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REVIEW

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# Quantifying extracellular vesicle heterogeneity: the effect of process conditions on protein cargo for skin therapy

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## Abstract

Extracellular vesicles (EVs) contain a variety of proteins with anti-inflammatory and immunomodulatory properties that offer promising benefits in skin therapy applications. An influx of EV proteomic studies in recent years has created the opportunity for a detailed comparison of EV heterogeneity between studies in the context of therapeutic applications. Although several process conditions are known to cause variability in EVs, little has been done to quantify the impact of these factors on the nature of EV protein cargo. This review aims to both compile publicly available EV proteomics data and quantitatively estimate. the impact of process conditions on protein cargo—particularly in the context of skin therapy applications. Of roughly 400 articles, 52 relevant proteomic studies were identified within the last 15 years. Across studies, 40% of the 13,000 observed proteins were identified in only a single study. EVs in general were found to be highly variable, with mixed effects models only able to account for 25–60% of variance when considering factors such as EV source, medium, isolation method, LC-MS ionization, and protein search algorithm. Overall, MSC-derived EVs contained a greater fraction of proteins within pathways associated with wound healing and skin therapy (immune system, hemostasis, extracellular matrix organization, and cellular response to stress) as well as the most number of unique proteins when compared to all other analysed EVs. Although EVs are a promising tool within skin therapeutics, the overall variability in protein cargo underscores the need for standardized methodologies to fully elucidate the impact of process conditions on EV cargo.

**Keywords** Extracellular vesicle, Proteomics, Stem cell

## Introduction

Small extracellular vesicles (sEVs), including exosomes and ectosomes, are a subclass of extracellular vesicles (EVs) less than 200 nm in diameter that play an active role in cell-to-cell communication and the transfer of bioactive cargo [1, 2]. This role in molecular transport has been linked to a number of regenerative, immunomodulatory, and anti-inflammatory properties, which have led to applications in tissue regeneration, wound repair, and treatment of skin diseases, such as psoriasis [3–5]. In addition to good stability, low toxicity, and low immunogenicity, sEVs have also demonstrated the potential for higher levels of therapeutic effectiveness than their parental cell in cell therapy applications [6]. These properties have positioned them as a promising and safer alternative to traditional cell therapy [7]. Among their many possible applications, sEVs have shown specific promise for skin therapy—multiple animal studies have shown that sEVs accelerate or

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promote wound healing [8–10], with human studies also demonstrating that sEVs reduce the appearance of wrinkles and generally improve skin appearance [11–16]. Although there are no EV-based therapies currently approved by the Food and Drug Administration (FDA) in the United States, at least 10 companies have released sEV-based skincare products (as of 2023) based on these promising results, with more product lines in development [17]. Despite this rapid commercialization, therapeutic approval has been hindered by concerns surrounding the standardization of sEV isolation and characterization [18, 19], emphasizing the need for quantifying and improving reproducibility.

As the therapeutic potential of sEVs, and EVs more broadly, is driven primarily by the microRNA (miRNA) and protein they transport, characterization efforts naturally focus on EV cargo [20], which has generally been observed to be highly variable. Not only does EV cargo reflect both the state and identity of the parental cell [21], but it is also influenced by process conditions, such as growth medium, induction, and isolation [22–24]. Indeed, the effect of isolation on EV purity is a well-established concern, with many, including the International Society for Extracellular Vesicles (ISEV), calling for the standardization of methods used and reported to increase reproducibility and comparability of collected data, with the Minimal Information for Studies of Extracellular Vesicles (MISEV) serving as a critical framework for achieving this [2, 18, 25, 26]. As it stands, the few molecules that are known to be present consistently across EV samples are practically limited to EV biomarkers [25, 27].

The question of EV cargo has received significant attention over the past several years. A number of EV cargo review articles have been published on topics that include broad perspectives (such as general contents and purification methods) [28, 29], the function or application of common EV proteins [30], and the impact of EV cargo on specific diseases and biological processes [31, 32]. Although some reviews have attempted to compare the specific results of EV proteomics between studies, they have generally been restricted by cell type or limited by the scope of their analysis [33–36]. A review by Poupardin et al. [33], for example, employed machine learning to text-mine 20,364 EV research articles, uncovering correlations between EV source, isolation methods, cargo, and function, but limited its analysis of cargo to six general categories. The aim of this review is to build and expand on these studies by directly assessing the quantitative consistency of EV proteomics across a variety of parental cells as well as determining the impact of EV source, culture conditions, isolation techniques, and analytical methods on EV cargo. Although both proteins and miRNA are important to EV function, this review places its focus on proteomics, as the number of transcriptomic studies was found to be insufficient for quantitative analysis.

## Methods

### Eligibility criteria

Within the literature search, EV articles were included if they were a) written within the last 15 years, b) made use of human cells, and c) reported identified proteins in an accessible format, e.g., via UniProt accession number. The search primarily focused on mesenchymal stem cells (MSCs), platelets, keratinocytes, and fibroblasts based on their known benefits in skin therapy applications; however, studies making use of other cell types were not excluded from the results, although they were not explicitly used as search terms. The majority of published articles were excluded based on the last of the inclusion criteria—data availability (or lack thereof). Many articles neglected to include protein identities entirely or gated the relevant information behind requests for author contact. As none of our requests for supplementary data yielded a response, any data not included with the article itself was deemed effectively unavailable. Although some articles provided raw spectrometry data, most commonly via the ProteomeXchange consortium, this was found to be of limited help as it was rarely sufficient to reproduce results (due to missing reference databases/settings and unavailable software).

### Search strategies

Keyword searches were conducted using a combination of "extracellular vesicle", "exosome", "proteomics", and various cell types including "MSC", "platelet", "keratinocyte", and "fibroblast" using the Scopus database—selected for its broad coverage of available literature [37]. Additionally, the EV-specific database, EV-Zone [33], was used to find EV articles containing the keywords "proteomics" along with one of the aforementioned cell types.

### Data collection and analysis

These literature searches returned 384 unique articles from Scopus and 30 unique articles from EV-Zone. Of the 414 identified articles, 52 met a subset of the stated criteria, with 36 articles providing accessible protein identities and an additional 16 articles providing only the total number of proteins identified. All 52 articles were utilized during analysis involving total protein counts, with the latter 16 excluded from comparisons that utilized specific proteins. A flowchart detailing the literature review strategy has been provided in Fig. 1.

