Proteomic Insights into Extracellular Vesicle Biology – Defining Exosomes and Shed Microvesicles

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Abbreviations

CM: conditioned medium

DC: differential centrifugation

DDA: data dependent acquisition

DG: density gradient

DIA: data independent acquisition

ELISA: Enzyme linked immunosorbent assay

ESCRT: endosomal sorting complexes required for transport

EV: extracellular vesicle

Exos: exosome

FBS: fetal bovine serum

iTRAQ: isobaric tagging technology for relative quantitation

LC-MS: liquid chromatography–mass spectrometry

MS/MS: tandem mass spectrometry

MRM: multiple reaction monitoring

MVB: multivesicular body

MVE: multivesicular endosome

nLC: nano liquid chromatography

PTM: post-translational modification

RP: reverse phase

SILAC: stable isotope-labelling with amino acids in cell culture

sMV: shed microvesicle

SRM: selected ion monitoring

SWATH: sequential window acquisition of all theoretical mass spectra

TMT: tandem mass tag

UF: ultrafiltration

UHPLC: ultra-high performance liquid chromatography

Abstract

Introduction: Extracellular vesicles (EVs) are critical mediators of intercellular communication, capable of regulating the transcriptional landscape of target cells through horizontal transmission of biological information, such as proteins, lipids, and RNA species. This capability highlights their potential as novel targets for disease intervention.

Areas Covered: This review focuses on the emerging importance of *discovery* proteomics (high-throughput, unbiased quantitative protein identification) and *targeted* proteomics (hypothesis-driven quantitative protein subset analysis) mass spectrometry (MS)-based strategies in EV biology, especially exosomes and shed microvesicles.

Expert Opinion: Recent advances in MS hardware, workflows, and informatics provide comprehensive, quantitative protein profiling of EVs and EV-treated target cells. This information is seminal to understanding the role of EV subtypes in cellular crosstalk, especially when integrated with other 'omics disciplines, such as RNA analysis (e.g., mRNA, ncRNA). Moreover, high-throughput MS-based proteomics promises to provide new avenues in identifying novel markers for detection, monitoring, and therapeutic intervention of disease.

1. Introduction

Cell-cell communication is important for all multicellular organisms and can occur proximally in the local microenvironment or distally. Cells exchange information through direct interaction, diffusible factors such as cytokines, growth factors, neurotransmitters, and extracellular matrix molecules, and/or by membrane-derived vesicles [1, 2]. Within the past decade, extracellular vesicles (EVs) have emerged as critical mediators of intercellular communication, particularly involved in the transmission of biological signals and select cargo between cells, thereby regulating various pleiotropic biological processes [3]. EVs exert diverse physiological and pathophysiological functions by horizontal transfer of protein, DNA, and RNA species between cells [4, 5]. Importantly, EVs can interact with target cells through specific receptor–ligand interactions, altering target cell behaviour not only directly but also by the transfer of surface receptors and selective sorting of bioactive cargo to these cells [6]. These processes can influence invasion of tumour cells, stimulate antigenic T-cell responses, modulate cell polarity, and influence the developmental patterning of tissues [4, 7-9]. Furthermore, EVs can be released from the surface of normal healthy cells as a fundamental physiological mechanism, dependent upon stimulus such as cell activation, pH, hypoxia, irradiation, injury, exposure to complement proteins, and cellular stress [10-12]. Whilst EVs have been primarily isolated, purified and studied *in vitro* using cell culture media, they have also been identified *in vivo.* Notably, they have been observed in diverse body fluids, such as semen, synovial fluid, saliva, urine, breast milk, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid and blood [13]. EVs have now been recognized as playing critical roles in cancer, infectious diseases, neurodegenerative disorders, as well as normal physiological processes such as haemostasis, inflammation, embryo receptivity and

implantation, coagulation and angiogenesis. Collectively, these studies have unveiled novel targets for EV-mediated therapeutic intervention [14, 15].

Proteins are critical bioactive constituents of EV cargo [16, 17] which directly regulate signalling cascades thereby influencing the transcriptional landscape of recipient cells (**Table 1**), especially during cancer progression. For example, the truncated and oncogenic form of the epidermal growth factor receptor (known as EGFRvIII) [18], hepatocyte growth factor receptor (Met) [19], mutant KRAS [17], extracellular matrix metalloproteinase inducer (EMMPRIN/CD147) [20], glypican 1 (GPC1) [21], macrophage migration inhibitory factor (MIF) [16], and selected integrins associated with lung and liver metastases [22, 23] have been identified in EV-mediated intercellular transfer. Provocatively, this horizontal transfer of oncogenic proteins can lead to the propagation of an oncogenic phenotype among subsets of those cancer cells in a tumour lacking this phenotype [18].

Proteomics can be described as the large-scale study of the structure and function of proteins in complex biological samples – this discipline encompasses post-translational modifications (such as phosphorylation), protein spatio-temporal localization and, importantly, protein-protein interactions (referred at as the interactome) [24]. The fundamental workhorse of modern high-throughput proteomics is mass spectrometry (MS). The human genome comprises 20–25,000 protein encoding genes [25]; whereas the total number of human protein products, including splice variants and PTMs, has been estimated to be close to one million (i.e., ~1.5% of genome) [25, 26]. Needless to say, most of the functional information encoded by genes resides in the proteome [27]. MS-based proteomics allows a distinct snapshot into biological perturbations through various proteomic strategies; the scope, specificity, and discovery potential. Indeed, there are two predominant MS-based approaches broadly

categorised as *discovery* and *targeted* proteomics [28, 29] Discovery proteomics focuses on strategies for high-throughput and unbiased identification of large protein datasets [28]. To this end, *discovery* proteomics often requires large sample quantities and multi-dimensional fractionation. In contrast, *targeted* proteomic approaches enable the precise quantification of a finite list of proteins (hypothesis-driven) across a wide range of samples/time-points/biochemical treatments. Importantly, *targeted* proteomics has augment sensitivity (relative to *discovery* proteomics), reproducibility, and absolute quantification. Discovery- and targeted-based strategies are available for both relative (direct comparative analysis) and absolute quantitation (spiked in labelled peptides). While *discovery* proteomics is most often employed to identify and categorize proteins in a sample or detect differences in the abundance of proteins, *targeted* quantitative proteomics enables the detection of pre-selected group of proteins/peptides in complex samples. Importantly, *targeted* proteomic assays establish a multiplexed platform for absolute quantitation - a fundamental facet of hypothesis-driven proteomics research [30]. In essence, *targeted* proteomics promises to extend our understanding of signalling cascades, the biological relevance of distinct network states, and advance targeted biological questions pertaining to EV research. This review focuses on the contribution of both *discovery* and *targeted* proteomics for the cataloguing, characterisation, and quantification of protein constituents across EV subtypes and more importantly, their biogenesis, trafficking, uptake, and biological function.

2. Defining extracellular vesicles

Classification of EVs are is typically based upon their cellular origin, biological function, and/or mechanism of biogenesis (**Table 2**). Employing accurate nomenclature and appropriately defining EVs is a vexing issue extensively discussed in the international EV

community (International Society for Extracellular Vesicles (ISEV)) [31]. Typically, cell-derived vesicles are enclosed by a lipid bilayer, ranging from ~30-1,500 nm in diameter depending on their origin [32]. EVs are currently distinguished on the basis of size, density, and protein expression [3, 32, 33]. Distinct biogenesis pathways lead to different types of EVs, and their cellular origin can be reflected in their cargo [34, 35]. For recent developments in the understanding of the major pathways of EV biogenesis and how these vesicles contribute to the maintenance of physiological homeostasis see Shifrin et al. [36]. Indeed, EVs from different cellular origins have been observed to sequester a distinct repertoire of molecules that are essential for their biogenesis, structure and trafficking. EVs most often contain membrane transport proteins, metabolic enzymes, fusogenic proteins, tetraspanins, heat shock proteins, lipid-related proteins, and phospholipases [32]. Additionally, cell-type specific proteins that reflect the phenotype of donor cells can be sorted into EVs, resulting in heterogeneity between distinct cell origins [37].

The content of EVs includes lipids, RNA species, DNA, and proteins that have been shown to be transferred to recipient cells [11, 38, 39]. Notably, EVs comprise messenger RNA (mRNAs), non-coding RNA (ncRNAs) including miRNAs, long non-coding RNA (lncRNA), single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), mitochondrial DNA, and oncogene amplifications (i.e., c-myc) [38, 40-43]. In addition, protein constituents within EVs (e.g., kinases, GTPases) can convey signalling messages to recipient cells in both local and distal environments [4]. EV surface proteins including EpCAM, CD24, CA-125, CA19-9, Met, A33, EGFR, CLDN3, and heparan sulfate proteoglycans have been shown to be significantly enriched in cancer EVs [19, 44-48]. Importantly, MS analysis of EVs has indicated enrichment in proteins that undergo various PTMs, including phosphorylation, glycosylation, ubiquitination, and acetylation [49-51]. In the field of EV research, it is becoming increasing

important to differentiate between EV subtypes enabling that distinct molecular attributes (i.e., luminal or surface components) to be accurately assigned to EV functionality [52].

3. Extracellular vesicle subtypes: Exosomes and shed microvesicles

It is well recognized that cells release essentially two EV subtypes: - (i) microparticles/shed microvesicles (sMVs), which are heterogeneous in diameter (~100-1,500 nm) and sediment at ~10-20,000*g*, and - (ii) exosomes, which are smaller in diameter (30-150 nm), relatively homogeneous, and sediment at ~100,000*g* [32] (**Figure 1**). Despite the existence of other types of EVs, including apoptotic bodies [53], large oncosomes [54], and migrasomes [55], this review will restrict its focus to exosomes and sMVs (**Table 2**).

3.1 Exosomes

Exosomes have endocytic origins being formed as intraluminal vesicles (ILVs) by inward budding of the limiting membrane of late endosomes or multivesicular bodies (MVBs) that escape lysosomal degradation [32]. MVBs subsequently fuse with the plasma membrane and release their ILV contents as exosomes into the extracellular environment. Exosome biogenesis has been linked with several distinct mechanisms including endosomal sorting complexes required for transport (ESCRT)-dependent [56], and ESCRT-independent [57]. Members of the Rab small GTPase family have been shown to modulate exosome secretion and thought to act on different MVBs facilitating their trafficking and release [58, 59]. However, it is not clear if each of these pathways act on different MVBs or, concomitantly, on the same MVB, or whether this is cell-type or origin-specific effect. (For excellent reviews of EV biogenesis mechanisms, see [35, 60]). Emerging evidence suggests that different apical and basolateral sub-populations of exosomes exist, based on distinct protein [61] and miRNA

[62] signatures from human cancer organoids. More recently, subpopulations of exosomes with distinct molecular compositions and biological properties have been identified [63]. Overall, these studies raise several important questions as to the underlying mechanisms responsible for the selective packaging of select cargo, and biological relevance of these exosomal subtypes.

3.2 Shed microvesicles

sMVs are generated by the direct outward budding from the plasma membrane, particularly involving cortical actin reorganization, outward protrusion of plasma membrane domains and subsequent detachment [64]. sMVs can be released either by activation, including cytokine-stimulation [51]) or other stimuli [65]. The molecular machinery for regulating outward budding of the plasma membrane and sMV release has been shown to involve ADP-ribosylation factor 6 (ARF6), Rac, RhoA, Cdc42, acid sphingomyelinase activity, and some ESCRT components [66-69]. Additionally, studies highlighting the role of contractile proteins, phosphorylated MLCK2; a kinase that activates myosin II, allowing for contraction of the actin cytoskeleton [67], and MYO1A in sMV biogenesis [70] have been reported. Definitive mechanisms underlying their biogenesis and formation are only beginning to emerge (reviewed [32]).

4. The importance of defining homogeneous extracellular vesicle subtypes

With the functional implications being defined for EVs, it is vital to understand these vesicles themselves. A seminal challenge in the field is to establish methods and identify stereotypical markers that will allow for homogeneous discrimination between exosomes, sMVs, and other EVs [32]. Importantly, this knowledge will eventually enable identification of molecular tools to specifically isolate, modulate secretion, and potential function of a select EV subtype. This

knowledge is also necessary to identify which EVs should be targeted for any therapeutic approach. The lack of biochemical and biophysical validation [52], along with disparate isolation strategies and nomenclature has distorted the parameters of defining EVs, raising the question of whether these vesicles represent discreet entities with specialized functionality or whether they merely a size continuum of the same entity. These issues have engendered immense interest in improving methodologies for EV isolation thereby identifying select markers that will discriminate between exosomes and sMVs (**Figure 1**).

