Comprehensive Proteomic Characterization of Breast Cancer-Derived Small Extracellular Vesicles for Biomarker Discovery

by

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Abstract

Breast cancer (BC) is the most prevalent cancer and the second leading cause of cancer-related mortality among Canadian women. Early detection and treatment significantly improve survival rates. The potential use of small extracellular vesicles (sEVs) as biomarkers for early BC diagnosis has gained increasing interest, mainly due to their promise as a non-invasive detection method. This study compared the sEVs proteome derived from a highly metastatic BC cell line, MDA-MB-231, with its parental cell line and exosomes derived from a non-cancerous breast epithelial MCF-10A cell line. sEVs isolation was performed using a less common approach involving precipitation with polyethylene glycol (PEG)-8000, separation via size exclusion chromatography (SEC), and concentration using filtration. Bioinformatic analyses revealed multiple enriched pathways in MDA-MB-231-derived sEVs contributing to cancer growth and proliferation. The proteins of these pathways can be a good source for BC biomarkers. In this study, three proteins were selected based on their unique presence in MDA-MB-231-derived sEVs and their contribution to the detected pathways related to BC: peroxidasin homolog (PXDN), glutathione hydrolase 5 proenzyme (GGT5), and plasminogen activator inhibitor 1 (SERPINE1). These proteins were validated through mass spectrometry-based parallel reaction monitoring, utilizing isotopically labelled synthetic peptides. This study highlights the promise of sEV-based proteins as non-invasive biomarkers for early BC diagnosis, potentially improving detection methods and patient outcomes.

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Dedication

To

My beloved family, for your unwavering support and love,

I always dedicate my work to you.

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List of Abbreviations

BC	Breast cancer
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
CID	Collision-induced dissociation
DDM	n-Dodecyl-β-D-maltoside
DEPs	Differentially expressed proteins
DGC	Density gradient centrifugation
DTT	Dithiothreitol
dUC	Differential ultracentrifugation
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ESCRT	Endosomal sorting complex required for transport
ESI	Electrospray ionization
ETD	Electron transfer dissociation
EVs	Extracellular vesicles
FASP	Filter-aided sample preparation
FDR	False discovery rate
FFF	Field-flow fractionation
GGT5	Glutathione hydrolase 5 proenzyme
GSH	Glutathione

HCD	Higher-energy collisional dissociation
HDI	Hydrostatic dialysis isolation
HPLC	High-performance liquid chromatography
IA	Immunoaffinity
IAA	Iodoacetamide
ILV	Intraluminal vesicles
ISEV	International Society of Extracellular Vesicles
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
lEVs	Large extracellular vesicles
LFQ	Label-free quantification
mEVs	Medium extracellular vesicles
miRNA	MicroRNA
MMPs	Matrix metalloproteinases
MS	Mass spectrometry
MVs	Microvesicles
MVBs	Multivesicular bodies
MWCO	Molecular weight cut-off
m/z	Mass-to-charge
PCA	Principal component analysis
PEG	Polyethylene glycol

PRM	Parallel reaction monitoring
PXDN	Peroxidasin homolog
Q-tof	Quadrupole-time-of-flight
Rab	Ras-associated binding
RIPK	Receptor-interacting protein kinases
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion chromatography
SERPINE1	Plasminogen activator inhibitor 1
sEVs	Small extracellular vesicles
SPR	Surface Plasmon Resonance
SRM	Selected reaction monitoring
TCEP	Tris(2-carboxyethyl)phosphine

Chapter 1

Introduction

The detection of most human diseases at early stages and the administration of early treatments increase the survival rates among patients[1]. Consequently, continuous efforts by the scientific community are directed toward developing new screening and diagnostic methods with increased sensitivity and specificity while concurrently minimizing the invasiveness of the procedures, alleviating the burden on patients associated with the procurement of clinical samples[2,3].

Cancer, a diverse and heterogeneous group of malignancies, can be characterized by unique molecular signatures that may originate from any tissue within the human body. However, rapidly proliferating cells are more susceptible to oncogenic transformations[4]. Despite the variability among cancer subtypes, several fundamental hallmarks underlie their pathogenesis, including sustaining proliferative signalling, evading growth suppressors, avoiding the immune system, enabling replicative immortality, promoting tumour-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, exhibiting genome instability and mutation, resisting cell death, and deregulating cellular metabolism[5]. Among the diverse cancer types, breast cancer (BC) exemplifies a prime condition that could significantly benefit from the development of novel, non-invasive early detection approaches. BC is the most prevalent cancer diagnosed globally and ranks as the second leading cause of cancer-related mortality in North America[6,7].

Extracellular vesicles (EVs) have been proposed to play crucial roles in BC pathogenesis and have been investigated as potential sources of biomarkers for early BC detection[8–10]. EVs hold a promising future for non-invasive cancer detection strategies because they can be isolated and quantified, and their surface characteristics reflect those of their parent cells. These membrane-bound particles are released in measurable quantities into various human biological fluids, such as urine, saliva, and serum[11]. Notably, oncogenically transformed cells exhibit elevated EV secretion compared to healthy control cells, influencing tumour initiation, progression, metastasis, and drug resistance mechanisms[11,12]. Elucidating the intricate relationship between EVs and cancer can provide insights into the disease profile by analyzing EV signatures, circumventing the need for direct tumour sampling.

1.1 Extracellular Vesicles

EVs is a term that refers to membrane-enclosed particles released by all cells into the extracellular environment[13,14]. The understanding of their functions has evolved significantly since their initial characterization in the mid-20th century. Initially, EVs were thought to serve primarily as a cellular waste disposal mechanism. However, they are now recognized as crucial mediators of intercellular communication, facilitating the transfer of biomolecules between cells, both locally and at distant sites[13]. The earliest evidence of EVs' existence dates to 1945 when Edwin Chargaff acknowledged the presence of "minute membrane debris" in plasma supernatants during blood coagulation experiments[13–15]. The following year, Chargaff and West further characterized these particles as "particulate fractions" resulting from "minute breakdown products of blood corpuscles[16]." In 1967, Peter Wolf employed electron microscopy to visualize these observed particles, describing "platelet dust" images and hypothesizing that the material originated from platelets, distinguishing them from intact ones[17,18]. Aaronson officially introduced the term "extracellular vesicles" in 1971, which used electron microscopy to reveal the complex subcellular structures of the flagellated alga *Ochromonas danica*[19]. This work provided initial insights into diverse intra- and extracellular membranous structures, establishing the biological nature of EVs[13,14,19]. Today, EVs are classified into three main subtypes – exosomes, microvesicles (MVs), and apoptotic bodies – based on their biogenesis within cells and release mechanisms[13,14]. During the 1990s, the field substantially expanded through studies demonstrating that EVs carry out essential immunomodulatory functions and mediate intercellular communication in physiological and pathological processes such as inflammation, proliferation, and neural signalling[14]. Other works investigated EVs' therapeutic potential by showing that dendritic cell-derived EVs loaded with tumour antigens can eliminate cancer cells. More recent research has implicated EVs in various mechanisms of cancer drug resistance and their potential as biomarkers and therapeutic targets in various diseases[13,14]. The field of EV continues to evolve rapidly, shedding light on the critical roles these nanoparticles play in health and disease.

1.1.1 Characterization of EVs

All EVs are defined to be enclosed by a phospholipid-bilayer membrane, secretion methods from cells into the extracellular space, lacking the capacity for self-replication and carrying diverse biomolecules such as proteins, nucleic acids, and metabolites[20]. A central distinction between exosomes and microvesicles lies in their biogenesis mechanisms, implying various biological roles[21]. With the advancement in isolation and characterization methodologies, unique properties and functions were assigned to distinguish individual EVs[22]. However, the biophysical overlapping nature between the subtypes of EVs yields more than one EV subclass that can be isolated simultaneously[23]. In 2018, the shared characteristics between non-apoptotic EVs caused the International Society of Extracellular Vesicles (ISEV) to publish guidelines discouraging the interchangeable usage of “exosomes” and “microvesicles.” Instead, ISEV endorsed the employment of general terminology independent of biogenesis pathways, such as small EVs (sEVs), medium EVs (mEVs) and large EVs (lEVs), or descriptions of density[24]. This nomenclature aims to elucidate the field’s absence of knowledge of precise biogenesis routes for vesicles through standardizing EV classification.

Exosomes

The term “exosomes” was initially introduced to the field by Trams et al. and Heine, who described them as EVs shedding from the cell surface[13,14,25]. However, the term exosomes, known today, was used by Johnstone and colleagues in 1987 to describe the formation of multivesicular bodies (MVBs) structures in reticulocytes[13,14,26]. After fusion with membranes, these structures released contained vesicles into the medium to remove unwanted proteins, causing the reticulocytes to mature into red blood cells[26]. They are the smallest EV subtype, ranging from 30 to 150 nm[27].

Their biogenesis is based on the endolysosomal pathway, where exosomes begin forming with the inward budding of the plasma membrane, leading to the formation of early endosomes[27,28]. These early endosomes then undergo further membrane invagination and budding, resulting in the accumulation of intraluminal vesicles (ILVs) within the endosomal lumen[29]. As more ILVs form, the early endosomes mature into late endosomal structures called MVBs, which can contain dozens of ILVs. Upon fusing the MVBs with the cell plasma, the ILVs will be released into the extracellular space as exosomes[29] (**Figure 1.1**). While the mechanism determining the outcome of MVBs is poorly understood, exosome formation and loading are well characterized[30]. Exosome cargo loading can depend on the endosomal sorting complex required for transport (ESCRT) machinery or be independent[31]. ESCRT-dependent cargo loading involves four complexes, which are ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III and their associated proteins such as (Alix, TSG101, HSC70, HSP90β)[32]. ESCRT-0 sorts ubiquitinated cargo proteins into the lipid domain, while ESCRT-I and ESCRT-II induce membrane deformation to form a stable membrane neck[33]. The ESCRT-III complex then recruits the Vps4 complex, which drives vesicle neck scission and recycling of the ESCRT-III complex, allowing the MVBs to separate into individual ILVs[33]. The fate of MVBs can be degraded in the lysosomes, released into the extracellular space as exosomes by fusing with the cell membrane, or undergo endosome recycling[33,34].

While ESCRT complexes play a significant role in MVB biogenesis and cargo sorting, it is now recognized that MVB biogenesis and EV formation can also occur through ESCRT-independent mechanisms[35]. Even simultaneously silencing all four ESCRT complexes, The generation of EVs was still observed to occur through ubiquitin-independent pathways such as the Ras-associated binding protein 31 (Rab31)-dependent, ceramide-dependent, and tetraspanins-dependent pathways[35–39]. Briefly, upon phosphorylation of Rab31, it recruits flotillin to traffic protein cargos into MVBs for packaging inside the ILVs in an ESCRT-independent manner mediated by lipid rafts. Rab31 also recruits a GTPase-activating protein to inactivate Rab7, a key regulator of late endocytic transport, interrupting the lysosomal degradation of MVBs and enabling the release of ILVs from the cell[37]. Additionally, the ceramide-dependent pathway showed that sphingolipid ceramide plays a role through its cone-shaped structure, possibly facilitating ILV membrane invagination by generating raft-based microdomains[38]. Studies have demonstrated the involvement of sphingomyelinases, which generate ceramide from sphingomyelin, in exosome biogenesis. Specifically, acid sphingomyelinase contributes to vesicle release from glial cells[40,41]. Tetraspanins provide an additional ESCRT-independent route for cargo sorting during MVB biogenesis and ILV formation[39]. Tetraspanins (CD9, CD63, CD81) are a family of membrane-bound scaffolding proteins that incorporate other proteins into tetraspanins-enriched microdomains, thereby controlling vital cellular processes and signal transduction pathways[42]. For example, CD81 tetraspanins have been shown to organize into protein-enriched microdomains that selectively sort cargo molecules into ILVs independently of ESCRT machinery[42].

Microvesicles

The term "microvesicles" was first used in 1971 by Schrier and colleagues to describe blebs released from maturing reticulocytes and platelets during membrane remodelling[43,44]. MVs, also called ectosomes, shedding vesicles, or microparticles, represent a medium-sized subclass of extracellular vesicles ranging from 50-1000 nm[45]. Their biogenesis occurs through direct budding and fission from the plasma membrane, though the precise mechanisms are less well characterized[46] (**Figure 1.1**). Subsequent studies revealed some of the molecular pathways governing the formation of microvesicles. One mechanism involves the recruitment of TSG101 and VPS4 to the plasma membrane by ARRDC1, promoting the generation of ARRDC1-

microvesicles[47,48]. Another study reveals that small GTPases such as ARF1, ARF6 and RhoA enable EV budding directly from cancer cell plasma membranes[47,49–51]. Activation of acid sphingomyelinase on the plasma membrane has also been implicated, as it triggers EV release from reticulocytes through ceramide production[47,52]. Together, these findings demonstrate that ESCRT-dependent and independent pathways centred on the plasma membrane can regulate the biogenesis of microvesicles.

Apoptotic bodies

Apoptotic bodies, the largest subtype of extracellular vesicles, were first observed by Kerr and colleagues in 1972 during the study of apoptosis, a programmed cell death pathway[53]. Apoptotic bodies range from 50 to 5000 nm in size and are formed through plasma membrane blebbing[54] (**Figure 1.1**). The molecular mechanism driving this blebbing involves cleavage of ROCK I by caspase-3 during apoptosis[55]. Cleaved ROCK I phosphorylates myosin light chain, inducing contraction of cortical actin-myosin and building hydrostatic pressure within the cell. This causes the plasma membrane to detach from the cytoskeleton and swell outward upon the uptake of cytosolic contents, forming apoptotic blebs[55].