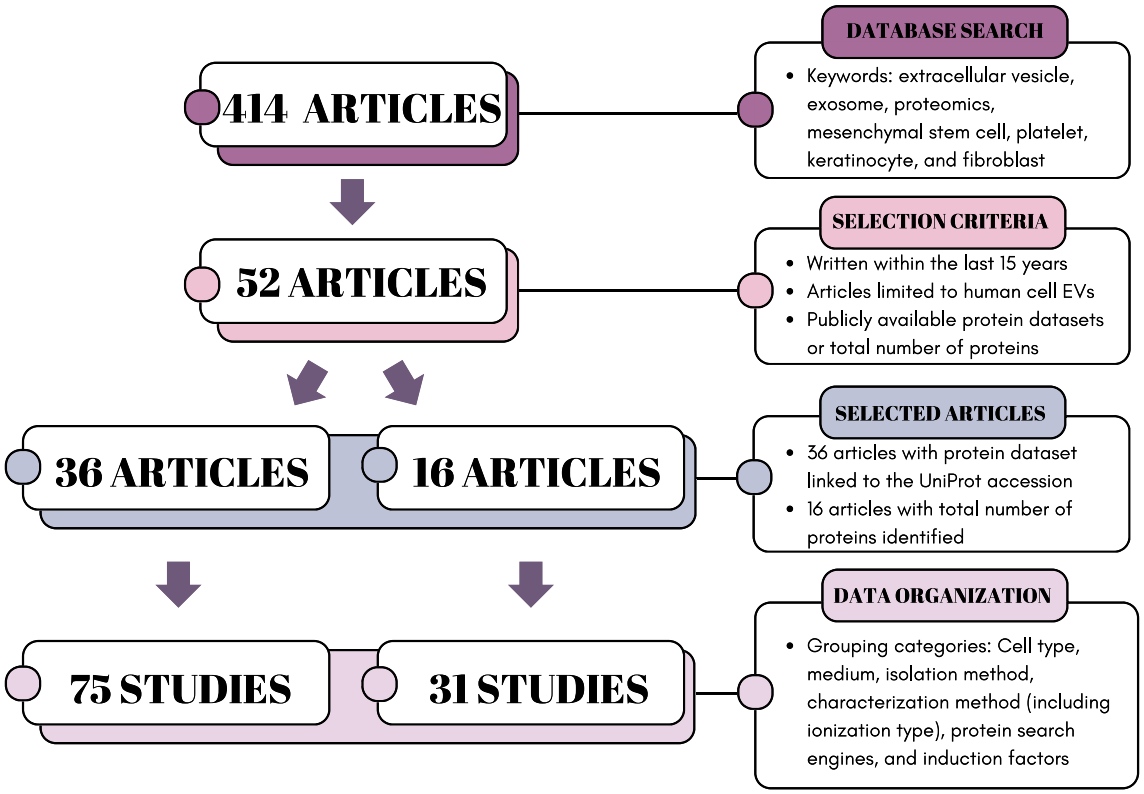


**Fig. 1** Overview of the identification, screening, and inclusion of literature results

All protein data was organized into a consistent tabular format using the R programming language (version 4.4.0), mapped to UniProt accession number, protein name, presence or absence of the protein, EV source, and an author-date code. In addition, the medium, isolation method, characterization method, protein search engines/analysis program, and induction factors were recorded for all articles. The following text will use the term "study" to refer to a consistent set of process parameters, with some articles reporting on multiple such "studies". As such, there are 75 studies within the 36 articles containing specific protein details and 31 studies within the articles only specifying the total number of proteins. While the majority of studies referred to the subject of their analysis as exosomes, approximately 20% referred to them as EVs or microvesicles; therefore, the term 'EV' will be adopted for consistency throughout the review in accordance with MISEV 2024 guidelines. All data collected and tabulated as part of this review have been made available at https://doi.org/10.5281/zenodo.13870014. It should be noted that although attempts were made to include data from a wide range of EV topics, preference for cell types with therapeutic potential may have restricted the scope of the proteomic studies identified in the search. As a result, the trends observed in this review may be specific to the proteomic studies analysed within and may not reflect the full range of EV proteomic studies.

## General overview

### EV source

Overall, 30 different EV sources were observed across all reported studies. These were broken down into six main categories described in Table 1. MSC cells were considered in the largest fraction of studies at 57%, while the remaining data is split between cancer cell lines (12% of studies), platelets (8%), immune cells (7%), keratinocytes (4%) and others (12%). While cancer cell lines are not used in skin therapy, they were included in the analysis to serve as a baseline for comparison. The large fraction of MSC cells stands out in comparison to other cell lines, underscoring the perceived benefits of MSC cells as EV producers [38, 39]. As MSC cells vary depending on the tissues they are derived from as well as the associated age of their donors [40], these were further split into "old"—adipose tissue (AD), bone marrow (BM), dental pulp (DP), endometrial, and olfactory mucosa (OM)—and "young"—umbilical cord (UC), Wharton's jelly (included in the analysis as a subset of UC), and

**Table 1** Category breakdown of EV sources

| Source category | EV source | Studies |
|---|---|---|
| Cancer cell line | A549, DKs8, H1975, H1993, U87, HeLa, Huh7 | [42–45] |
| Immune cell | T-Cell, plasma, natural killer (NK) | [46–50] |
| Keratinocyte | Keratinocyte | [51–53] |
| MSC | Adipose tissue (AD), bone marrow (BM), dental pulp (DP), endometrial, ESC-derived MSC, liver, olfactory mucosa (OM), umbilical cord (UC), undisclosed MSC | [29, 54–84] |
| Other | Fibroblast, jurkat, CD133 +, CPC, embryonic stem cell (ESC), HDC, iPSC, lymphatic endothelial, saliva | [46, 57–59, 75, 85–88] |
| Platelet | Platelet | [89–92] |

embryonic stem cell (ESC)—categories based on the characterization of Zhang et al. [41]. Of all the MSC studies, approximately three-fifths utilized old MSC cells and the remainder young MSC cells. Furthermore, old MSC cell studies predominantly consisted of BM (at approximately half), while young MSC cells were predominantly UC cells (approximately a third).

### Media

Cell culture media serves as a source of nutrients to support cell growth for EV production in vitro. In this study, the media were categorized into three main types: classical media, undefined commercial media, and natural sources. Classical media was used in 50% of the reviewed studies. These include standardized formulations like Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 Medium, which account for three-fifths and one-fifth of studies in this category, respectively. Undefined commercial media comprised of undisclosed proprietary formulations was used in 35% of the studies. 15% of studies did not make use of growth media, having isolated EVs directly from biological fluids, such as plasma or saliva, and are referred to as "natural" in this work. Of the studies that used media, 52% used EV-depleted serum to reduce EV contamination. However, EV-depleted serum may still contain residual EVs that can be inadvertently collected during isolation procedures [6, 93].

### Isolation method

Among the isolation methods summarized in Table 2, centrifugation (including differential centrifugation, differential ultracentrifugation, and density gradient ultracentrifugation) was found to be the primary isolation method for 68% of studies, with an additional 2% using ultracentrifugation in combination with precipitation. The next most popular methods were precipitation (15% of studies), SEC (6%), and filtration (5%), with the remainder associated with other less common methods, including protein-based affinity, membrane affinity, magnetic capture, and fluorescence-activated cell sorting. These observations are somewhat comparable to previous surveys of EV isolation reporting approximately 80% of studies relying primarily on ultracentrifugation [94], with perhaps a slight drop in popularity. The popularity of centrifugation may account for some of the observed variability in reported proteins, as centrifuge samples are more likely to be contaminated by non-EV proteins [28]. However, most methods observed large variability in the total number of proteins identified (Table 2), where affinity and SEC observed particularly large confidence intervals due to how infrequently the methods are employed. For studies employing ultracentrifugation to isolate EVs, the most common relative centrifugal force was 100,000 g (71%), followed by 120,000 g (10%). However, the time duration used had a much greater variability, ranging from 60 min to 18 h. Among studies employing precipitation methods, 72% used the ExoQuick-TC kit (SBI, EXOTC50A-1). Overall, there was a lack of consistency in the preprocessing steps employed by all methods, highlighting the need for standardized protocols.

