input for UMAP analysis to visualize clustering of publications. 2D spatial organization of clusters illustrates similarity between publications (**Figure 1**).

We identified 20 major clusters representing specific patterns of parameter combinations (Figure 1A). Clusters were numbered according to cluster size. Publications with a higher number of reported parameters tended to cluster together, indicating methodological or analytical commonalities (Figure 1B). For detailed analysis of cluster composition, we determined the proportion of publications containing a specific parameter within each cluster to identify parameters acting as cluster drivers (Table S1, Supporting Information). The clustering identified manuscripts with similar methods used concerning EV source, isolation, cargo characterization and functional analysis-related parameters thus enabling an overview of EV research areas. As one example, cluster 16 and 17 both have in common that most

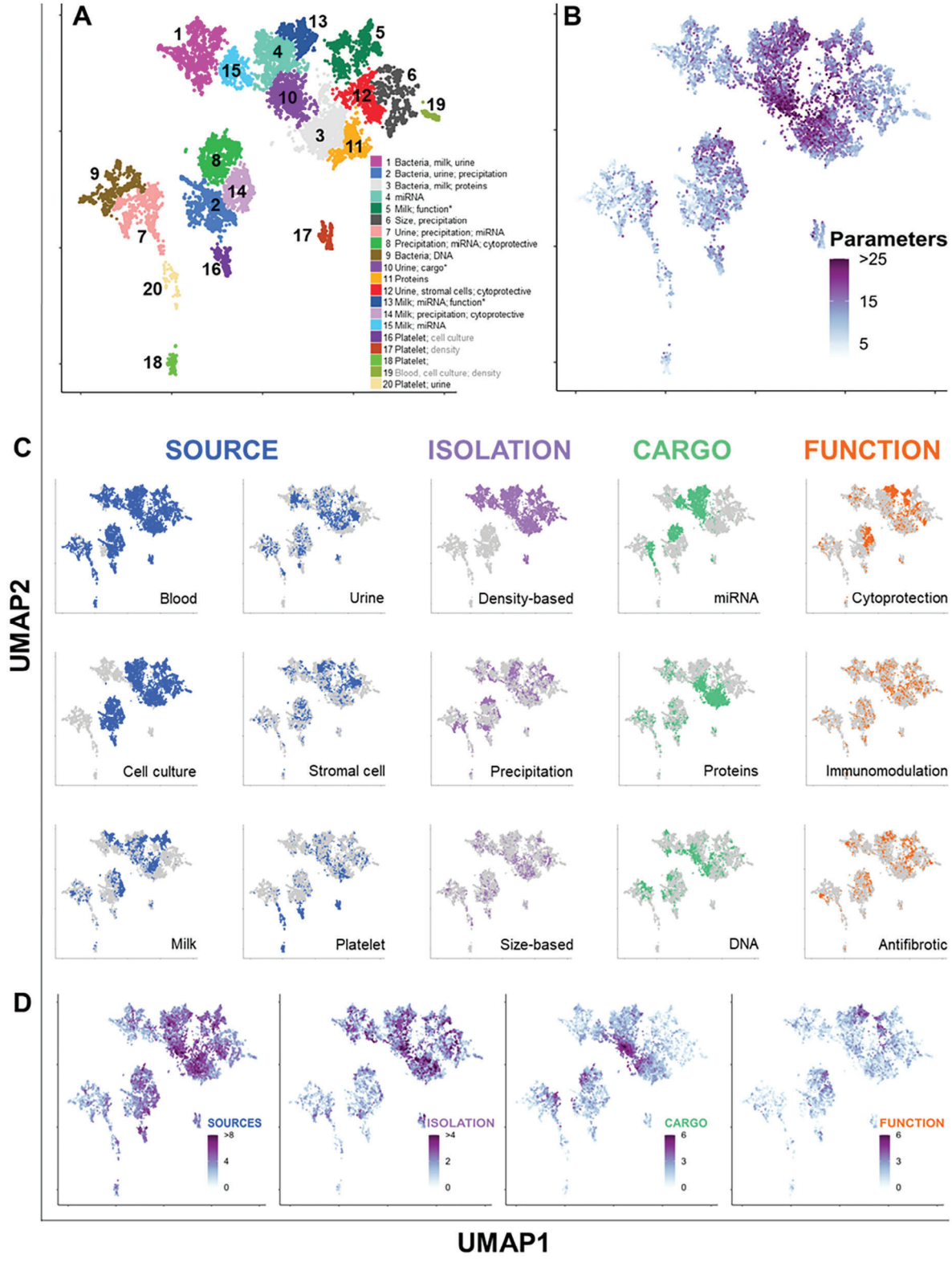

Figure 1. Uniform EV research data maps. Data are based on 20 364 original EV research articles, identified in PubMed for 2013–2022. Each point in the plots represents a single publication. A) We identified 20 clusters using hierarchical clustering of uniform manifold approximation and projection (UMAP), colored as indicated. Spatial distribution reflects the high-dimensional data structure. Color code legend indicating main drivers of clustering as shown. Parameters and categories separated in the color code legend by comma and semicolon, respectively. The percentage of publications containing individual parameters in single hierarchical clusters is shown in Table S1 (Supporting Information). Grey text in the legend indicates preferential contribution of the overall dominant driver parameters "blood", "cell culture," and "density"-based isolation. B) Unsupervised 2D depiction of the total

manuscripts investigated EVs derived from platelets. Most publications in cluster 17 used density-based isolation methods, that were not used at all in cluster 16. The most outstanding clustering phenomenon resulted from use of density-based isolation methods that seem to separate the EV field in two "hemispheres". The absence or presence of density-based methods separated the cluster landscape in a "northern hemisphere" (using density-based isolation methods) comprising clusters (1, 3, 4, 5, 6, 10, 11, 12, 13, 15, 17, and 19) and a "southern hemisphere" (7, 8, 9, 14, 16, 18, and 20) not using these methods–with platelet-EV cluster 17 standing separated.

Certain parameters are present across multiple clusters. Density-based isolation methods are absent in clusters 2, 9, 14, 15, 18, and 20. Other parameters, such as platelets as a source, showed more limited distribution (clusters 16, 17, 18, and 20, derived from 100 manuscripts on platelets). Additional parameters, e.g. cytoprotective function, show more subtle differences which might represent different approaches and applications in the field. Overlaps between parameters among publications in a given cluster highlight similarities regarding study design and focus. As an example, publications in cluster 10 reported on multiple parameters of the category cargo while in cluster 5 reports were focused on several aspects of EV function. This may indicate a common analytical interest among publications within each of these clusters. As another example, studies reporting on platelets (cluster 16, 17, 18, and 19) are separated by reports focusing on cargo (cluster 16) and publications reporting methodological similarities (multiple parameters of the category isolation were identified per publication, cluster 17).