A significant challenge in the field of EV biology is to enhance and standardize methods for EV isolation and analysis [13, 32, 71-76]. Whilst much has been reported about the unique chemical and biological properties of distinct EV subtypes, methods for their rigorous isolation and characterization are still largely empirical. With the added consideration, there is a large body of literature describing protocols for purifying EVs [47, 72, 73, 75, 77], and assessing their purity and concentration [78] are emerging as critical issues. Various recommendations on discovery research, characterization, and diagnostic research have been discussed and updated by the research community [13, 31, 52, 73, 79]. At present, no 'one-size-fits-all' isolation and characterisation strategy exists, with all available methods having advantages and disadvantages (reviewed [32]). It is important to note that the isolation/purification method of choice take into account the sample source/volume, purity, application, integrity and yield of EVs required for subsequent analyses, available instrumentation and processing time. For example, use of exosomes as drug delivery agent, as compared to their use in diagnostics, would require highly-purified and well characterized material [14, 15, 80].

5. Proteomic strategies reveal insights into EV biology

While MS-based proteomics is an important tool for identifying EV proteins that contribute towards physiological and pathological processes these methods have their particular strengths and weaknesses (**Table 3**). This section focuses on *discovery*- and *targeted*-based strategies - including both relative (direct comparative analysis) and absolute quantitation (spiking unlabelled samples with known concentrations of isotopically labelled synthetic peptides) (Table 4). For excellent reviews detailing workflows for the molecular characterization of EVs (both mammalian and non-mammalian systems) see Kreimer *et al.*, [81], Pocsfalvi *et al*., [82], and Choi *et al*., [83].

5.1 Discovery proteomics (label-free)

Put simply, *discovery* proteomics employs fundamental parameters such as tryptic peptide spectra to identify proteins in a high-throughput manner - and yields relative quantification for such identified proteins based on their corresponding peptide spectral numbers [84]. In a variety of EV-based studies, relative protein quantitation has been extensively employed [47, 61, 75, 85]. Label-free quantification compares proteins or peptides between different samples, usually through spectral counting [48, 61, 76, 85-90], peak area or intensity measurements [23, 63, 91]. Label-free approaches are highly-dependent on instrument reproducibility and are often of low throughput, thereby requiring enhanced instrument time in contrast to label-based approaches [92]. Moreover, there is a requirement for samples to be prepared and analysed separately to evade cross-contamination and augment reproducibility of label-free methods, which is often attenuated compared to metabolic and isobaric chemical labelling strategies [93]. Affinity-based techniques such as aptamer protein arrays (SOMAmers) have been increasingly applied to discovery EV-based proteomics [94]. These strategies require minimal sample volume, provide increased sensitivity, enable quantification and multiplexing albeit being

limited by the availability of protein array targets and optimized binding constants for each target.

Label-free spectral counting has been used to comprehensively evaluate different exosome isolation methods including ultracentrifugation, density-based separation, and immunoaffinity capture using anti-EpCAM coated magnetic beads [76]. In a study by Tauro *et al*., label-free mass spectrometry based on spectral counting using LTQ-Orbitrap was used to identify and selectively categorise known exosome markers and proteins associated with their biogenesis, trafficking, and release (i.e., ESCRTs, RabGTPases, tetraspanins), in addition to several new specific markers. Based on this approach, immunoaffinity was shown to be the most effective method for purifying (enriching) exosomes from cell culture extracts. Nevertheless, the authors highlight the use of density-based separation in providing significant advantages for exosome isolation in the event the use of immunoaffinity capture is limited (due to antibody availability and suitability of exosome markers) [72]. These studies highlight the importance of sample preparation and EV purification to verify the effectiveness of the selected strategy via the robust characterization of the obtained vesicles prior to proteomic applications.

In a report investigating distinct subtypes of exosomes (A33 glycoprotein-positive and EpCAM-positive) [61], GeLC-MS/MS (LTQ-Orbitrap) and label-free quantification (normalised spectral counts) was utilised. Each subtype had a distinct protein profile consistent with release from either apical (EpCAM-positive) or basolateral (A33-positive) surfaces of polarized human cancer cells. Furthermore, both exosome protein profiles were clearly distinguishable from that of sMVs, isolated from the same cell type. Further, label-free quantification was used to characterise protein differences between all known EV subtypes, prior to extensive biophysical characterization through methods such as dynamic light

scattering, cryo-electron microscopy, and by microfiltration [47]. Using gel-based fractionation and high-resolution nLC-MS/MS (Orbitrap Elite), this seminal study reports selective enrichment of proteins in sMVs (in comparison with exosomes), many of which have not been previously described in EVs. Critically, these observations support the notion of EV heterogeneity and identifies the requirement for discriminating between distinct EV subpopulations. Willms et al., utilised ultracentrifugation and sucrose density-gradient fractionation to purify subpopulations of exosomes (low-density: 1.12–1.19 g/mL and high-density: 1.26–1.29 g/mL), and further validated their observations through ultrafiltration and size-exclusion chromatography (UF-SEC) [63]. Importantly, this analysis reinforced the notion of exosome heterogeneity and utilising label-free quantitative proteomics using nLC-QExactive and a combination of Proteome Discoverer/SequestHT analyses, identified proteomic differences based on protein area normalisation. Of note, proteins enriched in low-density exosomes (compared with high-density exosomes), included established exosome marker proteins. An elevated level of similarity was identified in the high-density exosomes and sMVs, following RNA analysis. Strikingly, the exosome subpopulations induced differential effects on gene expression profiles in recipient endothelial cells (11 genes upregulated in response to low-density vs high-density exosomes). Whether subpopulations of EVs induce distinct proteomic programmes, differential uptake and/or targeting propensity in recipient cells warrants further investigation.

Comparative proteomic analyses of exosomes released by macrophages exposed to *Leishmania* and *Mycobacterium avium* have demonstrated that the vesicles from infected macrophages displayed distinct “signatures” of immune response, particularly in composition and abundance of inflammatory proteins [95, 96]. These studies substantiate the paradigm of selective sorting of cargo (proteins and RNA) into exosomes, particularly in response to biological stimuli.

Immunomodulatory mechanisms that include antigen presentation, immune activation, immune suppression and immune surveillance have also been reported to influenced by EVs [8, 97, 98]. Consistent with this, Graner et al. [99] demonstrated that exosomes derived from glioblastoma cells induced protective immunity and anti-tumour immune responses in syngeneic mice. A salient observation in the proteomic assessment of exosomes from glioma-derived patient sera in comparison to normal, patient-matched sera was the enrichment of EGFRvIII and TGF-β. These reports highlight that tumour-derived exosomes are able to induce effector immune responses, enabling exosomes to escape the blood-brain-barrier that may exert immunogenic responses in target cells. More recently, human gamma oncogenic herpes viruses have shown to reprogram the tumour microenvironment, particularly through exosomes released by infected cells [100]. Through the use of quantitative proteomics (2D difference gel electrophoresis, LTQ-Velos Orbitrap, spectral counting), Meckes and colleagues delineated distinct protein repertories in exosomes secreted by virally-infected and non-infected cells. Specifically, 360 proteins were identified unique to the viral exosomes, associated with modulation of cell death and survival, ribosome function, protein synthesis, and mammalian target of rapamycin signalling. It is tempting to speculate that cancer-derived exosomes obtained from infected cells can induce distinct signalling cascades and metabolic pathways in recipient cells.

An emerging paradigm in developmental biology involves intercellular communication during maternal-embryo crosstalk mediated via EVs [101, 102]. Both the contribution of exosomes [102] and sMVs [101] and label-free proteomics have been attributed to understanding maternal-embryo crosstalk. Greening and colleagues purified human endometrial exosomes in the presence of menstrual cycle hormones (estrogen and progesterone) and performed label-free proteomic profiling (LTQ-Orbitrap Elite, spectral counting) [102]. Progesterone-induced

endometrial exosomes exhibited fundamental differences in implantation-related protein networks (i.e., adhesion, migration, invasion, and extracellular matrix remodeling), and more importantly these salient differences were recapitulated in human primary uterine epithelial cell-derived exosomes under hormonal regulation. Endometrial exosomes were internalized by human trophoblast cells and this potentiated their real-time adhesive capacity; a biological response mediated partially through active focal adhesion kinase (FAK) signaling. Therefore, exosomes were identified to contribute towards endometrial-embryo interactions within the human uterine microenvironment, particularly by modulating adhesion required for receptivity and implantation. Moreover, proteomic analysis (triple quadrupole linear ion trap, 4000 Q Trap) has been performed to identify the protein cargo of sMVs isolated from a pluripotent embryonic stem cells, demonstrating a correlation between such cargo proteins and extracellular matrix proteins (lamin and fibronectin) known to be expressed in the blastocyst at the time of implantation [101]. Importantly, the contribution of sMVs to implantation was shown to directly affect implantation rates after embryos are transferred into the uteruses of female mice. Both studies raise important and exciting possibilities regarding the use of EVs subpopulations in therapeutic applications for promoting the natural ability of embryos to implant and establish a successful pregnancy.

In cancer biology, several studies have enlisted label-free quantitative spectral counting to assess proteomic differences between primary and metastatic cell-derived EVs [48, 88]. Critically, these global profiling approaches reveal selective enrichment of metastatic factors and signalling pathway associated components, enhancing our understanding of the cross-talk between tumour and stromal cells in the tumour microenvironment. As an integral facet of intercellular communication during cancer, EVs can mediate key biological processes such as epithelial-to-mesenchymal transition (EMT), invasion, migration, angiogenesis and metastasis

[7]. Recent studies by Tauro and colleagues report the comparative protein profiling (using nLC-LTQ-Orbitrap) of purified exosomes released from parent epithelial (MDCK) cells and oncogenic (H-Ras) transformed MDCK (21D1 cells) [85]. Notably, expression of EMT-related transcription factor (YBX1), and core splicing complex components were selectively enriched mesenchymal cell (21D1 cells) –derived exosomes (compared to parent MDCK cell-derived exosomes) based on label-free normalised spectral counting [85]. This proteomic study, revealed several proteins enriched in mesenchymal cell-derived exosomes associated with the development of the 'metastatic niche' and tumorogenesis onset. In line with this, exosome-mediated intercellular communication between oncogenic cells undergoing EMT and endothelial cells was investigated [103]. Exosomal Rac1 andPAK2 were subsequently identified as angiogenic promoters, that may function during the initial phases of the metastatic cascade. Podoplanin (PDPN), a transmembrane glycoprotein vital in propagating malignancy was identified to be selectively packaged into sMVs and exosomes [104]. Label-free proteomic analysis utilising nLC-Orbitrap Elite revealed that PDPN-induced EMT is associated with the upregulation of oncogenic proteins and diminished expression of tumour suppressors in exosomes. Notably, exosomes containing PDPN were shown to promote endothelial lymphatic vessel formation, with this effect mediated by surface-bound PDPN. The induction of an EMT-like state in A431 cells, through blockade of E-cadherin and EGFR stimulation resulted in the reprogramming of the EV proteome (distinct from that of parental cells) [89].

In another recent study label-free proteomics (significant spectral count and fold change ratios) was employed to profile exosomes derived from several different human malignant mesothelioma models - this study used a strategy based upon short-range SDS-PAGE/ UPLC/ LTQ Orbitrap Elite mass spectrometer and in-depth bioinformatics analyses of known reported cancer exosomal cargo (i.e., reported in databases and extensive literature review) to define a

select malignant mesothelioma protein signature that reflects oncogenic cargo [105]. Importantly, this study identified a mesothelioma exosomal signature (mEXOS) which has the potential for use as a diagnostic marker of the disease.

Proteomic profiling has been used to identify selected proteins in exosomes, particularly in melanoma metastatic exosomes that were responsible for the transfer of metastatic potential, thereby ascribing a role for exosomes during pre-metastatic niche formation [19]. Exosomal Met, in addition to other candidates with known roles in tumorigenesis and metastasis, were identified by proteomic profiling (LTQ-Orbitrap) and shown to be responsible for bone marrow education and initiating a shift in phenotype (pro-vasculogenic and pro-metastatic). Critically, an exosome-specific melanoma signature was identified in patient sera with prognostic and therapeutic potential. More recently, quantitative mass spectrometry-based proteomics (nLC-QExactive) of exosomes from lung-, liver- and brain-tropic tumour cells identified integrin subsets as fundamental determinants of metastatic organotropism, thereby furnishing mechanistic insights into organ-specific metastasis [23]. Employing label-free quantification proteomics (Proteome Discoverer/ Percolator/ average area of the three most abundant peptides for a matched protein used to gauge protein amounts within and between samples) revealed distinct exosomal integrin repertoires distinct to metastatic subsets. Specifically, integrins α6β4 and α6β1 were identified to be associated with lung metastasis, whilst exosomal integrin αvβ5 was found to be associated with liver metastasis. The integrin profile of each exosome subtype permitted selective targeting and uptake into organs, whereby which an abundance of ligand for each specific integrin was observed. A salient finding of this study was that integrin expression may further predict metastatic dissemination, identifying the possibility of harnessing exosomal integrin profiles in organ-specific cancer diagnostics. Cancer-derived exosomes have more recently been shown to promote liver metastasis by eliciting pre-

metastatic niche formation through a multi-step process [16]. This involves uptake of cancer exosome-derived factors by liver cells to generate a fibrotic microenvironment with immune cells to promote metastasis. Label-free mass spectrometry (nLC-QExactive, peak area) was employed to gain insights into protein targets and inflammatory mediators through which such exosomes promote liver cell activation and induce pre-metastatic niche formation. Specifically, the study utilised proteomics to identify exosomal MIF as an important mediator of liver pre-metastatic niche initiation.

