# The proteomic analysis of exosomes from breast cell lines reveals potential biomarkers of breast cancer

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# Abstract

### Background

Breast cancer is the most commonly diagnosed cancer in women worldwide. The identification of breast cancer molecular biomarkers would provide a more accurate assessment of individual disease risks and prognosis. Exosomes, small extracellular vesicles, have been shown to contribute to various aspects of cancer development and progression. Within the last decade, the content of exosomes has been increasingly explored as a new source of potential biomarker molecules for early disease detection.

### Methods

Exosomal proteomes of MDA-MB-231, a metastatic breast cancer cell line, and MCF-10A, a non-cancerous epithelial breast cell line, were compared. Proteomic analysis was conducted using nano-liquid chromatography coupled to tandem mass spectrometry. The expression of proteins in MDA-MB-231cells was analyzed using label-free protein quantification methods. For the selection of potential biomarkers, the following criteria were used: (i) proteins must be unique to MDA-MB-231 cells when compared to MCF-10A cells, ii) localized on the membrane, (iii) abundant in breast cancer and (iii) are reported to increase in expression as the disease progresses. The presence of selected proteins on exosomes was verified using flow cytometry methods.

### Results

In total, 1,107 exosomal proteins were identified in both cell lines, 726 of which were unique to the MDA-MB-231 breast cancer cell line. The biomarker selection process identified three

exosomal proteins (glucose transporter 1, glypican 1, and "disintegrin and metalloproteinase domain-containing protein 10") as potential breast cancer biomarkers. The presence of these three proteins was validated using flow cytometry methods. The proteomics dataset was also rich in other interesting breast cancer proteins, such as 16 metastasis-associated proteins and two kinases.

**Conclusion**

We demonstrate that breast cancer exosomes are a rich source of protein biomarkers that may be beneficial for diagnosis and prognosis.

# Acknowledgements

I would like to thank my supervisor Dr. Maxim Berezovski for the great opportunity of doing research at his lab. I am grateful for the trust and freedom he put in me; I had the chance to explore many different avenues and paths throughout the two years of my master's degree. I also like to thank Dr. Zoran Minic for his never-ending support and encouragement. His mentorship and advice have helped me overcome many difficulties and will leave a mark for the many years to come. Thank you, Dr. Minic, for always speaking your mind and honest opinion.

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## List of Abbreviations

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| ADAM10 | A disintegrin and metalloproteinase domain-containing protein 10 |
| BC | Breast cancer |
| CA15-3 | Cancer antigen 15-3 |
| CA27.29 | Cancer antigen 27.29 |
| CCP | Clathrin coated pits |
| CK5/6 | Cytokeratin 5/6 |
| CK7 | Cytokeratin 7 |
| CK8 | Cytokeratin 8 |
| CK14 | Cytokeratin 14 |
| CK18 | Cytokeratin 18 |
| CK19 | Cytokeratin 19 |
| CK20 | Cytokeratin 20 |
| CID | Collision-induced dissociation |
| DDA | Data-dependent acquisition |
| DDM | n-dodecyl β-D-maltoside |
| DMBT1 | Deleted in malignant brain tumours-1 protein |
| DMEM | Dulbecco’s modified Eagle’s medium |
| EE | Early endosome |

| | |
|---|---|
| EGF | Epidermal growth factor |
| ER | Estrogen receptor |
| EPB41L3 | Erythrocyte membrane protein band 4.1 like 3 |
| ESI | Electrospray ionization |
| EVs | Extracellular vesicles |
| FACS | Fluorescence-activated cell sorting |
| FBS | Fetal bovine serum |
| FC | Flow cytometry |
| FDA | The Food and Drug Administration |
| FDR | False discovery rate |
| FLNA | Filamin-A |
| FN1 | Fibronectin |
| GLUT-1 | Glucose transporter 1 |
| GO | Gene ontology |
| GPC-1 | Glypican 1 |
| HER2 | Human epidermal growth factor receptor 2 |
| HSP70 | Heat shock protein 70 |
| ILV | Inter-luminal vesicle |
| LFQ | Label-free protein quantification |

| | |
|---|---|
| LRG | Leucine rich α-2-glycoprotein |
| MCF | MCF-10A or Michigan Cancer Foundation-10A human mammary epithelial cell line |
| MDA | MDA-MB-231 or M.D. Anderson - Metastasis Breast cancer cell line #231 |
| MDSC | Myeloid-derived suppressor cells |
| MS | Mass spectrometry |
| MVBs | Multivesicular bodies |
| nLC-MS/MS | nano-liquid chromatography coupled to tandem mass spectrometry |
| NSCLC | Non-small cell ling cancer |
| NTA | Nanoparticle tracking analysis |
| PBS | Phosphate buffered saline |
| PIN1 | Peptidyl-prolyl cis/trans isomerase NIMA interacting protein |
| ROBO1 | Roundabout homolog 1 |
| STMN1 | Stathmin-1 |
| T-DM1 | Ado-trastuzumab emtansine |
| TEM | Transmission electron microscopy |
| TFRC | Transferrin receptor protein 1 |
| t-SNE | t-distributed stochastic neighbour embedding |
| UC | Ultra-centrifugation |
| UF-UC | Ultra-centrifugation combined with ultra-filtration |

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## Introduction

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# 1 Introduction

The early detection of human diseases significantly improves patients' survival rates and treatment options. New diagnosis methods are always sought after for better sensitivity and specificity. Many techniques rely on human biofluids for minimally invasive sampling and accurate diagnosis.

This is especially true for breast cancer (BC), which continues to be the most frequently diagnosed cancer among women (24.2%). With an estimated 2.1 million new cases in 2018, BC accounts for 15% of cancer-related female mortalities [1].

Several classification systems are used to distinguish between the different types of BC; these include receptor status, genome sequencing, grading, histopathology and staging. The staging system mainly relies on anatomical features, encompassing tumour size, nodal status, metastasis and hormone-receptor expression [2]. The stage of cancer at the time of diagnosis enables physicians and patients to assess the potential success of treatments and measure prognosis. Stage I is used for small and non-invasive tumours with negatives lymph nodes. Stage II and III denote relatively large tumours or nodal reach, while stage IV defines metastasized tumours [3].

Lowest overall survival rates are observed at the final stages when BC metastasizes [4]. BC overall survival has been strongly correlated to early detection and treatment [4-6]. Current BC detection and diagnosis techniques include self-examination, mammography, tomographies, ultrasound and biopsies [7,8]. These techniques are not only time consuming and costly, but they can also be invasive, examiner dependent and lack specificity and sensitivity [8]. Better and earlier detection methods are always sought after to increase survival rates.

## 1.1 Biomarkers

Recent efforts to develop new methods for disease detection have focused on finding biological molecules capable of identifying diseased individuals. Such molecules are referred to as biomarkers. The World Health Organization, in accordance with the United Nations and the International Labor Organization, defines a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” [9]. These molecules must have specific attributes. A biomarker should be a) clinically relevant, reflecting a change in measurement of a physiological or pathological state, b) sensitive and specific, c) reliable, d) practical as not to be very invasive and e) measured at a low cost [10].

Several BC biomarkers have been identified for prognosis and therapy assessment. The hormone estrogen receptor (ER), when expressed, is a biomarker for invasive BCs [11]. Breast neoplasms that are ER+ generally respond to antihormonal therapy with tamoxifen or an aromatase inhibitor [12]. The expression of CK7, CK8, CK18 and CK19 can be found in 90% of BCs while the expression of CK5/6, CK14 and CK20 are indicative of high-grade tumours [13]. The overexpression of the epidermal growth factor receptor II, also known as HER 2, can be found in 20% of BC cases [14]. It is indicative of aggressive BC subtypes and leads to worse prognosis than cases that do not overexpress HER 2 [15]. The FDA approved trastuzumab, lapatinib, T-DM1 (ado-trastuzumab emtansine) and pertuzumab for targeting the HER 2 receptor [12]. Several body BC fluids biomarkers have also been approved by the FDA. The CA15-3 and CA27.29 proteins can be used to monitor patients. These markers are to be used along with imaging and clinical examinations to assess treatment response [16]. Furthermore, the external fragment of the HER 2 protein can be shed into the bloodstream and used by clinicians as a prognostic marker of the disease state independent of therapy [12].

Unfortunately, beyond prognosis and therapy assessment, there currently are no FDA approved biomarkers for BC diagnosis and screening [12,17,18]. The search for BC screening biomarkers is still ongoing using new biomolecule sources such as small extracellular vesicles, also known as exosomes.

### *1.1.1 From the Lab to the Clinic*

Translating biomarkers from the laboratory to a clinical setting is a long and arduous process. This is reflected in the number of FDA approved biomarkers [19].The process can be roughly divided into two main stages: discovery and qualification, which includes analytical and clinical validations.

The discovery phase aims to identify potential candidate biomarker proteins that are differentially expressed between normal and diseased states. At this stage, factors that might complicate the analysis, such as proteins from other diseases, are to be minimized as much as possible. The proper selection of biological samples can ensure only disease-relevant proteins are studies. Such samples can include fluids close to or in direct contact with the diseased site, cell culture models or animal models' plasma [20].

Beyond the discovery phase, targeted quantitative approaches are to be done [20]. The aim of the qualification stage is to assess the quality of the biomarker measurement and its correlation to the outcome of interest [21]. In the analytical phase, the correlation of the biomarker to a disease state ahs to be established in the intended sample of use, if that was not used in the discovery phase [20]. Sample collection, processing and storage protocols are to be established and properly assessed [21]. Furthermore, the marker's biological variability and the minimum change in magnitude needed for decision making are to be determined, in addition to the biomarker's sensitivity and specificity [21].

Clinical biomarker qualification is also meant to evaluate the correlation of the biomarker with the outcome of interest. The clinical and analytical validation stages are iterative and build on each other, increasing the confidence level in the biomarker [21].

There is no clear path to be followed in order for a biomarker to be fully qualified [20]; this is due to the diversity of potential biomarkers. A marker with high negative consequences would require extensive validation in comparison to a candidate with low negative consequences [22].

## 1.2 Exosomes

Small extracellular vesicles (exosomes) have gained significant interest within the last decade as a novel source of biomarkers for the early detection of diseases. This is due to their ability to facilitate cellular commination between near and distant cells [23].

Exosomes are 30 – 120 nm in size and 1.13 – 1.19 g/L in density [24]. They are released by almost all cell types (reticulocytes, fibroblasts, macrophages, dendritic, placental, epithelial, neuronal, mast, T and B cells) and secreted in many bodily fluids (semen, urine, saliva, blood, breast milk, amniotic and cerebral fluid) [25-37]. Exosomes can house DNA, different kinds of RNA (microRNA, mRNA, and non-coding RNA), proteins and lipids, **Figure 1** [38]. Traditionally accepted exosomal markers are heat shock proteins (Hsc70 and Hsc90), tetraspanins (CD81), TSG101 and Alix. Other markers include CD63, CD9 and Rab family proteins, which are involved in exosome biogenesis [39].

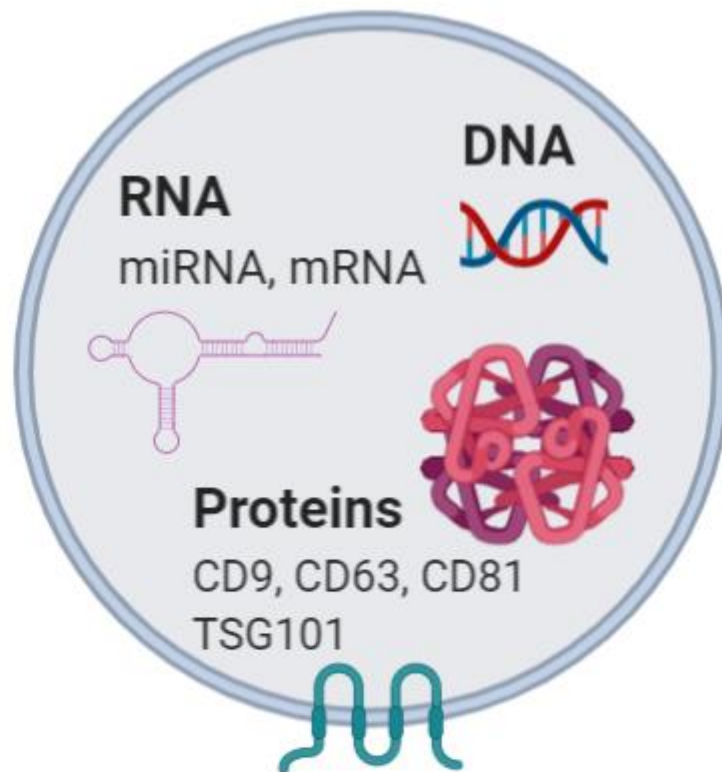



**Figure 1.** Exosomes hold a variety of biological molecules such as DNA, RNA, proteins and lipids. Proteins listed are exosome protein markers. Created with biorender.com.

### *1.2.1 Biogenesis*

The term "exosome" was initially used to describe particles in 40 -1000 nm in diameter [40]. Later, the term was used to refer to vesicles of 40 – 100 nm in diameter originating from multivesicular endosomes (MVE). The origin of exosomes was determined by following the path of gold nanoparticles that were taken up by different cell types then released in exosomes through the endosomal pathway [41]. The endosomal pathway is a mechanism for cells to interact with and regulate their environments. Cell surface glucose transporters, proton pumps and sodium channels, responsible for serum glucose levels, stomach acidification and cell homeostasis, respectively, are regulated by the endosomal pathway [42]. Moreover, endocytosis also modulates cell-cell and cell-

matrix interaction by regulating integrins and adhesion molecules, key players in cancer development, on the cell surface [43]. Exosome formation is just a part of the greater endocytic pathways, which include: targeting proteins for degradation, trafficking/recycling macromolecules to the plasma membrane, and for polarized cell, shuttling molecules across the cell [44].

The exosomal biogenesis pathway is presented in **Figure 2**. Briefly, the process starts with the inward budding of the cell membrane, through clathrin-dependent or independent endocytosis, to form endocytic vesicles that merge with the early endosome (EE) [45]. The EE matures into the late endosome, which is characterized by its acidic nature, as low as pH 4.5, and differentiated Rab-family GTPase proteins [46-47]. Late endosomes are also distinct from the EE by their ability to form multivesicular bodies (MVBs). MVBs are the result of the inward budding of the late endosome to form inter-luminal vesicles (ILVs). The endosomal sorting complex proteins (ESCRTs) are implicated in ILVs formation and packaging. ESCRT-0 binds ubiquitinated cargo followed by the recruitment of ESCRT-I and II, which helps bud the MVB into its lumen. Finally, ESCRT-III entirely separates the newly bud ILV from the MVB's membrane [48]. ILVs can also form through ESCRT independent processes involving tetraspanins and lipids [49]. Fully formed MVBs fuse with the cell membrane, facilitated by Rab-GTPase proteins, releasing their inter-luminal vesicles referred to as exosomes [50].