Blood as the most frequently used EV source showed minor contribution to clustering despite its high abundance in all clusters. It is worth mentioning that blood-EV numbers can surge due to platelet activation upon choice of an inappropriate platelet-activating anticoagulant (i.e., heparin and partly also EDTA) or extended blood sample storage. It is therefore mandatory to strictly adhere to existing guidelines to avoid major contamination of blood-EVs by artificially activation-induced platelet-derived EVs.[36–39] Guidelines exist for blood anticoagulation and EV collection,[40] and recommendations for blood-EV isolation have been published most recently.[41] Still, diverse isolation methods are required by specific downstream analysis techniques.[42]

Cell culture and density-based isolation are represented strongly in the majority of clusters (13 out of 20 and 12 out of 20, respectively), reflecting their prevalence in the field. Due to the overall high abundance of blood, cell culture, and density-based isolation within clusters, we focused on the remaining 33 parameters and used the most represented ones for detailed analysis of cluster composition and identification of putative cluster drivers (Figure 1C; Figures S4 and S5, Supporting Information). The three largest clusters (cluster 1–3) are enriched for bacterial, milk and urinary EVs, precipitation-based EV isolation and studies on protein cargo. Additional differences between the three clusters are mainly driven by the virtually complete absence of cell culture in cluster 1 and complete absence of density-based isolation in cluster 2 (Table S1, Supporting Information). The main drivers of cluster 4 are studies on miRNA cargo while cluster 5 is highly enriched for milk EV studies together with studies on cytoprotective function and endogenous regeneration. For cluster 6 the two most abundant parameters are size- and precipitation-based isolation. Cluster 7 and 8 are both enriched in studies on miRNA and precipitation-based isolation, separated by studies using urine-EVs in cluster 7 and studies on cytoprotective EV function in cluster 8. The main drivers of cluster 9 are bacterial EVs and DNA cargo analysis. Cluster 10 is enriched in studies investigating EV cargo including miRNA, proteins, DNA, and RNA, and the main source parameter urine. The principal driver of cluster 11 is protein cargo with 100% contribution. Cluster 12 contains a high proportion of urinary EV studies differing from cluster 10 by an increased number of immunomodulation, cytoprotective and antifibrotic function studies. Studies comprising cluster 13 mainly investigated miRNA, cytoprotective function and endogenous regeneration, with the majority using milk as an EV source. Drivers for cluster 14 investigated milk and cytoprotective function, and miRNA cargo for cluster 15. In clusters 16, 17, 18, and 20, 100% of studies used platelets as EV source. Differences between these clusters result from enrichment of cell culture and the absence of density-based isolation in cluster 16 and 17, and absence of both parameters in cluster 18 and 20. Cluster 20 is enriched for urine-EV studies in addition. Cluster 19 shows predominant enrichment of studies on blood- and cell culture-EVs, and density-based isolation, but no appreciable enrichment of any other parameter.

Publications reporting higher number of EV cargo parameters (cluster 10) did not cluster with those reporting higher number of functional parameters (cluster 5 and 13). This clustering pattern also reflects a preference on either cargo characterization or functional validation, as well as a focus on EV biomarkers versus therapeutic EV application (Figure 1D). This could be driven by distinct research questions, methods involved, or resources and expertise required for extensive cargo characterization versus functional validation. Clustering may also represent trends in terms of technology, EV sources, cargo of interest, and functional assay use.

3. Growing Combination of Sophisticated EV Isolation Methods

We next analyzed the range of methods combined to isolate EVs and analyze their cargo or function, to dissect their connectivity. Density-based methods were most often used to isolate EVs (in 10 258 of 20 364 studies; 50.4%) followed by precipitation (20.7%) and size-based isolation methods (13.4%). If methods like density-based isolation showed a high

number of parameters per publication. Color intensity represents the number of parameters detected within each publication as indicated in the color scale. C) Detection of specific category information (EV source, isolation method, cargo, function) within studies highlighted in color: blue represents sources (n = 18), purple isolation methods (n = 6), green cargo (n = 6), orange function (n = 6). Absence of parameters in a publication depicted as grey dot. Several parameters localize in more than one cluster (e.g., miRNA in six clusters). The remaining UMAP plots are shown in Figure S5. (D) Number of parameters per publication detected within the four categories EV source, isolation, cargo and function, as indicated in color scales.