In a salient study by Wrana and colleagues [106], the involvement of stromal-derived exosomes was found to promote protrusive activity, motility and metastasis of breast cancer cells, through the induction of autocrine Wnt-planar cell polarity (Wnt-PCP) signalling. Label-free proteomics (nLC-LTQ-Orbitrap/LTQ-XL, ProHits, spectral counting), identified exosomal tetraspanin (CD81) found to be responsible for uptake and internalisation in conjunction with functional analysis shown to directly regulate fibroblast-induced cancer cell motility and metastatic potential. These findings substantiate the role of exosomes as "agents of crosstalk" between stromal and cancer cells particularly, to facilitate cancer cell protrusive activity, motility and metastasis.

5.2 Discovery proteomics (label-based)

Various label-based approaches including chemical, proteolytic, isobaric, and metabolic-labelling techniques have been developed and applied to EV biology to overcome the issue of linearity (concentration:signal) and limited accuracy of label-free methods. Despite their significant quantitation advantages, several limitations confound their use [107]: (i) metabolic labelling strategies; inability for application in human tissue samples, limitation in diagnostic setting, time-consuming process, and costs of metabolic labelling approaches may be

substantial due to the amount of expensive labelled reagents, (ii) chemical labelling strategies; systematic errors in sample processing/digestion are introduced more readily, protein level labelling requires specific proteins such as cysteine/lysine, which makes peptides without these amino acids not quantifiable. In EV biology the most common labelling methods used to date include stable isotope-labelling with amino acids in cell culture (SILAC) [51, 108-110], multiplexed tandem mass tag (TMT) labelling [91, 111, 112], and multiplexed isobaric tagging technology for relative quantitation (iTRAQ) [113-116] (**Table 3**). Selected quantitation-based comparative proteomic analyses of EVs are summarized in **Table 4**.

SILAC based MS was used in combination with sodium carbonate treatment to investigate EV-mediated inflammatory and phosphorylated signalling proteins in sMVs and exosomes released by cytokine-stimulated β-cells. [51]. Both EV subtypes were isolated through sequential centrifugation whereby the sMV fraction was pelleted at 20,000*g* and subsequently the supernatant centrifuged at 100,000*g* to isolate exosomes [72]. Network analysis and quantitative proteomic validation highlight the involvement of TNFR1 and ICAM-1 in mediating cell signaling in cytokine-stimulated microvesicles. Subsequently, Cossetti *et al*., [117] interrogated neural stem/precursor cells and derived EVs using SILAC and an integrated pathway analysis. Of note, pro-inflammatory cytokine signaling pathways, specifically Stat1-associated ones, modulated the cellular proteome and induced selective sorting of proteins into EVs. This aspect of EV-mediated signaling is now emerging as an integral facet of intercellular communication between grafted stem cells and the host immune system.

A triple SILAC quantitative proteomic strategy was used to investigate the differential protein abundance in exosomes contributing to lung cancer progression [110]. This study enlisted non-small cell lung cancer (NSCLC) cell lines harboring distinct activating mutations in cell

signaling molecules KRAS or EGFR. Of the quantified 721 exosomal proteins identified, several distinct signaling proteins correlated with mutational status that contributed to lung cancer progression. Hence, this study might have potential clinical implications particularly towards exosome-based biomarker development for patients with NSCLC.

Recently, paracrine exosomal trafficking has been demonstrated to have a direct role in shaping the leukemic niche [112]. Huan and colleagues identified that leukemia-derived exosomes exert target cell specific effects that coordinate compartmental remodeling and successive loss of hematopoietic activity during leukemic invasion of the bone marrow. In addition to extensive molecular biology based analysis, murine xenograft studies, exosomal trafficking and high-throughput quantitative proteomic analysis was performed. Employing multiplex TMT mass spectrometry, 282 proteins were differentially expressed between exosome-treated and untreated cells. Candidate pathways such as ribosomal biogenesis, acetylation, cell proliferation and antioxidant activity were found to be activated, thereby ascribing a direct role for exosomes in the regulation of hematopoietic stem and progenitor cell (HSPC) function. These studies reinforce the fundamental role of EVs in mediating intercellular function and advocate for the importance of a thorough understanding of EV biogenesis, especially in the selective incorporation of proteins into distinct EV subtypes.

5.3 Targeted proteomics

Targeted MS is typically used to select, monitor, quantify and validate peptides/proteins of interest [118]. Multiple Reaction Monitoring (MRM) (or selected ion monitoring (SRM)) is a MS technique typically performed using a triple-quadrupole MS instrument [119, 120]. This technique involves the selection of a precursor ion (in this instance, a peptide functions as surrogate for the protein of interest) and the resulting fragment ions (products) comprise

transitions that are specific for monitored peptide sequences [120, 121]. Indeed, MRM assays require prior identification of peptides or proteins of interest, with the identification of potential candidate proteins generated during the discovery phase. The high sensitivity and specificity, coupled with a combination of unique precursor/transition ion pairs can result in the confident identification of a target protein in the a/fmol/μL (0.1−10 μg/mL) range [120]. Such targeted protein assays offer compelling advantages over immunoassays, including the ability to systematically configure a specific assay for essentially any protein or PTM form, without the requirement for an antibody, and the capability of multiplexed analyses of many peptides in a single analysis. A limitation of triple-quadrupole instruments is the relatively low resolution of precursor ion measurements, which may allow interference from nominally isobaric background contaminants in complex mixtures. Such instruments are also limited by their duty cycle, the rate at which transitions can be sampled with an acceptable signal-to-noise ratio. As such, variants in data acquisition, where an inclusion (target) list of peptide precursor ion/m/z values is used to direct data acquisition using the newer quadrupole-Orbitrap hybrid instrumentation, has allowed high-resolution analyses to be performed, and restricting interference from nominally isobaric contaminants. These modified modes of operation for targeted peptide analysis, the most powerful of which is termed parallel reaction monitoring (PRM), enable high-resolution, fast scanning, full scan MS/MS data, from which transitions can be derived [122, 123]. Recent reports suggest that PRM analyses exhibit performance characteristics (dynamic range, measurement variation, quantitative accuracy) comparable and above to those of MRM analyses performed on triple quadrupole instruments [124] (reviewed [125]).

In the context of EV biology, several targeted LC-MRM analyses have used in the validation of EV cargo, recipient cell uptake and development of multiplexed assays towards interrogation

of clinical samples. Demory-Beckler *et al*., [17] utilised targeted LC-MRM to monitor exosome internalization and protein incorporation in target cells. This study identified the elevated expression of various exosomal markers in select recipient cells following their treatment with parent cell-derived exosomes. Peptides specific for wild-type and mutant KRAS were designed to assess intercellular exosomal transfer of mutant KRAS. Expression of mutant and wild-type KRAS was monitored by LC-MRM in recipient cells and their respective exosomes (fmol/µg protein). Moreover, biological assays demonstrated that the mutation of KRAS can modify the signals released by cells via exosomes, resulting in the acquisition of a growth advantage for surrounding wild-type KRAS-containing cells.

Recently, an exosomal membrane protein (Gap junctional protein Cx43) was reported to facilitate the interaction between exosomes and human kidney HEK293 cells, and further assist in the transfer of exosomal cargo into target cells [126]. In this study, immunopurification [127] coupled with targeted-SWATH-MS was used to identify a set of Cx43-specific peptides, along with known exosomal proteins. Specifically, SWATH-MS was used to the integration of MS-based data independent acquisition (DIA) and targeted data analysis [128], thereby generating a distinct library of precursor masses and fragment ions for Cx43. Targeted MS further revealed the presence of Cx43 present in exosomes isolated from rat coronary perfusates, culture medium of organotypic heart slices and human plasma, postulating further biological and physiological roles for Cx43 in kidney cell-exosome communication.

Proteomic analyses of urinary microparticles from bladder cancer and hernia patients revealed 107 differentially expressed proteins in the discovery-phase, specifically by isotopic dimethylation labelling [129]. Differences in the expression of 29 proteins (41 signature peptides) was accurately quantified by LC-MRM in urine samples of bladder cancer, hernia,

and urinary tract infection/hematuria. Notably, a selected cell-surface glycoprotein (TACSTD2) was further validated in urine specimens by using a commercial ELISA, suggestive of a role in the diagnosis of bladder cancer. *Mycoplasma tuberculosis* infected macrophages were shown to secrete exosomes comprising pathogen-derived antigens [130]. Importantly, these exosomal components were detected by targeted proteomics assays in the serum of patients with acute and latent tuberculosis infection. Application of targeted LC-MRM assays to exosomes isolated from active *M. tuberculosis* patient-derived human serum led to the detection of 76 peptides representing 33 unique proteins. Of note, these proteins were related to mycobacterial adhesins and/or proteins that augmented the intracellular survival of *M. tuberculosis*. With the added consideration of these critical observations substantiating diagnosis, these proteins could also serve as active or latent phase virulence markers.

5.4 Intact (top-down) proteomics

Top-down proteomics [131], in contrast to the aforementioned 'bottom-up' (tryptic peptide-based) proteomics, identifies proteins in their intact state, without the need for proteolytic digestion into peptides. By these means, the intact polypeptide sequence is preserved, allowing for the identification of sequence variants and protein modifications. Currently, there are limited reports on EV-based top-down proteomics. Recently, Geis-Asteggiante and colleagues [132] used top-down proteomics to investigate exosome protein cargo derived from myeloid-derived suppressor cells, that participate in intracellular signalling within the TM. This report investigated low mass proteins (<30 kDa), identifying multiple proteoforms of the pro-inflammatory mediators S100A8 (oxidation/acetylation) and A9 (acetylation/methylation) known to suppress the immune response, and various proteolytically cleaved histones (H3 and H4). Furthermore, membrane protein-enriched EVs, specifically three cell-cell fusion type I transmembrane proteins (AFF-1 and EFF-1 and the glycoprotein B (gB), were generated and

validated by MS [133]. Importantly, this approach allows for further application in antibody generation, protein-protein interaction assays, and studies understanding differential expression of proteins and proteoforms.

Despite the vital insights afforded by assessment of complex protein networks in EVs, analyses of such quaternary interactions and multiprotein complexes by MS remains an area which is not well studied. Notwithstanding, a report by Nazarenko *et al.*, observed that the overexpression of exosomal Tspan8 influenced the assembly and functionality of a Tspan8/CD49d complex [134]. Based on comparative proteome analysis, this study utilised two-dimensional electrophoresis or prehydrated plastic sheet gel strips for separating hydrophobic proteins followed by matrix-assisted laser desorption/ionization–time-of-flight MS analysis. More recently, Ji *et al.*, employed the isogenic human colorectal cancer cell lines SW480 (primary carcinoma) and SW620 (lymph node metastasis), to identify known cellular complexes (e.g., TNIK-RAP2A and EpCAM-CLDN7-CD44) in exosomes derived from these cell lines [48]. Using global profiling based on GeLC-LTQ-Orbitrap, spectral counting, and network analyses, proteins involved in cellular complexes (e.g., Met-Grb2-β-catenin-1), were observed to co-localise in exosomes – these findings were confirmed by immunoprecipitation [48]. Understanding distinct protein complexes in EVs will have significant biological implications for target cell selection and internalisation in recipient cells. Recent technological advances such stabilised affinity mass spectrometry [135] which involves the application of flash freezing and affinity-based purification and analysis, and preserves native interactions in EVs, promise to bridge the gap between proteomics and understanding complex protein cascades in EVs [136, 137].

5.5 Post-translational modifications

The analysis of EV protein PTMs has been gaining traction due to their significance in EV biology (e.g., biogenesis, sorting, trafficking, protein localization, and functionality). In this regard, several types of PTMs have been investigated using MS-based proteomics - these include palmitoylation, phosphorylation, ubiquitylation, sumoylation, and glycosylation [138, 139]. For further reading of protein PTM involvement in EV biology see Szabó-Taylor *et al.*, [139] and Kreimer et al., [81].

Several proteomic studies have addressed the significance of PTMs, specifically phosphorylation, glycosylation, sulfation, and ubiqutinylation in EV biology [17, 46, 49-51, 140]. A differential centrifugation and density-based isolation strategy was employed to investigate the phosphoproteome of human urinary exosomes [140], prior to phosphopeptide enrichment. Through the use of a neutral loss scanning approach on a linear ion trap mass spectrometer (LTQ FT) in combination with PhosphoPIC analysis, 14 phosphoproteins and multiple novel phosphorylation sites, including serine-811 in the thiazide-sensitive Na-Cl co-transporter, NCC were identified. In line with this, Palmisano et al., [51] purified phosphorylated peptides and *N*-linked sialylated glycopeptides from membrane proteins derived distinct EV subtypes using titanium dioxide prior to nLC-LTQ-Orbitrap XL analysis. Importantly, select analysis pipelines were developed for both phosphorylated and sialylated peptides using a multistage activation approach, with quantitation performed using event detector and precursor ions quantifier in ProteomeDiscoverer and sequence motif analysis Motif-X. For sMVs, a total of 188 sialylated *N*-glycosylation sites from 104 glycoproteins and 130 phosphorylation sites from 67 phosphoproteins were identified. In contrast, 51 sialylated *N*-glycosylation sites from 38 glycoproteins and 21 phosphorylation sites from 15 phosphoproteins were reported in exosomes. Christianson et al., [46] recently demonstrated that exosome uptake in recipient cells by flow cytometry and confocal fluorescence microscopy

was dependent upon the surface exosomal expression of heparan sulfate proteoglycans 2-O-sulfation and N-sulfation. Enzymatic depletion of cell-surface heparan sulfate proteoglycans attenuated exosome uptake. However, exosome-associated heparan sulfate proteoglycans were shown to have no direct role in exosome internalization. Extending proteome analyses to interrogate protein sulfation during EV biogenesis, internalisation and towards influencing distinct EV subtypes would provide critical insights into the biology of EVs.