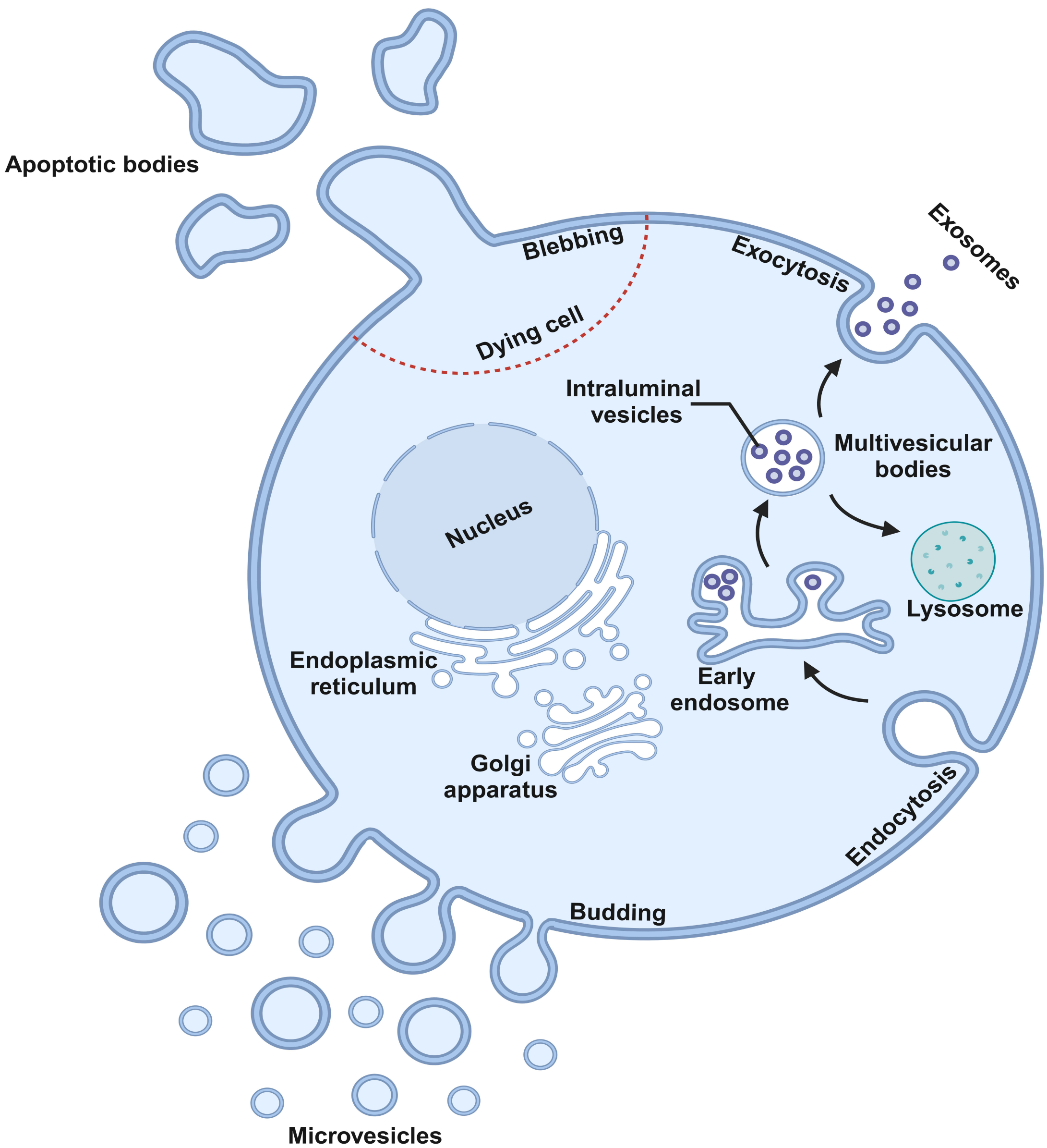

Figure 1.1 Overview of the biogenesis of the subtypes of EVs. Created in BioRender.com.

1.1.2 Isolation Methods of EVs

As interest in studying EVs has grown rapidly, researchers have made substantial efforts to develop techniques for isolating EVs from biological samples. These isolation methods take advantage of the unique biophysical properties of EVs, including their size, charge, density, and the presence of specific surface markers[56]. Several techniques have been employed to isolate EVs, such as differential ultracentrifugation (dUC), density gradient centrifugation (DGC), precipitation, ultrafiltration (UF), size-exclusion chromatography (SEC), and immunoaffinity (IA) capture[57].

The dUC technique is widely regarded as the gold standard method for isolating EVs, separating them into a pellet based on their sedimentation coefficient through sequential centrifugation spins applying varying centrifugal forces[57,58] (**Figure 1.2 A**). This coefficient depends on characteristics such as size, density, and shape[58]. While heavily utilized, dUC yields a heterogeneous mix of EVs attributable to their overlapping nature[59]. Separation through density gradient allows for even higher purity as an alternative. DGC employs preconstructed gradients to profile EVs by their intrinsic density, separating them into subpopulations[57]. The density medium facilitates isolation according to particles' buoyant properties rather than size alone, allowing for more purified enrichment of homogeneous EV subtypes.

UF and SEC are commonly used techniques that exploit size distribution properties inherent to EV subtypes. UF is the most used size-based isolation technique for separating EVs based on size or molecular weight[59]. UF utilizes a semi-permeable membrane to separate EV subtypes based on differential passaging rates attributed to size and molecular weight. Small molecules pass through membrane pores while EVs of larger size and mass are retained[60] (**Figure 1.2 B**). UF is frequently coupled with dUC to enrich particular EV populations since drawbacks like pore clogging reduce its effectiveness when used alone[61]. SEC isolates EVs solely based on size, utilizing a porous stationary phase column that allows for differential penetration – molecules with small hydrodynamic radii pass more profoundly through the porous beads, resulting in late elution.

Conversely, molecules with large hydrodynamic radii are excluded from entering the porous stationary phase, allowing them to elute earlier[62] **Figure 1.2 C**). This separation mechanism preserves EV integrity and biological functionality[63]. Sepharose (agarose) and Sephacryl (polyacrylamide) are commonly used as porous stationary phase beads for such separation. These have improved mechanical strength and are suitable for high flow rate separation in fast purification liquid chromatography applications[64].

The precipitation of EVs works by manipulating EV dispersibility by using volume-excluding polymers like polyethylene glycol (PEG)[65]. Upon addition of such compounds, EVs are precipitated out of solution via reductions in volume (**Figure 1.2 D**). A vital advantage of this technique is that it preserves the biological activity of EVs and yields more isolated EVs than centrifugation, making it among the best options for low-sample applications[66]. However, a subsequent centrifugation or filtration step is necessary to remove any contaminants or excess polymer introduced during the precipitation process[67]. Failure to do so can dry the sample, causing the precipitation of other molecules[22]. Also, it can interfere with downstream analytical techniques like mass spectrometry (MS) analysis by yielding a low number of detected proteins if polymer remnants are carried over[68].

The IA capture-based techniques utilize antibody-antigen interactions to isolate EV subpopulations. Specific antibodies target membrane-bound proteins (antigens) on the EV surface[69] (**Figure 1.2 E**). This approach provides a very highly purified and homogeneous EV sample population[70]. However, a fundamental limitation is that it yields a minimal harvest rate of EV subtypes due to dependencies on antibody availability for antigens of interest. The technique is also limited by the selection of antibodies that can be employed. Additionally, IA capture often requires pretreatment of samples, especially plasma, using techniques such as ultracentrifugation or ultrafiltration prior to antibody incubation[71]. This concentrates EVs and increases the likelihood of capture for optimal yields when working with dilute sample types like plasma.

While the techniques discussed above are among the most commonly employed, the field continues exploring novel methods that leverage EV biophysical traits. For example, sequential filtration applies the principles of ultrafiltration in a multi-step process using sequentially smaller pore membrane filters to concentrate EVs at each stage[72]. Another size-based option is field-flow fractionation (FFF), which injects a sample into a chamber subjected to a lateral flow, pushing larger particles toward walls and allowing the elution of smaller ones earlier. This allows the differentiation of various EVs and even tiny entities[73]. Hydrostatic dialysis isolation (HDI) also capitalizes on size, utilizing a membrane that small particles pass through via diffusion while larger EVs are retained[74].

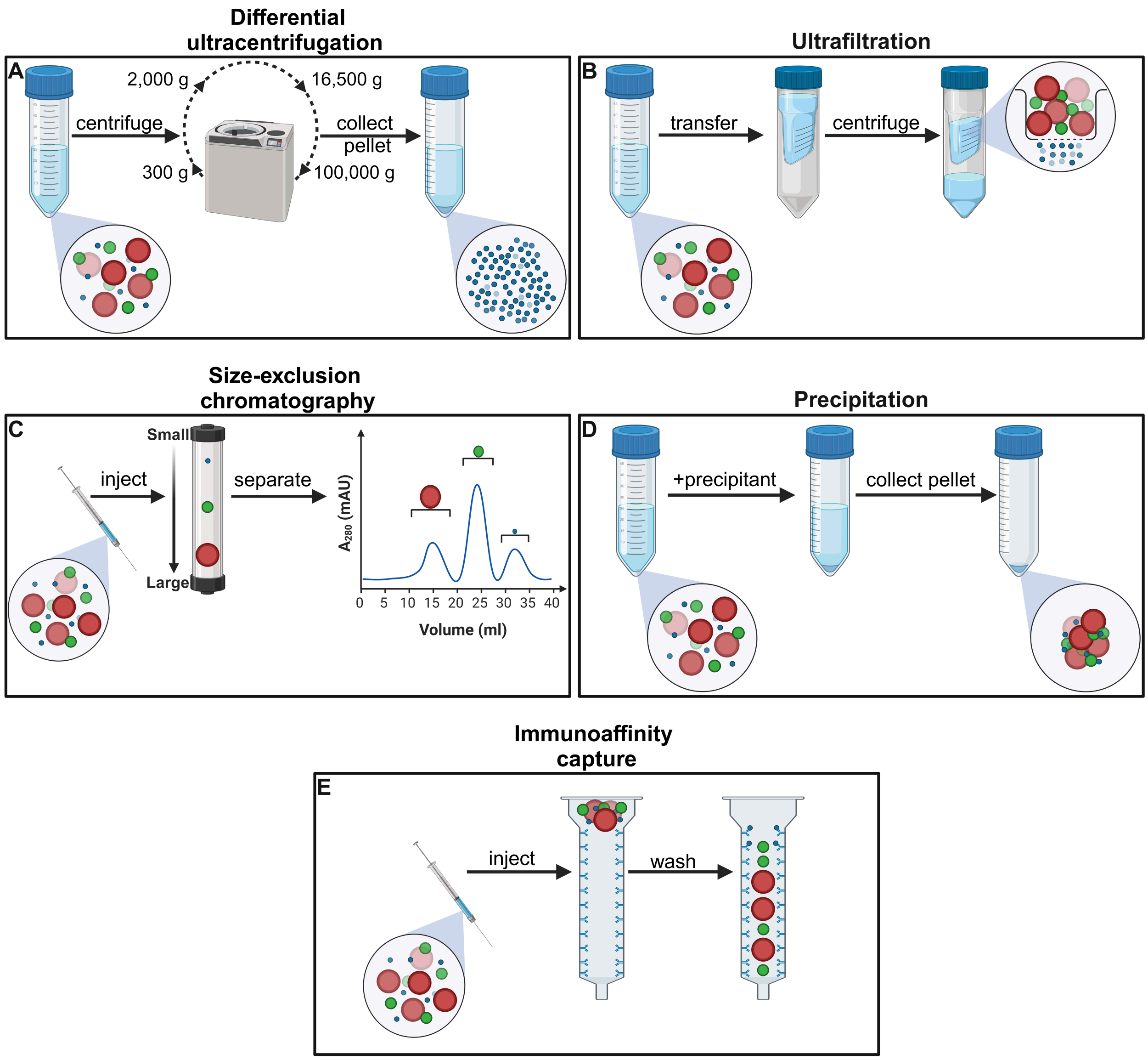

Figure 1.2 Common isolation methods of exosomes. (A) EV populations are subjected to multiple rounds of centrifugation at various forces and speeds. (B) Subtypes of EVs are isolated and retained on a semi-permeable filter where smaller molecules pass through. (C) EV subpopulations are separated based on their elution time due to their sizes. (D) EVs are captured as pellets through the addition of a precipitant molecule. (E) Subclasses of EVs are enriched based on the antigen-antibody interaction. Created in BioRender.com.

1.1.3 Role of EVs in Breast Cancer

It has been established that EVs play an integral role in intracellular trafficking, cellular signalling, and intercellular communication[75]. In recent years, substantial evidence has demonstrated EVs as crucial mediators in BC initiation, progression, metastasis, and acquisition of drug resistance through their roles in intercellular signalling pathways[76–78]. Cancer progression and advancement rely heavily on complex signalling networks within the tumour microenvironment[79]. For example, exosomes secreted by tumour cells have been shown to reprogram the cell microenvironment to support metastasis by inducing stromal cell plasticity, transdifferentiation, and promoting angiogenesis, all contributing to tumour growth[80]. Also, cancer-derived EVs play an essential role in promoting the formation of pre-metastatic niches that enable the spread of tumours by transforming target cells into a more malignant phenotype through transferring the EVs' bioactive contents, such as nucleic acids, proteins, and metabolites. Such transformation allows EVs to influence cells to support tumour cell colonization and growth[79].

Integrins, a family of 24 transmembrane heterodimeric receptors formed by combinations of 18α and 8β subunits, are the primary cell adhesion receptors for extracellular matrix components[81]. Notably, several integrin subunits, such as α1, α2, α3, α4, α5, α6, α9, αv, β1, β3, and β5, are implicated in physiological and pathological angiogenesis[82]. Studies have shown that BC exosomes expressing high levels of integrins such as αVβ5 tend to disseminate to liver tissue rich in fibronectin-enriched extracellular matrix, while those expressing high levels of α6β4 disseminate preferentially to lung tissues containing laminin-enriched extracellular matrix[83]. The proposed mechanism underlying the formation of these pre-metastatic niches involves the activation of pro-inflammatory S100 genes present in lung and liver tissues. Although the exosomal transfer of intact integrin-signalling complexes has not been demonstrated, exosomal-transferred integrin proteins are hypothesized to trigger signalling cascades leading to S100 activation by utilizing Src Kinases derived from either exosomes or target cells. Through this mechanism, cancer-associated exosomes establish organotropic pre-metastatic niches. These findings suggest that disrupting integrin functions and ligand-dependent signalling pathways could be a promising therapeutic strategy for combating various cancers[83,84].