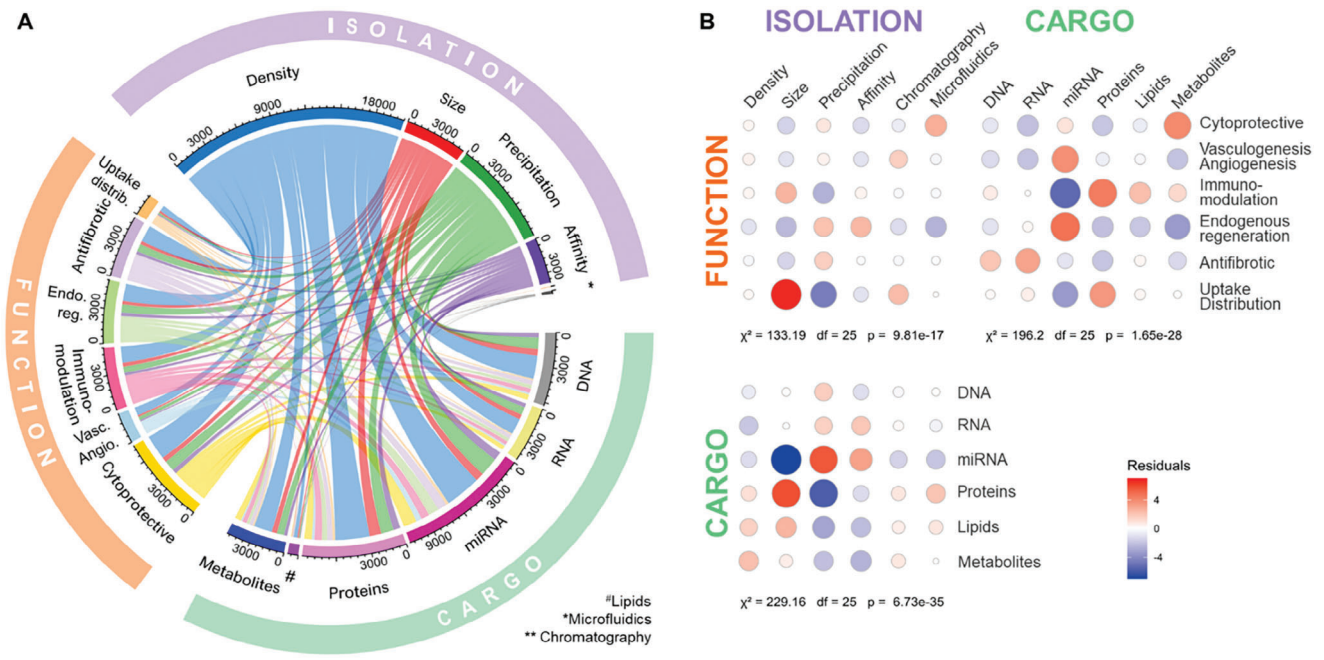

Figure 2. Connectivity of EV isolation method with cargo and function analysis. A) Results identified by text mining of 20 364 primary EV research articles, identified in PubMed for 2013–2022, were grouped in three color-coded categories: EV isolation method (purple), cargo (green), function (orange), and identified as indicated. The ring scale shows the number of combination pairs or "links" connecting the categories. Scale numbers don't match numbers of publications because most publications (16 046, 78.8%) used several methods. Example: density-based isolation methods were linked with cargo and function data 20 410 times, found in 10 258 articles. B) Chi-square (X^2) analysis showing significant association between isolation and function, isolation and cargo, and cargo and function, respectively. The standardized residuals derived from correlation between observed and expected values are shown as correlation plots. Positive residuals in red, negative in blue; dot size corresponding to color heat. Example: size-based isolation showing positive correlation with EV uptake/distribution (function) analysis as well as protein (cargo), and negative correlation miRNA (cargo). Symbols indicating #lipids, *microfluidics, **chromatography; abbreviated categories: uptake and distribution; endogenous regeneration; vasculogenesis, angiogenesis. An interactive Sankey plot is available online to highlight the flow between the categories.

percentage of use in the field, we also found a high number of interactions with other methods. The most frequently used combinations of isolation methods were density + precipitation (8.2%), density + size-based (5.9%) and density + affinity-based methods (2.3%; **Figure** 2A; Figure S6A, Supporting Information).

Density-based methods were consistently used in roughly half of the studies over the entire 10-year observation period. Use of size-based methods increased over the last decade, either reflecting methodological preferences or indicating that nano-sized vesicles are meanwhile obtained effectively based on their size rather than their variable density (Figure S6B, Supporting Information). We also detected a significant decrease ($p = 0.025$) in studies that rely on one single method for EV isolation (Figure S6C, Supporting Information). Combining orthogonal isolation methods is meanwhile considered to increase EV purity, presumably at the expense of yield. A recent study analyzed the impact of combining isolation strategies in 896 studies published in 2019 and found that the most used isolation methods, i.e., differential ultracentrifugation with or without ultrafiltration, did not necessarily result in the highest yield and/or high purity, partly varying with EV source.[43] The impact of combining various isolation methods on identity, purity, cargo stability and functionality of EVs isolated from different starting sources has not been systematically studied so far.

4. EV Cargo and Function

We found miRNA to be the most-analyzed cargo (in 5098 of 20 364 studies, 25.0%), and we detected 11 200 connections with other isolation and function analysis methods. Protein (20.6%) and DNA (16.4%) were the next most frequently measured cargo types. In studies addressing just one cargo entity, miRNA alone was the most studied cargo (10.9%, 2213 publications) followed by proteins (7.2%, 1461 publications), DNA (3.6%, 734 publications) and metabolites (2.8%, 576 publications) (Figure S7A, Supporting Information). This is consistent with our analysis of 46 123 publication keywords, in which "miRNA" and "proteomics" were third and tenth in the top fifty of the most used standardized keywords (Figure S8A, Supporting Information). EV studies testing miRNA, RNA and metabolites significantly increased, while DNA, protein and lipid analysis remained unchanged (Figure S7B, Supporting Information). Publications analyzing a combination of four different cargo entities showed a significant increase over the last decade ($p < 0.03$; Figure S7C, Supporting Information).

Regarding EV function, cytoprotection assays were most used (16.2% of articles) followed by endogenous regeneration (11.0%), anti-fibrotic function (10.9%), immunomodulation (10.6%), vasculogenesis/angiogenesis (5.4%) and uptake/distribution assays (3.1%) (Figure 2A; Figure S9AB, Supporting Information). Most

functional categories significantly increased over the past 10 years (Figure S9B, Supporting Information). We also found that 4409 publications (21.7%) relied on just one functional readout, while 14.8% (3018 publications) relied on two or more. In 12 937 publications (63.5%), we could not identify specific methods related to functional readouts, either due to a lack of functional characterization or because the selected panel of most common keywords did not cover all possible function-related methods. We found a significant increase of publications using a combination of two ($p < 0.001$), three ($p < 0.001$), and four ($p < 0.001$) methods for characterizing EV function (Figure S9C, Supporting Information). Inflammatory cytokine analysis followed by proliferation assays, angiogenesis and wound healing were the most published readouts (Figure S9D, Supporting Information).

5. Significant Association of Isolation Method, Cargo and Function

Ideally, one would be able to validate all promising and/or rationally arguable combinations of isolation methods to achieve optimal recovery of cargo and determine the efficiency of EVs with respect to the experimental/diagnostic function in question. Due to the number of possible combinations, this will not be feasible experimentally in the foreseeable future but may be partially modeled by computational tools. In a first series of tests, we therefore searched for significant association between isolation, cargo and function, using a Chi-squared (X^2) test of independence hypothesis. We displayed the residuals in correlation plots illustrating specific combinations of methods contributing to the X^2 significance deviating positive or negative from expected values. Density-based EV isolation showed moderate cargo or function correlation residuals. Size-based EV isolation showed positive correlation with EV uptake and distribution as well as protein analysis and negative correlation with miRNA data. EV isolation by precipitation negatively correlated with uptake/distribution analysis and protein cargo, and positively with miRNA (Figure 2B). In this regard, it was recently demonstrated that size-based isolation methods such as tangential flow filtration and size-exclusion chromatography provided higher EV protein recovery.[19,21,44,45] Mechanistically, size-based methods can produce purer EVs compared to a higher yield of less pure EV fractions obtained by precipitation.[46] Impurity of EV preparations can impede EV uptake and/or function.[47] Several additional less prominent but significant associations between isolation method and cargo or function were identified (Figure 2B).