Recently, Demory-Beckler et al., [17] identified an association between ubiquitination, previously implicated in exosomal sorting [141], and the number of identified abundant peptides detected in exosomes using a combination of peptide isoelectric focusing/ nLC-LTQ-Orbitrap hybrid mass spectrometer analysis. Exosomes from parental DLD-1 cells that contain both wild-type and G13D mutant KRAS alleles and isogenically matched cells: DKO-1 (mutant KRAS allele only) and DKs-8 (wild-type KRAS allele only) were isolated. Notably, mutant KRAS was shown to affect the composition of the exosome proteome based on normalized spectral count ratio and various statistical considerations. The authors hypothesized that ubiquitin-modified active KRAS might be specifically sorted to these vesicles, advocating for a non-random association. Future work in the field of EV research will investigate how selected components are trafficked to exosomes, elucidate the role of PTMs in regulating protein trafficking to EV subtypes, and identifying enzymes that direct PTMs across various cellular origins. Villarroya-Beltri et al., [142] discovered that sumoylated hnRNPA2B1 could recognize a motif in miRNA sequences and facilitate the sorting of specific miRNAs into exosomes. Furthermore, two hnRNP proteins (hnRNPA1 and hnRNPC), identified using an LTQ XP Orbitrap mass spectrometer, were shown to bind to exosomal miRNAs using a combination of immunoprecipitation-qPCR and electrophoresis mobility shift assay, suggesting that they might be candidates for the selective sorting of miRNAs.

Argonaute2 (Ago2), a fundamental miRNA binding protein, has been identified in cancer cell-derived exosomes particularly, through the use of immunoblotting [143, 144]. Strikingly, deep sequencing analysis revealed that knockout of AGO2 selectively attenuated expression (abundance) of several exosomal miRNAs (e.g., miR150, miR-451, miR-486), advocating for a potential mechanism in selective packaging of exosomal miRNAs [145]. Whether Ago2 protein is found within select EVs such as exosomes [144] as a consequence of co-purification [74], or the complex containing Ago2 associated with [146] or bound outside of vesicles [147], remains to be further investigated. It is tempting to speculate that distinct proteins and their modified forms might control the sorting of exosomal miRNAs and mediate their selective incorporation into exosomes. Recently, regulation of Ago2 exosomal sorting in colon cancer cells through oncogenic KRAS to promote phosphorylation of Ago2 has been shown to control the levels of three candidate miRNAs in exosomes (let-7a, miR-100, and miR-320a), implicating a key regulatory signalling event that controls Ago2 secretion in exosomes [144]. Hence, contribution of protein identification using mass spectrometry in investigating PTMs and their importance in EV biology, specifically in sorting regulation, will be a definitive focus of future research.

6. Databases as catalogues for extracellular vesicle research

Proteomic reports of EVs have yielded extensive catalogues of proteins in various types of EVs. Currently, three online public integrated databases detailing EV cargo that includes EVpedia [83], ExoCarta [148], and Vesiclepedia [149] are available. EVpedia (www.evpedia.info) provides a comprehensive list of EV-associated proteins, mRNAs, miRNAs and lipids from prokaryotic, non-mammalian eukaryotic and mammalian sources. In contrast, ExoCarta (www.exocarta.org) and Vesiclepedia (www.microvesicles.org) detail the

vesicular components of non-mammalian eukaryotic and mammalian EVs. Both Vesiclepedia and EVpedia catalogue data from multiple EV types whilst ExoCarta represents a primary resource for exosomal cargo and comprises annotations on the isolation and characterization methodology. A caveat in the use of these databases lies in the experimental detail provided by the authors with regards to the method of purification and heterogeneity of EVs analysed [73, 78]. To this end, these databases emphasise caution when employing such datasets, given that many published studies have limited purification protocols employed, and are often misrepresented by analysing heterogeneous vesicle subtypes. Further development will permit these databases to serve as valuable resources in providing content focussed on protein–protein interactions, post-translational modifications, lipid composition and biological relevance for analyses of distinct EV subtypes.

7. Proteomics provides insights into EV therapeutics

Recent high-throughput proteomic studies of EVs have accelerated the discovery of disease-specific biomarkers and the development of novel EV-associated therapeutic tools [115, 150-154]. Towards the application of biomarker discovery, whereby verification and validation is of utmost importance, elements such as absolute protein quantification, high throughput and multiplexing capabilities become increasingly significant. To this end, mass spectrometry facilitates the detection of multiple candidate peptides, provides high specificity and requires minimal sample volume (10-500 μL) with no added antibody development costs. Currently, most diagnostic based EV-proteomic studies have either employed discovery label-free based profiling to identify candidate EV protein target(s) in the circulation of patients or used targeted proteomic analyses to quantify candidate EV protein(s) in patient bio-specimens. Several clinical studies have employed multiplexed protein markers (e.g., 37 proteins [155]) and a

panel of proteins and miRNAs (4 and 4, respectively) [156] for disease diagnosis. Melo and colleagues [21] reported that the cell surface proteoglycan, glypican-1 (GPC1) was anchored to circulating exosomes and identified by proteomic profiling (based on nLC-MS and peak area quantitation). This salient observation has critical implications for the diagnosis of pancreatic cancer given that only 250 μL of blood is required for analysis. Notably, analysis using flow cytometry of $GPC1^+$ exosomes indicated optimal sensitivity and specificity (100%) in each stage of pancreatic cancer (carcinoma *in situ*, stage I and stages II-IV), substantiating its potential utility as a biomarker for all stages of pancreatic cancer and its potential for early detection. Further, Sze and colleagues utilised proteomic profiling (nLC-LTQ-FT Ultra linear ion trap mass spectrometer) to reveal 252 EV proteins that were modulated (based on precursor ion intensity of three most abundant unique peptides per protein) in plasma after myocardial ischemic injury [153]. The study prioritised six up-regulated biomarkers with potential for clinical applications; these reflected post-infarct pathways of complement activation and validated using antibody-based assays. Interestingly, the study further demonstrated that EV-derived fibrinogen components were paradoxically down-regulated, suggesting that a compensatory mechanism may suppress select pathways associated with post-infarct pathways, indicating potential for therapeutic targeting.

Proteomic profiling of EVs has been shown to reveal select markers that determine the extent of malignancy and stage of different human cancer types [19, 155-157]. Lyden and colleagues [16] used global mass spectrometry profiling (exosome purification using differential ultracentrifugation, nLC-QExactive mass spectrometry, and differential expression based on average area of the three most abundant peptides for a matched protein) was used to identify MIF as a candidate mediator of liver education. Current and developing methods in targeted mass spectrometry establish the foundations for proteomic platforms that could be used for

high-throughput analysis, verification and validation. In particular, proteomics could be used for rapid quantitative analyses of EV protein panels, signalling pathways and pharmacokinetics. This could be further extended for use in multiplexed assays, drug discovery and clinical applications for EV biomarkers [14, 158-160].

8. Expert commentary

Mass spectrometry is a powerful technology for the quantitative identification of protein components of EV subtypes – information that is fundamental for understanding their biogenesis, function, as well as discovery of stereospecific protein markers that might allow EV subtype discrimination.

Quantitative proteomics has elucidated complex protein networks in EVs associated with the establishment and regulation of an assortment of pathologies. To date, limitations of the use of proteomics in EV analysis is largely focused around analysis of heterogeneous EVs. Currently-available EV isolation methods are often heterogeneous and often do not allow differential analysis between subtypes. Recently, our group [47] and other laboratories [63, 75] observed that highly-purified EV subtypes (exosomes and sMVs) from the same cellular origin are biochemically and functionally distinct. Through the use of key strategies to enrich select subpopulations of EVs, and differential expression using MS, we identified proteins selectively and significantly enriched in EV subtypes. The universality for such proteins as markers of distinct EV subtypes must await further studies, specifically in an extensive range of cell types/ body fluids. Hence, reporting of methods used for isolation, purification, as well MS-based metrics for EV subtype characterisation, is essential. Further, monitoring of EV-enrichment and potential protein contaminants as standard operating procedures will ensure that sub-

optimal sample quality, artefacts and false positive identifications are minimised. This information will improve our understanding of EV biology at the protein level and, importantly, allow cross-laboratory comparisons and validation. Taken together, these advances in EV subtype purification and characterisation – assisted by recent advances in MS-based proteomics – such as protein sensitivity and dynamic range [161, 162], protein isoforms/variants [163, 164], subcellular localization, tissue distribution [165, 166], spatial localization [167], PTMs [168], multiplexed, quantitative assays, computational tools, and protein-protein interactions [169] - will accelerate EV-based diagnostics and therapeutics.

9. Five Year View

In the past decade we have witnessed great advances in the purification and characterisation of EVs. It is now evident that the major EV subtypes (exosomes and sMVs) can now be further dissected into multiple subpopulations of exosomes [61, 63, 75] and sMVs (Greening, Simpson, unpublished). Whilst this review focuses on exosomes and sMVs, there are other EVs such as large oncosomes whose biogenesis is yet to be revealed. Clearly in the next five years, further sub classification and characterisation will occur and the field will be challenged with the task of determining their biochemical and functional properties. Needless to say that the recent advances in high-performance MS hardware and software, as outlined in this review, are up to the task of determining the proteome profile of vanishing small amounts of material.

As highlighted in this review, proteomics is further indicating accumulating evidence of protein complexes in EVs. To date, these observations are based largely on immunoprecipitation [48, 134, 170]. With the improvement in methods to rapidly and stably isolate protein complexes using cryogenic lysis, it is now possible to obtain protein complexes containing both direct and

distant (transient) interacting partners via crosslinking. This technology promises to impact on EV biology, and will help answer the question of whether EVs contain preformed, biologically-active protein complexes. Needless to say, if proteomic approaches can be coupled with other omic approaches (e.g., RNA species), our understanding of RNA-protein complexes will be enhanced immeasurably.

Discovery and targeted proteomics now holds the promise of identification, quantification, and validation of EV proteins and determining EV subtype-specific markers. Using standardised, stringent purification measures, these established markers will enable rapid and effective monitoring of EV isolation and their subtypes, labelling and imaging protocol developments, biological insights, and more importantly, assay development across various pathological conditions. Improvements in discovery proteomics are presently focused towards increased multiplex quantitation and overcoming sample complexity through fractionation, increased mass ranges through improvements in labelling efficiency, and reduced user costs. Advances in targeted proteomics is being directed towards biomedical research and clinical applications, where large-scale quantification, improvements in method development, throughput, data processing and analysis and the utilisation of fast scanning high-resolution accurate-mass instruments to analyse low abundant proteins in complex biological matrices [125]. Further developments in data-sharing resources such as SRMAtlas, will provide resources of verified high-resolution spectra and multiplexed SRM assays [171].

This ability to evaluate a quantitative and multidimensional view of the proteome will not only be crucial to enhance our understanding of basic extracellular physiology and regulation but for pre-clinical, therapeutic and drug development studies. We predict an innovative and bright

future for expanding the application of MS-based proteomics methods to researchers in the EV biology community.

10. Key issues

- Non-standardized EV isolation protocols (for exosomes and sMVs), resulting in often heterogeneous EV mixtures and variation between studies, thereby making comparative conclusions between studies challenging
- Multidimensional proteomics for EV biology, where biochemical fractionation to be applied to reduce complexity and increase protein coverage (i.e., EV membrane /lumen proteome)
- Advent of technological innovation in proteomics (minimal sample material and preparation, microfluidic devices, biological dynamic range, reproducibility and specificity of techniques, PTMs, membrane/surface enrichment)
- Utilisation of an in-depth top-down proteomics to identify sequence variants and bioactive protein modifications in EVs
- New advances in "interactome profiling" focusing on network-centred strategies for EV analysis to highlight protein interactions and the influence of protein structures during biogenesis, trafficking and function
- Complementing experimental developments and purification strategies with refinements in data-sharing resources to ensure that the availability and quality of data for such experimental approaches is accurate
- The contribution of proteins and their modified forms in controlling the selective sorting of exosomal miRNAs/other cargo and selective incorporation into EVs

- Integrated proteomic, genomic, lipidomic, and metabolomic investigations to provide an integrative systems biology approach of EV biology
- Understanding the contribution of protein complexes in EV biology, and identifying and characterising these regulated processes, and their effects on function, stability and localization
- Extending our understanding of distinct mechanisms that underpin cell-type specific EV recognition and entry into cells
- An ongoing challenge is to precisely assign EV-mediated phenotypic change to one and/or multiple EV constituents

References of Importance

(*=of importance, **= of considerable importance)

** Ref 47 (Xu 2015)

Parallel purification strategy to isolate exosomes and shed microvesicles from cancer cells, label-free proteomics and in-depth characterisation to reveal such EVs are biochemically and functionally distinct.