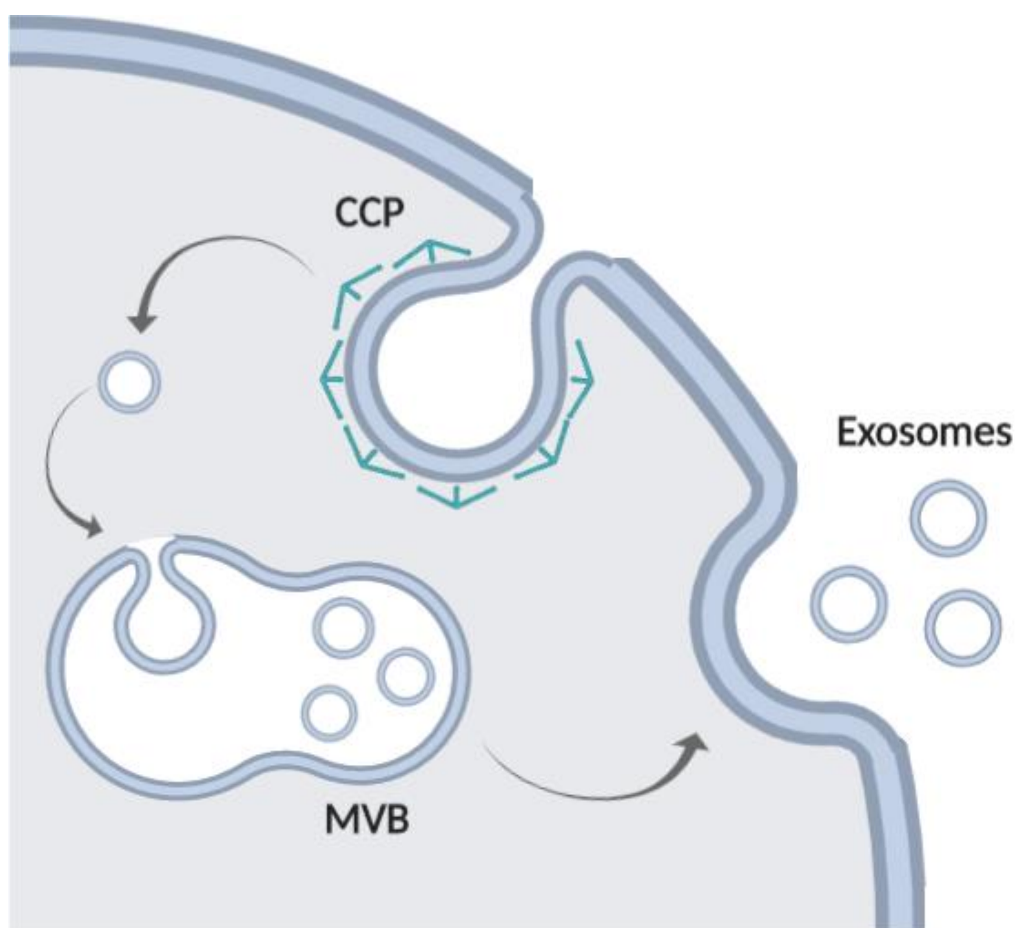



**Figure 2.** The figure depicts a portion of the exosomal biogenesis process. The process begins when clathrin-coated pits (CCP) help the plasma membrane internally bud to later form early endosomes (not shown). The internal budding of early endosomes gives rise to multivesicular bodies (MVBs), which go on to fuse with the plasma membrane releasing their contents (known as exosomes). The figure was created with biorender.com.

*1.2.2 Exosomal Packing*

Multiple studies have shown the selective enrichment of RNA and protein molecules in exosomes compared to parental cells. T, B, and dendritic cell-derived exosomes were found to be enriched in microRNA 760, 632, 654-5p and 671-59 compared to their cells of origin [51]. Long non-coding

RNAs that regulate gene expression at different levels also undergo exosomal enrichment in HeLa, and MCF-7 breast cancer cell-derived exosomes [52]. B-lymphocyte derived exosomes are enriched with a variety of tetraspanin proteins, including CD37, CD53 and CD86 [53]. Tetraspanin proteins play roles in cell adhesion, motility, signalling, membrane fusion and protein trafficking [54]. The cellular adhesion proteins, integrins, were also found to be enriched in exosomes [62].

The mechanisms of exosomal loading are still unclear. A 25-nucleotide stem-loop forming sequence in the untranslated 3'UTR region of mRNAs was found to be enriched in exosomal mRNA. The overexpression of microRNA-1289, which binds to the 25 nucleotides in the 3'UTR region, enhanced mRNA localization into exosomes [63]. Moreover, specific sequence motifs in microRNA controlled their enrichment into exosomes by binding the heterogeneous nuclear ribonucleoprotein A2B [55]. While the ESCRT complexes help package ubiquitinated cargo, tetraspanins also play a role in exosomal loading independently of ESCRTs. CD9 interacts with metalloprotease CD10 and facilitates its packaging into exosomes [56]. Furthermore, the Epstein-Barr virus LMP1 protein is loaded into exosomes by binding CD63 [57].

## 1.3 Breast Cancer-Derived Exosomes

Exosomes mediate indirect cell-cell communication. There are three possible mechanisms for exosomal signalling: 1) they can release their contents into the cytoplasm of recipient cells; 2) exosomes can interact with cell surface receptors and lipids, or 3) they can be internalized through endocytosis or transcytosis [58]. Exosomal protein signalling plays a variety of roles in BC disease, affecting cancer's ability to metastasize, evade the immune system and become drug resistant.

### *1.3.1 Invasion and Metastasis*

Exosomes confer phenotypic traits to recipient cells that mimic their cells of origin. Exosomes from the highly invasive and migratory breast cancer cell line Hs578Ts(i)8 increased the proliferation, migration and invasion capabilities of three less aggressive BC cell line variants: SKBR3, MDA-MB-231, and HCC1954 [59]. Moreover, Fibroblast derived exosomes boost breast cancer cells' motility through the WNT/PCP protein signalling pathways [60]. The WNT/PCP pathway controls cellular orientation, migration and proliferation capacities [61].

Exosomes can remodel the extracellular space making it more favourable for tumour cell seeding. The uptake of exosomes establishes metastatic niches in distant sites capable of supporting migrating breast cancer cells. The surface integrin profile of exosomes has been shown to influence their organotropic uptake; BC-derived exosomes enriched with integrin $\alpha_6\beta_4$ and $\alpha_6\beta_1$ were more likely to be taken up by the lungs, while integrin $\alpha_v\beta_5$ was associated with the liver [62].

### *1.3.2 Immune System Evasion*

Myeloid cells are part of the innate immune system and are essential in initiating, sustaining and inhibiting T cell immunity. Under certain circumstances, these cells are transformed into myeloid-derived suppressor cells (MDSCs), promoting tumour growth, metastasis and recurrence [64]. Breast cancer-derived exosomes have been shown to differentiate myeloid cells into MDSCs in the tumour's microenvironment. Moreover, exosomal proteins, prostaglandin E2 and TGF-β, were necessary for MDSCs' supported tumour progression [65].

Moreover, long term conditioning of mice with exosomes from the highly metastatic EO771 breast cancer cell line accumulated MDSCs in the lungs and livers, common BC metastasis organs. Furthermore, EO771-derived exosomes suppressed the proliferation of CD8 and CD4 T-cells.

Exosomes transformed T-cells into “exhausted T-cell,” which exhibit immune inhibitory receptors, causing cancer cells to evade the immune system in their microenvironment [66].

### *1.3.3 Drug Resistance*

The role of exomes in cancer drug resistance is increasingly being explored. Exosomes derived from HER2-overexpressing tumour cell lines SKBR3 and BT474 were shown to carry active HER2 protein. HER2-positive exosomes, and not HER2-negative vesicles, were shown to bind the chemotherapy drug Trastuzumab and inhibit its effects on SKBR3 cell proliferation [67]. Exosomes were also shown to transfer docetaxel resistance from MCF-7 resistant cells to drug-sensitive ones. This is hypothesized to take place by the exosomal transfer of P-glycoprotein, which disposes of many foreign substances out of cells [68].

### *1.3.4 Exosomes as BC Diagnostic Markers*

Due to their unique contents and selective enrichment, exosomes are being explored as diagnostic and therapeutic devices for breast cancer treatment. The presence and stability of exosomes in body fluids make them great candidates as diagnostic markers. Tissue biopsies are an invasive procedure that results in small samples that might not provide a full picture of the genetic heterogeneity of the tumours. The collection of exosomes, on the other hand, is minimally invasive, and their diverse populations can provide a detailed picture of a patient’s tumour status [69].

Several studies have explored exosomes for breast cancer biomarkers. Using the enzyme-linked immunosorbent assays, researchers found the exosomal protein fibronectin to be elevated in BC patients’ plasma at all stages of the BC disease. Fibronectin levels dropped after the surgical removal of the tumours, and its diagnostic accuracy was better for exosomes than direct plasma analysis [70]. In another study, the developmental endothelial locus-1 protein was identified as an

early-stage BC biomarker. Its elevated levels distinguished BC from benign breast tumours and noncancerous diseases [71].

## 1.4 Exosome Isolation

### *1.4.1 Isolation Methods*

Exosome isolation techniques are very different in their methodologies and characteristics, with some more developed and accepted than others. Current exosome isolation techniques rely on five principles: density sedimentation, size-based exclusion, particle precipitation, immunoaffinity selection and microfluidics methods [99].

### *1.4.2 Ultracentrifugation (UC)*

It is estimated that 56% of all exosome studies rely on the density-based differential ultracentrifugation isolation (UC) technique [100]. This method separates small vesicles based on their size and density. Using a series of centrifugation speeds, ranging from 3,000 *g* to 100,000 *g*, exosomes are separated from cells and particles such as apoptotic bodies and larger extracellular vesicles. Due to the diversity of EVs and their overlapping size ranges and densities, UC isolates a heterogeneous population of EVs, some of which might not originate form the endosomal pathway [99].

A variation on UC protocols is isopycnic ultracentrifugation. It utilizes a density gradient medium, such as sucrose or iodixanol, to separate vesicles based on varying densities. After loading the heterogenous isolated EVs population onto the medium, the sample is ultracentrifuged during which vesicles and particles are separated into different sections of the density gradient medium based on their respective densities. The addition of a density gradient enables the isolation and concertation of high purity small EVs while preserving their physical integrity [176]. Small EVs

like exosomes are collected at a density of 1.15 to 1.19 g/mL [114]. The density gradient medium, however, makes certain downstream mass spectrometry analysis difficult due to its stickiness to exosomes. Density gradient UC is also more labour intensive than UC and requires extensive washing and longer run times [115-117]. Moreover, studies on normal and density gradient UC methods have shown the presence of other non-exosomal vesicles and contaminating proteins that may be isolated along with exosomes [118].

### *1.4.3 Ultrafiltration*

Size exclusion-based methods using filters are able to separate EVs based on size and molecular weight exclusion limits. Ultrafiltration methods are faster than ultracentrifugation. Sequential filtration steps were successfully used to separate microvesicles and exosomes derived from the human colon cancer cell line LIM1863 [176]. Furthermore, ultrafiltration has also been used to isolate exosomes from as little as 0.5 mL of urine [177]. The pressure applied to EVs during ultrafiltration, however, could rupture EVs and clump them together and to the filtering membrane [105, 106].

### *1.4.4 Immunoaffinity Selection*

Other methods for exosome isolation include immunoaffinity capture techniques. They rely on capturing exosomes expressing specific markers, usually exosomal surface markers [99]. Such methods have been gaining prominence with some techniques rivalling UC [100]. Immunoaffinity techniques result in the most uniform population of exosomes [105]. There are several disadvantages to these techniques that are beyond the expensive cost and intricate sample preparation of antibody-coated beads. Due to the heterogeneity of extracellular vesicles, which overlap in size and surface markers, choosing an appropriate surface target can be difficult and may lead to the isolation of other kinds of vesicles, such as microvesicles, alongside exosomes

[119]. Furthermore, tumour cells are heterogeneous, and it may be that not all of them produce exosomes with similar markers, making it disadvantageous to isolate only a narrow range of exosomes of potentially low quantity [101].

### *1.4.5 Microfluidic Methods*

In addition to the classical methods of exosome isolation, based on size, density and immunoaffinity, new methods are being developed to separate exosomes using acoustic and electrophoretic microfluidic methods [99]. Lee at al. were able to separate vesicles above and below 200 nm using ultrasound standing waves with a separation efficiency of 90% [120]. Porous silicon nanowire-on-micropillar structures with ciliated micropillar array were tested for the isolation of exosomes. These structures trapped vesicles 40 – 100 nm in size with a median participle size of 83 nm. The trapped exosomes were separated from larger particles and protein contaminants with a retention rate of 60%. [121]. Microfluidic devices are quick to isolate small vesicles and cheap. However, they lack standardization and are hard to scale. Moreover, sample quantity might be insufficient for downstream analysis, such as proteomics [99].

### *1.4.6 Polymer Precipitation*

Polymers have also been explored for small EVs isolation. Water excluding polymers, such as polyethylene glycol, could entangle small EVs within the polymer's chains. Upon low-speed centrifugation, the polymer, along with the sequestered small EVs, form a pellet, excluding water and other soluble molecules in the sample [178]. Exosome polymer precipitation kits are offered by Systems Biosciences (SBI) and Life Technologies. The ExoQuick Kit, provided by SBI, was found to isolate 300 times the concertation of small EVs isolated by UC [179].

There is not a one size fits all exosome isolation technique, and no consensus on an ideal isolation technique has been established [122]. Downstream analysis of exosomes, purity and quantity are decisive factors in the choice of an appropriate isolation method.