6. Significant Association of EV Source with Isolation Method, Cargo and Function

The complexity of inter-relationship of EV isolation, cargo, and function data further increased when visualizing their connectivity with the respective EV sources. Multiple parameters can be considered when selecting EV source, isolation and analysis methods.[43,48] Downstream applications need to be compatible with source material and isolation method producing certain EV quality and quantity with or without documented contamination by nonvesicular nanoparticles including lipoproteins or protein aggregates.[27,48–50] Reproducibility and reliability based on selection and qualification of preparative and analytic methods are essential, ensuring consistent results.[51] The choice of EV isolation methods ideally would be based on a thorough evaluation of these factors, as well as the specific research question(s) and experimental design.

Source-function correlation showed most significant association ($X^2 = 1114.95$, $p = 3.63^{-179}$), followed by source-cargo ($X^2 = 908.65$, $p = 4.75^{-138}$) and source-isolation ($X^2 = 346.76$, $p = 2.53^{-33}$) (Figure S10, Supporting Information). To further dissect association patterns between various EV sources, isolation methods, cargo and function, we displayed the residuals in a network diagram, illustrating specific combinations of parameters that showed stronger or weaker association compared to the expected distribution (**Figure 3**). The outer node size in the network analysis corresponds to frequency of use of a source, isolation method, cargo or function test in the 20 364 original research articles published over the past decade. Most prominent nodes identified blood and cell culture as predominant EV sources, and a preference for density-based isolation. Line thickness indicates number of interactions between two parameters. Line color highlights positive (red) or negative (blue) associations indicating if interactions were higher or lower than the expected frequency. Line color saturation highlights strength of association.

As the most common EV source, blood showed the highest number of interactions with the most used isolation method, density-based isolation, but below the expected frequency (negative association). Precipitation as the second most used isolation method associated positively with blood EV studies, possibly due to its versatility at limited start volume. For cell culture-derived EVs, density-based isolation methods were preferentially used as indicated by a positive association. Precipitation showed positive association with the three major sources blood, cell culture and milk, but was less frequently used than expected for most other sources as indicated by a high number of negative associations. Size-based isolation methods were negatively associated with blood- and cell culture-EVs. Affinity purification was positively associated with nine out of the 18 sources, including cerebrospinal fluid, bronchoalveolar lavage fluid (BALF), and urine. Concerning cargo, blood-EVs had more than expected interactions with miRNA and less with protein. The latter result matches data described in a recent review.[42]

Regarding blood-EV function, cytoprotection was more and immunomodulation less frequently investigated than expected. Cell culture-derived EVs shared the same type of association with blood-EVs for cargo and function analyses with a positive association of miRNA analysis and cytoprotection. Most significant associations were found for immunomodulation as an over-represented functional test in studies with cerebrospinal fluid and bacteria. Strong association between bacteria and cerebrospinal fluid as EV sources and immunomodulation testing may reflect a focus of interest on immune system-related EV function.[1] DNA analysis in bacterial EVs and miRNA in stromal cell-derived EVs showed strongest positive association. Conversely, miRNA showed the expected strong negative association with bacterial EVs, presumably due to limited miRNA species discovered so far in bacteria and/or limited availability of commercially available assay formats (Figure 3). These results may also parade prevalent practices and potential bias in the EV re-

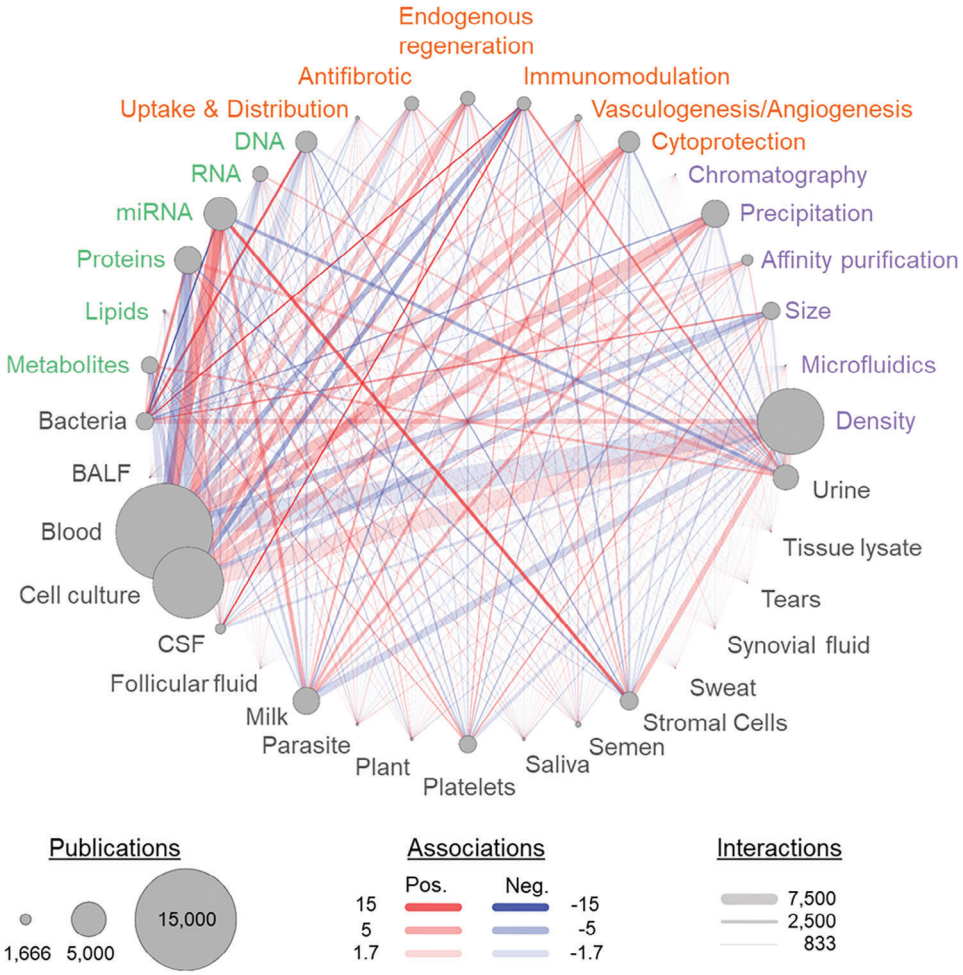

Figure 3. Association between EV source, isolation method, cargo and function. Each node in the network analysis represents a distinct parameter of the categories EV source, isolation method, cargo, and function as indicated by color code. Node size correlates with publication number as indicated in the lower left legend. Lines (termed edges) between nodes represent associations. Positive associations are shown in red and negative associations in blue with thickness proportional to the number of interactions found between different parameters. Color saturation of edges scaled to the residuals corresponding to stronger association as indicated. Abbreviations: cerebrospinal fluid (CSF); bronchoalveolar lavage fluid (BALF).