** Ref 32 (Xu 2016)

comprehensive overview of the properties, biogenesis, current methodologies, functions, and challenges associated with exosome research.

** Ref 17 (Demory-Beckler 2013)

comprehensive proteomic analysis of exosomes utilising discovery and targeted approaches to reveal the oncogenic composition of such vesicles directly modulates tumour progression

** Ref 51 (Palmisano 2012)

Specific isolation and characterization of membrane proteins from secreted EVs (exosomes and sMVs) using fractionation, protein-based labelling, and high accuracy mass spectrometry, in addition to peptide phosphoration and glycosylation enrichment strategies

** Ref 24 (Larance 2015)

Comprehensive review focusing on advances in multidimensional analysis in proteomics and how these approaches are transforming understanding of various cellular and physiological processes.

** Ref 16 (Costa-Silva 2015)

Utilised in-depth molecular biology and discovery proteomics to reveal the mechanism of how cancer exosomes regulate metastasis, eliciting pre-metastatic niche formation through a multi-step process.

* Ref 127 (Martins-Marque 2015)

Integration of immunopurification and MS-based data independent acquisition of purified human-derived exosomes to reveal interacting network

* Ref 75 (Kowal 2016)

Performed density-based fractionation and immuno-isolation to comprehensively characterization of heterogeneous populations of EV subtypes to reveal novel markers

* Ref 61 (Tauro 2013)

Sequential immunocapture and in-depth label-free-based mass spectrometry was used to identify distinct populations of exosomes (from apical and basolateral surfaces), which were different in biophysical characteristics and proteomic profiling to sMVs .

* Ref 86 (Zubiri 2015)

Performed tissue and urinary proteomics, utilising discovery and targeted strategies, to reveal exosome cargo as select markers for diagnosis and progression monitoring of diabetic kidney disease

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Figures

Figure 1 – Extracellular vesicle (exosome/sMVs) biogenesis and enriched protein markers

(A) Proposed molecular machineries involved in exosome and sMV biogenesis and release. Components of donor cells are selectively incorporated into extracellular vesicles (exosomes, sMVs) that contain diverse cargoes such as signalling proteins, transcriptional regulators, various RNA, DNA, and lipids species. Multiple components are involved in exosome biogenesis of ILVs (intraluminal vesicles) and MVBs (multivesicular bodies). ESCRT components are involved in an ESCRT-dependent intracellular pathway that selectively traffics MVBs and their ILV contents (exosomes) out of the cell, while other ESCRT-independent pathways (lipid-dependent) have also been described. Members of the Rab GTPase family have been shown to modulate exosome secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. It is unknown whether each of these pathways acts on different MVBs or on the same MVB concomitantly, or how exosome/ILV-loaded MVBs differ from those destined for lysosomes. For sMV biogenesis, there is limited understanding of the molecular machinery regulating outward budding of the plasma membrane and sMV sorting and vesicle release, apart from a requirement of ARF6, Rac, RhoA, Cdc42, acid sphingomyelinase activity, and some ESCRT components (ESRCT-I) (adapted from [32]). *(B) Proteins selectively enriched in exosomes and sMVs based on proteomic analysis of cancer-derived EVs.* Enriched categories and specific proteins in exosomes, sMVs, and common proteins to each EV subtype are shown. EV studies and protein selection category based on references [45, 47, 48, 61, 76, 85, 102]. Enriched categories in exosomes include tetraspanins, trafficking/sorting GTPases, ESCRT/ESCRT accessory components, and the NED proteins involved in cargo selection. In contrast, enriched categories in sMVs include cytoskeletal/microtubule related components, mitochondrial proteins, and

various enzymatic proteins. For categories commonly expressed between each EV subtype include the integral membrane proteins, various RNA binding proteins, and select cytoskeleton/ microtubule proteins.

Figure 2 - Workflows for discovery and targeted EV-based quantitative proteomics

Quantitative proteomics is a powerful approach used for discovery and targeted proteomic analyses to understand global and select proteomic dynamics in EV biology. Discovery proteomics focuses on optimized protein identification by improving the sensitivity and scope of proteomic analysis, requiring large sample quantities and extensive pre-fractionation, thereby limiting throughput. Alternatively, targeted proteomics strategies focus on improved sensitivity and throughput, although are limited by the number of components that can be accurately monitored and quantified.

DISCOVERY PROTEOMICS: Discovery proteomic strategies compare the levels of individual peptides in a sample (e.g., purified EV subtypes) to those in an identical, but experimentally modified, sample. Several different approaches for discovery label-free quantitation are available, and include spectral counting, and ion peak intensity/area. Spectral counting compares the spectra from different samples to determine peptide abundance between samples [48, 61, 75, 85, 102]. An increase in protein abundance typically results in an increase in the number of proteolytic peptides, protein sequence coverage, identified unique peptides, and the number of identified total MS/MS spectra (spectral count) for each protein [172]. In contrast, relative quantitation by ion peak intensity relies on LC-MS only (no MS/MS), with correlation between chromatographic peak area and the peptide/protein concentration [173]. Unlike quantitation by peak intensity, spectral counting does not require algorithms or other peak alignment and comparison processing tools, although significant normalization is required [48, 61]. These discovery label-free approaches offer a direct, sensitive platform, with no sample pooling, which can be integrated into different workflows without any extra effort or cost, best suited for quantification of enrichments in biochemical purifications in which fold changes to be quantified are reasonably large. However, such label-free workflows must be tightly controlled to avoid bias, due to the fact that unlabeled samples are individually analyzed.

Metabolic labeling is characterized by either the isotopic or isobaric labeling of proteins, after which samples are combined and processed for quantitative analysis. For stable isotope labeling by amino acids in cell culture (SILAC), cells are cultured in growth medium that contain $^{13}C_6$-lysine and/or $^{13}C_6$-arginine, resulting in selective proteotypic tryptic peptide labelling for at least one amino acid, resulting in a constant mass increment in labeled in comparison to non-labeled samples [174]. A key advantage of this SILAC-based metabolic labelling approach is the capability of highly sensitive protein identification and quantification, due to the fact that heavy and light samples are combined before sample preparation, minimizing the level of quantitation bias from processing errors, allowing identification of relatively small changes in protein abundance. Importantly, metabolic labeling may not be amenable to cell lines difficult to culture, or sensitive to changes in culture conditions, or may influence how the organism/model functions, as growth conditions are changed to allow incorporation of heavy compounds.

In addition to metabolic labelling, chemical or enzymatic isotopic labeling strategies are available - particularly suited for tissue samples derived from animals or humans where metabolic incorporation is difficult. In EV research, several different approaches have been employed including dimethyl-labelling, ^{18}O labelling, and isobaric labeling with mass tags. Dimethyl-labelling uses formaldehyde in deuterated water to label primary amines with deuterated methyl groups, allowing for a rapid, relatively inexpensive approach for diverse sample types, preferentially used for size-limited human tissue specimens [175]. However, this approach requires relative homogeneous samples or sample preparation to reduce the biological complexity. For ^{18}O labelling, samples are digested with either trypsin and ^{18}O water or ^{16}O water, and samples extracted and combined [176]. Although relatively easy to implement, the method is limited by inhomogeneous ^{18}O incorporation and inability to compare multiple samples within a single experiment. Further, isotope incorporation can be performed

at the protein or peptide level using, for example, ^{2}H, ^{13}C, ^{15}N or ^{18}O, as heavy isotopes [177]. Isobaric tags offer the significant advantage of having identical masses and chemical properties that allow heavy and light isotopologues to co-elute, resulting in concurrent peptide identification and relative quantitation [178]. Following fragmentation-induced tag cleavage, the tags are used to quantitate relative peptide intensities, with the peptide fragment ions sequenced for protein identification. The method allows for no increased complexity at the MS level, increased throughput for LC-MS analysis (up to eight experimental conditions in one analytical experiment), is expensive, and applicable to a diverse range of sample types. There are two different available isobaric tags: tandem mass tags (TMT) and isobaric tags for relative and absolute quantitation (iTRAQ), with iTRAQ based on the covalent labeling of the N-terminus and side chain amines of peptides from protein digestions with tags of varying mass.

TARGETTED PROTEOMICS: For targeted proteomics, selection of proteins and specific peptides and peptide fragments are determined, with the intensity of each transition analysed. For absolute quantification, the use of spiked-in isotopically labelled peptides generated by sample digestion, allows for co-elution with the target peptide and are concomitantly analyzed. The concentration of analytes is determined based on pre-determined calibration-response curve. This selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) approach allows for a very high level of reproducibility and sensitivity, although requires extensive assay optimization, selection of appropriate and specific tryptic peptides, and is expensive [121]. Recently, parallel reaction monitoring (PRM) provides an alternative to quantify targeted proteins and peptides with the Orbitrap analyzer - in comparison with the quadrupole analyzer used in SRM - providing a higher selectivity due to higher resolution [123]. Further, sequential window acquisition of all theoretical mass spectra (SWATH) has been implemented as a DIA strategy, where data is sequentially acquired in precursor-ion selection windows over a large window range allowing for retention time, fragment ion m/z,

and intensity to be acquired. SWATH therefore provides a permanent, entire record of fragment ion spectra of all peptides detectable in real time, retrospective capability, and the ability to perform a significant larger number of SRM-like experiments concurrently [179]. However, SWATH-MS data is incompatible with conventional databases, and instruments used to acquire the data are limited based on mass resolution and accuracy. The combination of high specificity fragment ion maps and targeted data analysis using information from spectral libraries of complete organisms offers unprecedented possibilities for the qualitative and quantitative proteome analyses.

Tables

Table 1 – Protein cargo directly influence extracellular vesicle function

Functional Category	Protein Cargo	Donor cell	Recipient (target) cell	Study Description	Reference
Oncogenic EV proteins	MIF	Pancreatic cancer cells	Liver Kupffer cells	Promotes liver pre-metastatic niche formation that enhance pancreatic cancer cell metastasis to liver	[16]
	Angiogenin, IL-8, VEGF	Glioblastoma cells	Endothelial cells	Promotes tube formation	[39]
	αvβ6 Integrin	Prostate cancer cells	Prostate cancer cells	Promotes tumour cell migration	[22]
	Met	Melanoma cells (highly metastatic)	Bone marrow derived cells (BMDC)	Enhance metastasis by mobilizing BMDC to lungs to establish a pre-metastatic niche	[19]
	KRAS	Colon cancer cells (mutant KRAS)	Colon cancer cells (wild-type KRAS)	Enhances anchorage independent growth of recipient cells	[17]
	integrin's α6β4, α6β1,αvβ5	breast and pancreatic cancer cells	Lung fibroblasts, epithelial cells, brain endothelial cells	Select integrin's promote adhesion by fusing with specific resident cells (α6β4 and α6β1 associated with lung metastasis, with integrin αvβ5 to liver metastasis), activate Src signalling pathways and inflammatory responses (pro-inflammatory S100 gene expression) in target cells	[23]
Sequestered tumour suppressor proteins	Hsp72	Murine thymoma, mammary carcinoma, colon carcinoma cells	MDSCs	Induces immunosuppression and enhances tumour growth	[180]
	PTEN	Astrocytes	Metastatic tumour cells	Brain (astrocyte)-derived exosomes mediate an intercellular transfer of PTEN-targeting microRNAs to metastatic tumour cells, while astrocyte-specific depletion of PTEN-targeting microRNAs or blockade of astrocyte exosome secretion rescues the PTEN loss and suppresses brain metastasis	[181]
EV cell surface proteins	HSPGs (Heparin sulfate proteoglycans)	human glioblastoma cells	CHO cells	HSPGs function as internalizing receptors of cancer cell-derived exosomes. Enzymatic depletion of cell-surface HSPG or pharmacological inhibition of endogenous proteoglycan biosynthesis significantly attenuates exosome uptake	[46]
Regeneration-related EV proteins	Annexin A1	Intestinal epithelial cells	Intestinal epithelial cells	ANXA1-containing EVs activate wound repair circuits.	[182]
	Wnt4	Umbilical cord mesenchymal stem cell	Skin cells	Exosome-delivered Wnt4 provides new aspects for the therapeutic strategy of MSCs in cutaneous wound healing	[183]
EV neurodegeneration disease proteins	PrP^{C}	Prion-indected MoRK13 cells	A2 MoRK23 cells	Mediate intercellular transfer of prions	[184]
	SOD1	NSC-34 motor neuron-like cells	NSC-34	Mediate intercellular transfer of misfolded WT SOD1	[185]
	tau	Microglia	Cortical neurons	microglia spreading tau via exosome secretion to contribute progression of tauopathy	[186]