## 1.5 Proteomics for Exosome Biomarker Discovery

Modern mass spectrometry (MS) proteomic methodologies have been extensively used for biomarker discovery studies, offering high sensitivity capable of identifying low abundant proteins over wide dynamic ranges [72]. This technology has been extensively applied for the identification of cancer protein biomarkers. A panel of 12 proteins was proposed as biomarkers for prostate cancer using quantitative proteomic techniques applied to biopsies taken by expert pathologists. The 12 markers could predict the aggressiveness and lethality of the cancer independent of surgical sampling errors [73]. Furthermore, HSP70, isoform 2 of UTP-glucose-1-phosphate uridylyltransferase and argininosuccinate synthase proteins could differentiate hepatocellular carcinoma patients with high rates of metastatic relapse from non-relapse patients [74].

The study of proteins has helped understand the role of exosomes and their proteins [60,62,65,67,68]. Several studies have also utilized high throughput proteomics in the hunt for exosome disease biomarkers. For example, analysis of urinary exosomes from non-small cell lung cancer (NSCLC) patients identified leucine-rich α-2-glycoprotein (LRG) as a prospective biomarker. The expression of LRG was found to be upregulated in urinary exosomes and lung tissues of NSCLC patients compared to control patients [75]. Moreover, the urinary exosomal proteins fatty acid-binding protein 5 (FABP5) was proposed as a biomarker for prostate cancer. FABP5 was found upregulated in prostate cancer diseased patients compared to the control group and was significantly associated, p-value = 0.011, with the high Gleason score tumours [76]. Using salivary exosomes, cystatin B, triosephosphate isomerase, and “deleted in malignant brain tumors-

1 protein” (DMBT1) were proposed as biomarkers for gastric cancer. These three proteins could differentiate gastric cancer patients from healthy controls with an 85% sensitivity and 80% specificity [77]. Furthermore, exosomes have also been found to be a source of early disease biomarkers in neurological diseases. The Alzheimer’s disease biomarker tau protein, phosphorylated at Thr-181, has been detected in exosomes early in the disease progression when patients were exhibiting mild symptoms [78]. The proteomic profiling of plasma exosomes from Parkinson’s disease patients identified four proteins (Clusterin, complement C1r subcomponent, apolipoprotein A1 and fibrinogen gamma chain) that not only are biomarker candidates but also correlated with the various stages of Parkinson’s disease progression [79].

## 1.6 Rational

BC treatment has improved significantly over the past several decades, mainly due to better treatment options and increased screening among women [80,81]. Such improvements, however, did not shake BC's position as the most frequently diagnosed cancer among women accounting for 15% of female cancer-related mortalities [1]. Molecular biomarkers for BC are hypothesized to offer a more accurate diagnosis and prognosis of the disease [82]. Several proteins have already been established as prognosis biomarkers. Unfortunately, there still are no diagnostic biomarkers for BC [12-18].

Proteins are essential biomolecules involved in many aspects of life, from energy storage and metabolism to regulation of cellular functions [83]. Abnormal protein folding or expression levels can lead to the development and progression of human diseases [84-86]. Modern global protein analysis techniques can identify the human proteome to great depths, making it possible to identify protein disease biomarkers [87,88]. There are several difficulties in protein biomarker discovery research [83]. Firstly, the identification of low abundance proteins is difficult. Unlike DNA and

RNA, which can be replicated exponentially, proteins cannot be amplified. Secondly, the detection of low abundance proteins can be masked by highly abundant ones. Thirdly, proteins have diverse properties and are sensitive to their biological environment making their extraction and solubilization difficult.

Exosomes can help overcome some of the challenges associated with protein biomarker discovery. They protect their contents from enzymatic degradation processes common in biological fluids, expanding the range of accessible molecules [89]. Furthermore, the analysis of isolated exosomes makes low abundance proteins more likely to be identified by the reduction/elimination of highly abundant secreted proteins from samples during the exosome isolation process. Furthermore, these small extracellular vesicles can be selectively enriched by cells with specific signalling molecules and proteins [44,46,62]. The analysis of such proteins can help determine the health state of parental cells by revealing protein signatures associated with specific diseases. These signatures can be used for disease diagnosis and prognosis.

# 2 Hypothesis and Aims

## 2.1 Hypothesis 1

Exosomes isolated from cell media using different methods (ExoQick kit, UC and UF-UC) will have different concentrations, sizes and protein contents.

### *2.1.1 Aims 1-2*

1- Compare the size and concentration of exosomes isolated using ExoQick, UC and UF-UC.

2- Analyze and compare the total protein content of exosomes isolated using the three methods.

## 2.2 Hypothesis 2

BC-derived exosomes carry proteins capable of driving cancer progression and acting as potential biomarkers.

### *2.2.1 Aims 1-4*

1. Compare the ability of different detergents (DDM, digitonin and Triton X-100) at solubilizing exosomal protein.
2. Analyze and compare the exosomal proteomes of cancerous and non-cancerous cell lines.
3. Develop a strategy for the selection of potential BC biomarkers.
4. Validate the presence of selected proteins on the surface of BC derived exosomes.

# 3 Methods

## 3.1 Cell Culturing

MDA epithelial breast cancer cells (ATCC HTB-26) and MCF non-tumorigenic epithelial breast tissue cells (ATCC CRL-10317) were used in this study. MDA cells were maintained in high-glucose Dulbecco's Modified Eagle's medium (DMEM, Gibco) and supplemented with 10% fetal bovine serum (FBS, Corning). MCF cells were grown in DMEM/Nutrient Mixture F-12 (DMEM/F12) supplemented with 5% horse serum (HS), 20 ng/mL epidermal growth factor (EGF), 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin. Cell culture media, for both cell lines, were supplemented with 100 units/mL penicillin and 0.1 mg/mL streptomycin (Pen Strep, Gibco). Cells were incubated at 37°C with 5% $CO_2$.

## 3.2 Exosome Isolation

Cells were grown in complete media. When cells reached 40-50% confluency, they were rinsed with Dulbecco modified phosphate buffered saline (PBS) and incubated with exosome free media for 48 hr. Exosome free media was prepared by ultra-centrifuging FBS or HS at 100,000 *g* for 17 hr. The vesicle enriched media was then collected and processed for the isolation of exosomes. Isolation optimization tests were carried out on MDA derived exosomes.

### *3.2.1 Differential Ultracentrifugation (UC)*

Cell culture media was harvested after 48 hours of incubation and subjected to differential ultracentrifugation (UC) to obtain isolated exosomes (**Figure 3**). 100 mL of cell culture supernatant was collected and immediately centrifuged at 300 *g* for 10 min. This was followed by a 3,000 *g* spin on a Sigma13190 rotor (MBI) for 10 min, then a 15,000 *g* centrifugation for 35 min (SW28 Ti rotor, Beckman Coulter). The supernatant was further centrifuged at 100,000 *g* for 2

hours (SW28 Ti rotor, Beckman Coulter). Exosomes were pelleted and collected after the 100,000 *g* spin. The collected exosomes were washed by centrifuging at 100,000 *g* for 1 hr (SW55 Ti rotor, Beckman Coulter) and finally resuspended with PBS.

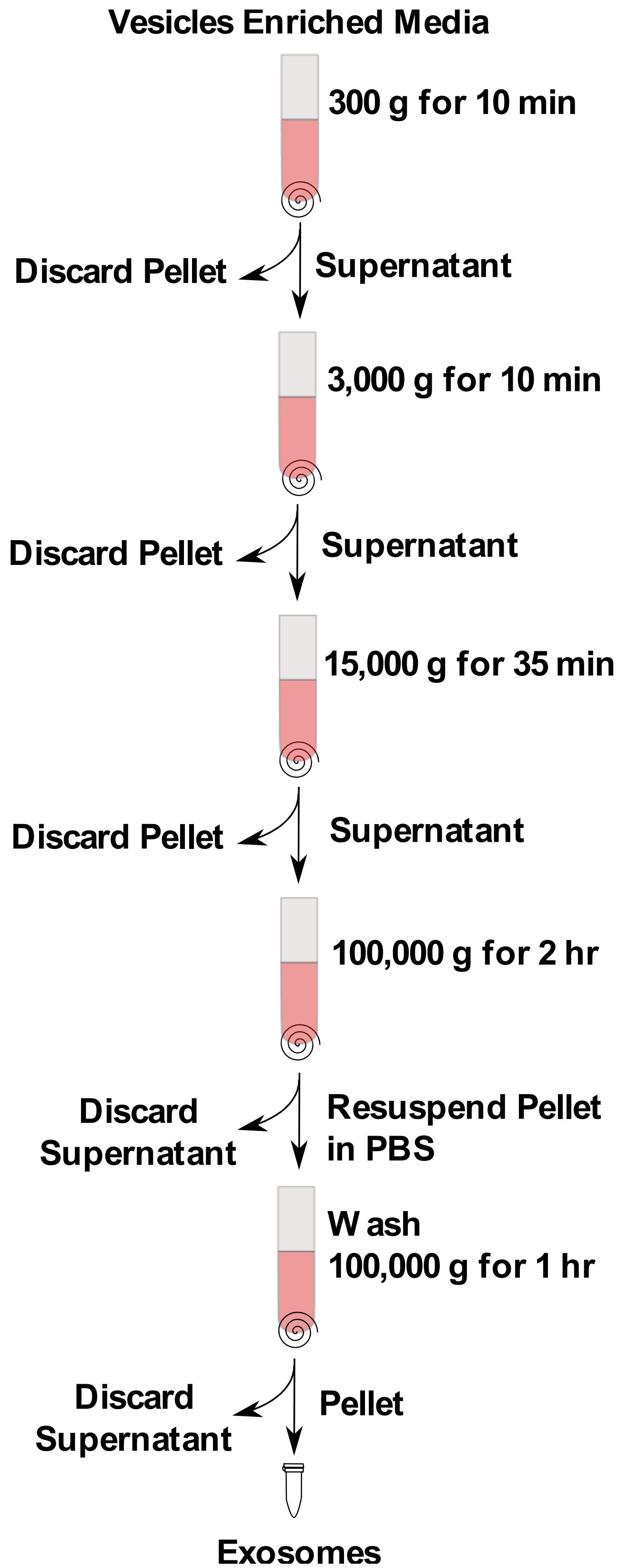



**Figure 3.** Exosome UC isolation protocol.

### *3.2.2 ExoQuick*

The ExoQuick-TC PLUS kit (Systems Biosciences) was tested for the isolation of exosomes from the MDA cell line. The isolation procedure was done according to the manufacturer's protocol [90]. In brief, 10 mL of exosome enriched media was centrifuged at 3,000 g for 15 min. This was followed by the addition of 5 mL of Isolation Reagent, vortexing for 5 min and overnight incubation at 4$^{o}$C. After 12 hours of incubation, the sample was centrifuged at 1,500 *g* on a Sigma 13190 rotor for 30 min. The supernatant was discarded, and the pellet resuspended in 250 μL of Resuspension Buffer. One unit of washed Microsphere Beads was added to the resuspended sample and placed on a shaker, at 450 rpm, for 15 min. Finally, exosomes were isolated by spinning the sample at 8,000 *g* for 5 min and collecting the pellet.

### *3.2.3 Ultracentrifugation – Ultrafiltration (UC-UF)*

After centrifuging exosome enriched media at 15,000 *g* for 35 min, the supernatant was filtered through a 100 kDa filter (Amicon Ultra-15, Millipore Sigma) by centrifuging at 3,000 *g*. The filter concentrated media was then washed by centrifuging at 100,000 *g* for 1 hour (**Figure S1**). The pelleted exosomes were resuspended in PBS.

## 3.3 Nanoparticle Tracking Analysis (NTA)

The ZetaView nanoparticle tracking microscope PMX-110 (Particle Metrix) was used for determining the concentration and size distribution of exosomes at 85 and 40 camera shutter speeds. Polystyrene beads 102 nm in size (Microtrac 900383) were used to focus the camera and calibrate the instrument.

## 3.4 Transmission Electron Microscopy (TEM)

Isolated vesicle fractions were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The fixed suspension containing exosomes were spotted on Formvar® coated copper grids (200 mesh; Canemco, Lakefield, ON, Canada) for 30 seconds. Samples were negatively stained with 2% uranyl acetate in water for 6 minutes and dried with filter paper. Vesicles were examined on a transmission electron microscope (JEOL JEM 1230, Japan) operated at 50 kV. The Feret's diameter of the particles was analyzed using ImageJ software [91].

## 3.5 Protein Concertation Measurements

The Bradford assay was used to asses sample protein concentrations. 100 µL of the detergent-solubilized sample was added to 900 uL of Coomassie Blue solution (ThermoFisher, 23236). The sample was added to cuvettes (7591 50, Brand) and absorption was read at a wavelength of 595 nm using an Ultraviolet-visible spectroscopy instrument.

## 3.6 In-solution Digestion

Purified exosomes, in 200 µL of PBS, were lysed with 50 µL solubilization buffer consisting of 8 M urea, 100 mM HEPES, 5% glycerol and a surfactant (any of 0.5% n-dodecyl β-D-maltoside (DDM), 0.5% Digitonin or 0.075% Triton X-100). Samples were reduced using 4 µL of 0.1 mM TCEP solution and incubated at 25°C for 55 min on a 450 rpm shaker. This was followed by alkylation with 4 µL of 0.5 mM iodoacetamide (in $H_2O$) solution and incubated at 25°C for 55 min on a 450 rpm shaker. Finally, proteins were digested using 1.5 µL of 0.3 µg/µL trypsin/LysC solution (Trypsin/LyC mix, Promega V5072) and incubated at room temperature for a minimum of 20 hours. 1.5 µL of 100% formic acid was added to samples that were vortexed and centrifuged at 10,000 *g* for 30 seconds. Samples were desalted using C18 TopTips (Glygen) columns,

following the manufacturer's instructions. Briefly, desalting columns were washed three times, with 70% acetonitrile then thrice with water. Samples, 266 µL, were slowly added to the column, after which columns were washed three times with water and proteins eluted with 70% acetonitrile. All solutions used for desalting consisted of 0.1% formic acid. Finally, samples were vacuum dried before mass spectrometry analysis.