search field. Additional strong association was found for platelet-EVs with size-based isolation and vasculogenesis/angiogenesis function (Table S2, Supporting Information). The regulation of angiogenesis by platelet-EVs has been attributed to the transfer of miRNAs.[52–54] Other studies demonstrated the role of stromal cell-derived EVs in angiogenesis.[21,55–57]

The most used sources for EV isolation over the past decade were blood (serum or plasma; in 14 934 publications, 73.4%) followed by cell culture (including conditioned media and culture supernatant; in 10 921 publications, 53.6%), milk (in 4139 publications, 20.3%) and urine (in 3947 publications, 19.4%). Together, these percentages exceed 100% because most publications studied EVs from more than one source in 14 254 of the 20 364 publications (70.0%; **Figure** 4A). The proportions match recent survey results indicating plasma, serum-free cell culture media, cell culture media enriched with serum and serum as the most used EV sources in 2019.[58] We also analyzed the EV-TRACK[34] (https://evtrack.org) database entries to cross-check predominant EV sources. Both the number of publications using specific sources and individual samples/experiments listed in EV-TRACK were consistent with our analysis. Blood (including serum and plasma) and cell culture (supernatant and conditioned media) were the most frequently studied EV sources among the studies registered in the EV-TRACK database (Figure 4B).

Plasma and serum (i.e., defibrinated blood plasma) are attractive sources of blood-born EVs obtained by minimally invasive procedures. Bio-banked blood/plasma/serum are valuable EV sources for retrospective studies, with restrictions for biomarker research due to variable preanalytics.[44] Since the quality of bio-banked blood samples can differ considerably, it is possible that not all samples can be used for EV analysis.[59–62] The anticoagulant heparin, for example, can block EV uptake and inhibit enzymes used for nucleic acid assays.[63,64] For cell culture, the second most used EV source, guidelines are much harder to define, as different cell types have different requirements for growth, and downstream applications are much broader, ranging from understanding basic EV biology to large scale therapeutic applications

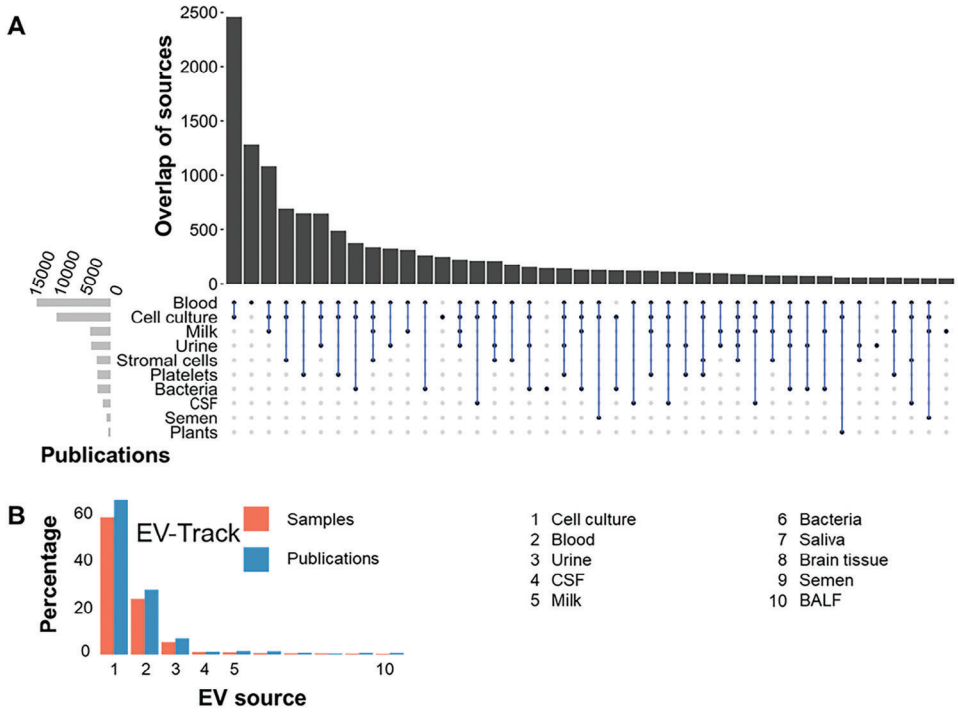

Figure 4. Most commonly used EV sources. A) The upset plot shows on the left the number of publications using EV sources as ranked from the most used on top to the least-used on the bottom. EV sources were identified in "material and methods" and/or "results" sections by keyword search. The upper histogram (in descending order) and the dot chart show the most occurring overlap of sources. Blue lines highlight the overlaps between sources. B) Percentage of the ten most-used sources for all samples/experiments (red) or publications (blue) listed in the EV-TRACK database for the period 2013–2022. Abbreviations: cerebrospinal fluid (CSF); bronchoalveolar lavage fluid (BALF).

with very different targets.[51,65] Milk EVs are an easily obtainable and scalable source of EVs for alimentary engineering and drug delivery.[66,67] The noninvasively obtainable bio-fluid urine represents a very attractive target for diagnostic applications. Recently published urine-EV isolation guidelines provide state-of-the-art recommendations.[68]