Table 2 - Classification and characteristics of extracellular vesicles

*EV type**	*Characteristics*				
	Biogenesis	***Size (nm)***	***Density (g/mL)***	***Protein markers***	***Cargo / Contents***
Exosomes (Exos)	Endosomal pathway; intra-luminal budding of multivesicular bodies, and fusion of multivesicular body with cell membrane. Involvement of ESCRT-dependent and ESCRT-independent (tetraspanin / lipid) mechanisms of formation	30–150	sucrose: 1.13-1.21 iodixanol: 1.10-1.12	Tetraspanins (CD81, CD63), ESCRT components, PDCD6IP/Alix, TSG101, flotillins, integrin's	mRNA, microRNA (miRNA), other non-coding RNAs, ssDNAs, dsDNAs mitochondrial DNA, and oncogene amplifications; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules
Microvesicles (shed microvesicles, ectosomes) (sMVs)	Plasma membrane/cell surface; direct outward budding of cell membrane. Cortical actin reorganization and various contractile proteins and distinct lipid species involved in formation of sMVs	100–1,500	sucrose:1.16 iodixanol: 1.18-1.20	KIF23, RACGAP, CSE1L, ARF6, EMMPRIN	mRNA, miRNA, non-coding RNAs, dsDNAs, cytoplasmic proteins and membrane proteins, including receptors
Large oncosomes	Non-apoptotic plasma membrane derived blebbed microvesicles by "amoeboid" migrating tumour cells	1,000-10,000	Unknown	CAV1, ARF6, DIAPH3, EGFR	miRNA, proteins
Apoptotic bodies	Plasma membrane/cell surface; direct outward blebbing of apoptotic cell membrane	500–2,000	Sucrose: 1.16-1.28	Phosphatidylserine, histones, calnexin, cytochrome C	Nuclear fractions (dsDNAs), cell organelles

Table 3 – Quantitative proteomic strategies utilised for extracellular vesicle biology

	Method	Application	Accuracy (process)	Quantitative proteome coverage	Linear dynamic range	Advantage(s)	Limitation(s)	References (relating to EVs)
Discovery	Label-free (spectral counting)	○ Simple biochemical workflows ○ Whole proteome analysis ○ Comparison of multiple states	+	+++	2-3 logs	Convenient, easy to implement and integrate, direct, sensitive platform, cost effective, no sample pooling	Relative quantification, sample bias, replicate analyses, sample throughput	[16, 48, 61, 100, 106, 154]
	Label-free (ion intensity)	○ Simple biochemical workflows ○ Whole proteome analysis ○ Comparison of multiple states	+	+++	2-3 logs			[23, 63, 91]
	Metabolic protein labelling	○ Complex biochemical workflows ○ Comparison of 2-3 states ○ Cell culture systems only	+++	++	1-2 logs	High sensitivity and accuracy, high reproducibility, low sample bias, identification of relatively small changes in protein abundance	Cell culture only, may not be amenable to cell lines difficult to culture, or sensitive to changes in culture conditions, may influence how the organism/model functions, expensive	[51, 108-110, 117]
	Chemical isotope labelling (MS)	○ Medium to complex biochemical workflows ○ Comparison of 2-3 states	+++	++	1-2 logs	Rapid, relatively inexpensive approach, suitable for diverse sample types	Limited sample complexity, rapid sample processing,	[114, 116, 187]
	Chemical isobaric labelling (MS/MS)	○ Medium complexity biochemical workflows ○ Comparison of 2-8 states	++	++	2 logs	Suitable for diverse sample types, MS2, multiplex capability, no increased complexity at the MS level	Expensive, mass ranges	[91, 111, 112]
Targeted	MRM / SRM / PRM	○ Low/medium complexity biochemical workflows ○ Targeted analysis of few proteins (precise quantification) ○ Assay development, clinical monitoring	++/+++	+	4-5 logs	High level of reproducibility and sensitivity. Provides good relative peptide quantification and can be coupled with heavy labelled reference peptide for absolute quantification. Most	Extensive assay optimization, selection of appropriate and specific tryptic peptides (peptide selection, transition and collision energy optimization, and interference-free transition selection), and is expensive. Requires prior identification of peptides and, in MRM, selection of reproducible	[17, 86, 129, 130]

						sensitive method because of high signal-to-noise ratio	fragments that do not exhibit interference	
	SWATH-MS (DIA)	○ Global identification analysis of all sufficiently abundant peptides based on repeatedly selecting mixtures of peptide species within large, pre-specified mass ranges ○ Biomarker discovery, monitoring, clinical assays	++/+++	+++	4-5+ logs	Unbiased precursor-ion collection, high analyte coverage, retrospective analyses. Requires user selection of precursor m/z windows for MS1 and MS2 scans.	Informatics and database searching (deconvolution and analysis), current instrument mass resolution and accuracy. Requires multiple steps from multiple experiments to compile spectral libraries, with more parameters to choose in recently developed, not-yet-established pipelines. Issues of variation caused by interference from other peptides. Sensitivity dependent on sample complexity, isolation window width, and desired coverage on sensitivity	[126, 128]

Table 4 – Selected quantitative proteomic studies investigating extracellular vesicles

	EV Category	EV Isolation Strategy	EV Marker Proteins	Proteomic Sample Preparation	Proteomic Analysis Strategy (Discovery/Targeted)	Study Overview and Contribution of Proteomics	References
Discovery Proteomics (Label-free)	Exos	DC (12,000*g* 20min, 100,000*g* 70min)	CD63, CD9, CD81, TSG101 (Proteomic)	10 µg sample (cell culture/ plasma), 8M urea, endoproteinase Lys-C and trypsin, C-18 StageTips	Discovery - label-free based (spectral counting) nLC-MS/MS, QExactive Orbitrap (3-5 µg sample), Proteome Discoverer/Mascot	Proteomics identified differentially enriched protein cargo in cancer-derived exosomes. MIF was significantly enriched, which led to investigate the role of exosomal MIF in pancreatic-derived exosomes in cancer progression and metastatic education in liver. MIF as a prognostic marker in plasma exosomes in pancreatic cancer patients who later developed liver metastasis	[16]
	Exos	0.2 µm filter + DC (100,000*g* 2h)	CD9, CD81, FLOT1 (WB/ ImmunoTEM)	Samples (cell culture), 8M urea/2.5% SDS with 200 µl of methanol spiked with internal standard tryptophan-d5	Discovery- label-free based (ion intensity) UPLC-MS/MS, TargetLynx, MassLynx	Proteomics of exosomes derived from cancer, fibroblast, and non-tumorigenic cell lines (MDA-MB-231, HDF, NIH/3T3, E10, and MCF10A) identified 48 proteins exclusive to cancer exosomes: membrane-anchored glypican-1 (GPC1) selected and validated. GPC1 identified in circulating exosomes as a selective candidate marker for diagnosis of early-stage pancreatic cancer	[21]
	Exos	DC (12,000*g* 20min, 100,000*g* 70min)	PDCD6IP/Alix (WB)	20 µg samples (cell culture), 8M urea, endoproteinase Lys-C and trypsin, C-18 StageTips	Discovery - label-free based (spectral counting, ion intensity) nLC-MS/MS, QExactive Orbitrap (3-5 µg sample), Mascot/MaxQuant	Proteomics of brain-, lung- and liver-tropic metastatic exosomes, identified six integrin's among the most abundant adhesion molecules. Functional studies of these select integrin's further demonstrated that exosomal integrin expression as a potential indicator for patient organ-specific metastasis	[23]
	Exos	0.2 µm filter + DC (10,000*g* 30min) + DG (30% sucrose, 100,000*g* 1h)	CD63, FLOT2, TSG101 (WB)	100 µg samples (cell culture), 6-9M urea, ultrafiltration, 55 mM iodoacetamide, trypsin, C-18 StageTips (also validated using 2D-DIGE)	Discovery - label-free based (spectral counting) nLC-MS/MS, LTQ-Velos Orbitrap, Proteome Discoverer/Mascot, Scaffold	Identified distinct effects of exosomes induced by virally-infected carcinoma cells on exosome content by quantitative proteomics. Analysis by proteomic profiling (and further validated using differential gel electrophoresis) identified multiple significant changes compared with the uninfected control cells and between viral groups. These findings suggest that virally-infected cancer-derived exosomes can activate cellular signalling and metabolism.	[100]
	Exos	DC (20,000*g* 20 min, 100,000*g* 70 min) + DG (40% sucrose cushion, 100,000*g* 70min)	PDCD6IP/Alix, TSG101, HSP70 (WB)	Cell culture, 10mM dithiothreitol, 50mM iodoacetamide, trypsin, C-18 StageTips	Discovery - label-free based (spectral counting) LTQ-Orbitrap, LC-MS/MS, Proteome Discoverer/SEQUEST	Proteomics of metastatic melanoma B16-F10 (high-metastatic) and B16-F1 (low-metastatic) exosomes, identified select cargo highly expressed in high-metastatic cell subsets (including Met oncoprotein). Functional (*in vitro* / *in vivo*) studies led to exosomal Met identified to directly contribute to education of bone marrow progenitor cells to establish pre-metastatic niche regions prior to onset of metastasis in lung/bone. Based on discovery proteomics and functional studies, developed a melanoma-specific circulating exosomal signature to predict stage and prognosis.	[19]
	Exos	0.2 µm filter + DC (120,000*g* 70 min)	CD63 (WB, FACS, TEM)	Cell culture, 2D-PAGE, in-gel trypsin	Discovery - label-free based (spectral counting), 7-Tesla LTQ-FT nLC-MS/MS, Mascot (analysis of MC/9 exosomes)	Proteomics identified three distinct mouse proteins (CDC6, ZFP271, CX7A2) expressed in human cells that were not present in mouse MC/9-derived exosomes. Contributed to the development of exosomal mRNAs which can be transferred and translated in recipient cells following exosomal uptake	[38]

					MALDI-TOF, PDQUEST, Mascot (*In vivo* translation analysis)		
	Exos	DC (10,000*g* 40min, 100,000*g* 2-14h) + DG (continuous 0.25-2M sucrose, 210,000g 20h)	CD63, CD81, CD82 (Proteomics), CD81 (EM, WB)	Cell culture, size/ion-exchange chromatography, 5 mM dithiothreitol, 50 mM iodoacetamide, trypsin	Discovery - label-free based (spectral counting), LTQ-Orbitrap/LTQ-XL, LC-MS/MS, ProHits	Proteomics identified highly abundant tetraspanins CD63, CD81, CD82, as well as their interacting partners, Ptgfrn and Igsf8 in stromal fibroblast-derived exosomes. These findings led to further investigate $CD81^{+}$ exosomes secreted from stromal fibroblasts promote breast cancer cell motility and metastasis through Wnt-PCP signalling, and establish the role of exosomes in mediating stromal-cancer communication during cancer development and progression	[106]
	Exos	0.1 μm filter + DC (10,000*g* 30min, 100,000*g* 1h), DG (5-40% iodixanol, 100,000g 18h), IAC (EpCAM-Microbeads, 100,000*g* 1h)	PDCD6IP/Alix, HSP70, TSG101	20 μg sample (cell culture)/SDS buffer, in-gel 10 mM dithiothreitol, 25 mM iodoacetic acid, trypsin	Discovery - label-free based (spectral counting), LTQ-Orbitrap, nLC-MS/MS, Mascot	This study used proteomics to evaluate 3 different methods to isolate exosomes from cancer cells. Identified known exosome markers and proteins associated with exosome biogenesis, trafficking, sorting and release. Label-free spectral counting was used evaluate the effectiveness of each method. Based on significant enrichment of exosome markers relative to each of the other methods, immunoaffinity was determined the most effective method to isolate exosomes	[76]
	Exos/ sMVs	Exos: 0.1 μm filter + Sequential IAC (A33-Dynabeads, flow through, EpCAM-Microbeads) sMVs: DC (10,000*g* 30min)	Exos: PDCD6IP/Alix, TSG101, EpCAM, A33 (WB)	20 μg sample (cell culture)/SDS buffer, in-gel 10 mM dithiothreitol, 25 mM iodoacetic acid, trypsin	Discovery - label-free based (spectral counting), LTQ-Orbitrap, nLC-MS/MS, Mascot	Using sequential immunoaffinity isolation to target A33 glycoprotein and EpCAM on the surface of polarised cancer-derived exosome subtypes, proteomics was used to characterise these distinct subpopulations of exosomes. Based on cell biology and proteomic profiling, A33-derived exosomes were shown to be released from the apical surface of cancer cells, while EpCAM-exosomes were shown to be released from basolateral surfaces. Further markers were identified which were selective and common between these subpopulations, in addition to characterisation of sMVs	[61]
	Exos	DC (10,000*g* 30min, 100,000*g* 1h) + DG (5-40% iodixanol, 100,000*g* 18h)	PDCD6IP/Alix, TSG101, FLOT1, CD9 (WB, Proteomics)	30 μg sample (cell culture)/SDS buffer, in-gel 10 mM dithiothreitol, 25 mM iodoacetic acid, trypsin	Discovery - label-free based (spectral counting), LTQ-Orbitrap, nLC-MS/MS, Mascot	Proteomics identified enrichment of metastatic factors, signal transduction molecules, and lipid-raft and lipid-raft-associated components in exosomes derived from human isogenic cancer cells (metastatic SW620, compared with primary SW480). Based on endothelial cell proliferation, exosomes were shown to transport functionally active cargo. Further identified exosome protein complexes EpCAM-CLDN7 and TNIK-RAP2A in exosomes, providing insights into the cargo exosomes transport during tumour progression	[48]