## 3.7 Nano-LC-MS/MS

Samples were analyzed by an Orbitrap Fusion (Thermo Fisher Scientific) coupled with an Ultimate3000 RLSCnano system (Dionex, Thermo Fisher Scientific). Peptides were separated on an in-house packed column (Polymicro Technology), 15 cm x 70 µm ID, Luna C18(2), 3 µm, 100 Å (Phenomenex) employing a water/acetonitrile/ 0.1% formic acid gradient. Samples were loaded onto the column for 105 min at a flow rate of 0.30 µl/min. Peptides were separated using 2% acetonitrile in the first 7 min and then using a linear gradient from 2 to 38% of acetonitrile for 70 min, followed by a gradient from 38 to 98% of acetonitrile for 9 min, then at 98 % of acetonitrile for 10 min, followed by a gradient from 98 to 2% of acetonitrile for 3 min and wash 10 min at 2% of acetonitrile. Eluted peptides were directly sprayed into a mass spectrometer using positive electrospray ionization (ESI) at an ion source temperature of 250°C and an ion spray voltage of 2.1 kV. The Orbitrap Fusion Tribrid was run in a top speed mode. Full-scan MS spectra (m/z 350 – 2000) were acquired at a resolution of 60 000. Precursor ions were filtered according to monoisotopic precursor selection, charge state (+2 to +7), and dynamic exclusion (30 s with a ± 10 ppm window). The automatic gain control settings were $5x10^5$ for full FTMS scans and $1x10^4$ for MS/MS scans. Fragmentation was performed with collision-induced dissociation (CID) in the linear ion trap. Precursors were isolated using a 2 m/z isolation window and fragmented with a normalized collision energy of 35%.

## 3.8 Flow Cytometry (FC)

For the validation of selected proteins, isolated exosomes, in 500μL of PBS, were incubated for 40 min with one of the following primary antibodies: Glut-1 (Invitrogen, MA5-31960, SA0377), GLYP-1 (Invitrogen, PA5-86043, polyclonal) and ADAM10 (MyBioSource, MBS435195, polyclonal). The stained exosomes were subsequently incubated in the dark for 40 min with BV421 conjugated secondary antibody (BD Horizon, 565014, polyclonal) and the exosomal markers anti-CD63 (Biolegend, 353008, H5C6) and anti-CD81 (Biolegend, 349512, 5A6). Data were acquired by a MoFlo Astrios EQ Flow Cytometer (Beckman Coulter). Control samples included an unstained exosome sample and several fluorescence-minus-one samples (**Supplementary Table S1**). Flow cytometry data were analyzed using the FlowJo analysis software.

## 3.9 Data Processing and Statistical Analysis

MS raw files were analyzed using the MaxQuant software [92]. Peptides were searched against the human Uniprot FASTA database using the Andromeda search engine [93], integrated into MaxQuant. Oxidation and N-terminal acetylation were set as variable modifications, while carbamidomethyl was fixed. Trypsin and LysC proteases were chosen as the digestion enzymes with a maximum of 2 missed cleavages. Identified peptides had an initial precursor mass deviation of up to 7 ppm and a fragment mass deviation of 20 ppm. The false discovery rate (FDR) for peptides (minimum of 7 amino acids) and proteins was 1%. A reverse sequence database was used in determining the FDR. For label-free protein quantification (LFQ), only unique peptides were considered. A contaminant database provided by the Andromeda search engine was used. All proteins matching the reverse database or labelled as contaminants were filtered out. Label-free

protein quantification values were obtained through MaxQuant quantitative label-free analysis [92].

### 3.10 Bioinformatic Analysis

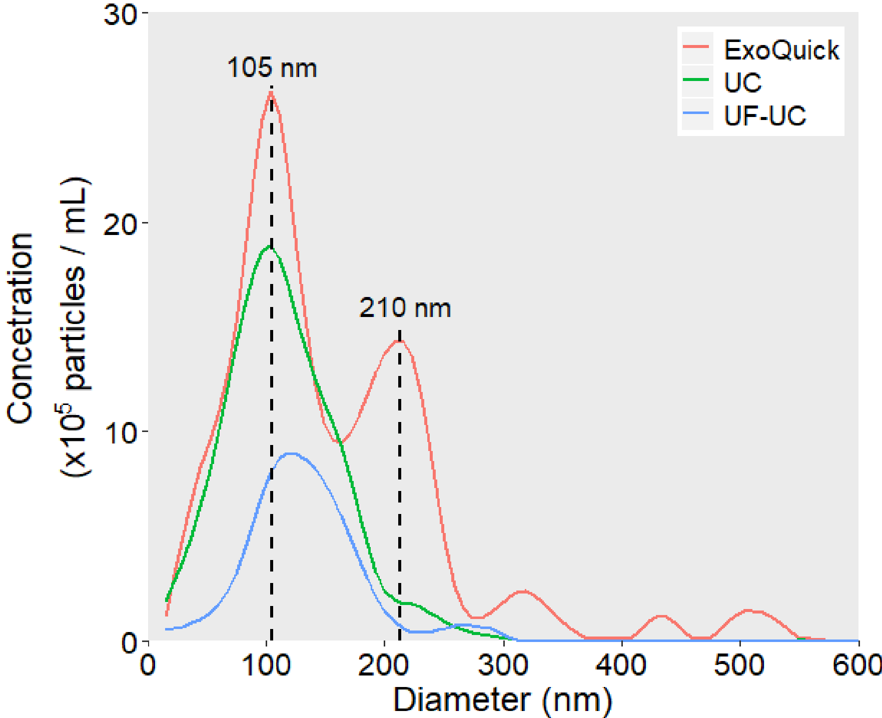
Gene Ontology (GO) functional annotations and Kegg pathway analysis were obtained using David 6.8 database [94]. DisGeNET Curated disease database was used to identify diseases associated with the identified proteins [95]. Information retrieved from previously mentioned databases was visualized using the “ggplot2” R program package [96]. Wilcoxon-Mann-Whitney test was used to assess significance; the test was conducted in the free software R environment [97].

# 4 Results

## 4.1 Comparison of Exosome Isolation Methods

In this study, three different isolation methods (UC, ExoQuick, UF-UC) were compared for their yield and specificity in capturing exosomes. NTA, TEM and MS were utilized for assessing the most suitable method. The highest concertation of vesicles was obtained using the ExoQuick kit ($26x10^5$ particles/mL) followed by UC ($19x10^5$ particles/mL) and finally UF-UC ($8x10^5$ particles/mL) (**Figure. 4a**). The average vesicle size of exosomes isolated by the UC method was found to be 105 nm, which is within the accepted 50-120 nm size range of exosomes [24]. In contrast, ExoQuick had a significant number of vesicles of size 210 nm, while the average particle size isolated by the UF-UC method was 135 nm. MS analysis of ExoQuick isolated exosomes identified only 5 proteins (**Supplementary Table S2**), while 986 and 503 proteins were identified using the UC and UF-UC methods, respectively. Even though the UF-UC method gave higher protein concentrations (**Table 1**), it resulted in fewer proteins being identified than UC. Out of the top 100 ExoCarta database exosomal proteins, the UC method identified 83 proteins, 40 more than the UF-UC method (**Supplementary Figure S2**). The specificity, high yield and number of identified exosomal marker proteins make the UC method the most suitable for exosome isolation. TEM images of UC isolated vesicles confirmed the presence of vesicles with an average size of 45.5 nm that ranged in size from 29 to 86 nm (**Figure. 4b**).

**a.**




**b.**

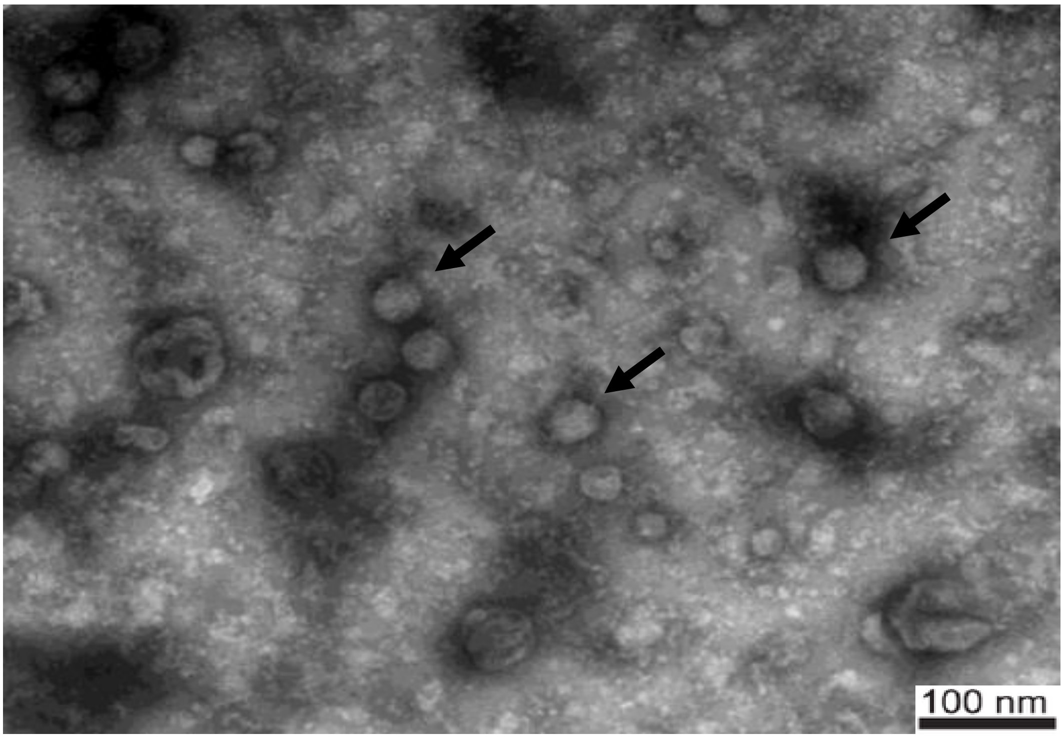


**Figure 4.** Characterization of isolated exosomes. **a.** NTA analysis of exosomes isolated by ExoQuick (red), UC (green) and UF-UC (blue). **b**. TEM images of exosomes isolated using the UC method show particles of an average size of 45.5 nm and a size range of 29-86 nm; arrows point to small vesicles.

**Table 1**: Protein concentration of isolated exosomes from UF-UC, UC and ExoQuick isolation methods. Samples were run in triplicate.

| *Isolation Method* | *Protein Concentration (µg/µL)* |
|---|---|
| ExoQuick | 0.080 ± 0.0240 |
| UF-UC | 13 ± 6.69 |
| UC | 0.030 ± 0.00780 |

## 4.2 Detergent Comparison

Three detergents - n-Dodecyl β-D-maltoside (DDM), Digitonin and Triton X100 - were tested for their ability to solubilize exosomal proteins in an in-solution digestion buffer. We observed that DDM was more effective at solubilizing exosomal proteins resulting in the identification of 986 proteins, compared to Triton X-100 and Digitonin that solubilized 861 and 778 proteins respectively. DDM also had the highest number of unique proteins, 470 proteins, not solubilized by the other detergents (**Supplementary Figure S3)**. The GO cellular localization analyses of proteins identified by the three detergents were similar. The term "Extracellular Exosomes" was the most highly enriched cellular localization term for all three detergents, with DDM having the highest number of proteins under this category (**Supplementary Figure S4**).

## 4.3 Global Protein Profiling of MCF-10A and MDA-MB-231 Exosomes

Each cell line was run in 3 biological replicates. Proteins were considered for analysis if found in at least two biological replicates. MDA and MCF exosomal proteomes were profiled to a depth of 986 and 381 proteins, respectively. There was a significant overlap between the cell lines with 260 exosomal proteins in common (**Figure. 5**). Out of 726 unique exosomal proteins found only in MDA, 31 were not reported in the exosomal protein database ExoCarta, while MCF had 12 (**Supplementary Tables S3 & S4**).

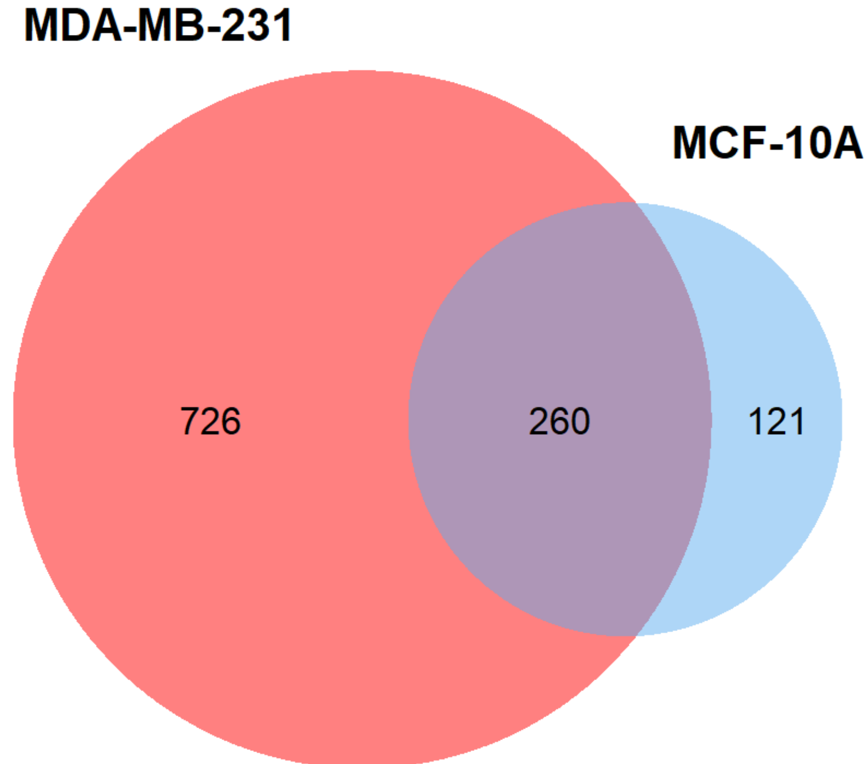



**Figure 5.** The overlap between MDA-MB-231 and MCF-10A exosomal proteomes. Out of the 986 MDA-MB-231 and the 399 MCF-10A exosomal proteins, 260 proteins were identified in both cell lines.

## 4.4 Functional Analysis of Identified Proteins

To understand the functional and biological role of the identified proteins, Gene Ontology and Kegg Orthology databases were used to annotate the identified proteins. The GO cellular compartment term Extracellular Exosome was the most highly enriched label for both MCF and MDA exosomal proteins (**Supplementary Figure S5**). GO terms associated with proteins only found in MCF derived exosomes were mainly implicated in housekeeping and maintenance of basic cellular functions, while annotations of the 726 unique MDA exosomal proteins were associated with two main themes: signalling and motility (**Supplementary Figure S5**). GO biological process terms for the MDA cell line related to signalling included: signal transduction, regulation of ERK1 and ERK2 cascade, positive regulation of protein phosphorylation and proteasome-mediated ubiquitin-dependent protein catabolic process. While, MDA cell line signalling pathways identified by Kegg included ErbB signalling, Proteoglycan, Rap1 signalling, T cell receptor signalling and PI3K-Akt signalling.