7. Counting EVs

EV quantification can be used for input normalization and thus precise execution and interpretation of assays.[51] Although total protein is often used for normalization, particle count, at least for a rather pure preparation, may reflect EV number more reliably.[56,69,70] Meanwhile, the particle: protein ratio of EV preparations is considered a surrogate of purity.[42] Of course, coisolating particles or abundant protein may confound any of these parameters, varying with isolation method. For example, in blood, lipoprotein particles are present at up to million-fold greater concentrations than EVs, and they often coisolate with EVs of similar size or density. Albumin is highly abundant in blood. Therefore, particle counts and particle: protein ratio can also be misleading.[27,71] To assess EV quantification strategies used in the literature, we next automatically selected publications reporting particle count and mode size for subsequent manual collection of records, because automated extraction of individual numbers from papers can be error-prone. We identified 2386 publications reporting particle counts. Just 104 of these studies (5.7%) reported particle: protein ratios in 297 datasets, and the remaining 2282 reported only particles/volume (**Figure 5**A). After manually extracting the particle counts to avoid false positive results, we individually analyzed the methods used to determine particle counts.[72] The vast majority (90.6%) of studies reporting particle: protein ratio used nanotracking analysis (NTA), 20 studies (6.4%) used resistive pulse sensing (RPS), six nanoparticle flow cytometry (NanoFCM), and two dynamic light scattering (DLS) to determine particle concentration (Figure 5B). Out of the 2386 studies, 538 also stated the mode size of their EV preparations as a size parameter less affected by outliers. Based on these data we created a general average mode size distribution of the EVs analyzed in original research articles over the past decade (Figure 5C). This size distribution interestingly showed a main peak with a mode size of 110 nm referring to small EVs (sEVs).[73] For the 104 publications providing the particle: protein ratio as an indicator of EV purity, the protein concentration was determined in the vast majority of 69.4% of the studies by bichinonic acid assay (BCA), 16.5% used Bradford assays, 5.4% used photometric protein measurement at 280 nm (e.g., NanoDrop; Figure 5D) and 4.7% Qubit. In studies reporting particle: protein ratio, EVs were most frequently derived from cell culture (46.4%), blood (plasma or serum; 21.9%) and tissue (8.3%) (Figure 5E). A 12-fold decreased mean particle:protein ratio was reported for density and precipitation-based isolation methods ($0.2 \pm 0.5 \times 10^{11}$ particles μg^{-1} protein) compared to size-based methods ($2.4 \pm 5.3 \times 10^{11}$ particles μg^{-1} protein; both mean ± SD) for cell culture and blood.[19,65,74] Density-based EV isolation was most commonly used in manuscripts

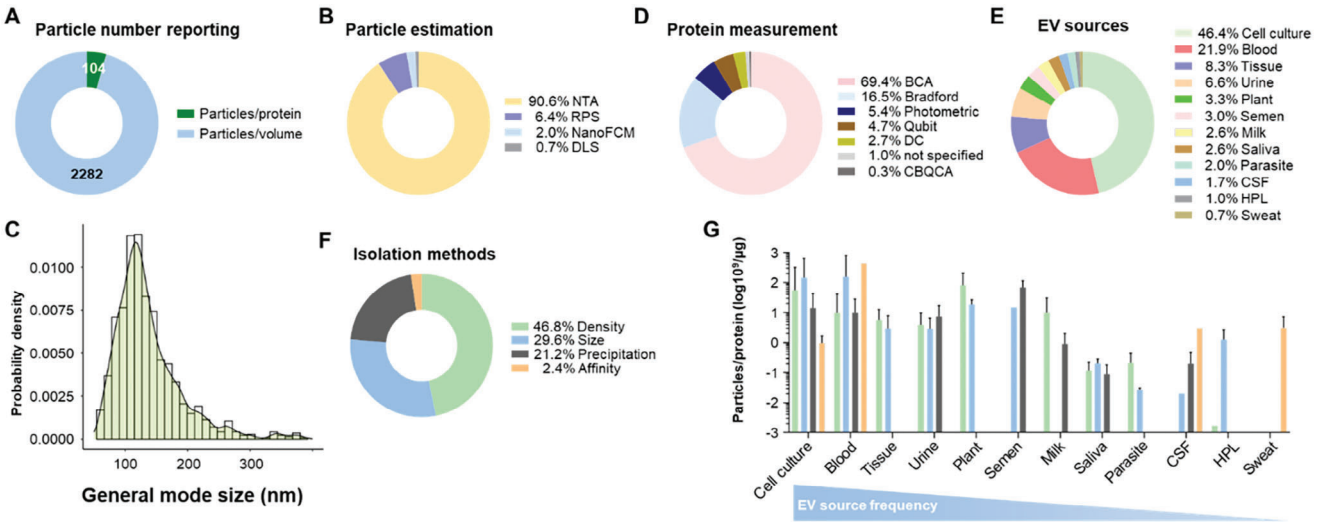

Figure 5. Reporting of relative versus protein-normalized particle quantification. A) Number of publications normalizing particle counts per volume or reporting particle: protein ratio. B) Detailed sub-analysis of the methods used for particle estimation in the 157 studies reporting particle: protein ratio. C) The computed mode size extracted from 538 publications (1185 values) accumulated into an average mode size of 110 nm. D–G) Supervised comparative analysis of 157 EV-related publications providing particle: protein ratio. D) Frequency of methods used for protein measurement. E) Percentage of sources used for EV isolation. F) Frequency of methods used for EV isolation. G) Log10 (particle/protein) ratio for EVs isolated from different sources shown in (E) by using density- (blue), size- (red), precipitation- (grey) or affinity-based (yellow) EV isolation methods as shown in (E). Color code in G) is the same as in F). Overall, 179 values were found in 137 publications; 15.33% were false-positive, as identified manually. Abbreviations: Nanotracking analysis (NTA); resistive pulse sensing (RPS); nanoparticle flow cytometry (NanoFCM); dynamic light scattering (DLS); bicinchoninic acid (BCA); detergent compatible (DC); 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA); conditioned medium (CM); cerebrospinal fluid (CSF).

that reported particle: protein ratio (46.8%). Another 29.6% used size-based isolation, 21.2% precipitation and only 2.4% used affinity-based methods for EV isolation (Figure 5F). Due to the limited number of particle: protein ratios reported, their variability and missing data for some isolation methods for other sources, stronger statements are not supported statistically (Figure 5G).

8. EV Identity

The MISEV2018 recommendations[33] and the most recently updated MISEV2023[75] regarding minimal information to be reported in studies of EVs highlighted the importance of proper characterization of EV fractions. In a previous analysis restricted to OA publications published until 2020, we already observed a constantly growing application of combined EV characterization methods.[76] Here, we found a continuation of this trend in all OA and non-OA primary research articles identified in PubMed 2013 – 2022 (Figure S11A–C, Supporting Information). Most publications (11 322 of 20 364 publications, 55.6%) employed two to four EV characterization categories (Figure S11A, Supporting Information). All categories were significantly more used over time (Figure S11B). Researchers increasingly used a combination of three or four characterization categories over time, following MISEV recommendations[33,77] (Figure S11C, Supporting Information). We also confirmed that most markers of EV identity belonged to MISEV category one: "*Transmembrane or GPI- anchored proteins associated to plasma membrane and/or endosomes*" including CD63, CD81 and CD9 and category two: "*Cytosolic proteins recovered in EVs*" including actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tumor susceptibility gene 101 (TSG101)[33] (Figure S12A,C, Supporting Information). At least two markers out of two different categories were used to identify EVs in 48.5% of publications indicating growing EV characterization efforts (Figure S12B, Supporting Information). The "top-ten" journals with the highest number of EV publications published a total of 6606 publications total (Figure S2C, Supporting Information).