	Exos	DC (10,000*g* 30min, 100,000*g* 1h) + DG (5-40% iodixanol, 100,000g 18h)	PDCD6IP/Alix, TSG101 (WB)	20 µg sample (cell culture)/SDS buffer, in-gel 10 mM dithiothreitol, 25 mM iodoacetic acid, trypsin	Discovery - label-free based (spectral counting), LTQ-Orbitrap, nLC-MS/MS, Mascot	Proteomics used to assess the contribution of exosomes following epithelial-to-mesenchymal transition (EMT): MDCK cells and MDCK cells transformed with oncogenic H-Ras. Proteomics identified typical EMT hallmark proteins seen in cells correlate with exosomes (reduction of characteristic inhibitors of angiogenesis, epithelial markers, with concomitant up-regulation of mesenchymal makers including vimentin). Mesenchymal exosomes shown to be reprogrammed with select proteases and integrin's implicated in regulating the tumour microenvironment to promote metastatic progression, in addition to key transcriptional regulators (e.g., YBX1) and core splicing complex components capable of inducing EMT in recipient cells	[85]
	Exos	DC (10,000*g* 30min, 100,000*g* 1h) + DG (5-40% iodixanol, 100,000*g* 18h)	PDCD6IP/Alix, TSG101 (WB - cell and primary)	10 µg sample (cell culture), SDS buffer, in-gel trypsin, C-18 StageTips	Discovery - label-free based (spectral counting) LTQ-Orbitrap Elite, nLC-MS/MS, Mascot, Scaffold	Purified human endometrial cell-derived exosomes in presence of menstrual cycle hormones (estrogen and progesterone) were examined for their potential to modify trophoblast function. Proteomics identified select cargo changes which contribute to endometrial-embryo interactions within the human uterine microenvironment to functionally regulate the adhesive capacity essential for successful implantation	[102]
	Exos	DC (10,000*g* 30min, 100,000*g* 1h) + DG (0.2-2.5M sucrose, 210,000*g* overnight)	CD9, CD63 CD81 (WB)	sample (cell culture)	Discovery - Aptamer-based Array (SOMAscan), 20 µg/mL, SomaLogic biomarker discovery assay (Agilent microarray 1129 proteins)	Novel affinity-based (aptamer) proteomics technology to allow simultaneous precise measurement of 1129 proteins enriched in exosomes. Identified various novel proteins not previously reported associated with cancer exosomes	[94]
	Exos/ sMVs	1) DC (10,000*g* 40min, 100,000*g* 90min) + DG (10%, 20%, 30% Iodixanol/Optiprep, 350,000*g* for 1h):2) IAC (CD63, CD9, CD81 coupled with protein A beads)	Exos: PDCD6IP/Alix, TSG101, CD81, CD63, ADAM10, Syntenin-1 sMVs: Actinin-4, Mitofilin, RACGAP1	SDS buffer, in-gel trypsin	Discovery - label-free based (spectral counting) , LTQ-Orbitrap XL, nLC-MS/MS, Mascot, Proteome Discoverer, *my*ProMS, peptide XICs, MassChroQ	EVs from human dendritic cells were separated by ultracentrifugation (10K and 100K pellets), and then by floatation on iodixanol gradients or by immuno-isolation. Extensive quantitative proteomic analyses between low- and high-density exosomes allowed for comparison of isolated EV populations, highlighting several classically used exosome markers, like MHC, flotillin, and HSP70, are present in all EVs. Identified proteins specifically enriched in small EVs, and define a set of protein categories displaying different relative abundance in distinct EVs. Demonstrate presence of exosomal and non-exosomal subpopulations within small EVs, and further isolate these subpopulations by immuno-affinity using either CD63,CD81, or CD9.	[75]

	Exos/ sMVs	DC (10,000*g* 30min, 110,000*g* 70 min) + DG (0.4-2.5M sucrose, 200,000*g*, 16h);SEC: (HiPrep Sephacryl S-400 HR 16/60, AKTA prime system)	Exos: PDCD6IP/Alix, TSG101 sMVs: Actinin-4, CCNY	8M urea, trypsin, strata-X-C-cartridge	Discovery - label-free based (peak area), QExactive, nLC-MS/MS, Proteme Discoverer, SequestHT	Density-gradient centrifugation of isolated melanoma-derived exosome subpopulations revealed the presence of distinct ubtypes (LD and HD based on low- and high-density), differing in biophysical properties and their proteomic and RNA profiles. Further, exosomes from other cell sources (neuroblastoma, squamous carcinoma, heart endothelial cells, mesenchymal stem cells, plasma) evaluated the presence of LD and HD exosome subpopulations on a sucrose density gradient Interestingly, the subpopulations mediated differential effects on the gene expression programmes in recipient endothelial cells.	[63]
	Exos	DC (20,000*g* 75min, 100,000*g* 90 min)		SDS PAGE, in-gel trypsin	Discovery - label-free based (spectral counting) LTQ-FTMS (secretome), nLC-MS/MS, Sequest, Scaffold; QExactive (exosomes), nLC-MS/MS, MaxQuant	Demonstrated that apoptosis-resistant primary AML blasts, as opposed to apoptosis-sensitive cells, were able to up-regulate BCL-2 expression in sensitive AML blasts in contact cultures. Using secretome proteomics identified novel proteins in apoptosis regulation. This analysis revealed that major functional protein clusters engaged in global gene regulation, including mRNA splicing, protein translation, and chromatin remodelling, were more abundant in secretome of apoptosis-resistant AML. These findings confirmed by subsequent EV-based proteomics. Finally, confocal-microscopy-based colocalization studies show that splicing factors-containing vesicles secreted by high AAI (anti-apoptosis index) cells are taken up by low AAI cells	[90]
	Exos/ sMVs	SCUF (different pore-sized PVDF ultrafilters (0.65,0.45,0.22 and 0.1 µm), 3,000 *g*) (sMVs: >0.65 µm , Exos: <0.1 µm)	Exos: PDCD6IP/Alix, TSG101, CD81, CD63 sMVs: KIF23, CSE1L, RACGAP1	10 µg sample (cell culture), SDS buffer, in-gel trypsin, C-18 StageTips	Discovery - label-free based (spectral counting) LTQ-Orbitrap Elite, nLC-MS/MS, Mascot, Scaffold	Using an ultrafiltration approach to separate and isolate distinct EV subtypes (exosomes and sMVs) from culture medium, extensive biochemical and functional characterisation of these EVs was performed, demonstrating one subtype (fraction Fn1) comprised heterogeneous EVs with particle diameters of 30-1300nm, the other (fraction Fn5) being homogeneous EVs of 30-100nm diameter. Proteomics identified select and common marker proteins between the distinct EV subtypes: 350 proteins uniquely identified in sMVs, many have the potential to enable discrimination of this EV subtype from exosomes (notably, KIF23, CSE1L, and RACGAP1). Both EVs shown to be induce invasion of recipient fibroblast cells, with sMVs shown to promote invasion significantly greater than exosomes	[47]
	Exos	ExoQuick; Filter (100K NMWL) + ExoQuick	HSP60	cell culture/serum	Discovery- label -free based (spectral counting), LC-MS/MS, QExactive Orbitrap, X! Tandem/Scaffold	Mass spectrometry demonstrated that the protein content of cardiac exosomes differed significantly from other known types of exosomes and contained cytosolic, sarcomeric, and mitochondrial proteins	[188]
	Exos	DC (12,000 g 60 min, 200,000 g 18h, PBS wash, 200,000 g 18h)	CD9, CD81	Sample (plasma), 8M urea, Sep-Pak C18 cartridge	Discovery- label -free based (spectral counting), nLC-MS/MS, LTQ-FT Ultra linear ion trap, Proteome Discoverer/Mascot	Proteomic profiling identified 252 EV proteins that were modulated based on spectral count ratio after myocardial infarction	[189]

Discovery Proteomics (Labelling)	Exos/ sMVs	DC (10,000*g* 30min, 100,000*g* 70min) + DG (5-40% iodixanol, 100,000*g* 18h) 0.22µm filter + qEV SEC column	PDCD6IP/Alix, CD63, TSG101 (WB)	50 µg sample (cell culture), 4% SDS buffer, Lys-C/trypsin mix (1:20 w/w), TMT-labelling, StageTip SAX fractionation	Discovery – label-based (TMT-labelling), spectral counting, LTQ-Orbitrap XL, nLC-MS/MS, Trans-Proteomic Pipeline, Comet, QuantiMore	Using breast cancer cells, TMT quantitative proteomics approach and support vector machine were employed to identify 251 proteins from purified exosomal cargo proteins (density-based iodixanol-derived) exosomes compared with sMVs, and crude exosomes	[111]
	Exos	0.2 µm filter + DC (100,000*g* 70 min)	CD81 (WB, Proteomics)	Streptavidin-Sepharose microbeads enrichment, SDS-PAGE, in-gel trypsin, C-18 desalting, peptide ^{18}O labelling, isoelectric focusing fractionation (3100 OFFgel)	Discovery – label-based (^{18}O labelling), ion intensity, LTQ-Orbitrap, nLC-MS/MS, SEQUEST, QuiXoT	Proteomics identified intracellular interactome of tetraspanin-enriched microdomains (TEMs) in human lymphoblast cells and exosomes. CD81 shown to be important in TEMs and exosome cargo selection. This study suggests a pattern of interaction networks and a specialized platform for selection of exosome components during biogenesis	[187]
	Exos	0.2 µm filter + DC (12,000g 20min, 100,000*g* 2h) + DG (30% sucrose/D_2O cushion	CD9, CD63 (WB)	200 µg sample (cell culture), 5 mM tricarboxyethyl Phosphine hydrochloride, 20 mM methyl methanothiosulfonate, trypsin, Sep-Pak C-18, 4-plex iTRAQ labelling, SCX fractionation	Discovery - label-based (4-plex iTRAQ labelling), Q-TOF 6540, LC-Chip-MS/MS, MassHunter, ProteinPilot	iTRAQ-based quantitative proteomics used to identify proteins in cancer cell-derived exosomes, 140 proteins identified differentially expressed including various pro-angiogenic proteins. Cancer exosomes shown to significantly increase the tubulogenesis, migration and invasion of human endothelial cells, and validation of select pro-angiogenic proteins in recipient endothelial cells following exosomal internalization	[115]
	Exos	DC (15,000*g* 30 min, 20,000*g* 2h, 100,000*g* 2h) Fractionation (membrane and luminal): (Na_2CO_3 pH 11, ice, 150,000 2h, ultrafiltration	CD63, CD81, PDCD6IP/Alix (WB)	Cell culture (2-5% of total protein), Lys endopeptidase C, trypsin, Poros R2 and Oligo R3 purified, 4-plex iTRAQ labelling	Discovery - label-based (4-plex iTRAQ labelling), hydrophilic interaction LC (offline), nRP-LC-MS/MS, LTQ-Orbitrap Velos, Mascot	Developed a new strategy for quantitative proteomics of exosomal membrane and luminal proteins from *in vivo* metastasis model. iTRAQ proteomics identified several exosomal proteins associated with epithelial-to-mesenchymal transition: increase vimentin and hepatoma-derived growth factor in the membrane, and casein kinase IIα and annexin A2 in lumen of exosomes from metastatic cells	[114]
	Exos	DC (10,000*g* 30min, 100,000g 1h)	FLOT1 (WB), CD63 (ImmunoTEM)	100 µg sample (cell culture), acetone precipitation, trypsin, 8-plex iTRAQ labelling, SCX fractionation	Discovery - label-based (8-plex iTRAQ labelling, ion peak intensity), nLC-Ultra-MS/MS, LTQ-Orbitrap Velos, Mascot, ScaffoldQ+	Quantitative proteomics identified 1354 proteins in exosomes derived in response to different types of cellular stress (hypoxia, TNF-α-induced activation, high glucose and mannose concentration). Several exosomal proteins/mRNAs showed altered abundances after exposure of their producing cells to cellular stress, which were validated by immunoblot or qPCR analysis. Demonstrates that RNA and proteins cargo in exosomes are reflected by hypoxia and endothelial activation	[116]
	Exos	DC (10,000*g* 30 min, 100,000*g* 1h)	TSG101, PDCD6IP/Alix (WB)	cell culture, lysis (RIPA buffer: 1% Triton X-100) Method A: SDS-PAGE gel, in-gel trypsin	Discovery - label-based (SILAC labelling), nUPLC-MS/MS QExactive Orbitrap, MaxQuant, Perseus	Exosome protein content changes induced by Hepatitis B virus (HBV) and HBV oncogenic virion HBx were quantitatively analysed by SILAC/LC-MS/MS causing marked and specific changes in exosome protein contents, further validated in exosomes purified from HBV-infected patient sera	[108]