Furthermore, MDA cell line motility associated GO biological process terms included cell adhesion, migration and establishment of protein localization. Motility related Kegg pathways were associated with endocytosis, adherens junction, focal adhesion and regulation of actin cytoskeleton.

Interestingly, 36 protein kinases were identified in the MDA exosomal proteome with 19 and 2 being cancer and BC associated, respectively (**Supplementary Table S5**). In addition to protein phosphorylation, kinase GO biological process terms related to actin remodelling, cell migration, and signalling were among the top 10 terms with the lowest p-values. The Kegg pathway analysis of MDA exosomal kinases included several signalling terms, such as adherence and gap junctions

and NF-kappa B pathways, in addition to actin regulation and endocytosis motility terms (results not shown).

## 4.5 Biomarker Selection Process for BC-derived Exosomes

The DisGenet gene-disease database led to the identification of 247 unique MDA exosomal proteins associated with cancer. These cancer-related proteins were found to be significantly enriched in our dataset with a Wilcox p-value of $2.26x10^{-6}$. The protein list was further narrowed by selecting only breast cancer-related ones. The following terms were used as a query in the DisGenet database: "Breast adenocarcinoma", "Invasive Ductal Breast Carcinoma", "Malignant neoplasm of breast" and "Mammary Ductal Carcinoma". These terms reflect the metastatic mammary adenocarcinoma cancer subtype of the MDA cell line [98]. In total, 87 proteins from our dataset were predicted to be relevant to BC disease; these proteins were also found to be highly expressed compared to the rest of the proteome with a Wilcoxon test p-value of $5.5x10^{-8}$ (**Supplementary Figure S6**). The GO analysis of the 87 proteins identified 16 metastasis-associated proteins (**Supplementary Table S6**), and 62 membrane proteins according to their Biological Process and Cellular localization, respectively. The 62 membrane proteins were further analyzed for the selection of potential biomarkers. To confirm the plasma membrane origin of these proteins, the WoLF PSORT protein subcellular localization prediction tool was used. Only proteins with a plasma membrane score of 10 or more were considered. This resulted in 15 predicted plasma membrane proteins (**Table 2**). Furthermore, to ensure the study of easily detectable proteins, confirmed membrane proteins with a $\log_{10}$ LFQ intensities greater than 50% of all identified MDA proteins were selected (**Figure. 6, Table 2**). The final list of potential BC biomarkers consisted of 8 proteins: FN1, EPB41L3, TFRC, GPC1, FLNA, GLUT-1, ROBO1 and ADAM10, **Figure 6**.

An exhaustive literature search of the 8 proteins was performed to better understand their function in BC disease and assess their potential as biomarkers. Glypican 1 (GPC-1), glucose transporter 1 (GLUT-1), also known as solute carrier family 2 (SLC2A1), and disintegrin and metalloproteinase 10 (ADAM10) were picked for validation as biomarkers. On the other hand, fibronectin (FN1), band 4.1-like protein 3 (EPB41L3), filamin-A (FLNA), roundabout homolog 1 (ROBO1) and transferrin receptor protein 1 (TFRC) did not meet the set criteria described below.

**Table 2.** List of membrane proteins suggested as potential breast cancer biomarkers.

| UniProt ID | Gene | Description |
|---|---|---|
| **P02751** | FN1 | Fibronectin |
| **Q9Y2J2** | EPB41L3 | Band 4.1-like protein 3 |
| **P02786** | TFRC | Transferrin receptor protein 1 |
| **P35052** | GPC1 | Glypican-1 |
| **P21333** | FLNA | Filamin-A |
| **P11166** | GLUT-1 | Glucose transporter 1 |
| **Q9Y6N7** | ROBO1 | Roundabout homolog 1 |
| **O14672** | ADAM10 | Disintegrin and metalloproteinase domain-containing protein 10 |
| Q86Y82 | STX12 | Syntaxin-12 |
| Q16625 | OCLN | Occludin |
| Q9BY67 | CADM1 | Cell adhesion molecule 1 |
| Q13433 | SLC39A6 | Zinc transporter ZIP6 |
| Q04721 | Q04721 | Neurogenic locus notch homolog protein 2 |
| P19022 | CDH2 | Cadherin-2 |
| P33527 | ABCC1 | Multidrug resistance-associated protein 1 |

**Bolded UniProt IDs are for proteins considered for validation due to their relative high abundance.**

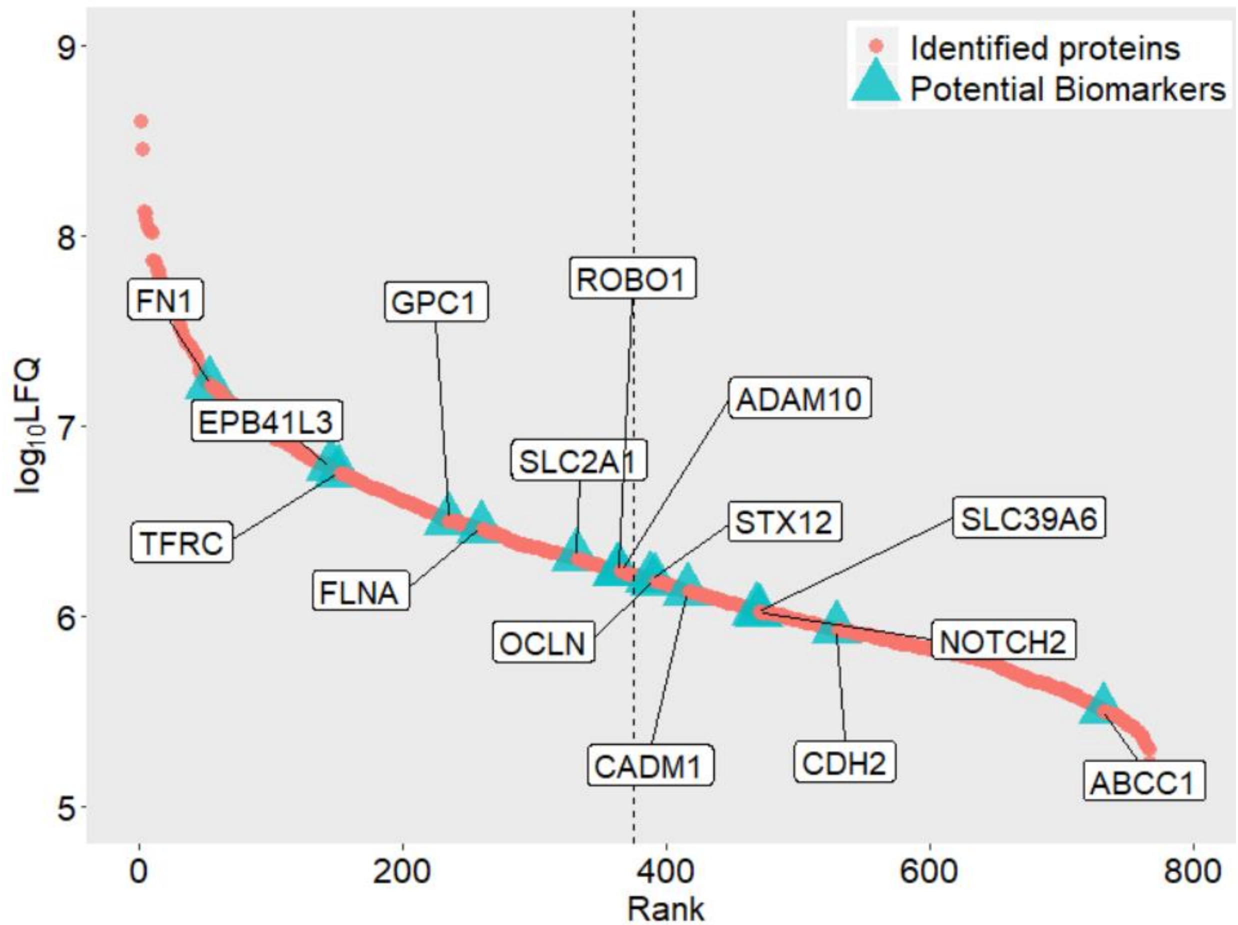



**Figure 6**. The abundance distribution of the 15 potential BC biomarkers over the whole identified proteome. Only abundant proteins, to the left of the dashed lines, were considered for validation.

## 4.6 Surface Expression of GPC-1, GLUT-1 and ADAM10 Validation

To confirm the presence of selected proteins on the surface of metastatic BC derived exosomes, flow cytometry experiments were conducted using anti-GPC-1 and anti-GLUT-1 antibodies along with two antibodies against exosomal markers CD63 and CD81. Several fluorescence-minus-one controls were used to ensure proper data analysis (**Supplementary Table S1**). The expression of both GLUT-1, GPC-1, and ADAM10 overlapped with CD63 and CD81 positive exosomes (**Figure. 7**). The exosomal marker CD63 stained 45.8% of vesicles, while 33.6% were positive for CD81. 8% of exosomes were positive for GPC-1, CD63, and CD81. 10% of exosomes were positive for CD63 and GPC-1 only, while 8.3% were stained with both CD81 and GPC-1. 10% of exosomes were stained positive for GLUT-1, CD63, and CD81. 10.9% and 10.2% of GLUT-1 positive exosomes were also positive for CD63 and CD81, respectively. 6.6% of CD63 and CD81 positive vesicles expressed ADAM10. 9.5% of vesicles were positive for both CD81 and ADAM10, while 8.6% expressed ADAM10 and CD63. These results confirm the presence of ADAM10, GLUT-1, and GPC-1 on the surface of BC cell-derived exosomes.

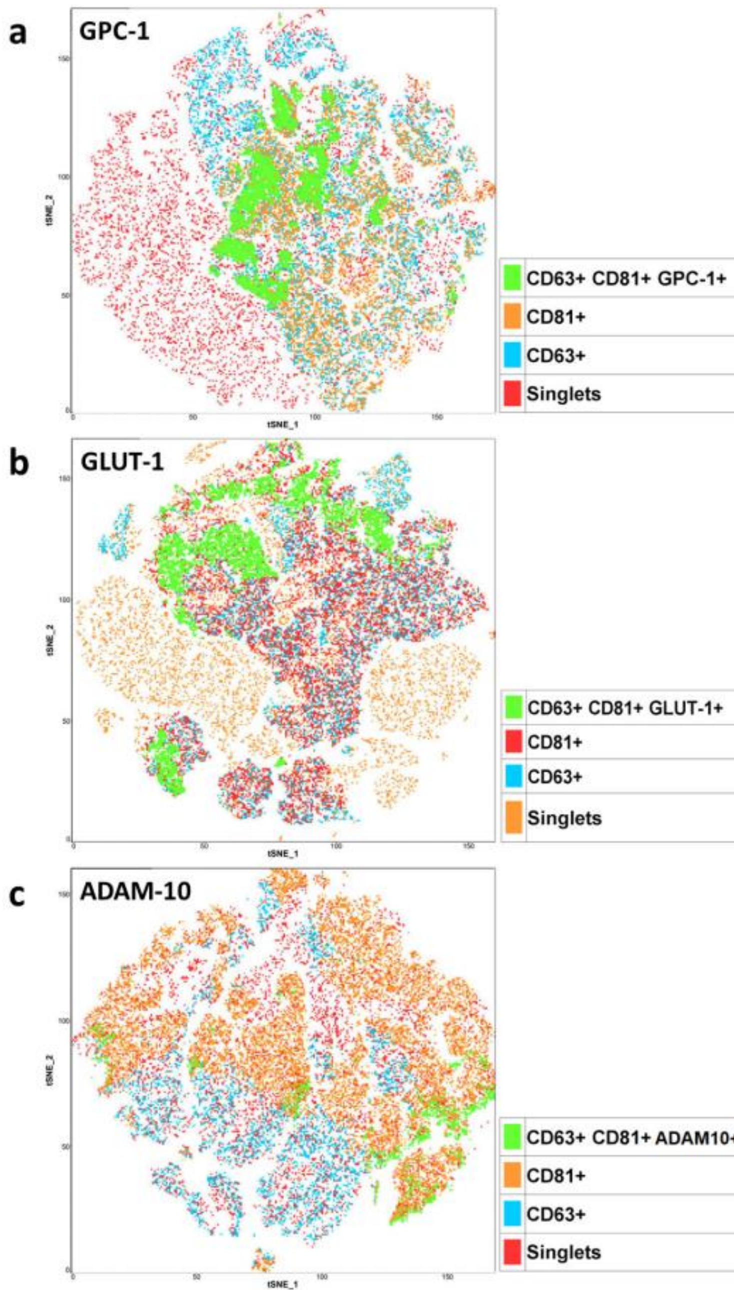



**Figure 7.** tSNE plots of exosomes analyzed by FACS showing the expression levels of GLUT-1 (**a**) GPC-1 (**b**) and ADAM10 (**c**) along with CD81 and CD63 on the surface of exosomes.

# 5 Discussion

## 5.1 Exosome Isolation

Current exosome isolation techniques currently rely on five techniques which are: ultracentrifugation, size exclusion, microfluidics techniques, immunoaffinity capture and exosome precipitation [99]. Three different exosome isolation techniques were tested for exosomal recovery and MS suitability. Differential ultra-centrifugation, ultra-filtration paired with ultra-centrifugation and ExoQuick precipitation kit were compared based on NTA and MS.

### *5.1.1 Size Based Characterization*

ExoQuick kit provided the highest yield of exosomes. It also captured a significant number of particles larger than exosomes at diameters of 225, 315, 435 and 525 nm. The minimal sample processing required for exosome isolation using the ExoQuick kit makes this method convenient for small and valuable samples but also unspecific [100]. The ExoQuick kit used a polyethylene glycol (PEG) polymer to capture exosome-like vesicles from cell culture media; the PEG polymer has the ability to precipitate not only exosomes but also larger vesicles (MVs and apoptotic bodies) and protein aggregates [101-104].

The UF-UC protocol isolated particles of a median size of 135 nm and had the lowest vesicle yield. The centrifugal force applied to vesicles during the filtration processes has been reported to skew results. The pressure applied to exosomes during filtration causes them to clump together and could potentially rupture the vesicles on the 100 KDa filter [105]. Exosomes could also stick to the filter's membrane and become unavailable for further analysis [106].

UC, on the other hand, provided a relatively high and uniform concentration of exosomes with a median size of 105 nm. This method continues to be the "gold standard" for the isolation of

exosomes [99]. It is, however, known to isolate contaminates such as vesicles larger than 100 nm and protein aggregates [101]. Moreover, the shear force applied to exosomes during centrifugation may damage the vesicles [107].