Several microRNA (miRNA) molecules have been identified as potent cancer metastasis promoters, particularly miR-105 and miR-181c[79]. These miRNAs play crucial roles in facilitating the spread of cancer cells to distant sites within the body. miR-105, which is characteristically expressed and secreted by metastatic BC cells, acts as an effective regulator of cell migration by targeting the tight junction protein ZO-1. In endothelial cell monolayers, which form natural barriers against metastasis, exosome-mediated transfer of cancer-secreted miR-105 efficiently disrupts these tight junctions and compromises their integrity. Overexpression of miR-105 in non-metastatic cancer cells induces metastasis and increases vascular permeability in distant organs, facilitating the spread of cancer cells. Notably, miR-105 can be detected in the circulation during the pre-metastatic stage, and its levels in the blood and tumour are associated with ZO-1 expression and metastatic progression in early-stage BC[85]. Another miRNA, miR-181c, delivered by cancer-derived EVs, disrupts the blood-brain barrier by causing abnormal actin localization by downregulating its target gene, PDPK1. The degradation of PDPK1 by miR-181c leads to the downregulation of phosphorylated cofilin, resulting in the activation of cofilin-induced modulation of actin dynamics. These findings reveal a mechanism by which EVs mediate brain metastasis by destroying the blood-brain barrier, allowing BC cells to metastasize to the brain[86].

The involvement of exosomal communication in tumorigenesis has highlighted the potential of developing diagnostic methods to identify tumorigenic-associated vesicle cargo as biomarkers for early detection of BC[87]. Early detection of cancerous cells is a critical factor in BC prognosis, as it can significantly reduce mortality rates[88]. However, current medical approaches such as mammography, ultrasound, and biopsies for detection and diagnosis are costly and time-consuming, posing challenges for healthcare systems and individuals[89]. These issues and inadequate sensitivity for identifying new tumours render most methods unsuitable for widespread BC screening. MS is one of the techniques commonly used for protein biomarker discovery because it can analyze and quantify various biological molecules simultaneously. This unique ability of MS to analyze proteins may make it a valuable tool for characterizing cancer-derived vesicle cargo, advancing the development of biomarkers for optimizing early BC detection[90].

1.1.4 Application of EVs in Biosensing

EVs have emerged as valuable tools in biosensing applications, particularly in cancer detection, owing to their distinctive properties and the crucial information they convey about their cellular origins. In this context, optical biosensors have become integral to EV-based cancer detection methodologies[91]. Surface Plasmon Resonance (SPR)-based biosensors have garnered significant attention. These sensors utilize the oscillation of surface electrons in response to light stimuli, enabling label-free, real-time detection of EVs. The utility of SPR-based biosensors extends to quantifying EV concentrations and elucidating EV-protein interactions, with promising applications in distinguishing HER2-positive breast cancer patients from healthy individuals[92]. The advantages of SPR-based biosensors are multifaceted. They circumvent the need for fluorescent or radioactive labels, thereby simplifying sample preparation and mitigating potential interference[93]. Furthermore, these sensors facilitate real-time observation of binding events, providing valuable kinetic information. Their capacity to detect minute changes in mass on the sensor surface renders them particularly suitable for nanoscale exosome detection[92,93]. However, it is essential to acknowledge the limitations of this technology, including sensitivity issues with low molecular weight compounds, constraints in sensor area and capacity, and susceptibility to non-specific binding interference[94].

In parallel with optical biosensors, electrochemical biosensors have gained prominence in cancer detection due to their high sensitivity and rapid response characteristics[95]. These biosensors operate by converting biological recognition events into quantifiable electrical signals. The mechanism involves a biorecognition element that binds explicitly to EV surface markers, an electrode surface for the recognition event, and a transduction mechanism that translates the binding event into an electrical signal[95,96]. An exemplary application of this technology is the development of a novel electrochemical biosensor for detecting CD147, a protein present on cancer-derived exosomes, potentially facilitating early colorectal cancer detection[97]. Electrochemical biosensors offer several advantages, including rapid response times, high sensitivity, amenability to miniaturization, and potential for portable or point-of-care applications. They also boast excellent detection limits, often in the parts-per-million range. However, these sensors have drawbacks, such as sensitivity to sample matrix effects, limited shelf life and operational lifespan, and comparatively shorter longevity than some alternative sensor types[98].

1.2 Mass Spectrometry-Based Proteomics

The discovery of the central dogma of molecular biology, which described the one-way flow of genetic information from DNA to RNA to proteins, was a transformative milestone in understanding heredity and cellular processes[99,100]. This breakthrough spurred the development of DNA sequencing technologies[101]. Advances in sequencing methods enabled researchers to decode the complete human genome sequence and the RNA transcripts produced from genes[102]. As these techniques became more widely adopted, genomics and transcriptomics fields emerged to systematically study an organism's complete set of genes and the transcribed RNA expressed by the genome, respectively[103]. While DNA and RNA carry the genetic instructions, proteins are the functional molecules that carry out essential biological processes within cells. Recognizing this led to the establishment of proteomics, a discipline aimed at comprehensively characterizing entire proteomes—all the proteins expressed by a genome, tissue, or organism—to gain molecular insights into biological mechanisms at the protein level[103]. For instance, proteomics has been vigorously pursued, particularly for investigating health conditions like cancer. Identifying biomarker proteins can offer perspectives into dysregulated cellular activities underlying these diseases[104].

Proteomics research heavily utilizes liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify and quantify proteomes[105]. Liquid chromatography (LC) separates molecules based on differential partitioning between a liquid mobile phase flowing through a column and a stationary phase immobilized to functionalized particles in the column. Initially, analytes interact with the stationary phase and are then eluted from the column when they strongly interact more with the mobile phase than the stationary phase[106]. Optimizing the mobile and stationary phase characteristics depends on the sample and the analysis objectives[107]. LC-MS/MS exploits differences in analyte solubility and phase interactions to separate compounds of interest from complex sample matrices, improving sensitivity, precision and specificity over mass spectrometry alone.

Various proteomics strategies are employed with LC-MS/MS to study proteins, including bottom-up, middle-down, and top-down workflows[108]. The traditional bottom-up approach involves fully digesting proteins into short peptides for high-throughput LC-MS analysis. However, complete digestion obfuscates the ability to differentiate between distinct protein isoforms. While this workflow yields comprehensive peptide mapping and high protein coverage, the isoform-specific information is inevitably lost[109]. The middle-down strategy circumvents this limitation by employing limited proteolytic digestion to generate longer peptides for LC-MS analysis. This enables interrogating a broader range of peptide fragments, with the longer and partially overlapping peptides retaining isoform-resolving information[110].

In contrast to bottom-up and middle-down techniques that rely on proteolytic digestion, top-down proteomics analyzes intact proteins directly via LC-MS without any prior digestion. This approach provides a more comprehensive characterization of each protein by preserving the molecular distinctions between different proteoforms and isoforms. Top-down proteomics is well-suited for unambiguously determining diverse protein modifications, mutations, and variant proteoforms[111]. However, characterizing heavily modified proteins, especially those larger than 30 kDa, is challenging, making this approach less effective for analyzing complex proteoforms. As the number of modifications increases, the complexity of analysis grows exponentially due to the vast number of theoretical combinations[112]. Each proteomics strategy confers distinct analytical advantages tailored to specific research objectives. Integrating complementary multi-dimensional approaches can synergistically leverage each technique's strengths to deliver the most in-depth insights into the protein composition of biological systems[113].

1.2.1 Bottom-Up Proteomics Workflow

The bottom-up proteomics approach fundamentally involves enzymatic digestion of proteins into peptides, which are then separated by reverse phase LC and analyzed by MS/MS. However, the specific workflow requires careful consideration based on the characteristics of the biological sample matrix. The process can be broadly divided into three key steps: generating identifiable peptides from crude biological samples (**Figure 1.3 A**), separating and analyzing the peptides by LC and then generating MS1 and MS2 spectra by a high-resolution mass spectrometer (**Figure 1.3 B**), and computationally identifying and quantifying proteins by matching the experimental spectra against in-silico generated spectra (**Figure 1.3 C**).

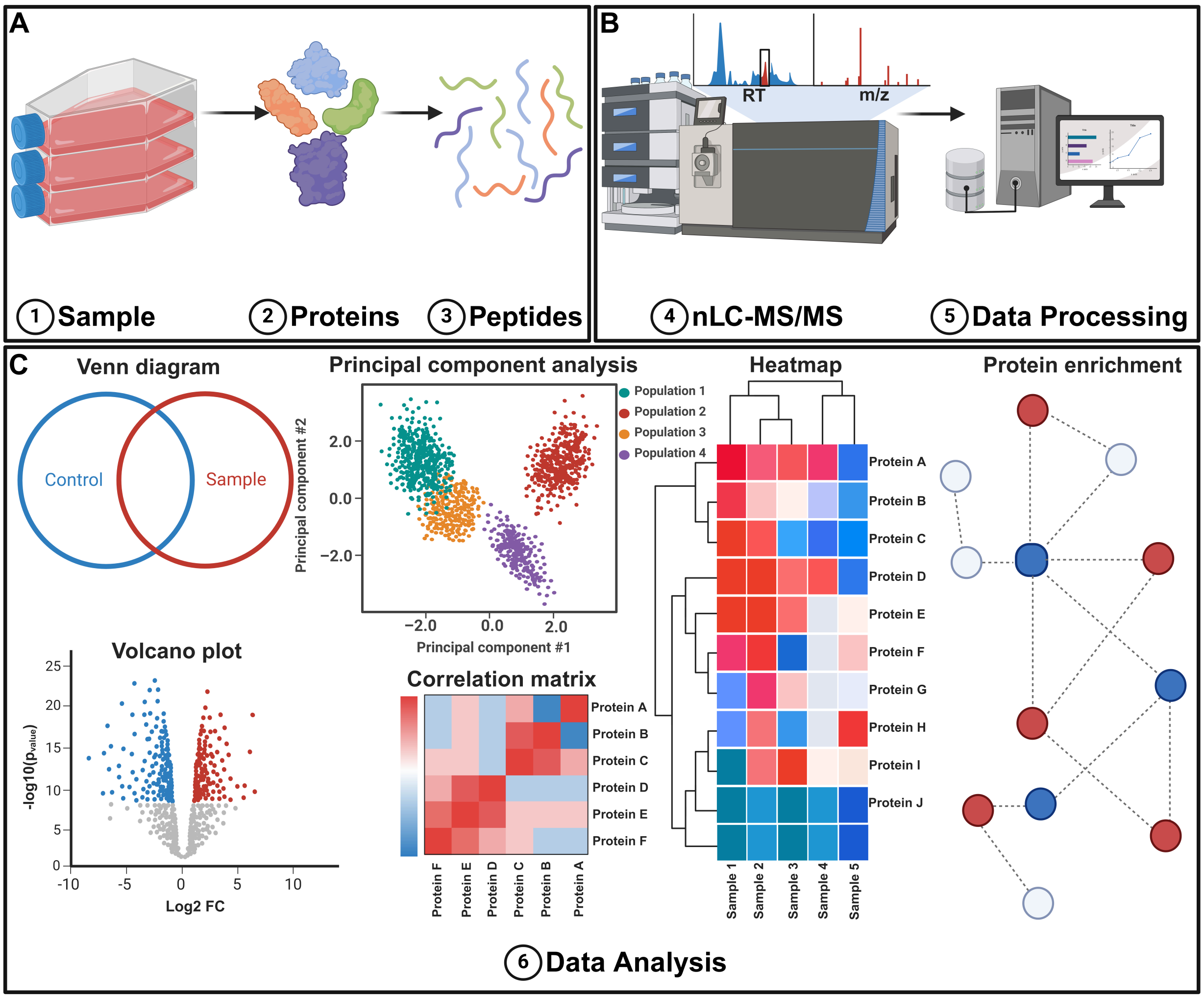

Figure 1.3 Schematic representation of a bottom-up proteomics experiment. (1) A biological matrix of interest is chosen (2) proteins are subjected to extraction (3) proteins are digested into peptides (4) peptides are separated and analyzed by LC-MS/MS (5) Generated mass spectra are processed to identify peptide and proteins (6) Proteins and peptides are manipulated, analyzed, and interpreted. Created in BioRender.com.

Peptide Generation

Enzymatic digestion to generate peptides is a critical preprocessing step in many bottom-up proteomics studies. Successful proteolysis requires optimizing various parameters during sample processing. The nature and composition of the biological sample must be initially considered before selecting appropriate methods for solubilizing and denaturing proteins. The in-solution digestion approach is commonly employed and will be briefly described here[114]. Cell lysis, achieved through heat, mechanical forces, or detergents, is the first step to extracting proteins from the biological matrix. Detergents like sodium dodecyl sulphate (SDS) or n-dodecyl-β-D-maltoside (DDM) are regularly used to solubilize proteins from subcellular complexes or membranes by disrupting intermolecular interactions while maintaining solubility. However, the specific detergent choice depends on the target protein characteristics and downstream application compatibility[115]. Chaotropic agents like urea and guanidine hydrochloride can complement detergents by denaturing proteins through interference with intramolecular hydrogen bonding[116].

Additional processing includes reducing disulphide bonds between cysteine residues using agents like dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) to prevent protein refolding[117]. Alkylating reagents like iodoacetamide (IAA) are also used to cap the reduced cysteines permanently[118]. Once the proteins are sufficiently solubilized and unfolded, enzymatic digestion employs one or more proteases like trypsin, which cleaves proteins with high specificity at the side of lysine and arginine residues. This specificity increases the density of cleavage sites and introduces positive charges into the resulting peptides, which is beneficial for downstream MS analysis. Lys-C protease, which cleaves at the C-terminal of lysine residues, is also commonly used to complement trypsin digestion[119]. Before MS analysis, the peptide mixture is desalted and purified from contaminants like detergents, reducing agents, and salts using solid-phase extraction with C18 resins.