				Method B: SDS-PAGE gel, 12% PAGE embedded, in-gel trypsin			
	Exos	DC (10,000*g* 30 min, 100,000*g* 70 min)	N/A	2 sample (cell culture), cellytic M reagent, in-gel trypsin	Discovery - label-based (SILAC labelling), nLC-MS/MS, LTQ-Orbitrap XL, Mascot, MaxQuant	Quantitative proteomics identified marked and specific changes in exosome protein contents derived from human lymphocytic cells induced by HIV-1-infection. Specific exosomal changes (regulatory molecules that impact the processes of cellular apoptosis (ANXA5 and LDHB) and proliferation (CD38)). Cells infected by HIV-1 suggested to impact their local environment through exosomal delivery of HIV-1-binding modulatory molecules	[109]
	EVs/ Exos	DC (100,000*g* 70min) Exos: DG (0.32-2M sucrose, 100,000g 1h)	PDCD6IP/Alix, HSP70, TSG101, CD63, CD9 (WB)	Samples (cell lysate, cell culture), cytosolic, membrane, nuclear and cytoskelatal fractions, in-gel trypsin, C-18 StageTips	Discovery - label-based (SILAC labelling), peptide intensity, (unknown instrument), Mascot, MaxQuant	IFN-γ is exchanged via stem cell-derived EVs/exosomes and induces specific activation *in vitro* of pro-inflammatory cytokine signalling in target cells. Identify specific proteome changes in cellular proteome and in EV/ Exos protein cargoes (e.g., protein metabolism cohort, IFN-γ signalling pathway, Stat1 signalling pathway). This study provides evidence that EVs sample the parental cellular response to the microenvironment through cytokine receptors and functionally transfer membrane vesicles	[117]
	Exos/ Large oncosomes (1-10μm diameter)	DC (10,000*g* 30min, 100,000*g* 60min) + DG (5-60% iodixanol, 100,000g 3h)	CD63, CD81, TSG101 (WB)	Samples (cell culture), SDS-PAGE, 10 mM dithiothreitol, 55 mM iodoacetamide, in-gel trypsin	Discovery - label-based (SILAC labelling), ion intensity, nLC-MS/MS, LTQ Orbitrap XL, MaxQuant	Quantitative proteomics identified differentially expressed and unique proteins in large oncosomes (1-10μm) and exosomes (~100nm) from prostate metastatic cells. CK18 is highly expressed in large oncosomes, detected in circulation and tissues of mice and patients with prostate cancer	[54]
	Exos/ sMVs	DC (10,000*g* 30min, 100,000*g* 70min) + DG (5-40% iodixanol, 100,000*g* 18h) 0.22μm filter + qEV SEC column	PDCD6IP/Alix, CD63, TSG101 (WB)	50 μg sample (cell culture), 4% SDS buffer, Lys-C/trypsin mix (1:20 w/w), TMT-labelling, StageTip SAX fractionation	Discovery – label-based (TMT-labelling), spectral counting, LTQ-Orbitrap XL, nLC-MS/MS, Trans-Proteomic Pipeline, Comet, QuantiMore	Using breast cancer cells, TMT quantitative proteomics approach and support vector machine were employed to identify 251 proteins from purified exosomal cargo proteins (density-based iodixanol-derived) exosomes compared with sMVs, and crude exosomes	[111]
	Exos	DC (10,000*g* 20min, 100,000*g* 120min)		Samples (cell were cultured with Exos), SDS-PAGE, in-gel trypsin	Discovery – label-based (10-plex TMT-labelling), ion intensities, Orbitrap fusion, nLC-MS/MS, Protein discovery 1.4	AML exosomes participate in the suppression of residual hematopoietic stem and progenitor cell (HSPC) function in a series of in vitro studies, with the helps from a novel multiplex proteomics technique, we identified candidate pathways involved in the direct exosome-mediated modulation of HSPC function.	[112]
	Exos/ sMVs	sMVs: DC (20,000*g* 2h) Exos: DC (20,000*g* 2h, 100,000*g* 2h) Fractionation (membrane): (Na_2CO_3 pH 11, 250,000 1h)		Samples (cell culture), 6M urea and 2M thiourea, Lys-C, trypsin, C-18 StageTips Phosphopeptide/Sialic acid glycopeptide enrichment on TiO_2	Discovery - label-based (SILAC labelling), nLC-MS/MS, LTQ-Orbitrap XL, ion chromatogram area, Proteome Discoverer, Mascot	Quantitative proteomics identified differentially expressed proteins in beta cell-derived EVs in response to cytokine-induced apoptosis. Further enriched and identified membrane-associated proteins including several cell death and cell signalling molecules. Quantified specific protein phosphorylation and N-linked sialylation sites in exosomes and sMVs	[51]

Targeted Proteomics	Exos	0.2 µm filter + DC (150,000*g* 2 h)	HSP70, FLOT1, TSG101 (WB) WT RAS, KRAS G13D, TSG101, CD9, PDCD6IP/Alix (LC-MRM)	Discovery - 50 µg samples (cell culture), ammonium bicarbonate/ trifluoroethanol, trypsin, isoelectric focusing	Label-free based (spectral counting), nLC-MS/MS, LTQ-Orbitrap, Myrimatch	Proteomics used to identify that mutant KRAS effects protein composition of exosomes released from cancer cells (including DLD-1 cells (WT and G13D mutant KRAS alleles), KDO-1 (mutant KRAS allele only) and Dks-8 (WT KRAS allele only)). Oncoproteins, including KRAS, EGFR, SRC family kinases, and integrin's were identified in exosomes from mutant KRAS cells	[17]
				Targeted – 50 µg samples, SDS-PAGE, 5 fractions per lane between 20-25 kDa, trypsin, isoelectric focusing (IEF)	Label-based (isotopically labelled), defined peptides spiked into standard/ sample (0.5 fmol/µl), LC-MRM, TSQ Vantage triple quadrupole, Skyline	LC-MRM proteomics used to identify/quantify between WT and mutant KRAS protein, showing enrichment of mutant KRAS in exosomes. Exosomal markers were detected in recipient cells via targeted LC-MRM. Exosomes shown to transfer mutant KRAS to cells expressing only wild-type KRAS, resulting in functional changes associated with three-dimensional growth	
	Exos	ExoQuick (1,500*g* 15 min)	CD63, TSG101, HSP70 (WB)	Discovery - cell culture, SDS-PAGE, trypsin, C-18 ZipTips	Label-free based (peptide identification/ion intensity), nLC-MS/MS, LTQ linear IT, SEQUEST, BioWorks	Proteomics used to identify secretory candidate biomarkers related to the pathogenesis of age-related macular degeneration (AMD) (from culture media and isolated exosomes). Common proteins led to further quantification using LC-MRM	[190]
				Targeted - 6M urea, trypsin, C-18 ZipTips	Label-free based (specific targeted peptides, peak area with internal standard), LC-MRM, QTRAP 5500 hybrid triple quadrupole/linear IT	LC-MRM used to quantify 6 selected/common proteins in exosomes derived from aqueous humor and AMD patients	
	Exos	DC (17,000*g* 30 min, 100,000*g* 70 min)	TSG101, CD9 (WB)	Discovery – 25 µg samples (urine), trypsin, dimethyl labelling (heavy/light), C-18 spin column Samples pooled (n=9), 50 µg for 2D SCX-RP	Label-based (isotopic dimethyl labelling), ion intensity, 2D SCX-RP nLC-MS/MS, LTQ-Orbitrap, MaxQuant, ProteinCenter	Proteomics used to identify candidate urinary biomarkers from exosomes for non-invasive bladder cancer (107 differentially expressed proteins)	[129]
				Targeted – 25 µg samples, trypsin, dimethyl labelling	Label-based (isotopic dimethyl labelling, ion intensity/peak area with internal standard), AB/MDS Sciex 5500 QTRAP, LC-MRM	Differences in the expression of 29 proteins (41 peptides) quantified by LC-MRM in urine samples (bladder cancer, hernia, and urinary tract infection/hematuria). Expression of 24 proteins identified as significantly different between bladder cancer and hernia, with TACSTD2 identified and further validated by ELISA in 221 individual urine samples	
	Exos	0.2 µm filter + Exoquick	N/A	Discovery – 20 µg samples (serum), SDS-PAGE fractionation, trypsin	Label-free based (spectral counting), nLC-MS/MS, LTQ linear IT, Mascot, SEQUEST, X! Tandem, Scaffold	Proteomics identified >250 protein candidate diagnostic biomarkers for active tuberculosis from human serum exosomes. Led to development of targeted proteomics assays employing LC-MRM	[130]
				Targeted - samples (serum, culture filtrate, whole cell lysate, or recombinant proteins), in-gel trypsin	Label-free based (peak area), TQ-S, UPLC-MRM, Skyline	Targeted MRM multiplexed assays applied to exosomes isolated from human serum samples to detect 76 peptides representing 33 unique active tuberculosis protein candidatures. These proteins are known mycobacterial adhesins and/or proteins known to contribute to intracellular survival of *Mycobacterium tuberculosis*	

	Exos	DC (17,000*g* 10 min, 175,000*g* 70 min) + Exoquick	PDCD6IP/Alix, TSG101 (WB)	Discovery – 5 µg samples (urine), SDS-PAGE, trypsin, C-18 micro-column	Label-free based (spectral counting), nLC-MS/MS, LTQ-Orbitrap, Proteome Discoverer, Mascot, SEQUEST, X! Tandem, Scaffold	Proteomics identified 25 urinary exosomal proteins differentially expressed in diabetic nephropathy. Three proteins were selected for LC-SRM validation	[86]
				Targeted - 30 µg samples, in-gel trypsin, C-18 micro-column	Label-free based (peak area) LC-SRM, 6460 Triple Quadrupole, Skyline	3 proteotypic proteins (AMBP, MLL3, and VDAC1) responding to diabetic nephropathy were validated in urine	
	Exos	DC (17,000*g* 10 min, 100,000*g* 70 min)	PDCD6IP/Alix (WB)	Discovery - 50 µg samples (urine), 2D-DIGE, in-gel trypsin	Label-free based (DIGE ratio), 4800 Plus MALDI TOF/TOF analyser, 4000 Series Explorer, Mascot	Proteomics identified 11 protein candidates differentially expressed in urine from control and chronic kidney disease patients. Regucalcin highest differential expression (down-regulated) confirmed by WB	[191]
				Targeted - in-gel trypsin, C-18 spin column	Label-free based (peak area), LC-Chip-SRM, 6460 Triple quadrupole (HPLC-Chip Cube), Skyline	LC-SRM identified regucalcin in serum exosomes in control (not in chronic kidney disease patient pooled urine)	
	Exos	Cell-derived Exos: 0.22 µm filter + DC (16,500g 20 min, 120,000*g* 70 min) + DG (sucrose 0.4-2.5M, 160,000g overnight) Plasma-derived Exos (3 mL): DC (12,000*g* 45 min, 110,000*g* 2 h) + DG (sucrose 0.4-2.5M, 160,000g overnight)	CD63, TSG101, PDCD6IP/Alix, FLOT1 (WB)	Discovery – immunopurified (Cx43), SDS-PAGE, in-gel trypsin, C-18 OMIX tips Targeted - label-free based (ion intensity) targeted SWATH-MS, Information Dependent Acquisition (IDA)	Label-free based (SWATH-MS) method was built for a set of Cx43-specific peptides previously identified in a Cx43-immunopurified sample, nLC-SWATH, OpenSWATH, PeakView, AB Sciex TripleTOF 5600+	To selectively target Cx43 (gap junctional protein), targeted-SWATH-MS was generated to identify a set of Cx43-specific peptides, in addition to exosomal proteins, CD63, Alix, Hsc70, and Hsp90. Targeted MS further revealed Cx43 present in exosomes isolated from rat coronary perfusates, culture medium of organotypic heart slices and human plasma, suggesting further biological and physiological roles for Cx43 in cell-exosome communication. Importantly, this study led to demonstrate hexameric exosomal Cx43 could modulate the interaction and transfer of information between exosomes and acceptor cells	[126]

DC; differential ultracentrifugation, DG; density-gradient fractionation, SEC; size exclusion chromatography, TEM; transmission electron microscopy, ImmunoTEM; immuno-transmission electron microscopy, WB; western blotting, MS; mass spectrometry, ELISA; enzyme-linked immunosorbent assay, FACS; Fluorescence-activated cell sorting, proteomic; identified using proteomics, DIGE; difference gel electrophoresis, FT; fourier transform, MALDI-TOF; matrix-assisted laser desorption/ionization time-of-flight, IAC; immunoaffinity capture, SEC; size exclusion chromatography, qEV; size exclusion column, LC-MRM; liquid chromatography– multiple reaction monitoring, SCX-RP; strong cation exchange-reversed phase chromatography.