### Characterization method

The vast majority of the studies relied on mass spectrometry (MS) for proteomic analysis. Only a single article used an alternative method—western blotting with a differential average (DAve) and differential confidence index (DCI) for protein identification/quantification [58]. The majority of MS studies (90%) used an unlabelled technique, while the remainder made use of either tandem mass tags (TMT) or isobaric tags for relative and absolute quantitation (iTRAQ) labelling. Between labelled and unlabelled MS, there was no significant difference ($p = 0.3$) observed in the number of proteins identified—where labelled observed an average of 1200 proteins and unlabelled observed approximately 970 proteins. The largest differences in MS analysis were based on ionization source, with 48% of the studies using spray ionization, 38% using collision ionization, and 14% categorized as "other" (including matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) or unspecified ionization methods). Analysis was also found to differ with respect to data analysis software—with Proteome Discoverer (39% of studies), MaxQuant (26%),

**Table 2** Outline of common EV isolation methods, with a comparison of the average number of proteins identified using each method (expressed as mean ± 95% confidence interval)

| Method | Description | Average protein count | Studies |
|---|---|---|---|
| Differential ultracentrifugation | Samples are centrifuged sequentially at increasing speeds to remove live cells (300-800 g), dead cells (1200-3000 g), and debris (10,000–20,000 g). Samples are then ultracentrifuged to isolate EVs (100,000–200,000 g). A density gradient can be used to increase EV purity by creating distinct density bands to suspend EVs in a band with similar density | 762 ± 142 | [42, 43, 46, 48–52, 54, 56–59, 61, 63, 67, 68, 73–77, 79, 81–85, 87, 89–91] |
| Precipitation | EVs are precipitated by lowering their solubility via the addition of super hydrophilic polymers, such as polyethylene glycol (PEG) | 1285 ± 684 | [44, 45, 47, 62, 64–66, 72, 80] |
| Size Exclusion Chromatography | The sample is passed through a porous column matrix with different sized channels. EVs are isolated based on size as large diameter particles cannot pass through small channels and elute out of the column faster, while small diameter particles pass through a maze of channels and elute later | 1385 ± 1025 | [29, 55, 92, 95] |
| Ultrafiltration | EVs are separated by size using membrane filters. Larger particles are first removed by passing through 0.8 and 0.45 μm filters. EVs are isolated by passing through 0.22 and/or 0.1 μm filters | 643 ± 565 | [69, 70] |
| Affinity | EVs are isolated based on surface marker recognition by antibodies. The sample is incubated with immobilized antibodies on magnetic beads or plates that selectively bind to EV markers | 2351 ± 4321 | [50, 53] |

Protein Pilot (9%) and Mascot Distiller (9%) as the most common[1] — as well as search engine, with Mascot as the most frequently used (45%), followed by Andromeda (23%) and SEQUEST (7%). As previous studies have shown that only 50–85% of identified proteins overlap between search engines [96], the variation in analysis methods is likely to be a substantial factor in inconsistencies that complicate comparison between studies.

## Protein counts

### Overall protein variability

Of the approximately 13,000 distinct proteins identified across all articles, 40% were exclusive to a single study (Fig. 2A). In fact, protein identification was found to be so variable that only 38 of the 13,000 proteins were present in more than half of the studies. These trends can be observed across isolation methods as well, with centrifugation and precipitation studies identifying a significant number of unique proteins (Supplementary Fig. 1). Although proteins were observed more frequently among studies in methods such as SEC and filtration, these methods were used less frequently overall. Therefore, the repetition of proteins may be more representative of the relationship between studies within the same article than the type of isolation method used. The most commonly identified proteins generally fell in line with previously reported top 100 proteins identified by ExoCarta [97] (Fig. 2B), corresponding to ubiquitous expression in cells and important roles in cellular processes such as apoptosis regulation and metabolism [98]. However, even these proteins were subject to considerable variability—18 of the 100 proteins generally considered the most frequently identified appeared in less than half of the studies. Furthermore, while CD9, CD81, and CD63 are commonly used in commercial kits to isolate EVs via immunoaffinity, they were only identified in approximately 50–70% of studies. Nevertheless, 95% of all studies included at least one protein indicative of EV presence from the first two categories of the five-category framework recommended

[1] Other softwares (10%) including Bioworks browser, Bruker Compass Data Analysis, FunRich, Partek Genomics Suite, Scaffold, and Spectrum Mill were rarely repeated between studies, and the remainder were unspecified or utilized in-house programs.

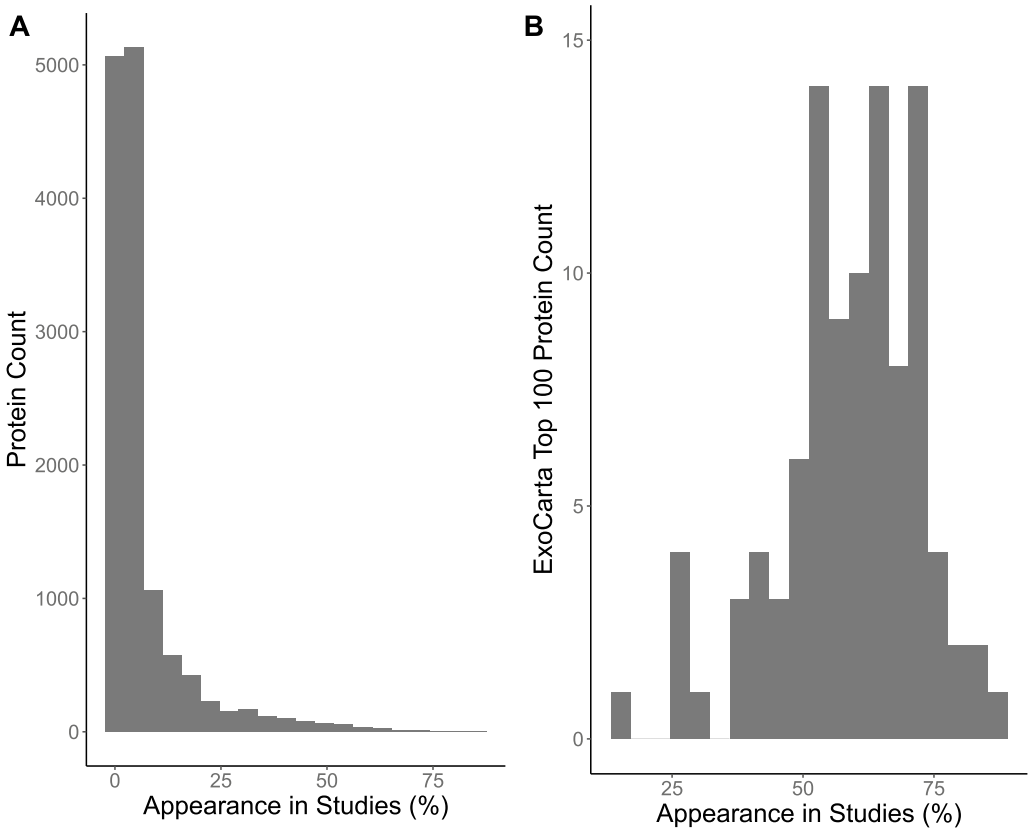


**Fig. 2** The total number of (**A**) proteins (based on UniProt accession number) and (**B**) top 100 ExoCarta proteins identified within studies.