Even though MVs have been depleted from media before further processing by either UC or UF-UC, both suffered from the isolation of a broad distribution of particles some of which are beyond the defined size of exosomes, 50-105 nm, indicating the inability of both methods to provide a narrow distribution of particles around the105 nm size.

### *5.1.2 Protein Analysis*

The lowest number of identified proteins was obtained using exosomes isolated by the ExoQuick kit, 5 proteins in total, **Table 2**. Several studies found it hard to fully solubilize ExoQuik's PEG polymer, making further downstream analysis (such as western blots and RNA extractions) difficult [100,101]. Moreover, the low protein count may also be due to PEG's protein precipitation ability [108]. PEG disrupts the hydrolytic layer of proteins by sequestering them and excluding their access to water [109,110]; this makes it hard to solubilize and digest proteins.

Even though UF-UC had a much higher protein concentration than UC, it resulted in a lower protein count, **Table 1**. This can be explained by the nature of the UF-UC sample. After the depletion of MV by a 15,000 *g* spin, the sample is filtered. The filtration step enriches the media with not only vesicles but also other biological molecules, such as protein aggregates [111]. This makes the UF-UC samples richer in abundant proteins than UC samples. The presence of highly abundant proteins can interfere with the identification and quantification of low abundance ones [112]. The DDA methodology, used for data acquisition, sends the top 10 highest intensity ions for scanning by the Orbitrap MS, excluding low abundant peptides, further exacerbating the issue [113].

### *5.1.3 Choice of Isolation Method*

UC was chosen for further sample analysis due to its high vesicle yield and specificity at isolating vesicles of the size of 105 nm. Furthermore, the higher number of identifiable proteins and exosomal markers the UC method provides, makes it a more suitable method for downstream MS analysis. There are, however, other exosome isolation methods that were not tested.

## 5.2 Detergent Comparison

Three non-ionic detergents (DDM, digitonin and Triton-X100) were tested for their ability to solubilize exosomal proteins. Since DDM recovered the most proteins and had the highest number of proteins under the "extracellular exosome" cellular localization label, all in-solution digestion experiments were carried out using this detergent.

DDM has not been considered for the solubilization of exosomes, to my knowledge. Previous studies of different human cell lines found SDS and Triton X-100 to be the most suitable for the solubilization of exosomes [123,124]. SDS was not tested, and Triton X-100 was used at a relatively low concentration due to its incompatibility with mass spectrometry analysis [125-127].

## 5.3 Biomarker Selection

Using UC, 1,107 proteins from MDA and MCF exosomes were identified in this study, of which 726 were unique to MDA. The significant difference in the number of exosomal proteins identified from each cell might be due to the low number of exosomes produced by MCF. MCF has been shown to produce significantly less small extracellular vesicles and proteins than MDA [128]. MDA exosomal protein annotations could be grouped into two themes: motility and signalling. This is inline with the reported function of cancer exosomes as promoters of cell motility and metastasis [129-133]. For the selection of potential BC biomarkers, the focus was on exosomal

proteins upregulated in BC that are reported to further increased in expression as the disease progressed. This led us to select GPC-1, ADAM10 and GLUT-1. The cell surface proteoglycan protein GPC-1 has been reported to be strongly expressed in BC tissue compared to healthy ones; its levels were also higher in patients' tissue with advanced stages of BC [134]. GPC-1 was found expressed in 98.8% of oesophageal squamous cell carcinoma patients' tumour tissue with higher levels associated with poorer prognosis [135]. ADAM10, also known as a disintegrin and metalloproteinase domain-containing protein 10 or CD156c, is a transmembrane protease protein. The knockdown and selective inhibition of this protein in BC MDA cells reduced their migration ability without affecting cell numbers. ADAM10 was also expressed at a higher rate in high-grade tumours compared to low-grade ones; higher levels of this protein correlated with poor outcomes for basal subtypes of BC patients [136]. While in NSCLC, ADAM10 was found to be significantly higher in cancerous tissue, especially in metastatic tissue ($P<0.05$), compared to adjacent noncancerous samples [137]. Finally, GLUT-1 is a solute carrier family 2 protein that facilitates the transport of glucose across the plasma membranes of mammalian cells [138]. GLUT-1 expression correlated with higher grade BC cancer and increased proliferative activity. The absence of this protein significantly increased disease-free survival in BC patients [139]. Extracellular vesicle levels of GLUT-1 protein have been found to double when A431 epidermal carcinoma cells are programmed to transition from epithelial to mesenchymal cells [140]. In gastric cancer, GLUT-1 levels significantly correlated with the invasion level and clinical state of cancer and its overexpression resulted in enhanced tumour growth in-vivo [141]. Taken together, these three functionally relevant and abundant exosomal proteins could be good candidates for minimally invasive BC detection.

Other membrane proteins within the eight selected proteins, such as FN1, EPB41L3, FLNA, ROBO1 and TFRC (**Table 2**), were not chosen for validation due to reasons described below. FN1 is a cell surface binding extracellular glycoprotein [142-144]. This protein has already been proposed as a BC biomarker; FN1 levels were elevated in BC patients' EVs with better diagnostic accuracy than its levels in plasma [145]. EPB41L3 and FLNA proteins connect the cytoskeleton to transmembrane proteins inside cells; these proteins are oriented towards the cellular lumen and do not have extracellular domains, making their detection difficult [146,147]. ROBO1 protein has not been reported in exosomes before this study according to our knowledge. High expression of this protein in BC cells, in-vitro, is associated with decreased tumorigenesis, while its low expression in invasive ductal carcinoma patients resulted in poor prognosis [148,149]. Finally, TFRC is a membrane glycoprotein that facilitates the cellular uptake of iron from the transferrin plasma glycoprotein via receptor-mediated endocytosis [150]. The TFRC protein has been explored for cancer therapy drug delivery due to its endocytic abilities [151,152]. In BC, the TFRC protein, along with nine other iron-related proteins, has been used to successfully distinguish between cancerous and non-cancerous BC lesions [153]. This protein was found differentially expressed between normal, benign, in-situ and invasive carcinomas in breast tissues. Its expression is reported to be elevated in invasive and aggressive BCs compared to other phenotypes [154]. Furthermore, the TFRC protein, along with three other proteins, has also been identified as a candidate biomarker for estrogen and progesterone positive (ER+/PR+) invasive ductal BC using pre-clinical samples [155]. TFRC has already been identified on the surface of MDA derived exosomes [156]; hence, it was not selected for validation.

## 5.4 Other BC Exosomal Proteins

The 87 identified BC proteins were rich with interesting proteins such as 16 metastasis-related proteins (**Supplementary Table S6**). Of the metastasis-associated proteins is Stathmin-1, also known as oncoprotein 18. It is a cytosolic phosphoprotein that regulates microtubule dynamics [157]. Immunohistochemical analysis of BC tissue exhibited an upregulation of Stathmin-1 compared to non-malignant tissue. High expression of Stathmin-1(STMN1: P16949) in BC patients correlated to lower survival rates [158]. Using Stahtmin-1 expression levels and phosphorylation status, BC patients were classified into high and low-risk groups, highlighting its prognostic value [159]. Furthermore, the Peptidyl-prolyl *cis/trans* isomerase NIMA-interacting protein (PIN1: Q13526) also drives BC metastasis. PIN1 mediates the isomerization of phosphorylated pSer/Thr-Pro motifs [160]. Its expression has been found to be higher in metastatic cancers compared to primary ones [161,162]. In BC cell lines, including MDA, PIN1 was found to increase cancer cell metastasis and invasion capabilities by activating the NOTCH pathway. PIN1 enhanced NOTCH1 tumorigenic activity by potentiating its cleavage by $\gamma$-secretase [163]. PIN1 also promoted the epithelial-mesenchymal transition of MCF-7 BC cells by increasing the transcriptional activity of STAT3, signal transducer and activator of transcription 3, which in turn upregulates the TWIST transcription factor [164]. This protein is being explored as a therapeutic target for cancer. Several PIN1 targeted microRNAs (miR296-5p, miR-200c, and miR-370) inhibit cancer progression by decreasing mRNA levels of PIN1 [165-167].

Kinase proteins play essential roles in cancer processes such as immune evasion, cell cycle regulation, and cell proliferation. The extensive role of protein kinase phosphorylation in most cell signal transductions makes them ideal therapeutic targets [168,169]. Out of the two BC associated kinases, the c-Src (SRC: P12931) was of interest (**Supplementary Table S5**). It is a proto-

oncogene tyrosine-protein kinase Src. The c-Src kinase regulates functions during tumour cell migration, invasion, proliferation and metastasis [170]. This kinase has been implicated in the late-onset of BC bone metastasis. c-Src is critical for the survival of BC cells in the bone marrow by suppressing apoptosis-related signals and activating the Akt survival-promoting pathway [171].

Based on the results of the present study, we hypothesize that GLUT-1, GPC-1 and ADAM10 proteins may be novel potential biomarkers for BC derived exosomes. Future studies should examine the exosomal presence of these proteins in samples from healthy and BC diseased populations. The best model system for such a study would be breast tumour-derived exosomes isolated from the blood of BC patients. Blood, however, is a source of exosomes from all tissue types and not only breast tumours. Conducting such a study on exosomes from diverse tissue sources would complicate the selection process and reduce its specificity to BC disease. Therefore, to identify clinically relevant biomarkers, all our selected proteins in Table 1 may be taken into consideration. Furthermore, such blood biomarkers can be efficiently validated. Other studies have proposed several biomarkers of BC [172]. Here, however, our proposed biomarkers are cell surface membrane proteins that can be easily accessible for diagnostic sampling. These membrane proteins could be integrated into effective and simple biomarker diagnostic assays based on monoclonal antibody and aptamer-based methods [173,174].

# 6 Future Directions

Significant differences were found between the BC cell line MDA and the non-cancerous breast cell line MCF. As this study was performed on only one advanced TNBC cell line, including more cell lines of the same type of BC disease would strengthen the protein selection process. Such cell lines might include BT-20 and MDA-MB-468 cell lines. Furthermore, adding early stage TNBC cell lines (SUM102PT cell line) to the analysis would also help in ensuring the progressive increase in expression of selected proteins along the disease stages and further strengthen the protein selection process.

Proper validation of the biomarker protein selection process has to be conducted on blood samples of healthy and BC patients. This study was not initially conducted on blood samples since blood is a source of exosomes from all tissue types and not only breast tumours. Conducting such a study on exosomes from diverse tissue sources would complicate the selection process and reduce its specificity to BC disease.

# 7 Limitations

## 7.1 Number of cell lines analyzed

This study was performed on a small number of cell lines. In the future, it would be valuable to include more aggressive and early stage TNBC cell lines. This would strengthen the protein biomarker selection by identifying proteins that increase in expression as the BC disease progresses. Adding more cell lines would decrease the reliance on the literature and enable more robust statistical analysis.

## 7.2 Lack of Specific Exosomal Markers

Exosomes are small vesicles generated when multivesicular bodies, produced through the endosomal pathway, fuse to the plasma membrane releasing their contents [41]. There are several accepted markers for exosomes, some of which include: TSG101, FLOT-1, CD9, CD63, CD81, HSP90AB1, ALIX, ESCRT-I/II/III and more [122].

Studies have shown several of the currently accepted exosomal markers not to be exosome specific, isolated using a density gradient ultracentrifugation, but are also found on other small vesicles [180]. Such findings could be attributed to the heterogeneity of extracellular vesicles, including small vesicles such as exosomes [181, 122].

In this study, we considered exosomes to be small vesicles isolated using ultra-centrifugation, at a centrifugal speed of 100,000 *g*, positive for CD81 and CD63 surface proteins. These proteins have the potential to be found on vesicles other than exosomes, complicating the conclusions drawn in this research. Furthermore, there could be small vesicles generated by the endosomal pathway that are not positive for CD81 or CD63. By excluding/including vesicles only positive for CD63 and

CD81, other endosomal small vesicles might not have been considered, or contaminant small vesicles not originating from the endosomal pathway might have been included in the MS and flow cytometry analyses.