9. Terminology and Application

Our analysis confirmed an apparent nomenclature change over time (**Figure 6**). Researchers increasingly adhered to recommendations by using the umbrella term "EV" (from 11.1% in 2013 to 48.6% in 2022). In contrast, use of the term "exosome" showed no significant change over the last decade, possibly reflecting a still-common but inaccurate use of the "exosome" term for various types of EVs.

The new term "small EVs" (sEVs), describing particles < 200 nm diameter,[1,33] was significantly more frequently used over the last years (< 0.1% in 2014 to 5.0% in 2022). A growing proportion of publications was found reporting diagnostic (e.g., biomarker) and therapeutic research (preclinical and clinical; the latter increasing from 10.2% in 2013 to 23.4% in 2022). Among terminology used by the authors we identified 94 keywords within the top 250 most frequently used keywords that demonstrated significant temporal variations. The use of the terms EV and sEV markedly increased, while the use of "microvesicle" declined. Notably, expressions associated with diagnosis, biomarkers, and therapeutics showed a significant rise over time (Kendall test, $p < 0.05$). A preponderance of terms linked to technological aspects (flow cytometry, nanoparticle tracking

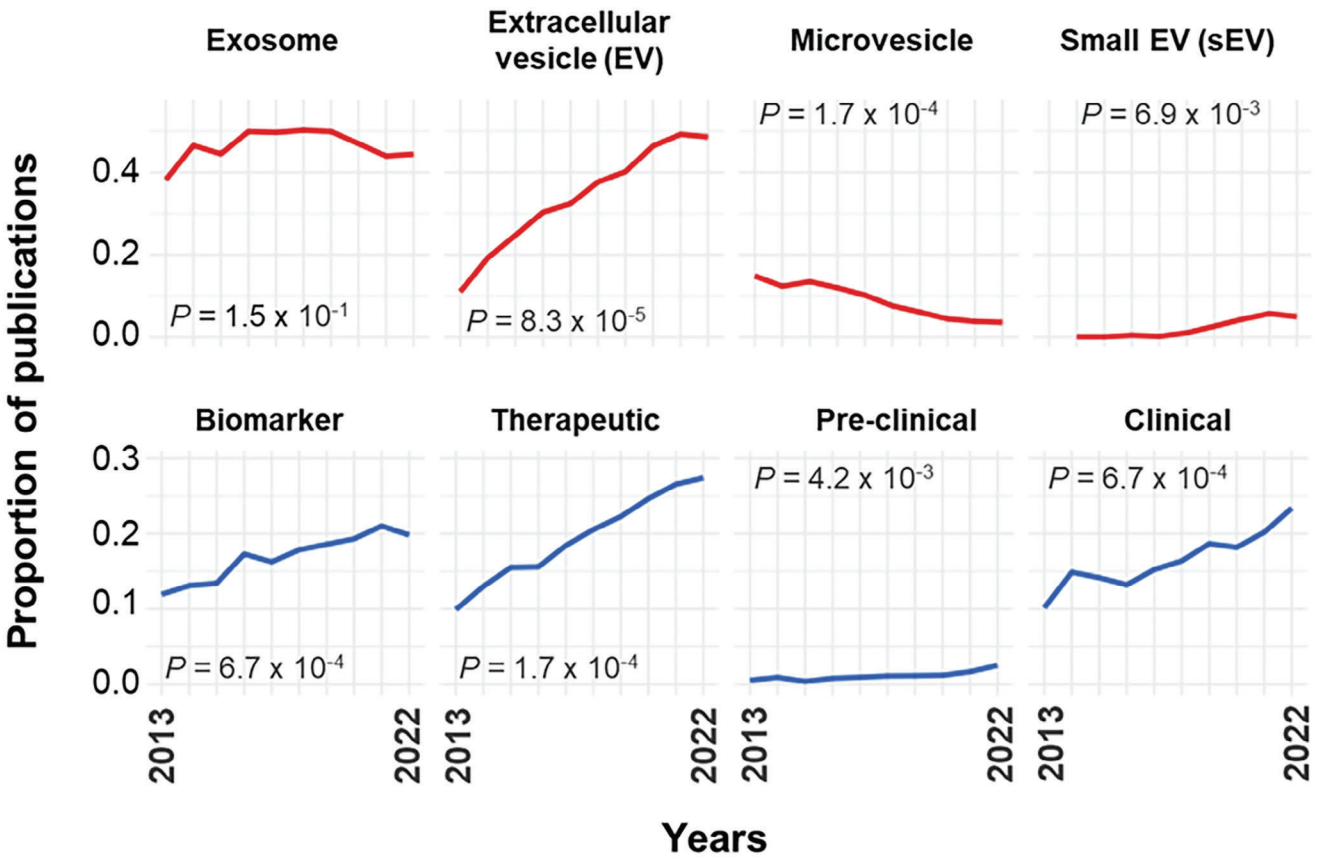

Figure 6. Selected changes of EV nomenclature and application over time. Line charts showing selected nomenclature terms (red) and application terms (blue) indicating significant changes over time; Kendall test, *p* values are shown within the respective plots. Keyword search was conducted only in title and abstract of the publications to reduce the risk of false-positives.

analysis, mass spectrometry, electron microscopy, ultracentrifugation) and EV cargo (microRNA, proteome, lipids) showed diminishing prevalence over the past decade. Terms related to applications (diagnosis, biomarkers, and therapeutics) increased. These trends paralleled the transformation of the field from initial discovery and characterization of EVs to their implementation in diagnosis and therapy, in clinical trials (Figure 6 and Figure S8B, Supporting Information).

10. Reporting and Information Tools

Research transparency including rigorous reporting of material, methods and result details is crucial, particularly in a technology-driven rapidly expanding field. For this purpose, the EV-TRACK database[34] was created in 2017 to record detailed parameters for EV isolation and characterization methods. Based on the amount of data reported, an EV-metric score is generated to reflect the degree of reporting and to motivate users to reach the highest standards. Several other web-based EV research expert resources are available, including ExoCarta, EVpedia, and Vesiclepedia, which catalog multiple datasets and cover interest areas from molecular mechanism to disease pathophysiology.[78]

However, there are no tools available supporting in-depth keyword search in the rapidly growing number of EV research articles. To address this gap, we created a searchable database (www.ev-zone.org). XML files downloaded from the PMC database (www.ncbi.nlm.nih.gov/pmc; currently 15 632 publications) will be automatically updated with segmentation for research articles, including searchable sections (abstract, introduction, materials, and methods, results/discussion, conclusion, figure captions). We provide an interactive and customizable text mining platform accelerating the search process. This approach offers an advantage over the conventional PMC search, which permits searching only within the entire text corpus and may identify keywords in introduction/discussion sections that otherwise do not present data related to these keywords. Our database not only allows classical searches using Boolean expressions, but also the use of regular expressions ("regex" or "regexp"). The latter, being composed of special characters (.*?+^$[]\{}()|) and literal characters, enable complex pattern-matching searches. An enhanced search extracts complete sentences containing the identified keyword(s) and provides the number of occurrences of the keyword within the manuscript, together with their location. Keyword selection is a critical factor, but defining multiple supplementary keywords can partly compensate for this limitation.