by the MISEV 2023 guidelines [2]. This underscores the limitation of relying on individual biomarkers, as they may not comprehensively capture the full spectrum of EVs. Despite evidence indicating EV presence in most studies, 91% also reported contaminants (in accordance with category 3 of the five-category framework), with L-lactate dehydrogenase and albumin being among the most commonly detected.

Protein uniqueness was also compared in terms of parental cell line (Fig. 3). Of all the cell lines, the largest number of overall proteins was identified in EVs derived from MSCs — approximately 10,300 (of 13,300 total distinct) proteins. Of those proteins, approximately 4450 were uniquely identified in MSC-derived EVs, indicating an overall heterogeneity within MSC EVs. On the other hand, only 528 unique proteins were identified in "other" sources despite the variety of different EV sources within the category. Despite the large number of uniquely identified proteins, hierarchical clustering revealed a close relationship between EVs derived from immune cells and cancer lines, with 70% of proteins in immune cell derived EVs overlapping with proteins in cancer cell derived EVs.

## Protein characterization

### Cellular localization

The large degree of heterogeneity in unique protein identity (and the general ubiquity of the few commonly identified proteins) begs the question of whether reported proteins are truly specific to EV cargo as opposed to other sources within the parental cells. As EV cargo is believed to result from a highly selective sorting mechanism that incorporates many targeted signaling and functional proteins from diverse cellular regions [99, 100], protein localization analyzed using gene ontology (GO) cellular component terms serves as a simple means of determining if observed patterns agree with expected trends. A summary of the top terms is presented in Fig. 4. Overall, approximately 40–80% of the identified proteins are localized to the cytoplasm, extracellular space, and cell membrane. This general pattern aligns with existing literature, which suggests that EV cargo predominantly originates from the extracellular space, cell membrane, or cytoplasm, with proteins from compartments such as the endoplasmic reticulum, nucleus, and mitochondria either under-represented or absent [101]. However, our results also suggest that approximately 30–60%

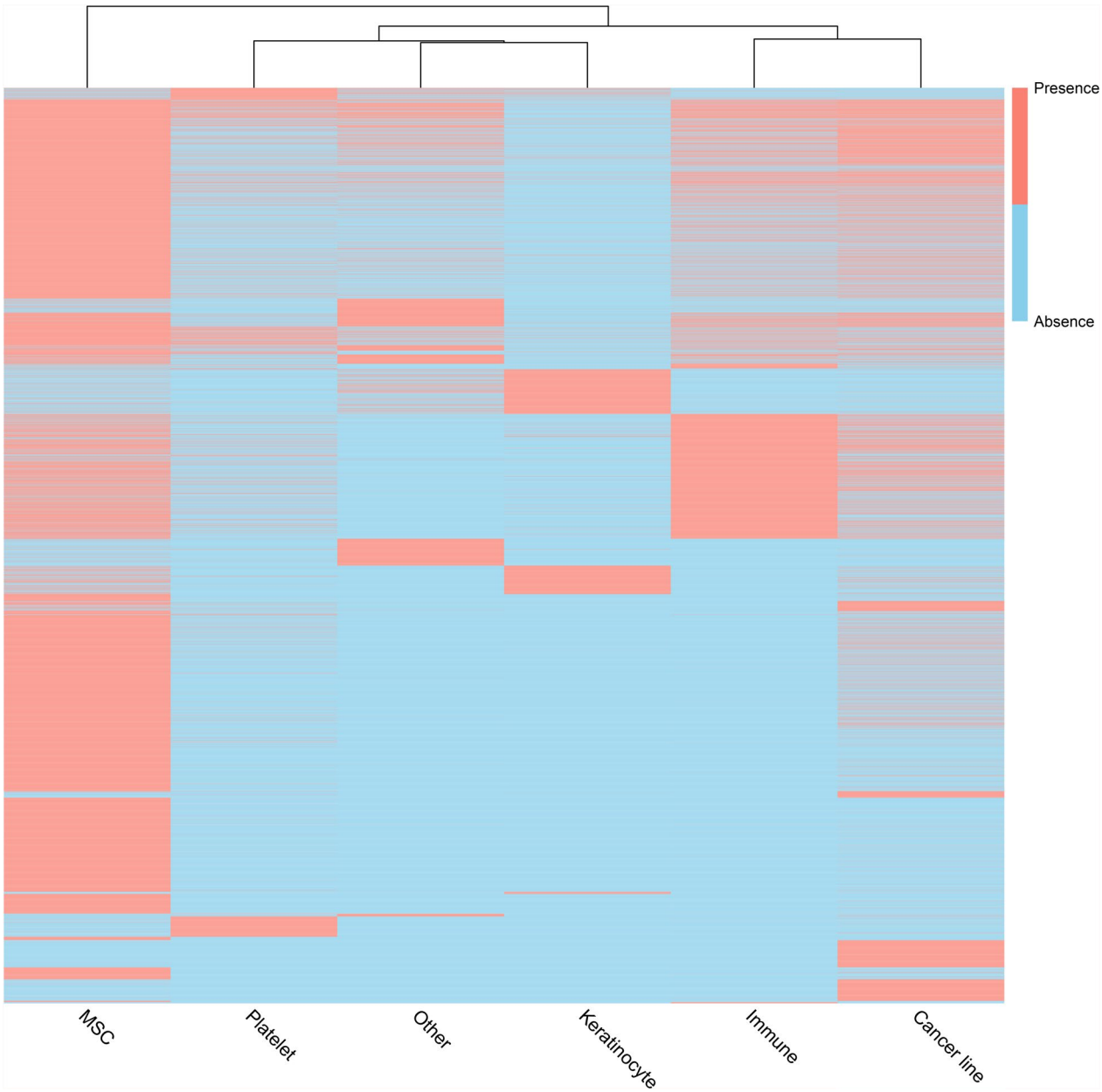


**Fig. 3** Heatmap of the proteins identified (but not present in all cells) across EV sources

of the identified proteins are localized to the nucleus, which does not agree as well with reported trends. EVs from cancer cells, in particular, as seen in Fig. 4A, were found to have, on average, more proteins localized to intracellular organelles, potentially due to metabolic changes and stress [102]. Overall variability was found to depend on cellular compartment, with membrane proteins showing the greatest consistency across all studies, with a coefficient of variance of 15%. In contrast, extracellular proteins showed the highest variability, with differences of up to 80% in EVs from the same cell type and those obtained from the same method of isolation. This variability could result from contamination of extracellular proteins in the conditioned media due to different purification strategies. For instance, methods such as ultracentrifugation and precipitation, which showed the greatest variability in extracellular proteins, are frequently associated with such contamination, as they