LC-MS/MS Analysis

Following desalting, the peptides undergo LC-MS/MS analysis. A mass spectrometer relies on (i) an ionization source to convert molecules into gas-phase ions, (ii) a mass analyzer that separates ions based on their mass-to-charge ratio (m/z) using electromagnetic fields, and (iii) a detector to measure the separated ion intensities. The characteristics of the ionization source, mass analyzer, detector, and supplementary components like ion traps or quadrupoles influence peptide ion intensity measurements[120]. Additional pre-MS separation using high-performance liquid chromatography (HPLC) is often employed to separate complex samples. A common bottom-up workflow couples nano-flow HPLC with MS/MS, where peptides are first separated based on hydrophobic interactions on a C18 column before electrospray ionization (ESI) generates positively charged gas-phase ions for MS analysis. In MS/MS, the precursor peptide m/z is first determined (MS1), followed by isolation, fragmentation, and sequence analysis (MS2) of selected precursor ions[121]. Different fragmentation techniques like collision-induced dissociation (CID), higher-energy collisional dissociation (HCD), or electron transfer dissociation (ETD) can be employed. CID, the most widely used method, relies on colliding peptide ions with inert gases like helium or nitrogen, converting the kinetic energy into vibrational energy that cleaves the weakest peptide bonds, generating b- and y-ion products. CID's rapid fragmentation enables high throughput MS2 acquisition suitable for routine proteomics studies. However, HCD and ETD can help preserve labile post-translational modifications or enable isobaric labelling strategies[122].

In addition to the standard LC-MS/MS workflow, targeted proteomics techniques can be employed to analyze specific proteins or peptides of interest quantitatively. Several methods have been developed, including selected reaction monitoring (SRM) and parallel reaction monitoring (PRM). SRM is performed using a triple quadrupole mass spectrometer, which includes three quadrupoles arranged in series: Q1, Q2, and Q3[123]. The first quadrupole (Q1) acts as a mass filter, selecting a specific precursor ion from a complex mixture based on m/z. The selected precursor ion is then fragmented through CID in the second quadrupole (Q2), a collision cell. The third quadrupole (Q3) selects specific product ions from the fragments generated in Q2, which are then directed to the detector for quantification[124]. SRM is widely used in targeted proteomics, especially in quantifying proteins by measuring specific peptides. It is beneficial in clinical research for biomarker validation due to its high-throughput, making it ideal for measuring hundreds of analytes in a single run concurrently[125]. Also, the method provides high reproducibility and reliability, which are critical for quantitative studies[126]. However, the method requires careful selection and optimization of transitions, which can be time-consuming and complex. Additionally, SRM can struggle with detecting proteins present in low abundance. This is particularly challenging in complex biological matrices where these proteins might be overshadowed by more abundant species[127].

Conversely, PRM is typically performed on high-resolution mass spectrometers, such as Orbitrap or quadrupole-time-of-flight (Q-TOF) instruments[128]. Like SRM, a specific precursor ion is selected in Q1 and then fragmented in the collision cell. Finally, instead of using Q3 to select specific product ions, an Orbitrap or TOF analyzer acquires a full MS/MS spectrum of the selected precursor ion, meaning all possible fragment ions are monitored simultaneously with high resolution and high accuracy, providing comprehensive data on the fragmentation pattern[129]. PRM is used in proteomics for targeted quantification, similar to SRM. Its ability to monitor all fragment ions simultaneously makes it particularly useful for complex samples where interference might be an issue[130]. PRM improves sensitivity for detecting low-abundance targets and provides high mass accuracy[131]. The use of high-resolution instruments enhances data accuracy and resolution. However, the high-resolution data generated by PRM can be complex and require advanced software tools for accurate interpretation and quantification[132].

MS Spectra Processing

Sophisticated software suites like MaxQuant and Proteome Discoverer enable the processing of complex MS data using spectral matching algorithms to compare the experimental MS1 precursor masses and MS2 fragmentation patterns against an in-silico-generated database of theoretical peptide sequences[133,134]. The primary objective is to derive peptide and protein identification and quantification information from the raw spectral data. However, the parameters for generating in-silico spectra are contingent upon the specific sample conditions under investigation. The appropriate reference proteome in FASTA format must be selected for the biological sample under investigation[135]. Secondly, the correct protease and potential variable or fixed modifications on residues must be considered, as protease specificity influences the generation of peptides. Variable modifications, such as methionine oxidation, occur optionally on residues, while fixed modifications, like carbamidomethylation of cysteines using agents like IAA, arise from sample preparation steps[136]. Decoy databases containing reversed sequences are incorporated to estimate false discovery rates (FDR) and validate identifications. Score distributions from target and decoy matches are compared, with decoys exhibiting lower confidence. An FDR-controlled threshold determines the confidence level required for peptide identification. Contaminant databases are also used to filter out non-sample identifications[135]. Ultimately, this computational workflow generates lists of identified and quantified peptides and proteins, which enable calculating statistical analyses and assessing differential protein abundance changes. This process allows for extracting maximum biological insights by converting the raw mass spectral signals into validated protein identifications and quantitation data.

1.3 Role of MS in Marker Detection

While LC-MS/MS can be used directly for biomarker detection, its primary value in EV research lies in comprehensive proteomic profiling to identify candidate biomarkers that can later be detected using more clinically practical platforms such as immunoassays or targeted assays[137]. MS-based proteomic approaches are utilized to analyze the protein composition of EVs derived from breast cancer cells. This analysis enables the identification of specific proteins that are overexpressed or uniquely present in cancer-derived EVs compared to those from healthy cells, making these proteins potential markers for diagnostic and therapeutic targets[138]. The method of EV isolation significantly influences the content of EVs and must be taken into consideration. A study examining the protein content of EVs derived from mesenchymal stem cells found that different isolation methods yielded varying proteomes[139]. High-speed centrifugation resulted in EVs with a higher abundance of ribosomal and mitochondrial proteins, which were found to induce early apoptosis in cancer cells, an effect not observed with EVs isolated by other methods[139].

In contrast, ultracentrifugation and sucrose cushion methods yielded EVs enriched with proteins involved in immune responses, cell-cell interactions, and extracellular matrix interactions, resulting in a higher abundance of proteins associated with inflammatory responses and coagulation pathways[139]. Another study investigated the effect of isolation techniques on the proteome of EVs derived from MDA-MB-231 TNBC cells[140]. Ultracentrifugation-derived EVs from breast cancer cell lines exhibited higher purity, marker expression, and a more significant number of sEV proteins compared to other methods. In breast cancer plasma samples, ultracentrifugation-derived EVs had the lowest protein contamination and the highest purity, with strong expressions of sEV markers like CD9 and CD81[140]. EVs obtained using a total exosome isolation kit exhibited the broadest size distribution and the highest protein contamination, indicating lower purity despite having the highest particle concentration[140]. The combined approach, which integrates the benefits of both ultracentrifugation and total exosome isolation kit, demonstrated the highest purity regarding particle number and protein contamination ratio. In breast cancer plasma samples, EVs obtained using the combined approach showed the highest proportion of sEV-related proteins and the lowest percentage of lipoprotein-related proteins, making it a promising method for isolating sEVs with improved purity[140]. Bottom-up proteomics is also adept at detecting post-translational modifications, which are often altered in cancer cells and can affect protein function and stability, playing a crucial role in cancer progression and having diagnostic and therapeutic implications. An investigation of the role of lysine acetylation on sEVs derived from MDA-MB-231, MCF-10A, and MCF7 cell lines showed that specific lysine-acetylated peptides could be considered as potential targets for MDA-MB-231[141]. Many acetylated proteins were found to be metabolic enzymes involved in critical pathways such as glycolysis and the tricarboxylic acid cycle, suggesting that the acetylation of metabolic enzymes indicates potential changes in their catalytic activities, which could alter metabolic flux in cancer cells. This modification may contribute to the metabolic reprogramming commonly observed in cancer, known as the Warburg effect, where cancer cells preferentially utilize glycolysis even in the presence of oxygen[141].

Similarly, an investigation of protein phosphorylation of sEVs derived from MDA-MB-231, MCF-10A, and MCF7 cell lines revealed 1,335 phosphopeptides and 587 phosphoproteins unique to MDA-MB-231 sEVs[142]. Among these phosphoproteins were enzymes involved in cancer progression, including ATP citrate lyase, phosphofructokinase-M, sirtuin-1, and sirtuin-6. Additionally, 60 novel phosphorylation sites were observed that can be used in diagnostic and therapeutic studies[142]. Overall, the isolation method and the state of sEVs should be considered when analyzing their proteome, as these factors significantly influence protein composition and, consequently, potential biomarker discovery.

1.4 Thesis Objectives

BC remains a significant public health concern, being the most prevalent malignancy afflicting women worldwide[143]. Despite substantial advances in screening and therapeutic strategies over recent decades, which have improved patient outcomes, a pressing need exists to elucidate the underlying molecular mechanisms driving tumour progression and metastasis[144]. EVs, particularly exosomes, have emerged as pivotal mediators of intercellular communication, facilitating the exchange of biomolecular cargo implicated in physiological and pathophysiological processes[145]. Early MS-based proteomic studies identified proteins such as CD9, CD63, and CD81 as exosomal markers, paving the way for their utilization in characterizing EV populations. Contemporary high-throughput proteomic techniques employing MS now permit extensive profiling of the human proteome, enabling the discovery of protein biomarkers for various diseases, including cancer[146].

The present study aims to comprehensively characterize and compare the proteomes of exosomes secreted by malignant MDA-MB-231 and non-malignant MCF-10A human breast epithelial cell lines utilizing nLC-MS/MS. Furthermore, the exosomal proteomes will be compared against the respective protein profiles of the parental cell lines, facilitating the delineation of potential mechanisms governing the selective sorting of protein cargo into exosomes. The primary objective is to identify differentially abundant biomarker proteins that modulate intercellular signalling and the tumour microenvironment during cancer progression. Additionally, the exosomes will be isolated via a combined methodology incorporating PEG precipitation, SEC for purification, and ultrafiltration for concentration, ensuring the preservation of vesicle integrity and morphology until enzymatic digestion.

Comparative analysis of exosomal proteomic profiles provides insights into the phenotypic state of parental cells through the revelation of disease-associated protein signatures. The discovery of such differentially abundant proteins facilitates the exploration of mechanisms underlying cancer metastasis and tumour progression. In addition, the characterization of exosomal cargoes enhances our understanding of how tumours prepare pre-metastatic niches and modulate the microenvironment to promote their advancement through remodelling cell-cell communication networks.

Chapter 2

Materials and Methods

2.1 Cell Culture

The MDA-MB-231 (ATCC®, Cat No. HTB-26™) and MCF-10A (ATCC®, Cat NO. CRL-10317™) cell lines were obtained from ATCC, Canda. The MDA-MB-231 cells were cultured in DMEM/F12 growth medium (Thermo Fisher, Cat No. 11330032) supplemented with 10% EV-depleted fetal bovine serum (Sigma Aldrich, Cat No. F1051), 100 µg/mL penicillin, and 100 µg/mL streptomycin, and 25 ng/mL of Amphotericin B (Thermo Fisher, Cat. No. 15240096). The MCF-10A cells were propagated in DMEM/F12 growth medium augmented with 5% EV- depleted horse serum (Sigma Aldrich, Cat No. H1138), 100 µg/mL penicillin, and 100 µg/mL streptomycin, and 25 ng/mL of Amphotericin B, 20 ng/mL epidermal growth factor (Peprotech, Cat No. GMP100-15), 0.5 mg/mL hydrocortisone (Sigma, Cat No. H-0888), 10 μ/mL insulin (Sigma, Cat No. I- 1882), and 100 mg/mL cholera toxin (Sigma, Cat No. C-8052). The EV-depleted media for fetal bovine serum and horse serum were generated by diluting them in 1:1 in a cell culture medium and subjecting them to ultracentrifugation at 100,000 g for 20 hours. The two cell lines were subsequently grown in the EV-depleted media within a humidified incubator maintained at 37 °C with 9% CO_2 until reaching 90% surface confluence.

2.2 Exosomes Isolation, Purification and Extraction

Upon both cell lines reaching 90% cell confluency, the cell culture medium was removed, and the cells were washed with phosphate-buffered saline (Thermo Fisher, Cat No. 10010023). Fresh medium was then supplemented to initiate a further 72-hour period of conditioned medium collection. After centrifugation at 2000 g for 20 minutes, the conditioned medium was harvested and incubated at 4 °C overnight with 12% PEG-8000 (Thermo Fisher, Cat No. 043443.A3). The mixture was spun at 4000 g for 20 minutes to pellet EVs, then resuspended in 1 mL of 150 mM ammonium acetate at pH ~7. The samples were injected onto a Sephacryl S-500 HR size-exclusion chromatography column (40 cm x 2 cm, Sigma Aldrich, Cat No. S500HR) and separated at 1 mL/min flow rate using 150 mM ammonium acetate at pH ~7 as eluent. Fractions were monitored with UV absorbance at 280 nm and collected in 2 mL increments. Fractions corresponding to EVs were pooled and concentrated to 500 μL using 30 kDa molecular weight cut-off (MWCO) centrifugal filters (Millipore, Cat No. UFC803024) at 4000 g. EVs were dried in a vacuum centrifuge overnight, producing dry pellets stored at -80 °C for further analyses.

2.3 Transmission Electron Microscopy

EVs were fixed in 2% glutaraldehyde for 30 minutes and applied to 200-mesh Formvar-coated copper grids (Ted Pella, Cat No. 01800, Redding, California, United States) for 10 minutes. After blotting excess liquid and rinsing twice with LC-MS grade water, the grids were negatively stained using Uranyless EM stain (Electron Microscopy Sciences, Cat. No. 22409) for 60 seconds. Excess liquid was removed, and the EVs were visualized using a JOEL JEM 1230 transmission electron microscope operated at 50 kV.