11. Conclusion

In this review we explored an unorthodox approach of clustering published results in the EV research field over the past decade

to identify connectivities between EV source, isolation method, cargo and function. Using this unsupervised strategy, publications reporting higher number of EV cargo parameters (cluster 10) did not cluster with those reporting higher number of functional parameters (cluster 5 and 13). Such a decoupled view on either EV cargo or function could be driven by distinct research questions with a focus on either EV biomarkers or therapeutic EV application. Methods involved, resources availability and expertise required for sophisticated EV cargo characterization versus functional validation can play a role. To a certain extend clustering may also represent trends in terms of technology, EV sources and cargo of interest, as well as function assay popularity. Weak clustering of studies with cargo and function parameters can also result from a lack of in-depth mechanistic studies. Additional correlation analysis detected a significant association between certain EV sources with defined isolation, cargo or function parameters. Our key observations and respective experimental recommendations are summarized in Box 2 and the unexpected significant correlations in Box 3.

Box 1: EV Commons—Names and Nomenclature

Extracellular vesicles (EVs) are lipid bilayer membrane-enclosed particles secreted by virtually all cell types. The term was first used in the 1970s and became more visible through research on bacterial outer membrane vesicles in the 1980s and cancer cell-derived vesicles in the 1990s. The use as an umbrella-term for the various types of cell-derived vesicles was endorsed by the research community-based recommendations on "minimal information for studies of EVs" (MISEV2014, updated MISEV2018).

Various types of "exosomes", derived from the intracellular endosomal compartment, and "ectosomes," derived from the outer cell membrane by highly orchestrated budding mechanisms, have been discovered so far (see glossary). Despite growing insight into EV biogenesis, the term exosome is still frequently used for all EVs, as is the term microvesicle for describing all outer cell-membrane-derived EVs, including those smaller than 200 nm, which are clearly below "micro." In light of the growing number of newly discovered EV subtypes, it would be appropriate to curtail misleading use of specific and clearly defined terms. We recommend using the umbrella-term EV unless a specific EV subtype can be clearly identified in a study.

Box 2: Key Observations and Recommendations

- Strong associations exist between EV source, isolation, cargo and function. Different EV sources may be considered when searching for a specific cargo, and before targeting a function of choice. Preanalytic parameters that can change source characteristics and impact EV quality and quantity need to be validated.
- EV isolation method matters. Size-based EV isolation and precipitation showed particularly strong positive and negative correlation with several cargo and function parameters, respectively.
- Combining isolation methods can increase EV purity, partly at the expense of yield. The impact of combining various isolation and analysis methods on identity, purity, cargo stability and functionality of EVs isolated from different starting material deserves systematic analysis.
- Parameters to be considered when selecting EV source and appropriate isolation and analysis methods include sample type, cell source and culture condition. Start volume as well as other physical parameters and biochemical composition may require specialized downstream methods.
- Particle size and density affect isolation methods for EVs and can result in various degree of coisolation of protein aggregates, lipoproteins or other nonvesicular extracellular nanoparticles.
- Nanoparticle number/concentration can differ from EV number/concentration in samples that contain nonvesicular nanoparticles. Blood-derived EV preparations might be checked particularly for lipoprotein content that can profoundly vary in individual blood plasma or serum samples.
- A decoupled view on either EV cargo or function as observed for the past decade could be driven by distinct research questions with a focus on either EV biomarkers or therapeutic EV application but may also indicate a lack of comprehensive mechanistic studies.
- More mechanistic studies are needed to provide a better understanding of the basic physiological functions of EVs and how they can be influenced in health and disease.
- Substantial methodological guidelines for EV isolation and analysis are available and deserve attention toward increasing scientific rigor already in advance of planning EV research studies.
- Nomenclature harmonization may be considered in view of the growing number of differently designated cell lineage-specific exosome and ectosome type of EVs discovered recently.
- AI tools can aid selecting most promising combinations of EV isolation methods for obtaining optimum cargo retrieval toward selected experimental, diagnostic or therapeutic function as highlighted by this comprehensive analysis.
- Core methods reporting could be considered as part of the initial quality check in scientific journals. Incentives are needed to motivate regular reporting of key parameters of academic EV studies. Editors in quality journals may consider requesting EV-TRACK or equivalent report summaries and define a metric threshold in advance of selecting articles for peer review.

Box 3: Unexpected Significant Correlations

- Persistent use of density-based isolation methods was observed together with a lack of correlation with cargo and function parameters identified in this study.
- The absence or presence of density-based methods separated the unsupervised cluster landscape in a "northern hemisphere" using density-based isolation methods and a "southern hemisphere" not using these methods.
- The use of size-based methods increased over the last decade, indicating that nano-sized vesicles can meanwhile be obtained effectively based on their size rather than their variable density.
- Size-based EV isolation showed positive correlation with EV uptake and distribution as well as protein analysis and negative correlation with miRNA data.

- Blood as the most common EV source showed the highest number of interactions with density-based isolation as the most used isolation method and protein cargo analysis, but below the expected frequency (negative association).
- Precipitation as the second most used isolation method associated positively with the three major sources blood, cell culture and milk, but was less frequently used than expected for most other sources as indicated by a high number of negative associations.
- EV isolation by precipitation negatively correlated with uptake/distribution analysis and protein cargo, and positively with miRNA.
- Most significant associations were found for immunomodulation as an over-represented functional test in studies with cerebrospinal fluid and bacteria.
- Source-function correlation showed most significant association, followed by source-cargo and source-isolation.
- Particle: protein ratio was determined only in a minority of reports over the past decade indicating space for improvement toward better understanding of EV purity and identity, and its impact on cargo and function analysis.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

extracellular vesicles (EVs), isolation methods, machine-learning, meta-analysis, nanoparticles, regenerative medicine, stem cells

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