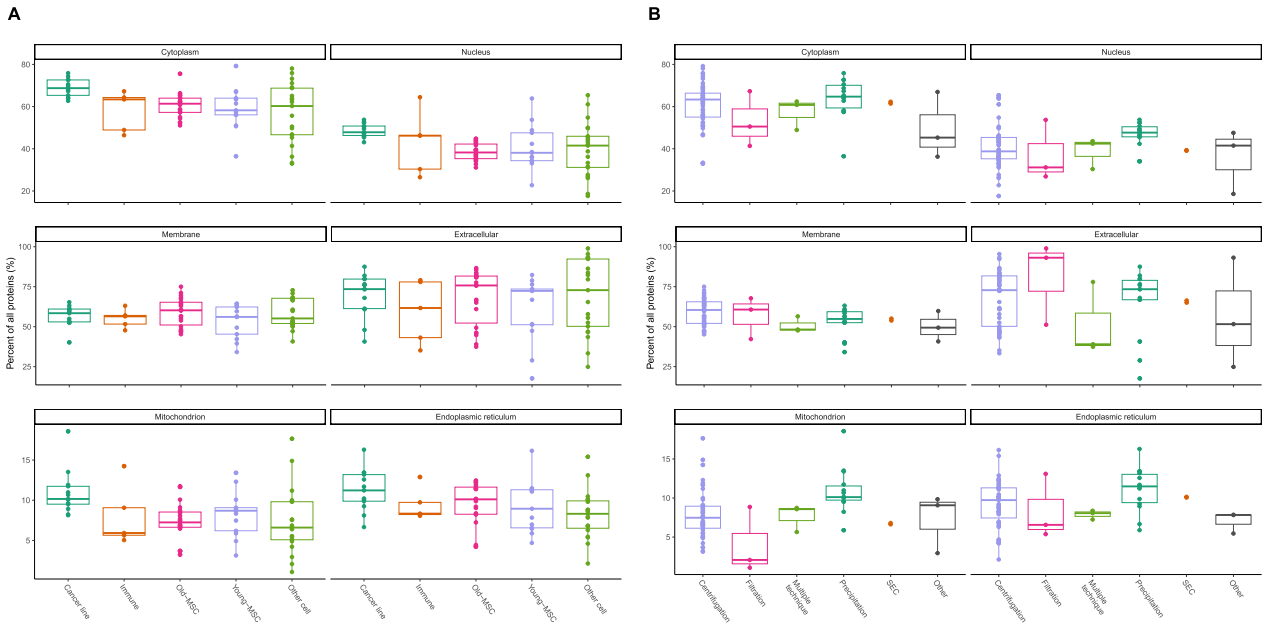


**Fig. 4** Percentage of observed proteins, based on A. EV source and B. isolation technique, originating from various cell locations. To simplify the analysis, GO terms 'cytoplasm' and 'cytosol' were combined to 'cytoplasm'; 'plasma membrane' and 'cell surface' to 'membrane'; 'extracellular region', 'extracellular space' and 'collagen containing extracellular matrix' to 'extracellular'; and 'nucleus', 'nucleolus', 'nucleoplasm', 'nucleosome' and 'nuclear envelope' to 'nucleus'

have a greater propensity to co-isolate soluble media proteins alongside EV [103, 104]. On average, studies that employed precipitation for isolating EVs also had a higher fraction of proteins localized to the cytoplasm, nucleus, mitochondrion, and endoplasmic reticulum.

### Protein function

As individual proteins can play multiple roles within biological systems, large variability in protein identity does not necessarily translate to function. Generalized protein function was estimated with pathway analysis using Reactome (Version 89 [105]). Reactome pathway analysis was chosen as it offers key insights not only on the functions of the proteins, but also on the pathways and reactions impacted by the cargo. Pathway selection for this analysis focused on anti-aging and the stages of wound healing as key clinical and commercial goals within the context of EV skin therapy. Anti-aging involves promoting the formation and rejuvenation of extracellular matrix (ECM) components, such as collagen, to improve skin elasticity and prevent free radical induced skin damage through antioxidant activity [106, 107], while the stages of wound healing involve hemostasis combined with an appropriate inflammatory and immune response [106–108]. With these functions in mind, EV sources were compared by determining the total number of proteins identified within several top Reactome pathways relevant to skin therapy, including extracellular matrix organization, cellular response to stress, hemostasis, and the immune system [109]. The analysis was limited to the presence/absence of proteins, as expression levels were not consistently reported and would further constrain the available data.

Similar to protein counts, the overall results were highly variable, with differences in fractions of observed proteins per pathway, ranging up to 45% across studies within a given cell type (Fig. 5), reflecting the variability in the number and types of proteins identified. This high degree of variability may not only highlight the impact of culture, isolation, and characterization methods, but also the state of the cell at the time of EV production. On average, MSC-derived EVs contained the highest fraction of proteins relative to all proteins in the aforementioned pathways when compared to other groups with more than 5 studies. In the ECM organization pathway, 13 of the 15 studies with the highest number of proteins belonged to MSC-derived EVs. On the other hand, keratinocytes consistently observed the lowest average fraction of proteins within all main pathways. Similarly, cancer cell derived EVs were found to consistently contain less than 20% of proteins in any given pathway with the exception of one study on HeLa cells [45]. While MSC-derived EVs generally had high fractions of proteins belonging to all four pathways, platelet-derived EVs were more enriched in proteins involved in hemostasis compared to others, while NK-derived EV studies had