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# 9 Appendix

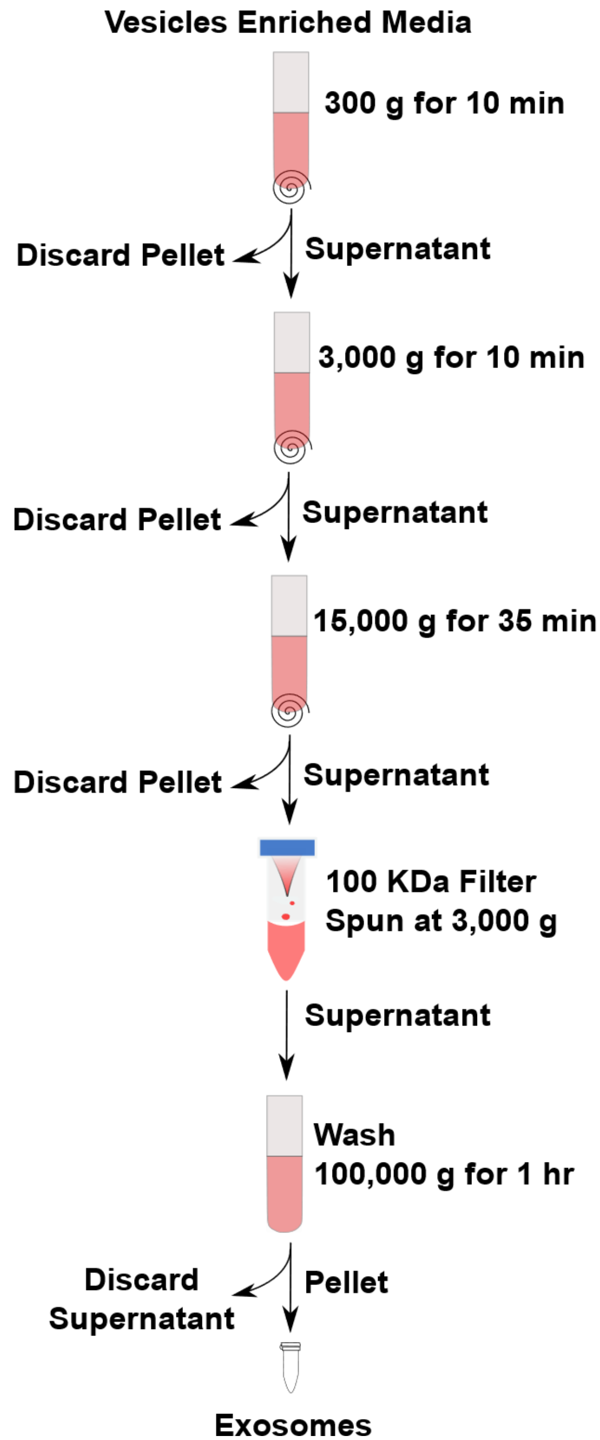



**Figure S1.** Experimental steps used for the UF-UC exosomes isolation method.

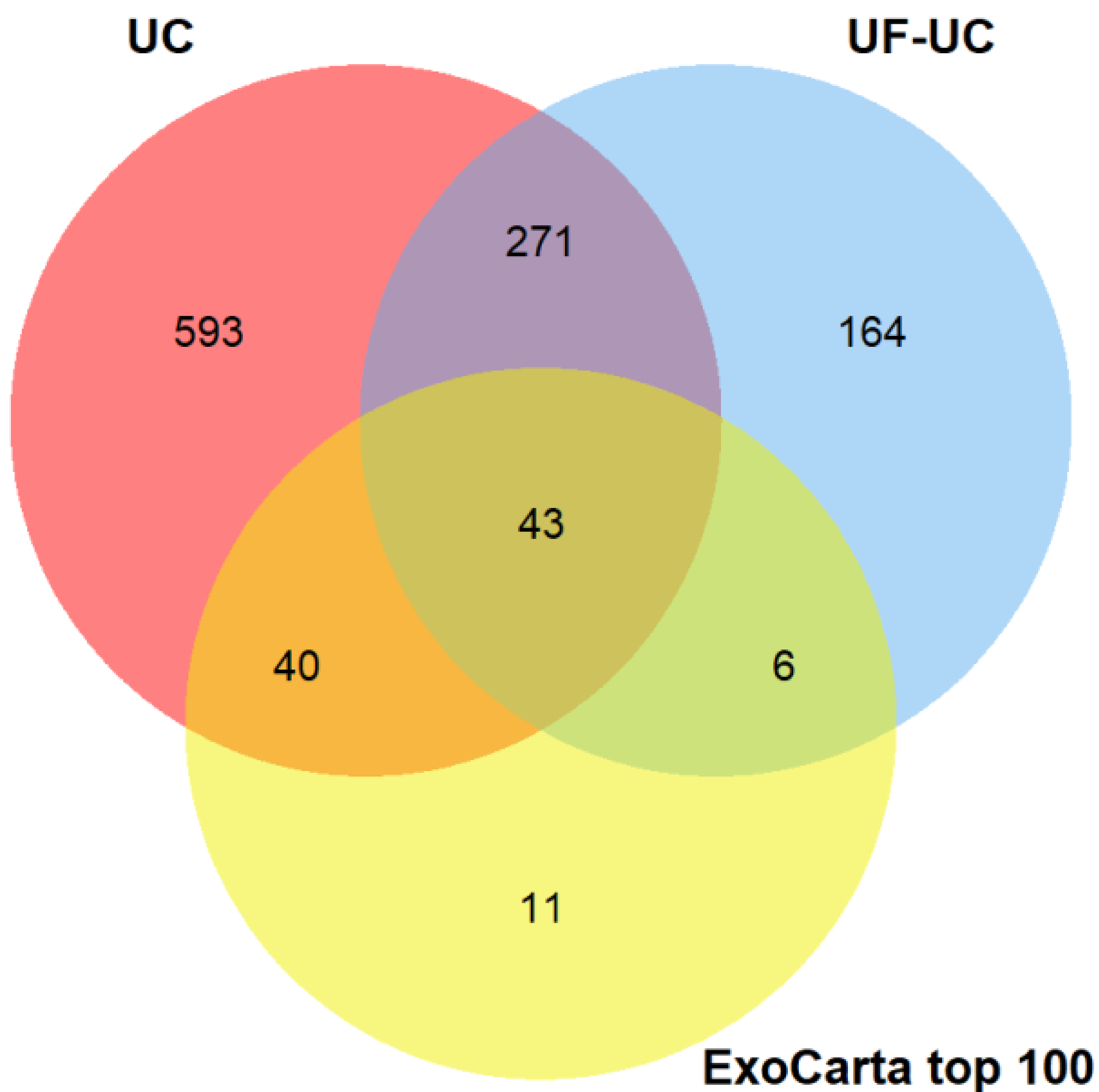



**Figure S2.** The UC exosome isolation method has more proteins in common with the ExoCarta database Top 100 identified proteins than the UF-UC method.

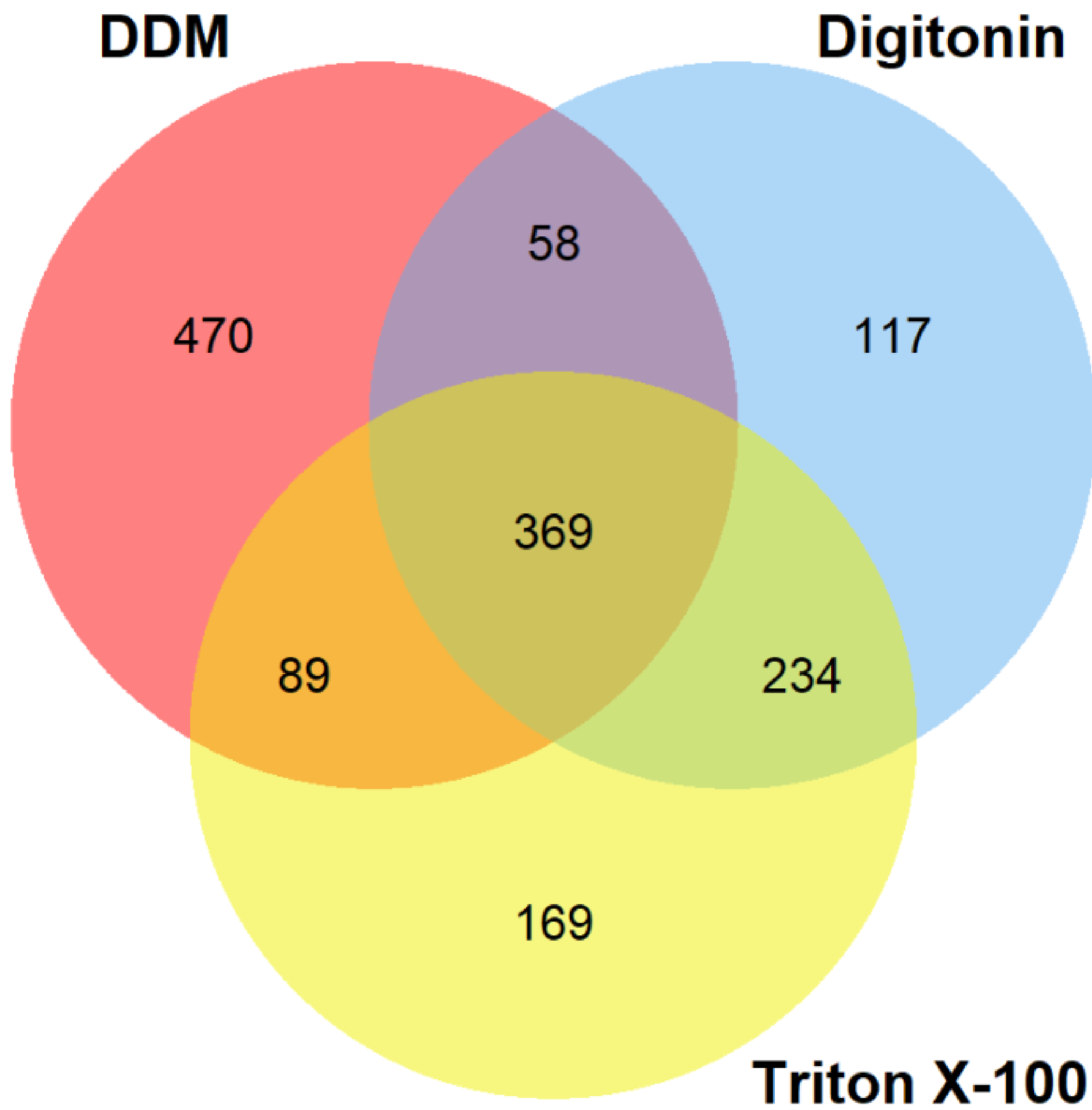



**Figure S3.** Venn diagram displaying the overlap in exosomal proteins identified using three detergents: DDM, Digitonin and Triton X-100.

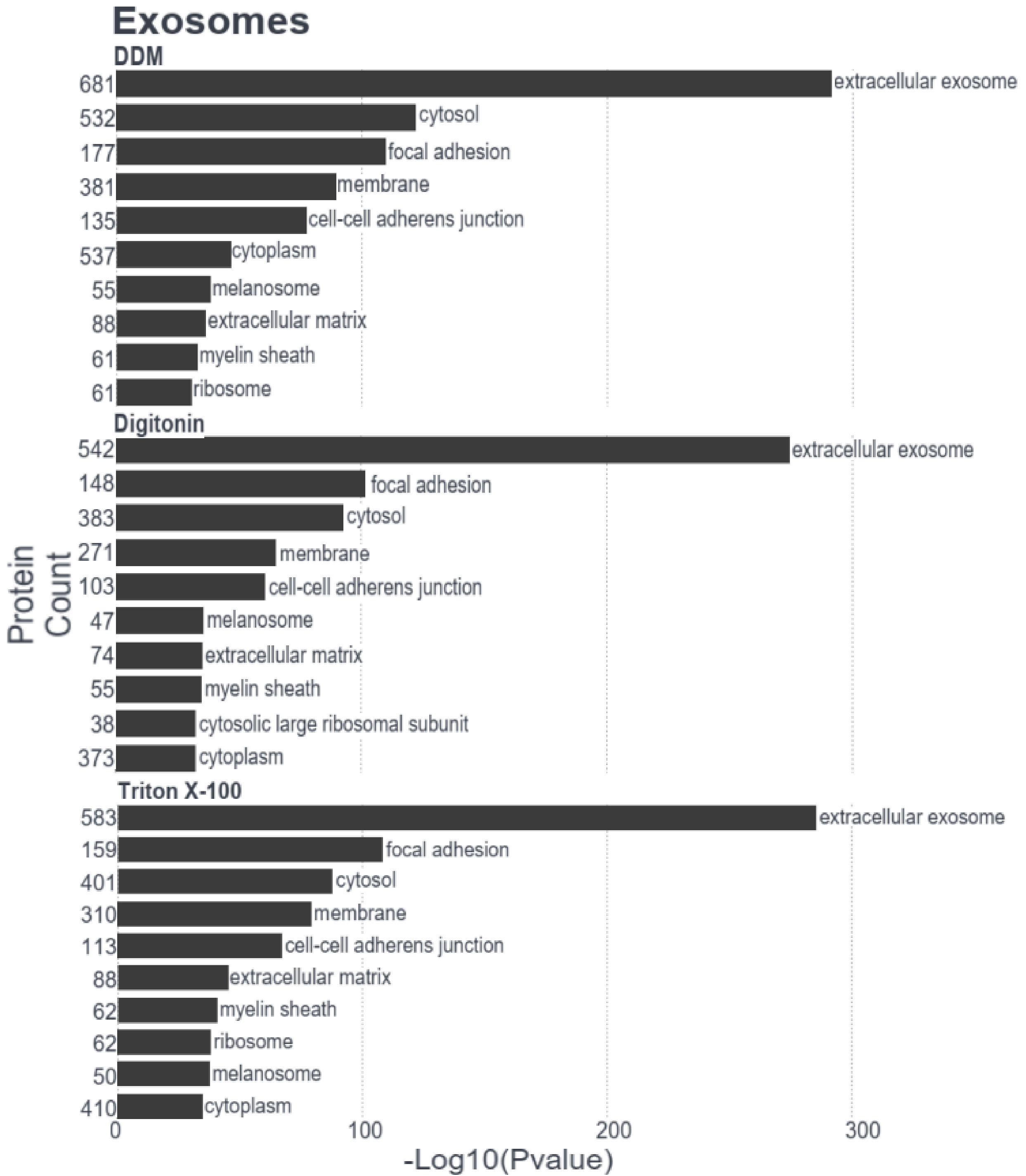
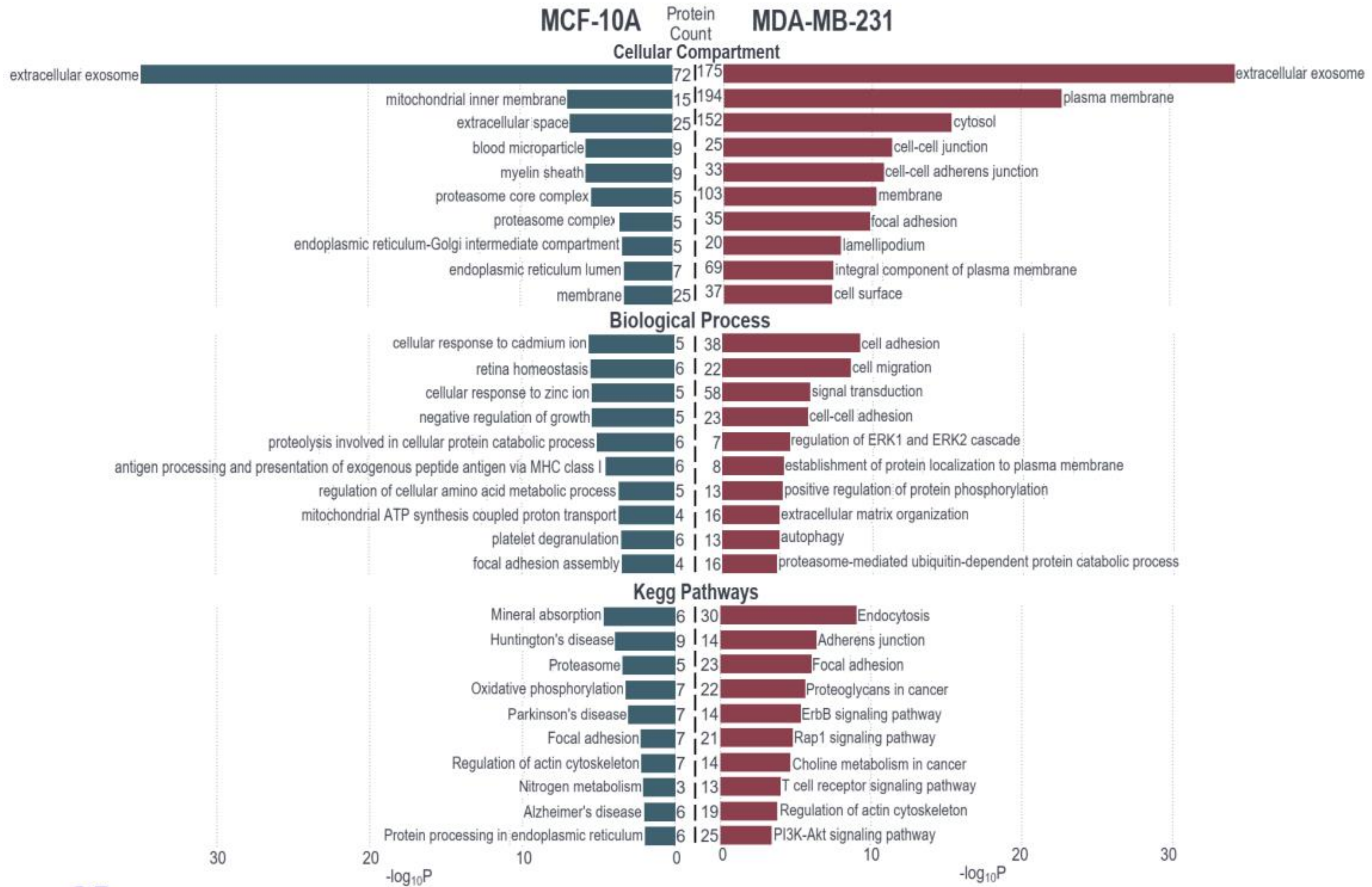



**Figure S4.** Top 10 GO cellular localization of proteins solubilized by DDM, Digitonin and Triton X-100. P values were calculated using Fisher's exact test and were provided by David's functional annotation tool.