2.4 Dynamic Light Scattering

Concentrated EVs were mixed with 150 mM ammonium acetate solution (pH ~7) in a 1:1 ratio within a cuvette. The mixture was allowed to equilibrate for 2 minutes at room temperature. Subsequently, the cuvette containing the EV-ammonium acetate mixture was measured in the Zetasizer Nano ZS (Malvern Instruments, United Kingdom).

2.5 Nanoparticle Tracking Analysis

EVs' size distribution and concentration profiles were characterized using a ZetaView nanoparticle tracking microscope PMX 110 system (Particle Metrix, Meerbusch, Germany). Measurements were acquired at two camera shutter speed settings, 85 and 40. Before sample analysis, the system was calibrated, and the camera was focused using a suspension of 102 nm polystyrene beads (Cat No. 900383). Serial dilutions from 1:10 to 1:10,000 were prepared from the original exosomal samples to identify the appropriate concentration range of 10^8 – 10^9 particles/mL for optimal instrument performance.

2.6 Total Protein Concentration

The protein content of individual cells and EV samples was quantified using the bicinchoninic acid (BCA) assay. 50 µL of solubilized samples were incubated with 150 µL of 50:1 reagent A:B (Thermo Fisher, Cat No. 23225) for 1 hour at 37 °C. Absorbance readings were taken at a wavelength of 562 nm using a Cytation™ 3 imaging reader. A standard calibration curve was constructed using serial bovine serum albumin (BSA) dilutions.

2.7 Sample Preparation for Proteomics Analysis

The sample preparation utilized the filter-aided sample preparation (FASP) methodology[147]. Frozen EV pellets were first resuspended in 200 µL of a solubilization buffer (1.5% SDS, 3 M urea, and 50 mM TRIS-HCl, pH 8). Conversely, cells were washed with PBS and resuspended in 15 mL of solubilization buffer. Solubilized samples were transferred to a 30 kDa MWCO centrifugal filter (Microcon® 30K device, Millipore, Cat No. MRCF0R030) and incubated there for 10 minutes. After incubation, 400 µL of denaturation buffer (8 M urea, 50 mM TRIS-HCl, pH 8) was added, and samples were centrifuged at 14,000 g for 15 minutes. The residual of SDS detergent was removed through iterative wash with 400 µL denaturation buffer. Subsequent protein reduction was achieved by mixing 8 µL of 100 mM TCEP with 200 µL denaturation buffer and incubating at room temperature for 30 minutes, followed by centrifugation at 14,000 g for 15 minutes. Alkylation of reduced cysteine residues occurred by adding 8 µL of 500 mM IAA to 200 µL denaturation buffer, followed by incubation in the dark at room temperature for 45 minutes. Samples were then centrifuged at 14,000 g for 15 minutes. Further buffer exchange involved the addition of 200 µL digestion buffer (50 mM TRIS-HCl, 0.6% glycerol, pH 8) followed by centrifugation at 14,000 g for 15 minutes. Residual urea was eliminated through repetitive washes with a solubilization buffer. The proteins were then digested using 200 µL of digestion buffer supplemented with 600 ng trypsin/Lys-C protease and incubated at 37°C for 12 hours. Peptides were collected via centrifugation at 14,000 g for 20 minutes. 40 µL of digestion buffer was added, and samples were centrifuged at 14,000 g for 20 minutes, maximizing the peptide collection. The peptides were acidified using 2 µL of 100% formic acid and desalted on in-house C18 TopTip microcolumns. The C18 microcolumns were first washed trice using 50 µL of 70% acetonitrile containing 0.1% formic acid, followed by a triple wash using 50 µL 0.1% formic acid. The samples were loaded onto the C18 microcolumns in 50 µL increments, followed by another triple wash of 50 µL 0.1% formic acid. Finally, the peptides were eluted thrice using 50 µL of 70% acetonitrile containing 0.1% formic acid. The purified peptides were vacuum-dried and stored at -80 °C.

2.8 LC-MS/MS Analysis

Protein samples containing a maximum of 50 µg, as determined by a BCA assay, were analyzed using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 nanoRSLC system (Dionex, Thermo Fisher Scientific). Peptides were separated chromatographically on an in-house packed column (Polymicro Technology) with dimensions of 15 cm × 70 µm ID, Luna C18(2), 3 µm, 100 Å (Phenomenex), using a gradient of water/acetonitrile/0.1% formic acid. The EVs peptides injection onto the column lasted 105 minutes at a flow rate of 0.30 µL/min. An initial 2% acetonitrile stage lasted 7 minutes, followed by a linear gradient to 38% acetonitrile over 70 minutes. The gradient then increased to 98% acetonitrile over 9 minutes and remained there for 10 minutes. Finally, the gradient backed down to 2% acetonitrile over 3 minutes, followed by a 10-minute wash. Conversely, the injection run for the cell peptides lasted for 140 minutes. The run consisted of an initial isocratic hold at 2% acetonitrile for the first 7 minutes. This was followed by a linear increase in the acetonitrile concentration from 2% to 38% over 100 minutes, from 7 minutes to 107 minutes. Subsequently, the gradient was ramped up from 38% to 98% acetonitrile over the next 5 minutes, from 107 to 112 minutes. An isocratic hold at 98% acetonitrile was maintained for 10 minutes, from 112 to 122 minutes. The acetonitrile concentration decreased from 98% to 2% over 8 minutes, from 122 to 130 minutes. Finally, a re-equilibration step at 2% acetonitrile was performed for 10 minutes, from 130 to 140 minutes. Eluted peptides were introduced by nanoelectrospray ionization (nESI) in positive mode at 250 °C and 2.1 kV. The Orbitrap Fusion mass spectrometer operated in top speed mode, acquiring full scan MS spectra from 350-2000 m/z at 60,000 resolutions. Precursors were selected based on monoisotopic mass, the charge state of +2 to +7, and 30-second dynamic exclusion with a 10 ppm window. Automatic gain control was set at $5x10^5$ and $5x10^3$ for MS and MS/MS scans, respectively. Peptide fragmentation was achieved using CID in the linear ion trap mass spectrometer. Precursor ions were isolated with a 2 m/z window and fragmented at a normalized collision energy of 35%.

2.9 Proteomics MS Data Processing

The raw mass spectrometry data was processed using MaxQuant software (version 2.3.0.0) and its integrated Andromeda search engine[133,148]. Peptide sequences were matched against a reference human UniProt FASTA database containing 20,424 entries as of August 30, 2023, supplemented with a file of common contaminants. Typical settings were applied except where noted. Variable modifications accounted for were N-terminal acetylation and methionine oxidation, while carbamidomethylation of cysteines was treated as a fixed modification. A minimum peptide length of 7 amino acids was enforced. False discovery rates of 0.01 were calculated and applied at both the protein and peptide levels using a target-decoy approach of searching reversed sequences. Trypsin specificity was set for cleavage C-terminal to arginine and lysine residues, allowing up to two missed cleavages. An initial mass tolerance of 10 ppm was permitted for precursor ions, while product ion tolerances were 0.5 Da. The 'Match between runs' feature was enabled across all samples to boost peptide identification[149]. Proteins and peptides matching the decoy database were filtered out. Label-free protein quantification required a minimum of two quantifiable peptide ratios.

2.10 Proteomic Data Analysis

The data analysis and visualization were carried out in the R environment. MaxQuant output files for exosomes and cells, proteinGroups.txt were imported into R, with proteins annotated as potential contaminants or reverse identifications by MaxQuant being excluded from further analysis. Proteins must be identified by at least one unique peptide in at least two samples to be considered for downstream analysis.

Principal component analysis (PCA), performed using the prcomp function in R, and complete hierarchical clustering were computed based on scaled label-free quantification (LFQ) intensity values. The analysis included only proteins with LFQ values present in at least 50% of the samples. Missing values were imputed by randomly drawing from a downshifted Gaussian distribution of log-transformed LFQ intensities (shift = 1.8 standard deviations, width = 0.3 standard deviations) to simulate low-abundance protein profiles. Imputed proteins LFQ intensities were used in an unpaired t-test to calculate p-values and the fold change.

2.11 Validation of Proteins with Isotopically Labelled Peptides

For each protein under investigation, a pair of peptides were synthesized with isotopic labelling (SpikeMix L, JPT Peptide Technologies, Berlin, Germany) and diluted in a series using 1% formic acid. Protein samples, not exceeding 50 µg, underwent digestion into peptides and were spiked with the isotopically labelled peptides. The peptide-enriched samples were subjected to chromatographic separation employing a Thermo Scientific™ EASY nLC II System (Thermo Fisher Scientific) equipped with a Thermo Scientific™ Acclaim PepMap™ 100 precolumn (dimensions: 2 cm × 100 µm ID; C18, 5 µm, 100 Å) and a Thermo Scientific™ Acclaim™ PepMap™ RSLC C18 column (dimensions: 15 cm × 75 µm ID; 3 µm, 100 Å). The peptides were loaded onto the columns for 100 minutes at a 0.25 µL/min flow rate. The separation utilized a gradient of water/acetonitrile/0.1% formic acid, progressing from 0% to 6% acetonitrile over 2 minutes then from 6% to 43% over 70 minutes. The gradient increased to 100% acetonitrile over 4 minutes, with a 10-minute hold at 100%. The acetonitrile concentration decreased from 100% to 10% over 4 minutes, followed by a 10-minute wash. The eluted peptides were introduced via nanoelectrospray ionization (Thermo Scientific™ EASY spray, Waltham, Massachusetts, U.S.) into a Q-Exactive Plus hybrid quadrupole-orbitrap mass spectrometer for parallel reaction monitoring (PRM) scanning, guided by an isolation list developed in Skyline software.

Chapter 3

Results

3.1 Isolation and Characterization of EVs

Cells were cultured in EV-depleted media until achieving approximately 90% confluence. The supernatant was harvested and centrifuged at 2000 g to remove cellular debris. The cell-depleted medium was then treated with PEG-8000 and incubated overnight. Subsequently, the PEG-containing medium underwent centrifugation at 4000 g, and the pellet was resuspended in ammonium acetate. The crude sample was fractionated using SEC columns (**Figure 3.1 A**). The initial peak, corresponding to the EVs, was collected and concentrated for subsequent experiments (**Figure 3.1 B**).

A combination of techniques, including DLS, NTA, TEM, and PRM, were employed to characterize the isolated EVs. DLS analysis revealed that EVs derived from MDA-MB-231 and MCF-10A had 106 and 122 nm median sizes, respectively (**Figure 3.1 C**). These findings were corroborated by NTA analysis, which revealed median sizes of 116 and 125 nm for MDA-MB-231 and MCF-10A-derived EVs, respectively (**Figure 3.1 D**). TEM imaging of UranyLess-stained vesicles from isolated fractions was performed to visualize them and examine their membrane structure (**Figure 3.1 E**). Both MCF-10A and MDA-MB-231 cell lines exhibited membrane-enclosed vesicles within the exosomal size range of approximately 100 nm. To further confirm the presence of exosomes in the isolations, bottom-up proteomic profiling was conducted on the collected fractions. The analysis identified 94 out of 100 commonly reported exosomal markers listed in the ExoCarta database (**Figure 3.1 F**). These findings provided strong evidence for the presence of exosomes in the samples, allowing for the progression to subsequent experiments.

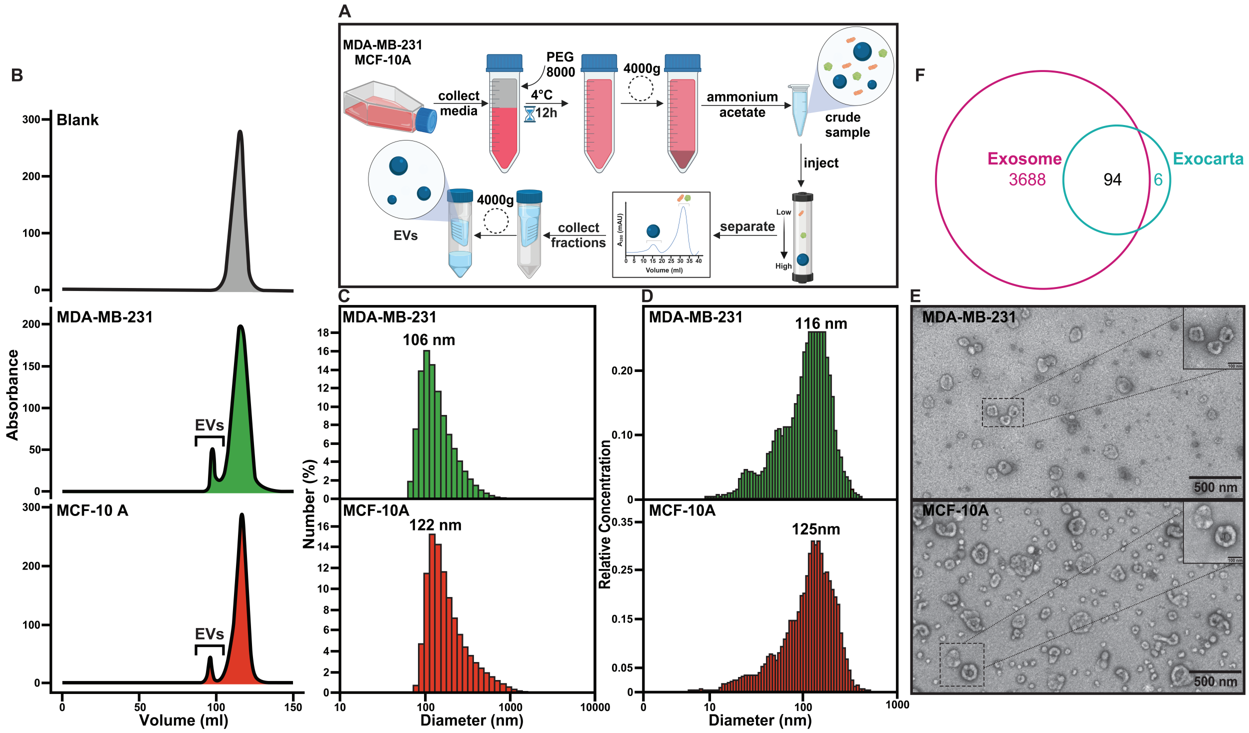

Figure 3.1 Characterization of EVs obtained using a combination of PEG-8000 precipitation, SEC separation, and filter concentration. (A) A schematic illustration of workflow for EV enrichment by PEG-8000, SEC, and filtration. (B) Chromatograms obtained from SEC, detected at A280, indicate EVs' presence within the small peak. (C) Size distribution of isolated exosomes measured by DLS reveals a median of 106 – 122 nm. (D) NTA analysis of the purified exosomes yields a size distribution with a median range of 116–125 nm. (E) TEM images of the enriched exosomes confirm the size and the presence of a membrane-enclosed structure. (F) Bottom-up proteomic analysis of the small peak demonstrates the detection of 94 exosomal markers listed in ExoCarta database.