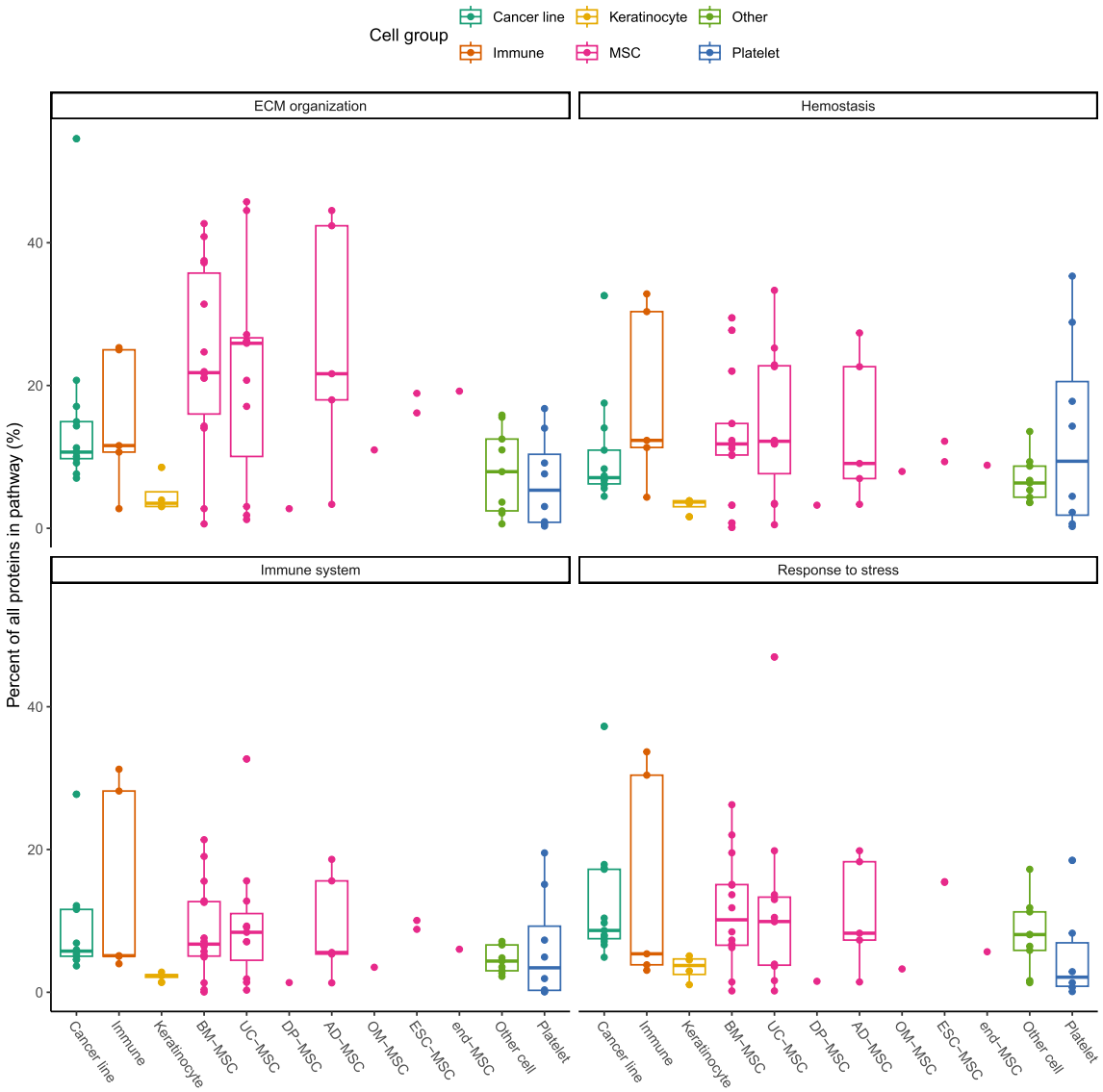


**Fig. 5** Comparison of EV sources by the fraction of proteins observed within Reactome pathways relevant to skin therapy

the second highest fraction of proteins in the immune system. This suggests that EVs from specialized cells have a higher fraction of proteins involved in their specialized functions, while MSC-derived EVs are enriched in proteins from a wider range of functions, supporting the claim that although specific cells contribute to each stage of wound healing, MSC may encompass the full spectrum [110]—potentially making them effective in diverse skin therapy applications. Furthermore, while, on average, MSC EVs exhibit higher fractions of pathway proteins, the large range in fractions observed for MSC cells indicate that not all EVs of a cell are created equal, and additional considerations are necessary to produce EVs with desired therapeutic benefits.

The unique functional protein cargo observed in MSC-derived EVs suggests higher biological activity and potentially greater effectiveness for skin applications, which appears to justify the preference for MSCs in this context. An analysis of preclinical studies in the field of skin therapy showed that, while none of the studies compared different EV types, over 60% of both animal and cell studies with positive results were performed on MSC-derived EVs and 30% on stem cell derived EVs [3, 17, 19, 111]. Moreover, five out of six clinical studies identified were performed using MSC-derived EVs, while only one used platelet derived EVs. Beyond MSC-derived EVs, platelet EVs stand out for application in wound

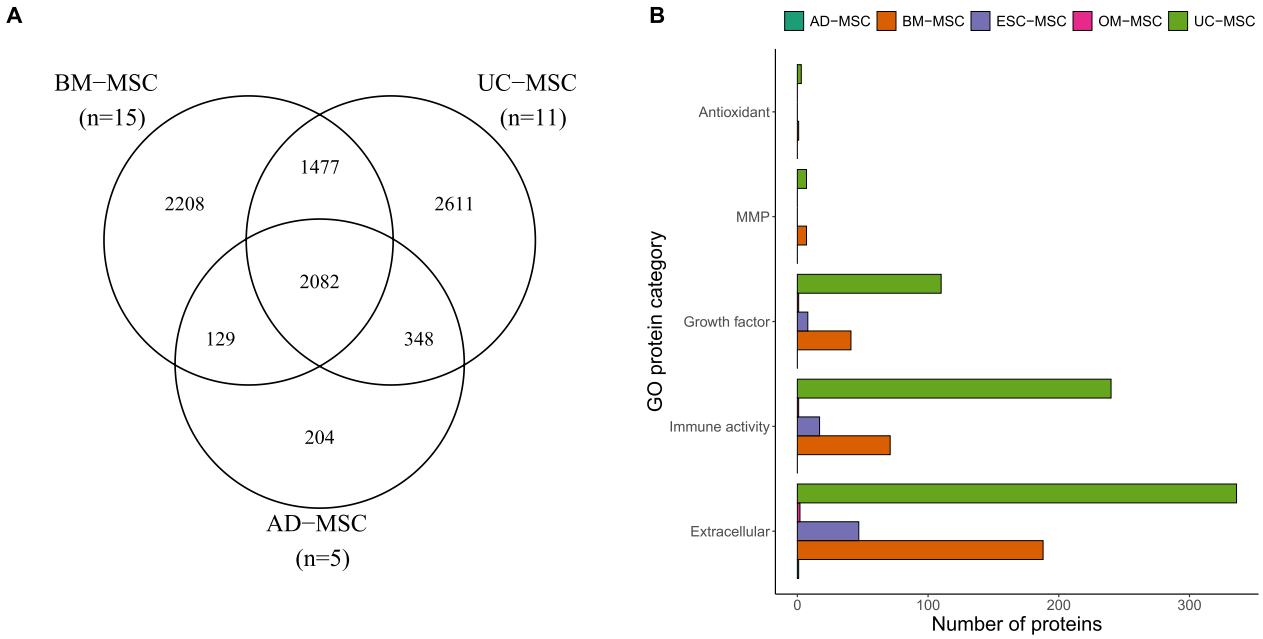


**Fig. 6** A comparison of the number of unique proteins identified in EVs from varying MSC tissues in terms of (**A**) number of proteins and (**B**) functionality

healing for their ability to impact hemostasis pathways, and NK EVs for promoting immune function.