MCF-10A
Protein Count
MDA-MB-231
Cellular Compartment
extracellular exosome
mitochondrial inner membrane
extracellular space
blood microparticle
myelin sheath
proteasome core complex
proteasome complex
endoplasmic reticulum-Golgi intermediate compartment
endoplasmic reticulum lumen
membrane
72
15
25
9
9
5
5
5
7
25
175
194
152
25
33
103
35
20
69
37
extracellular exosome
plasma membrane
cytosol
cell-cell junction
cell-cell adherens junction
membrane
focal adhesion
lamellipodium
integral component of plasma membrane
cell surface
Biological Process
cellular response to cadmium ion
retina homeostasis
cellular response to zinc ion
negative regulation of growth
proteolysis involved in cellular protein catabolic process
antigen processing and presentation of exogenous peptide antigen via MHC class I
regulation of cellular amino acid metabolic process
mitochondrial ATP synthesis coupled proton transport
platelet degranulation
focal adhesion assembly
5
6
5
5
6
6
5
4
6
4
38
22
58
23
7
8
13
16
13
16
cell adhesion
cell migration
signal transduction
cell-cell adhesion
regulation of ERK1 and ERK2 cascade
establishment of protein localization to plasma membrane
positive regulation of protein phosphorylation
extracellular matrix organization
autophagy
proteasome-mediated ubiquitin-dependent protein catabolic process
Kegg Pathways
Mineral absorption
Huntington's disease
Proteasome
Oxidative phosphorylation
Parkinson's disease
Focal adhesion
Regulation of actin cytoskeleton
Nitrogen metabolism
Alzheimer's disease
Protein processing in endoplasmic reticulum
6
9
5
7
7
7
7
3
6
6
30
14
23
22
14
21
14
13
19
25
Endocytosis
Adherens junction
Focal adhesion
Proteoglycans in cancer
ErbB signaling pathway
Rap1 signaling pathway
Choline metabolism in cancer
T cell receptor signaling pathway
Regulation of actin cytoskeleton
PI3K-Akt signaling pathway
30
20
10
0
$-\log_{10}P$
0
10
20
30
$-\log_{10}P$

**Figure S5.** Top 10 GO functional annotation labels, for biological process and cellular compartment, and Kegg pathway analysis for exosome MDA-MB-231 (right) and MCF-10A (left) unique proteins. P values were calculated using Fisher's exact test and was provided by David's functional annotation tool.

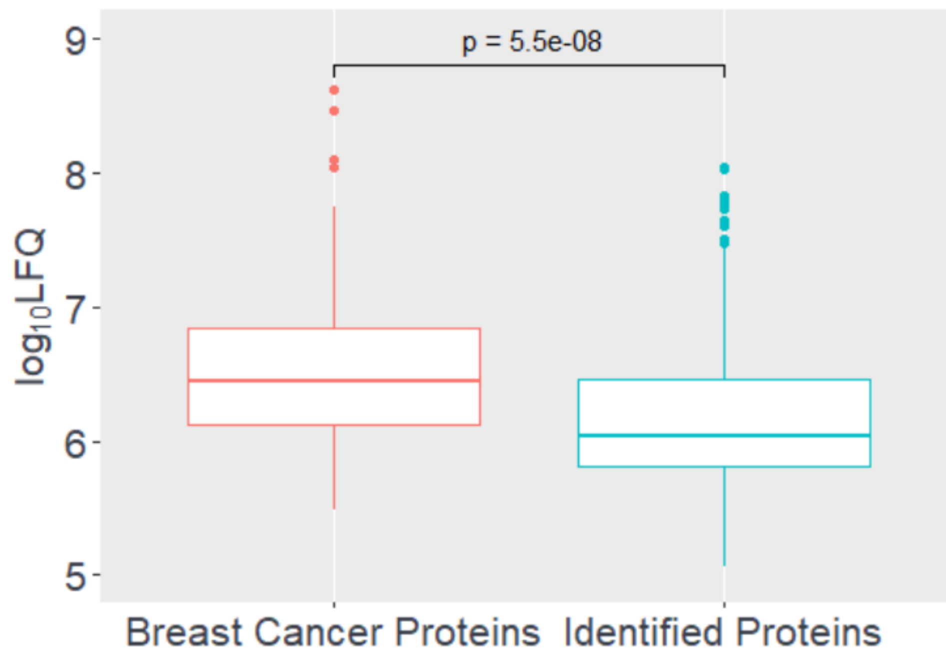



**Figure S6.** The graph shows the higher expression levels of DisGenet BC identified proteins compared to the rest of the MDA exosomal proteins. The difference was found to be significant according to Wilcoxon-Mann-Whitney test with a p-value of 0.00028.

**Table S1.** A list of the different control samples used for the validation of GLUT-1 and GLYP-1 using flow cytometry.

| Control # | Description |
|---|---|
| 1 | Exosomes unstained |
| 2 | Exosomes stained with CD81 and GLUT-1 |
| 3 | Exosomes stained with CD63 and GLUT-1 |
| 4 | Exosomes stained with CD81 and GLYP-1 |
| 5 | Exosomes stained with CD63 and GLYP-1 |
| 6 | Exosomes stained with CD81 and ADAM10 |
| 7 | Exosomes stained with CD63 and ADAM10 |
| 8 | Exosomes stained with CD81 and CD63 |

**Table S2.** List of proteins obtained by the mass spectrometry analysis of ExoQuick isolated exosomes processed using the in-solution digestion method.

| UniProt ID | Gene | Description |
|---|---|---|
| Q9NR48 | ASH1L | Histone-lysine N- methyltransferase ASH1L |
| P46100 | ATRX | Transcriptional regulator ATRX |
| Q8N987 | NECAB1 | N-terminal EF-hand calcium-binding protein 1 |
| P09874 | PARP1 | Poly [ADP-ribose] polymerase 1 |
| O75398 | DEAF1 | Deformed epidermal autoregulatory factor 1 homolog |

**Table S3:** MDA-MB-231 identified exosomal proteins not previously reported in the exosomal protein database ExoCarta.

| UniProt ID | Gene | Description |
| --- | --- | --- |
| P47813 | EIF1AX | Eukaryotic translation initiation factor 1A, X-chromosomal |
| O60232 | ZNRD2 | Protein ZNRD2 |
| P0DJJ0 | SRGAP2C | SLIT-ROBO Rho GTPase-activating protein 2C |
| O95218 | ZRANB2 | Zinc finger Ran-binding domain-containing protein 2 |
| O95235 | KIF20A | Kinesin-like protein KIF20A |
| P07358 | C8B | Complement component C8 beta chain |
| Q32P51 | HNRNPA1L2 | Heterogeneous nuclear ribonucleoprotein A1-like 2 |
| P42771 | CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| P49711 | CTCF | Transcriptional repressor CTCF |
| P49768 | PSEN1 | Presenilin-1 |
| Q8NEV1 | CSNK2A3 | Casein kinase II subunit alpha 3 |
| Q05519 | SRSF11 | Serine/arginine-rich splicing factor 11 |
| Q14527 | HLTF | Helicase-like transcription factor |
| Q15651 | HMGN3 | High mobility group nucleosome-binding domain-containing protein 3 |
| Q4VCS5 | AMOT | Angiomotin |

| | | |
|---|---|---|
| Q6FI81 | CIAPIN1 | Anamorsin |
| Q6P9B6 | MEAK7 | MTOR-associated protein MEAK7 |
| Q6PKG0 | LARP1 | La-related protein 1 |
| Q7Z2K8 | GPRIN1 | G protein-regulated inducer of neurite outgrowth 1 |
| Q8TB73 | NDNF | Protein NDNF |
| Q96GD4 | AURKB | Aurora kinase B |
| Q9BQ16 | SPOCK3 | Testican-3 |
| Q9H4F8 | SMOC1 | SPARC-related modular calcium-binding protein 1 |
| Q9H7B2 | RPF2 | Ribosome production factor 2 homolog |
| Q9NNW5 | WDR6 | WD repeat-containing protein 6 |
| Q9NQS7 | INCENP | Inner centromere protein |
| Q9NWQ8 | PAG1 | Phosphoprotein associated with glycosphingolipid-enriched microdomains 1 |
| Q9NZI8 | IGF2BP1 | Insulin-like growth factor 2 mRNA-binding protein 1 |
| Q9UET6 | FTSJ1 | Putative tRNA (cytidine(32)/guanosine(34)-2-O)-methyltransferase |
| Q9Y383 | LUC7L2 | Putative RNA-binding protein Luc7-like 2 |
| Q9Y6N7 | ROBO1 | Roundabout homolog 1 |

**Table S4.** MCF-10A identified exosomal proteins not previously reported in the exosomal protein database ExoCarta.

| UniProt ID | Gene | Description |
|---|---|---|
| P49448 | GLUD2 | Glutamate dehydrogenase 2, mitochondrial |
| P04179 | SOD2 | Superoxide dismutase [Mn], mitochondrial |
| P0C0L5 | C4B | Complement C4-B |
| P13928 | ANXA8 | Annexin A8 |
| P14927 | UQCRB | Cytochrome b-c1 complex subunit 7 |
| P18859 | ATP5PF | ATP synthase-coupling factor 6, mitochondrial |
| P24539 | ATP5PB | ATP synthase F(0) complex subunit B1, mitochondrial |
| P29034 | S100A2 | Protein S100-A2 |
| P62328 | TMSB4X | Thymosin beta-4 |
| Q15165 | PON2 | Serum paraoxonase/arylesterase 2 |
| Q5JTV8 | TOR1AIP1 | Torsin-1A-interacting protein 1 |
| Q9HC84 | MUC5B | Mucin-5B |

**Table S5. Kinase proteins in the BC MDA-MB-231 cell line.**

| | |
|---|---|
| **Unique MDA-MB-231, Kinases** | O94804, O95819, O96013, P06241, P06493, P07947, P07948, P10644, P12277, P12931, P13861, P17612, P19784, P30085, P42771, P43250, P48426, P68400, P78527, Q13308, Q16513, Q7KZI7, Q7L7X3, Q8N4C8, Q96B97, Q96GD4, Q99755, Q99986, Q9H2G2, Q9H8S9, Q9P289, Q9UIG0, Q9UKE5, Q9UKS6, Q9UNF0, Q9Y3F4 |
| **Cancer Kinases** | O95819, P06241, P06493, P10644, P12277, P12931, P14618, P15531, P17612, P19784, P22392, P29966, P48426, P68400, P78527, Q7KZI7, Q96B97, Q96GD4, Q9Y3F4 |
| **BC Kinases** | P12931, P42771 |

**Table S6.** List of BC and BC metastasis associated proteins identified using the DisGenet database.

| | |
|---|---|
| **BC Proteins** | O00299, O00571, O14672, O14745, O43175, O43390, O75340, P02751, P02786, P04075, P04899, P06733, P06744, P07195, P07741, P07900, P08670, P09211, P09874, P0DMV9, P11166, P11387, P11388, P11940, P12931, P13639, P13797, P14174, P14866, P16104, P16403, P16949, P17302, P17987, P19022, P21333, P22087, P23381, P23528, P24534, P26358, P33527, P35052, P35222, P35579, P42771, P43487, P46781, P48643, P49327, P52565, P55010, P61978, P62081, P62241, P62701, P62750, P62753, P62805, P62899, P67809, P84077, Q02952, Q04721, Q07955, Q08431, Q13433, Q13526, Q14112, Q15181, Q16625, Q16643, Q86VP1, Q86Y82, Q8IUE6, Q92597, Q99575, Q99816, Q9BY67, Q9NTK5, Q9UBP0, Q9UHI8, Q9UNF1, Q9Y281, Q9Y2J2, Q9Y4F1, Q9Y6N7 |
| **Metastasis Proteins** | **O14672**, O14745, O75340, **P02751**, P07900, P08670, P12931, P16949, P17302, **P19022**, P23528, P35222, P35579, P52565, Q13526, **Q9Y6N7** |

**Bolded proteins were among the 15 potential BC biomarkers.**

# 10 Contributions

**Yousef Risha** Designed the study, offered critical input, conducted the experiments, wrote the manuscript and analyzed the data.

**Dr. Zoran Minic** Designed the study, offered critical input, co-wrote the manuscript and analyzed samples using mass spectrometry

**Dr. Maxim Berezovski** Designed the study, offered critical input, and co-wrote the manuscript.

**Dr. Shahrokh Ghobadloo** Operated the MoFlo Astrios EQ Flow Cytometer instrument.

**Dr. Arkadiy Reunov** Operated the TEM for exosome imaging.

**Dr. Derrick Gibbings** Provided access to the NTA instrument.