3.2 Proteomic Profiling of Cell Line-Derived Exosomes

The proteomes of exosomes derived from MDA-MB-231 and MCF-10A were compared using five biological replicates for each cell line. Approximately 5 μg of total protein from each isolation was analyzed by nLC-MS/MS.

An average of 2,843 proteins were identified, excluding contaminant and reverse proteins, with at least one unique peptide in three or more replicates from either cell line (**Figure 3.2 A**). PCA based on the scaled LFQ intensity was used to compare the fractions of each cell line (**Figure 3.2 B**).

The first two principal components show the exosome separation from both cell lines. Similarly, hierarchical clustering of the proteins based on the scaled LFQ intensity revealed distinct clustering between the cell line-derived exosomes (**Figure 3.2 C**). A Venn diagram analysis identified 507 proteins unique to the MCF-10A cell line, 513 proteins unique to the MDA-MB-231 cell line, and 2,367 proteins common to both cell lines (**Figure 3.2 D**). Among these shared proteins, 166 and 175 proteins were considered upregulated (p-value < 0.05 and fold change ≥ 4) and downregulated (p-value < 0.05 and fold change ≤ 0.25), respectively, in MDA-MB-231 exosomes (**Figure 3.2 E**).

The differentially expressed proteins (DEPs) were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, and the top 10 pathways with the lowest p-values were plotted (**Figure 3.2 F**). The enriched pathways included those significantly contributing to cancer growth, such as the PI3K-Akt signalling pathway, ECM-receptor interaction, and pathways in cancer or those involved in cancer metastasis, small cell lung cancer, and neutrophil extracellular trap formation. In contrast, the downregulated pathways included those that tend to hinder cancer progression and metastasis, such as necroptosis and the P53 signalling pathway. The unique proteins from exosomes derived from MDA-MB-231 cells were also subjected to KEGG annotation enrichment analysis (**Figure 3.2 G**). A similar trend to the DEPs was observed, with enriched pathways involved in cancer progression and metastasis, including hepatocellular carcinoma, the Hippo signalling pathway, and the TGF-beta signalling pathway.

Conversely, the enriched KEGG pathways in MCF-10A-derived exosomes elucidated processes contributing to normal cellular homeostasis. Specifically, metabolic pathways were prominent among DEPs, while fatty acid degradation pathways were enriched in proteins unique to MCF-10A-derived exosomes. This dichotomy in pathway enrichment underscores the distinct molecular profiles of exosomes derived from non-tumorigenic and metastatic breast cancer cell lines.

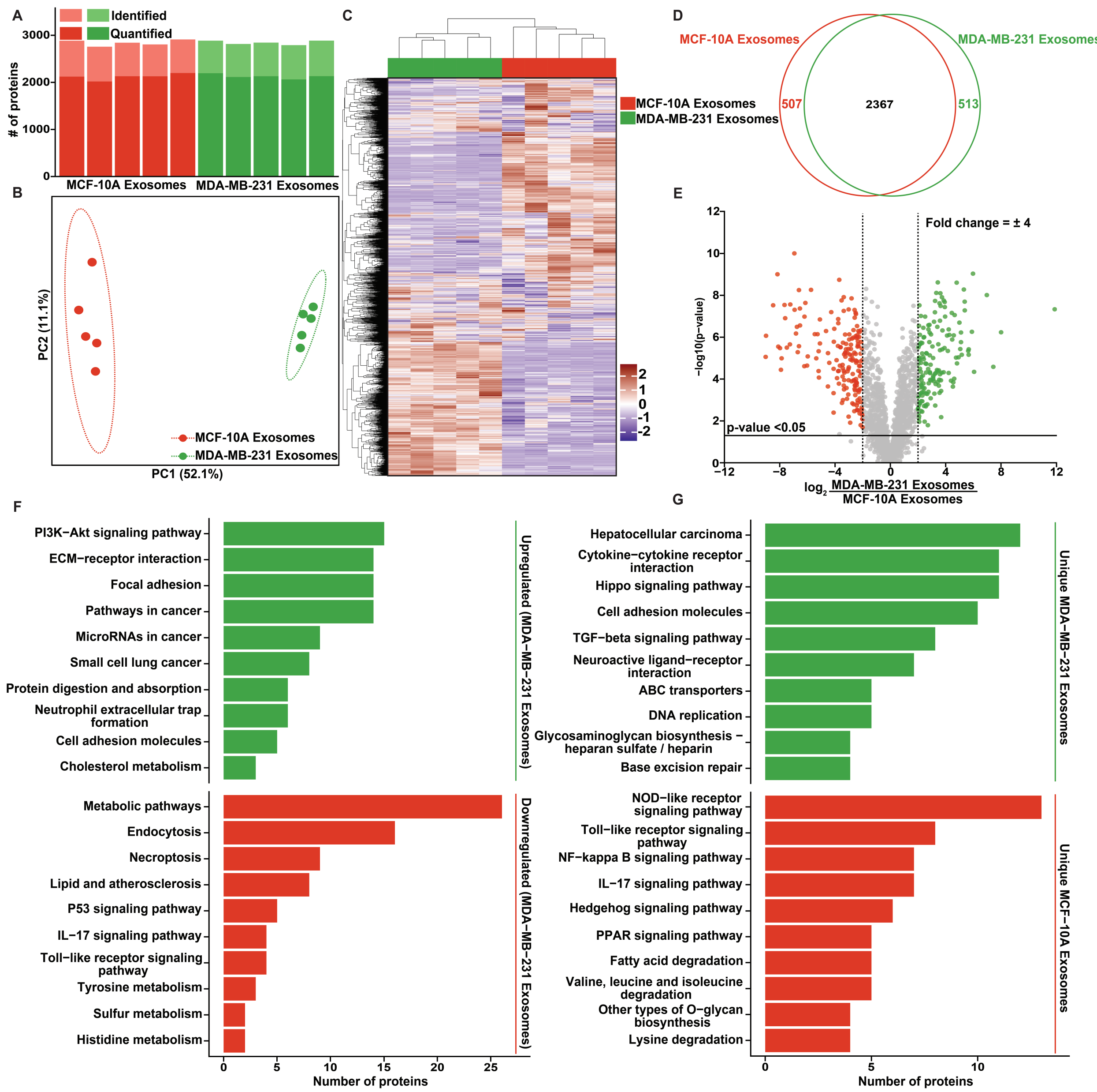

Figure 3.2 Proteomic analysis revealed enriched pathways in MDA-MB-231 exosomes. (A) The number of identified and quantified proteins detected in exosomes derived from MDA-MB-231 and MCF-10A cell lines. (B) The principal component analysis of exosomes from MDA-MB-231 and MCF-10A demonstrates a distinct separation between the cell lines. (C) Heatmap analysis of exosomes from two cell lines, showcasing complete clustering. (D) Venn diagram illustrating the overlapping proteins between the two conditions. (E) Volcano plot of quantified proteins common to both samples, highlighting significantly upregulated and downregulated proteins in MDA-MB-231 exosomes. (F) Top 10 KEGG enriched pathways based on DEPs. (G) Top 10 KEGG annotated pathways unique to each cell line.

3.3 Proteomic Profiling of Parental Cell Lines

A comprehensive proteomic analysis was conducted on the parental cell lines MCF-10A and MDA-MB-231 to elucidate unique and differentially expressed proteins that potentially contribute to cellular functions, signalling pathways, and phenotypic disparities between non-tumorigenic and metastatic breast cancer cells. This investigation employed methodologies analogous to those utilized in exosome profiling. The study identified an average of 4,208 proteins, excluding contaminants and reverse proteins, with a minimum of one unique peptide in three or more replicates from either cell line (**Figure 3.3 A**). Subsequently, PCA and hierarchical clustering were performed on the proteins based on scaled LFQ intensity (**Figure 3.3 B & C**). The initial two principal components demonstrated distinct differences between the cell lines, with clear clustering patterns observed.

The comparative analysis revealed 429 and 613 proteins unique to MCF-10A and MDA-MB-231 cell lines, respectively (**Figure 3.3 D**). Among the 3,829 common proteins, 117 were classified as upregulated (p-value < 0.05 and fold change ≥ 4) and 218 as downregulated (p-value < 0.05 and fold change ≤ 0.25) in MDA-MB-231 cells (**Figure 3.3 E**). KEGG enrichment analysis of the DEPs and unique proteins indicated that the top 10 pathways with the lowest p-values predominantly comprised defence and repair mechanisms enriched in MDA-MB-231 cells (**Figure 3.3 F**). These enriched pathways encompass mismatch repair, base excision repair, and nucleotide excision repair. Certain pathways highly enriched in exosomes, such as the ECM-receptor interaction, exhibited downregulation in MDA-MB-231 cells. Pathways associated with cancer progression and metastasis were enriched in proteins unique to MDA-MB-231 cells, including cancer-related pathways and hepatocellular carcinoma (**Figure 3.3 G**).

In concordance with the exosome proteome profiling, KEGG enrichment analysis of DEPs and unique proteins in the MCF-10A cell line demonstrated the enrichment of pathways contributing to normal cellular function. These pathways include metabolic processes, fatty acid metabolism, degradation, and biosynthesis in DEPs. Furthermore, pathways such as cofactor biosynthesis and fatty acid metabolism were enriched in proteins unique to the MCF-10A cell line.

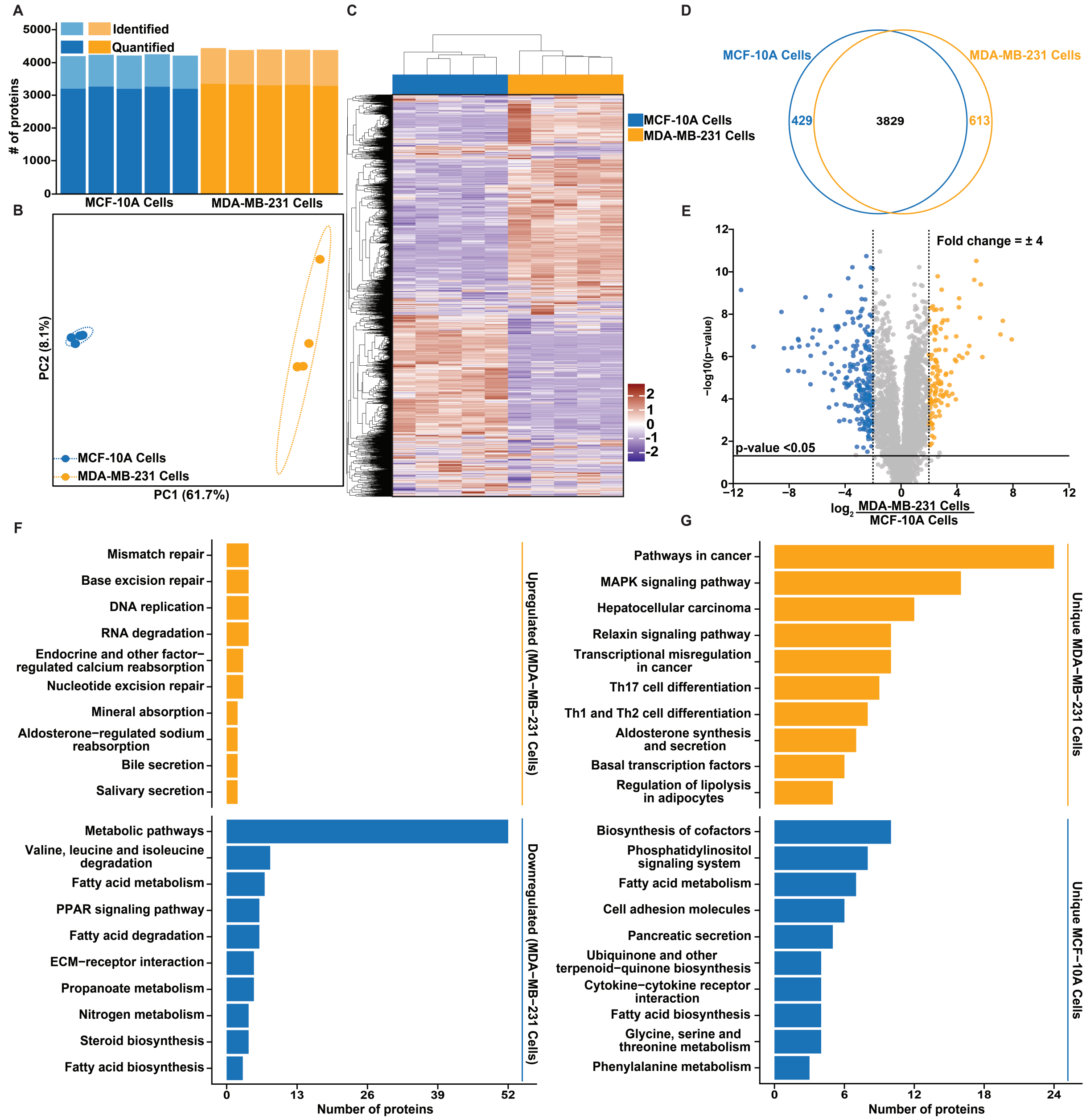

Figure 3.3 Comparative proteome profiling of MDA-MB-231 and MCF-10A parental cells. (A) Overview of protein identification and quantification across both cell lines. (B) Principal component analysis showing distinct clustering patterns between MDA-MB-231 and MCF-10A cells. (C) Hierarchical clustering visualized through heatmap representation demonstrates complete sample segregation. (D) Venn diagram of shared and unique proteins between cell types. (E) Differential protein expression depicted by volcano plot analysis, highlighting proteins with significant fold changes between MDA-MB-231 and MCF-10A cells. (F) Top 10 significant KEGG pathways based on DEPs. (G) Top 10 enriched KEGG pathways unique to each cell line.