The standout nature of MSC EVs justified further comparison between EV sources from old and new MSCs (as categorized in Sect. "EV source"). UC-derived EVs were found to have a greater number of unique and total proteins than in either of the old tissues (Fig. 6A), despite a greater number of studies reporting BM-MSC EVs. Figure 6B presents a comparison of EV functions by analysing the unique proteins of a given MSC type. Since this analysis focused on the uniquely identified proteins, the interactions between proteins within a pathway are limited. Therefore, GO analysis provided a closer inspection of individual protein function. Key functions analysed include antioxidant activity, immune activity, growth factors, matrix metalloproteinases (MMP), and ECM proteins since these protein types are known for their involvement in wound healing and skin therapy applications [109, 112, 113]. Of the unique proteins, UC-MSC EVs were shown to have a greater number of growth factors, immune activity proteins, and extracellular proteins than those observed in other MSC EVs. It is perhaps due to unique proteins with these functions why UC-derived EVs are seen as having greater therapeutic potential over other MSC sources [114, 115]. Within wound healing specifically, a comparison between AD, BM, and UC EVs previously showed that UC EVs were superior for the proliferation and migration of keratinocytes, while BM EVs were superior for fibroblasts [116]. While Hoang et al. [116] observed various growth factors for wound healing, transforming growth factor beta (TGF-β) was only observed in UC-derived EVs. On the other hand, we identified TGF-β across AD, BM, and UC EVs, suggesting that the beneficial properties are not exclusive to a single protein.

### Targeted protein analysis

More targeted protein analysis was also performed to assess the presence or absence of specific proteins identified in literature for their therapeutic potential. These proteins include ECM proteins (collagen, COL; elastin, ELN; fibronectin, FN1; and decorin, DCN), growth factors (epidermal growth factor (EGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and TGF-β, collagen promoting proteins (MCP1), MMP inhibitors (TIMP), and those promoting wound healing (PCNA, ANGPT2 and CD34) [17, 19, 106, 107, 111]. The full list of targeted proteins is provided as supplementary material in the Zenodo repository. Based on the presented results in Fig. 7, MSC-derived EVs once again stood out as the most enriched in targeted proteins compared to other cell groups. The MSC types

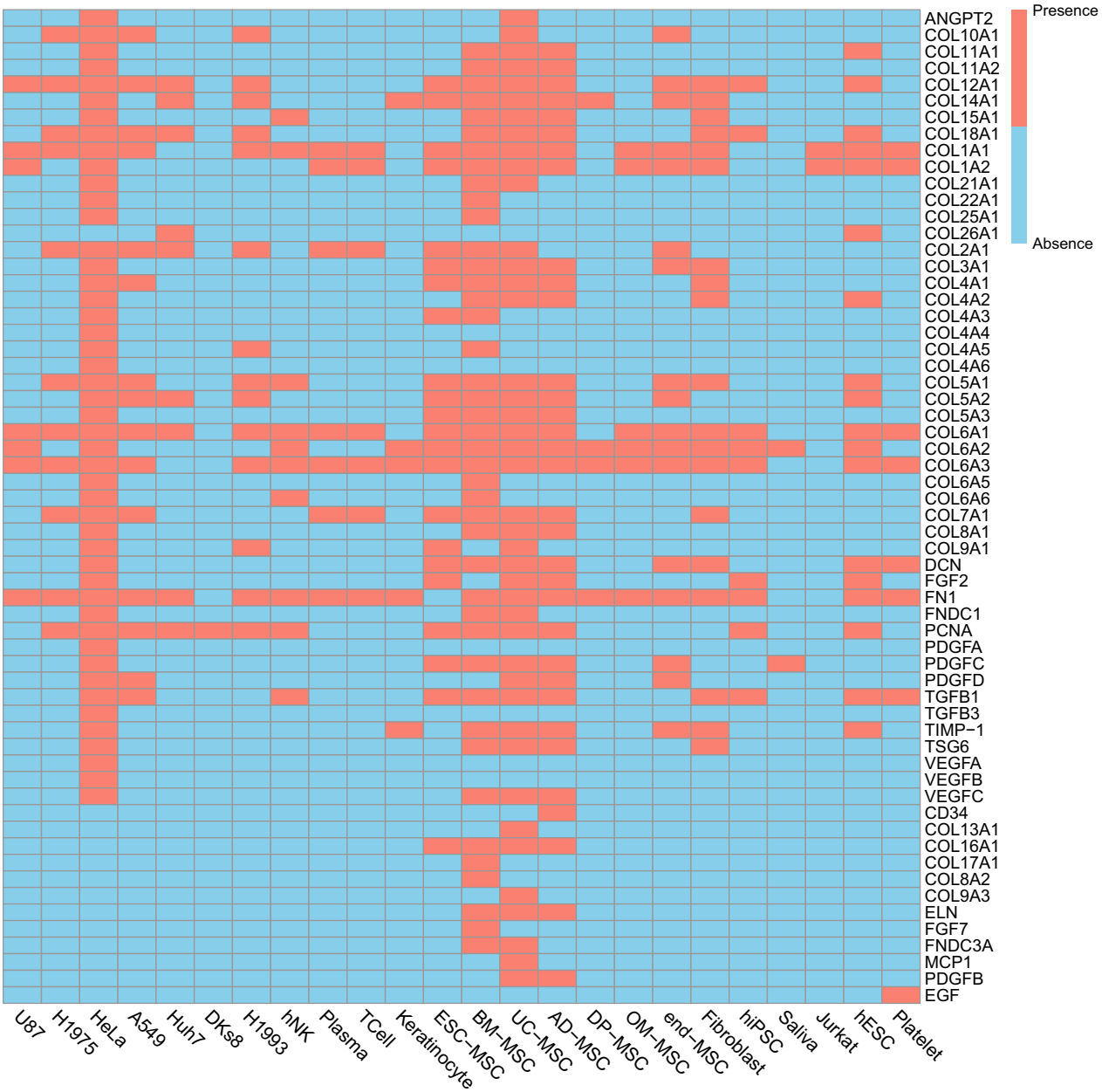


**Fig. 7** Presence or absence heatmap of skin therapy proteins by cell type

with the greatest number of targeted proteins include UC-MSC, BM-MSC, and AD-MSC. This trend was fairly consistent with four BM-MSC, two UC-MSC and two AD-MSC derived EV studies containing more than 25 targeted proteins. Most of the targeted ECM proteins, growth factors such as VEGFC, PDGFC and TGF-β, and important proteins such as TIMP-1 were found to be present in the top three MSC cell types. In contrast, protein angiopoietic-2 (ANGPT2), believed to promote wound healing through angiogenesis in a preclinical study using UC-MSC derived EVs [9], was only identified

**Table 3** Percentage of variance accounted for in sample models for the total observed proteins, wherein residuals refer to unexplained variance. *MMP: matrix metalloproteinase; AO: antioxidant

| | All | | | | MSC | | | |
|---|---|---|---|---|---|---|---|---|
| **Factor** | **Total (%)** | **Immune (%)** | **MMP (%)** | **AO (%)** | **Total (%)** | **Immune (%)** | **MMP(%)** | **AO (%)** |
| EV Source | – | – | 16.0 | – | 1.7 | – | – | – |
| Ionization | 0.9 | – | 6.5 | – | – | 4.9 | 13.7 | 0.8 |
| Isolation | 18.7 | – | – | 2.5 | 34.1 | 34.5 | – | 46.6 |
| Medium | 5.6 | 49.5 | 12.4 | 47.5 | 2.1 | – | 10.1 | – |
| Search Algorithm | 11.3 | 1.6 | – | – | 0.7 | 12.7 | – | 13.0 |
| Residual | 63.6 | 48.9 | 65.2 | 49.5 | 61.3 | 47.9 | 76.3 | 39.6 |

in UC-MSC among the MSC cell types. This highlights the potential of maximizing therapeutic efficacy by combining EVs from different MSC types, as some proteins appear to be unique to particular MSC types.