3.4 Identification of Potential Breast Cancer EV Markers

A comparative analysis of the proteome of parental MDA-MB-231 cells and their derived exosomes was conducted to identify potential breast cancer EV markers. The study profiled 613 proteins unique to MDA-MB-231 cells and 513 proteins unique to MDA-MB-231 exosomes to distinguish proteins exclusive to or shared between the cells and their derived exosomes. Comparing the proteins in exosomes and cells revealed 411 and 511 proteins unique to MDA-MB-231 exosomes and cells, respectively, with 102 common proteins (**Figure 3.4 A**). KEGG annotation analysis of the unique and common proteins between cells and exosomes revealed the enrichment of previously described pathways in both exosomes and cells. However, among the pathways uniquely enriched in MDA-MB-231 exosomes is the ABC transporters pathway, which has multiple roles in tumour growth and metastasis (**Figure 3.4 B**). Additionally, the glutathione metabolism pathway, which plays a role in protecting and maintaining the growth environment of cancer cells, became more enriched in common proteins (**Figure 3.4 C**). Conversely, 117 and 166 upregulated proteins in MDA-MB-231 cells and exosomes, respectively, were analyzed to identify potential breast cancer EV markers. Among the upregulated proteins, 144 were exclusively upregulated in MDA-MB-231 exosomes, while 95 were upregulated solely in MDA-MB-231 cells. Notably, 22 proteins were commonly upregulated in cells and exosomes (**Figure 3.4 D**). KEGG annotation of the uniquely upregulated pathways in MDA-MB-231 exosomes revealed the enrichment of previously mentioned pathways, such as ECM-receptor interaction and PI3K-Akt signalling pathway, as well as the HIF-1 signalling pathway, which is closely related to these two pathways (**Figure 3.4 E**). Thus, all these unique and enriched proteins from different pathways of MDA sEVs could be a potential source for TNBC biomarkers.

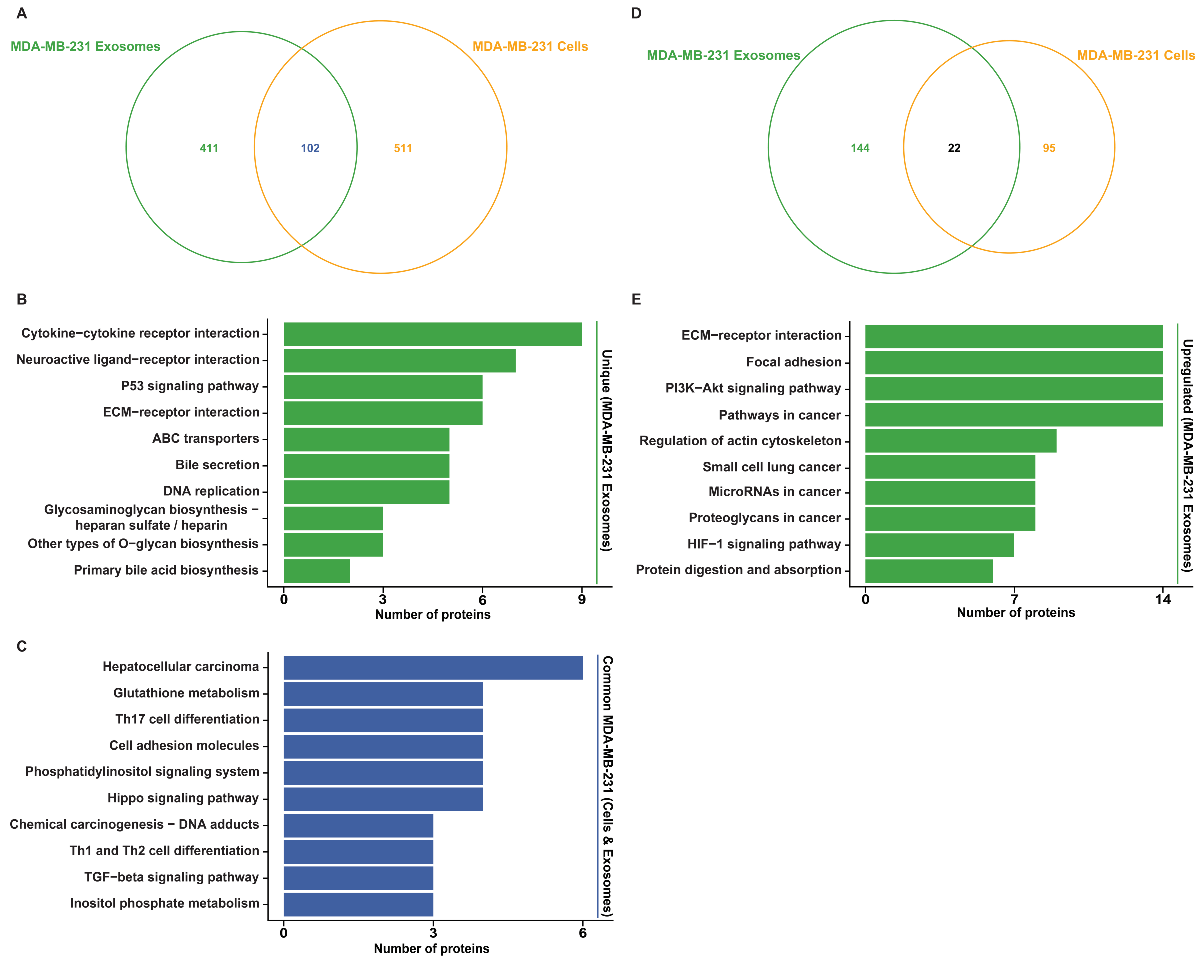

Figure 3.4 Proteomic analysis depicting unique pathways to MDA-MB-231 exosomes. (A) Venn diagram showing the overlapping between the unique proteins between MDA-MB-231 exosomes and parental cells. (B) The top 10 enriched pathways based of unique proteins found in MDA-MB-231 exosomes. (C) The top 10 enriched pathways based of common proteins found in MDA-MB-231 exosomes and parental cells. (D) Venn diagram depicting the overlapping between the upregulated proteins between MDA-MB-231 exosomes and parental cells. (E) Top 10 KEGG annotated pathways based on the upregulated proteins found in MDA-MB-231 exosomes and parental cells.

3.5 Protein Validation

Several exosomal markers found in the ExoCarta database, including CD9, CD81, PDCD6IP, and PKM, were selected for validation. Additionally, the selection criteria for potential proteins to be validated were based on their significant enrichment in MDA-MB-231 exosomes compared to MCF-10A exosomes, unique expression in MDA-MB-231 exosomes but not in MDA-MB-231 cells, and their previously investigated contribution to cancer progression, metastasis, and survival. Three proteins were chosen among the potential biomarkers: Plasminogen activator inhibitor 1 (SERPINE1), Glutathione hydrolase 5 proenzyme (GGT5), and Peroxidasin homolog (PXDN). SERPINE1 was observed in multiple interconnected pathways, such as the HIF-1 signalling pathway, the Hippo signalling pathway, and the p53 signalling pathway, which were highly abundant or unique in MDA-MB-231 exosomes compared to MCF-10A. GGT5 was highly expressed and unique to MDA-MB-231 exosomes and cells. PXDN was highly expressed in MDA-MB-231 exosomes compared to the parental cells and MCF-10A. Although it does not participate in a specific pathway, its role is related to cancer growth and metastasis. All selected proteins, including the exosomal markers and potential biomarkers, were validated by spiking samples with isotopically labelled synthetic peptides, which revealed identical retention times and similar fragmentation patterns (**Figure 3.5**). Ions were selected based on their high intensity and signal-to-noise ratio, ensuring reliable identification and quantification of the targeted peptides.

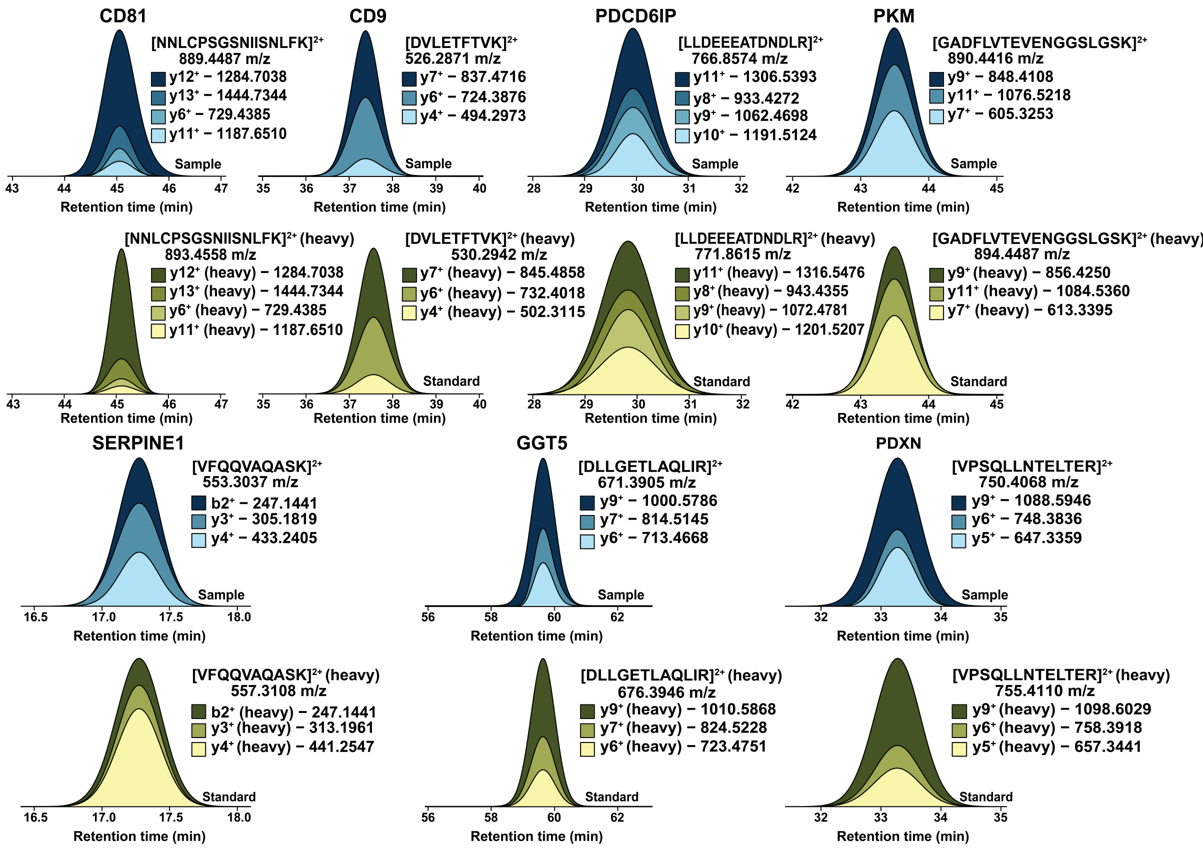

Figure 3.5 Validation of three candidate proteins. Internally labelled peptides of CD81, CD9, PDCD6IP, PKM, SERPINE1, GGT5, and PXDN spiked internally show chromatograms with similar retention times. Ions were selected based on their high intensity and signal-to-noise ration.

Chapter 4

Discussion

4.1 EV Isolation and Characterization

The isolation of EVs from biological matrices has been the subject of extensive research, with various methods developed to enrich EVs based on their size, density, or surface antigen markers[150]. These methods aim to separate EVs from whole cells, cellular debris, and soluble proteins, ideally distinguishing between EV subpopulations. However, the overlap in size, density, and biochemical composition of EV subpopulations poses a significant challenge in achieving complete separation[151].

dUC is the most common technique for EV separation, offering sufficient yields and purity. Nevertheless, this method has been reported to alter the morphology of the isolated particles[152]. Alternative techniques, such as polymer precipitation, preserve the biological activity of EVs[153] but may interfere with MS analysis due to incomplete removal of the precipitant, resulting in low protein detection. SEC allows for size-based separation of EVs; however, it fails to discriminate between exosomes and microvesicles due to their overlapping size ranges[154]. Filtration-based techniques also separate EVs based on a defined size range, but the recovery yield is often low due to membrane clogging[155]. Several techniques can be combined to enhance EV yield and purity, overcoming individual methods' limitations. For instance, filtration-based isolation is often combined with dUC[156], while in this study, precipitation-based isolation, SEC purification, and filtration-based methods were combined to enrich exosomes.

The SEC chromatograms revealed a small peak of around 100 mL of elution relative to the blank sample, suggesting the presence of exosomes. In contrast, the large peak represents the elution of media components for MDA-MB-231 and MCF-10A. The size distribution of exosomes determined by DLS and NTA had a median of 106 nm and 116 nm in MDA-MB-231 and 122 nm and 125 nm in MCF-10A, respectively, consistent with previous reports[157]. It is important to note that populations of exomeres, size ranging ≤50 nm, were also isolated[158]. This explains the different morphologies observed amongst smaller particles in TEM despite similar size frequency profiles in NTA and DLS. Additionally, the lower frequencies of larger particle diameters in the distributions indicate the potential isolation of microvesicle subpopulations, which share not only size distribution but also common protein content with exosomes[159]. This can lead to misattributing biological functions since these vesicles arise through distinct biogenesis pathways. However, unique markers for exosomes and microvesicles can be utilized to confirm the proper isolation of each subpopulation. In this study, spiking the sample with isotopically labelled peptides unique to CD9, CD81, PDCD6IP, and PKM protein markers validated the presence of marker proteins. These proteins are well-documented exosomal markers, indicating successful enrichment of the exosome population. Isotopically labelled peptides offer advantages over antibodies, bypassing the limitations associated with antibody generation, such as the difficulty in generating high-affinity antibodies for rapidly changing and aggregating intrinsic proteins. Additionally, isotopic peptides exhibit increased stability and reduced interference from cross-reactivity with other proteins and molecules.