## Mixed effects modelling

A mixed effects modelling framework was used to disentangle the underlying effect of cell type from variations in culture conditions, isolation methods, and analytical techniques.[2] Mixed effects models account for correlations between observations by incorporating both fixed and random effects, with random effects grouping elements that are from the same distribution. Mixed effects models were generated for the total number of observed proteins, and the fraction of identified proteins in the five key protein functions stated previously. Five model factors were initially considered for inclusion—EV source, growth medium, isolation method, characterization method, and ionization technique. Characterization was eventually excluded, as the vast majority of the studies focused on similar unlabelled liquid chromatography with tandem mass spectrometry (LC–MS/MS) techniques. In addition, a number of factors were combined to prevent a level from being represented by only a single study. For example, since many of the isolation methods were limited to a small handful of studies, combining the non-centrifugation methods into a single "other" category provided more overlap with the other factors. These methods were also repeated for a case study utilizing only the studies with MSC cells, where the cell type compares between young and old MSC cells. All models were compared using the Akaike Information Criterion (AIC), which resulted in similar values for all models of a given protein fraction or the total observed proteins. The extracellular matrix protein and growth factor models were excluded from further analysis due to lack of fit.

When considering all EV sources for the protein fraction models, growth medium and isolation were found to be the largest sources of variance among the factors considered, while isolation method, search algorithm, and medium featured as prominent factors for MSC cells depending on the desired protein type (Table 3). However, the mixed effects modelling approach also enabled us to quantify that approximately 60–65% of the variance for total protein counts and approximately 40–75% of the variance for protein fractions cannot be explained by commonly discussed sources of variance such as EV source, media, etc.[3] This suggests that a majority of the overall variance in EV cargo cannot be traced to a specific process variable, suggesting that either a) we have yet to uncover the main factor behind cargo variance or b) EVs possess a large degree of inherent heterogeneity beyond common process parameters.

Although the selected factors are all commonly perceived to impact cargo, it is likely that there are other influential factors, given the complex nature of cargo loading [117]. For example, twelve articles analysed the effect of induction factors or process conditions, such as hypoxia, cell storage, or passage number, on EV cargo. The individual studies showed changes in protein cargo, where, for example, EVs isolated from early-passaged cells versus late-stage passaged cells reported different protein profiles, including enriched extracellular proteins in early passages [67]. However, with typically unique conditions, and few studies analysing these conditions in general, the influence of induction factors cannot be accounted for within this model. On the other hand, it is important to quantify the role of inherent EV heterogeneity on overall variability. High EV heterogeneity poses challenges for commercial production of EVs, requiring standardized methods and rigorous controls and characterization

[2] Mixed effects modelling uses random effects to represent a distribution of categorical variables. While the overall effect of a factor (e.g. exosome source) is calculated as a single categorical variable, the levels of that factor (e.g. MSC or platelet) are calculated from the distribution of that factors effect.

[3] Any variance within a model that cannot be explained by the model factors (e.g., EV source) is represented by the residual values.

to ensure consistency between batches. Currently, direct comparisons between EVs have generally been considered difficult due to high variability in methods [75], which indicates a greater need for replicates within studies to determine overall heterogeneity. However, within the observed articles, only one performed an in-depth comparison between technical and biological replicates, where biological replicates showed greater variability between the proteins that are present [44].

## Conclusion

Although EV variability is frequently discussed in a qualitative manner, this review presents a significant attempt at quantitatively characterizing EV cargo. In general, MSC-derived EVs, commonly used in skin therapy, were found to contain a greater fraction of proteins corresponding to pathways associated with the four stages of wound healing and skin rejuvenation than other EV sources. However, overall variability in these protein fractions indicates impacts from other factors. Furthermore, EVs from various MSC tissues contained a significant amount of unique proteins. Unique proteins originating from UC-MSCs, for example, were found to have stronger associations with growth factor, immune activity and extracellular matrix protein functions as compared to other sources. While EV source, protein search algorithms, and process conditions, such as medium, isolation, and characterization, have all been perceived as influential on EV cargo, quantifying their role in EV protein content poses significant challenges. Growth medium, search algorithm, and isolation method were determined to be important factors in identifying proteins corresponding to immune, MMP, and antioxidant function. However, mixed effects models also identified that 40–75% of the overall variance could not be quantified, suggesting that additional factors may be influencing variability (such as methods of EV induction). The overall level of variability is also reflected in the fact that 40% of observed proteins were identified in just one of all the considered studies. Overall, highly variable EV cargo requires greater consistency in process and analysis techniques across studies to fully quantify their effect on cargo composition. However, despite the variability in protein content, the average function of an EV from any given cell line within MSCs trend towards the same general benefits in skin therapeutic applications.

### Abbreviations

| | |
|---|---|
| AD | Adipose tissue |
| AIC | Akaike information criterion |
| ANGPT2 | Angiopoietic-2 |
| AO | Antioxidant |
| BM | Bone marrow |
| COL | Collagen |
| DCN | Decorin |
| DMEM | Dulbecco's modified eagle medium |
| DP | Dental pulp |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| ELN | Elastin |
| ESC | Embryonic stem cell |
| EV | Extracellular vesicle |
| FGF | Fibroblast growth factor |
| FN1 | Fibronectin |
| GO | Gene ontology |
| iTRAQ | Isobaric tags for relative and absolute quantitation |
| LC–MS/MS | Liquid chromatography tandem mass spectrometry |
| MALDI TOF | Matrix-assisted laser desorption/ionization time-of-flight |
| MMP | Matrix metalloproteinases |
| MS | Mass spectrometry |
| MSC | Mesenchymal stem cells |
| NK | Natural killer |
| OM | Olfactory mucosa |
| PDGF | Platelet derived growth factor |
| PEG | Polyethylene glycol |
| RPMI | Roswell Park Memorial Institute |
| SEC | Size exclusion chromatography |
| TGF-β | Transforming growth factor beta |
| TMT | Tandem mass tags |
| UC | Umbilical cord |
| VEGF | Vascular endothelial growth factor |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04279-5.

Supplementary file1.

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### Author contributions

MC and KSI contributed equally to this paper. MC was responsible for data curation, analysis, and manuscript writing, KSI for data screening, analysis, and manuscript writing, JP for review and editing, and SS for supervision, review and editing.

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### Availability of data and materials

The dataset generated and analysed during the current study (along with corresponding R files) are available in the Zenodo repository at https://doi.org/10.5281/zenodo.13870014. If additional information is required, it is available upon request from the corresponding author.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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