4.2 Role of Enriched Pathways in Breast Cancer

The study investigates the differences in exosomal protein composition between cancerous and non-cancerous cell lines, focusing on pathways linked to cancer progression, metastasis, and invasion. In particular, MDA-MB-231 exosomes exhibit upregulation of pathways such as PI3K-Akt signalling and ECM-receptor interaction, which are downregulated in their parental cells and MCF-10A exosomes. The extracellular matrix (ECM) is pivotal in the tumour microenvironment, offering structural support and modulating cell behaviour. ECM components like collagen and fibronectin interact with cell surface receptors, such as integrins, activating the PI3K-Akt pathway, which enhances cell survival, proliferation, and migration—critical processes in cancer progression[160,161].

The PI3K-Akt pathway promotes epithelial-to-mesenchymal transition (EMT), which endows epithelial cells with mesenchymal characteristics, increasing their migratory and invasive potential. During EMT, ECM remodelling occurs through the degradation of the basement membrane and interstitial matrix by matrix metalloproteinases (MMPs), a process partly regulated by PI3K-Akt signalling[160,162]. A bidirectional relationship exists between ECM alterations and extracellular vesicle content and secretion; EVs, in turn, can modify ECM properties and signalling pathways like PI3K-Akt, forming a feedback loop that enhances tumour progression and metastasis[163,164].

Conversely, pathways such as necroptosis and the P53 signalling pathway are downregulated. These pathways typically suppress cancer progression. Necroptosis, a form of programmed cell death distinct from apoptosis, involves receptor-interacting protein kinases (RIPK1 and RIPK3) and mixed lineage kinase domain-like pseudokinase[165]. It directly induces cancer cell death, reducing tumour size and proliferation[166]. The P53 signalling pathway responds to cellular stress by triggering apoptosis in cells with damaged DNA, preventing mutation propagation and halting the cell cycle for DNA repair or apoptosis if damage is irreparable, thus inhibiting potential cancerous cell proliferation[167].

The Hippo and TGF-beta signalling pathways are uniquely enriched in MDA-MB-231 exosomes. These pathways contribute to cancer progression, metastasis, and aggressive invasion. Exosomes from MDA-MB-231 cells carry signalling molecules that enhance cancer's aggressive traits. For example, upregulated Hippo pathway components YAP and TAZ in exosomes can promote cell proliferation and migration, supported by findings that bone marrow mesenchymal stem cell-derived exosomes enhance these processes in breast cancer cells via the Hippo pathway[168]. The TGF-beta pathway has a dual role in breast cancer, acting as a tumour suppressor or metastasis promoter depending on the cancer stage. Initially, TGF-beta inhibits cell proliferation and induces apoptosis, but as cancer progresses, it can promote tumour invasion and metastasis[169]. The presence of metastasis-related pathways in exosomes, such as those linked to hepatocellular carcinoma, suggests that the cells are beyond the early stages, with TGF-beta signalling promoting metastasis.

Several pathways are uniquely or heavily upregulated in MDA-MB-231 exosomes compared to their parental cells, including glutathione metabolism, ABC transporters, and HIF-1 signalling. Glutathione metabolism produces glutathione (GSH), a crucial antioxidant that maintains cellular redox balance and plays a significant role in cancer biology. GSH metabolism is linked to cancer progression, treatment resistance, and metastasis. It helps cancer cells manage oxidative stress, detoxifies harmful substances, and contributes to treatment resistance by neutralizing reactive oxygen species (ROS)[170,171]. High GSH levels are associated with increased tumour growth and metastasis, supporting cancer cell survival and proliferation through enhanced antioxidative capacity[171,172].

ABC transporters, including ABCB1, ABCC1, and ABCG2, contribute to multidrug resistance by actively transporting a range of chemotherapeutic agents out of cancer cells, thereby decreasing drug accumulation and efficacy[173,174]. The expression levels of these transporters are linked to breast cancer prognosis, with higher expression often observed in less differentiated and more metastatic subtypes[173,175]. The HIF-1 signalling pathway, particularly the HIF-1α subunit, plays a crucial role in breast cancer. HIF-1α is a key transcription factor that responds to hypoxic conditions commonly found in tumours. It regulates angiogenesis, metabolism, and cell survival genes, which are essential for cancer progression. One of the hallmark effects of HIF-1α is the promotion of glycolysis in cancer cells, a process known as the Warburg effect. This metabolic shift allows cancer cells to favour glycolysis over oxidative phosphorylation, even when oxygen is present, supporting rapid cell growth and survival in the hypoxic tumour microenvironment[176].

4.3 Key Proteins in Enriched Breast Cancer Pathways

Several proteins emerged as significant in the identified pathways, with SERPINE1, PXDN, and GGT5 particularly noteworthy. SERPINE1, in particular, demonstrates involvement in multiple pathways crucial to cancer development and progression. These pathways include HIF-1 signalling, p53 signalling, and Hippo signalling, all of which contribute to various aspects of cancer pathogenesis, including growth, metastasis, and invasion. Notably, SERPINE1, a gene involved in cancer progression, is a target within the TGF-β signalling pathway. This pathway plays a critical role in breast cancer advancement. The TGF-β pathway facilitates EMT, a process through which cancer cells acquire invasive characteristics. This transition is orchestrated by the phosphorylation of SMAD proteins, which subsequently modulate SERPINE1 expression. Through this mechanism, SERPINE1 enhances the invasive and metastatic capabilities of breast cancer cells[177]. In hypoxic environments, breast cancer cells experience elevated levels of ROS, leading to increased SERPINE1 expression. This upregulation correlates with enhanced cellular motility, invasiveness, and activation of EGFR signalling cascades[178]. Importantly, elevated SERPINE1 levels have been observed in patients with advanced stage III and IV breast cancer, serving as an indicator of poor prognosis[179].

PXDN, an enzymatic protein, is pivotal in reinforcing collagen IV networks within the extracellular matrix, particularly in the basement membrane. This reinforcement is achieved through the formation of sulfilimine cross-links, which are fundamental to maintaining the structural integrity of the basement membrane[180,181]. PXDN facilitates the creation of these cross-links by generating and utilizing hypobromous acid, a reactive species that mediate the bonding between collagen IV molecules[182].

PXDN exhibits upregulation in ovarian cancer with elevated expression levels correlating with poor patient survival outcomes[183]. PXDN expression has been significantly associated with tumour size and pathological grade[184]. Experimental suppression of PXDN through knockdown techniques markedly reduced the proliferation, invasion, and migration capabilities of HEY cells[183]. Furthermore, silencing PXDN in these cells led to a notable decrease in the phosphorylation levels of critical proteins in the PI3K/Akt pathway, including p-PI3K and p-Akt, thereby inhibiting the activation of this pathway. This suggests an indirect role for PXDN in modulating ovarian cancer cell behaviour through regulation of the PI3K/Akt pathway, although the precise mechanism remains unclear[183].

Upregulations of PXDN in the normal mammary epithelial cell line D492 induced changes in marker expression, resulting in a more basal-like phenotype and inhibition of branching formation in 3D culture[185]. This upregulation also enhanced the migration and invasion capabilities of the cell line. Similarly, overexpression of PXDN in the HER2+ breast cancer cell line SKBR3 increased invasive potential[185]. In the TNBC cell line HCC1143, which has the capacity for EMT, PXDN overexpression resulted in a modest increase in SNAIL expression, a regulatory factor for TGF-β-mediated EMT. Although not statistically significant, this overexpression trended toward increased migration[185].

Interestingly, existing literature indicates that PXDN was not significantly expressed in breast-invasive carcinoma tissue, suggesting its potential utility as a cancerous marker in exosomes[186].

GGT5 modulates GSH and ROS levels within cancer cells. By catalyzing the cleavage of the gamma-glutamyl moiety from GSH, GGT5 influences the cellular redox balance, thereby promoting cancer cell survival under oxidative stress conditions[187,188]. GGT5 exhibits high expression in cancer-associated fibroblasts (CAFs), integral components of the tumour microenvironment that influence tumour growth and metastasis in lung adenocarcinoma[189]. This elevated expression correlates with poor patient survival outcomes. GGT5 contributes to tumour cell proliferation and drug resistance by augmenting intracellular glutathione levels and reducing ROS in cancer cells, thereby enhancing the survival and chemoresistance of lung adenocarcinoma cells[189]. Notably, inhibition of GGT5 using the small-molecule inhibitor GGsTop has demonstrated tumour growth suppression and increased cancer cell sensitivity to chemotherapy[189]. In gastric cancer, GGT5 is incorporated into a prognostic model that predicts poor outcomes, indicating its role in the dysregulated metabolic microenvironment of the tumor[190]. The expression of GGT5 in gastric cancer is associated with lipid metabolism and other metabolic pathways crucial for cancer cell survival and proliferation[190].

Furthermore, GGT5 expression is linked to immune cell infiltration in the tumour microenvironment. High GGT5 expression correlates with increased infiltration of M2 macrophages, regulatory T cells, and monocytes, often associated with immunosuppressive environments[190,191]. Additionally, tumours with elevated GGT5 levels exhibit higher expression of immune checkpoint genes such as PD1 and CTLA4, suggesting a potential role for GGT5 in modulating immune evasion mechanisms in gastric cancer[190]. GGT5 also facilitates cancer cell migration and invasion by inducing epithelial-mesenchymal transformation, an essential process in cancer metastasis[192]. Interestingly, GGT5 and other GGT family members in breast and colorectal cancers are expressed in control and tumour tissues without significant differences between cancerous and non-cancerous states[193].

Chapter 5

Conclusion

5.1 Summary

This study aimed to characterize the proteomic profile of extracellular vesicles, specifically exosomes, derived from the carcinogenic cell line MDA-MB-231, compared to its parental cells and the non-malignant cell line MCF-10A. The study employed a novel approach for exosome isolation, combining precipitation with PEG-8000, SEC, and filtration techniques.

The proteomic analysis of exosomes was conducted in two stages. Initially, the exosomal proteome was compared against MCF-10A exosomes to identify unique and upregulated proteins in MDA-MB-231 exosomes. These proteins were then enriched against the KEGG pathway database to elucidate the potential functional roles of the exosomal cargo. The second stage involved comparing the unique and upregulated exosomal proteins to those found in the parental MDA-MB-231 cells. This approach aimed to identify potential markers that could serve as clinical targets for treatment.

The identified proteins were further annotated against the KEGG pathway database to uncover pathways specific to MDA-MB-231 exosomes. These analyses identified three proteins as potential targets: SERPINE1, GGT5, and PXDN. These proteins were selected based on their upregulation, uniqueness, roles in the identified pathways, and potential involvement in cancer growth, metastasis, and invasion.

It is noteworthy that while SERPINE1 has been extensively studied in the context of cancer, the roles of GGT5 and PXDN in cancer-derived extracellular vesicles remain less explored. Previous studies have identified these proteins in other forms within cancer cells or non-cancerous extracellular vesicles. However, their specific functions in breast cancer-derived exosomes have not been thoroughly investigated.

This study's approach provides a novel perspective on the proteomic landscape of cancer-derived exosomes and highlights potential new biomarkers for cancer diagnostics and therapeutics. Identifying GGT5 and PXDN as proteins of interest in cancer-derived exosomes opens new avenues for research into their roles in cancer progression and their potential as biomarkers or therapeutic targets.

5.2 Limitations

This study exhibits several limitations. The primary focus on TNBC MDA-MB-231 cells and their exosomes partially characterized MCF-10A cells, with most enriched pathways and proteins reflecting normal cellular processes. The inclusion of additional breast cancer cell lines could have verified whether the identified markers are unique to MDA-MB-231 or common across various breast cancer types. A comparative analysis of these proteins' enrichment levels in different cell lines might have elucidated whether the presence or absence of HER2, progesterone, and estrogen receptors influences their role in cancer progression and metastasis. Another limitation, although not central to the study's primary objective, is the assumption that the isolated EV subpopulation consists purely of exosomes. While most isolated particles are likely exosomes, some macrovesicle-derived particles may also be present. Incorporating immunoaffinity capture techniques targeting common exosomal markers such as CD9, CD63, and CD81 could potentially enhance the purity of exosome enrichment. These limitations highlight areas for potential improvement in future studies, which could provide a more comprehensive understanding of exosomal proteomics in breast cancer and potentially reveal novel biomarkers and therapeutic targets across various breast cancer subtypes.

5.3 Future Work

The future work of this study encompasses several vital directions. Primarily, broadening the scope by incorporating a wider array of tumorigenic cell lines and expanding the number of control cell lines is essential. This expansion would provide a more comprehensive understanding of the observed phenomena across different cancer types and cellular contexts. A key focus should be placed on investigating the roles of the identified proteins, particularly PXDN and GGT5, which remain less explored in cancer biology. This investigation could be achieved through overexpression studies in control samples, observing whether these cells subsequently develop cancerous traits such as enhanced growth, invasion, and metastatic potential. Should the control cells exhibit these malignant characteristics, examining the EV content to elucidate their role in intercellular communication and cancer progression would be crucial.

Conversely, in cancerous cell lines expressing these proteins, applying inhibitors or knockout techniques to suppress their activity could provide valuable insights. Observing whether this intervention leads to a reduction in cancerous traits would be particularly informative. If a slowdown in malignant behaviour is observed in parental cells alongside decreased protein levels, it would be imperative to investigate the EV content. This analysis would help determine whether the cancerous cells have potentially packaged these proteins for intercellular transfer, which could explain the observed decrease in protein content within parental cells. Following thorough investigations into the effects of both inhibition and overexpression of these proteins, the ultimate goal would be to translate these findings into clinical samples. This final step would explore the potential of targeting these proteins to inhibit cancer growth and metastasis in patient-derived tissues, bringing the research closer to practical therapeutic applications.

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