THE ROLE OF EXTRACELLULAR VESICLES IN BREAST CANCER PROGRESSION AND DIAGNOSIS

THE ROLE OF EXTRACELLULAR VESICLES IN BREAST CANCER PROGRESSION AND DIAGNOSIS

By:

KHRYSTYNA PLATKO, H.B.Sc.Kin

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

McMaster University MASTER OF SCIENCE (2016) Hamilton, Ontario (Medical Sciences)

TITLE: The role of extracellular vesicles in breast cancer progression and diagnosis

AUTHOR: Khrystyna Platko, H.B.Sc.Kin (McMaster University)

SUPERVISOR: Dr. Khalid Al-Nedawi

NUMBER OF PAGES: xii, 107

ABSTRACT

Breast cancer (BC) is the second most commonly occurring malignant disease in women and one of the leading causes of cancer-related death worldwide, globally accounting for almost half-a-million deaths per year. In Canada, BC is the second leading cause of death in women preceded only by lung cancer. Invasion and metastasis are the most common causes of mortality in patients with BC. Studies show that extracellular vesicles (EVs) play an important role in immune system evasion, invasion and metastasis. Studies have shown a significant elevation of EVs in the serum of cancer patients compared to healthy subjects. Furthermore, elevated secretion of EVs has been correlated with cancer malignancy. Therefore, it has been suggested that EVs may be an important non-invasive diagnostic and prognostic tool for cancer. Herein our *in vitro* studies show that ER-α is secreted *via* EVs from MCF-7 cells. Furthermore, our mass spectrometry (MS)-based proteomic study showed that the proteomic profile of EVs from the plasma of BC patients differs from that of healthy subjects. In addition, we have also shown that vesicular abundance of proteins associated with tumour malignancy, such as tissue factor (TF), plasminogen activator inhibitor (PAI-1), a disintegrin and metalloproteinase 12 (ADAM12) and β-Catenin is different between primary tumour and metastatic disease.

AKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Khalid Al-Nedawi for allowing me to work on such an exciting project with great potential. Without his knowledge and guidance I would not be where I am today. I would also like to thank other members of my committee, Drs. Mark Inman and Paul Forsythe, for their advice and direction.

I would like to give my deepest gratitude to Talha Quereshi for teaching me everything about cell culture techniques. I would also like to thank Jolene Read for helping me through some of the more difficult parts of my project.

Several other researchers must be thanked. They include Sarka Lhotak for teaching me tissue histology techniques, Dr. Eric Bonneil, for assisting with proteomic analysis and Dr. Damu Tang for providing me with breast cancer cell lines.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADAM12	A disintegrin and metalloproteinase 12
AF	Activation function
AI	Aromatase inhibitor
ALIX	ALG-2 interacting protein
AR	Androgen receptor
ARF	ADP-ribosylation factor
ATCC	American type cell cultures
BC	Breast cancer
BSN	Bjerrum and Schafer-Nielsen
BSA	Bovine serum albumin
CBP	CREB binding protein
CST	Cell signalling technologies
CYP2D6	Cytochrome P450 2D6
DC	Detergent compatible
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DBD	DNA binding domain
ECM	Extracellular matrix
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
EM	Electron microscopy
EMT	Epithelial to Mesenchymal Transition
ER	Estrogen receptor
ERE	Estrogen response element
ESCRT	Endosomal sorting complex
EtOH	Ethanol
EVs	Extracellular vesicles
FA	Formic acid
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HER2	Human epidermal growth factor receptor 2
HKRC	Hamilton kidney research centre
HRP	Horseradish peroxidase
HSP	Heat-shock protein
ILV	Intraluminal vesicle
LBD	Ligand binding domain
LFQ	Label free quantification
MAPK	Mitogen activated protein kinase
MS	Mass spectrometry
MVs	Microvesicles

MVB	Multivesicular body
NCoR	Nuclear receptor corepressor
NLS	Nuclear localization signal
NRS	Normal rabbit serum
NTD	N-terminal domain
OICR	Ontario Institute of Cancer Research
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PC	Prostate cancer
PIK-3	Phosphoinositide 3-kinase
PTEN	Phosphate and tensin homolog
PR	Progesterone receptor
PM	Plasma membrane
PS	Penicillin Streptomycin
PS	Phosphatidylserine
RAC	Receptor associated coactivator 3
RCL	Reactive center loop
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SERPIN	Serine proteinase inhibitor
SERM	Selective estrogen receptor modulator
SNAP	Soluble N-ethylmaleimide-sensitive fusion attachment protein
SNARE	Soluble N-ethylmaleimide-sensitive fusion attachment protein receptor
TAM	Tamoxifen
TBS-T	Tris buffered saline-Tween 20
TCEP	Tris carboxyethyl phosphine
TF	Tissue factor
TfR	Transferrin receptor
TGS 101	Tumour susceptibility gene 101
TGF	Transcription growth factor
TIF2	Transcriptional intermediary factor 2
TNBC	Tripple negative breast cancer
TNM	Tumour-Node-Metastasis
uPA	Urokinase plasminogen activator
tPA	Tissue plasminogen activator
VTE	Venous thromboembolism
WB	Western blotting

CHAPTER 1: INTRODUCTION

Breast cancer (BC) is the second most commonly occurring malignant disease in women and one of the leading causes of cancer-related death worldwide, globally accounting for almost half-a-million deaths per year [1]. In Canada, BC is the second leading cause of cancer-related death in women preceded only by lung cancer [2]. It has been estimated that in 2015, 25,000 Canadian women were diagnosed with BC, and 5,000 died as a result of the disease [2]. Furthermore, one in nine Canadian women develop BC during their lifetime, and one in 30 die as a result of BC morbidities [2]. Several environmental [3], hereditary [4-7] and lifestyle [8-13] factors have been associated with increased risk of developing BC and poor patient outcome.

Invasion and metastasis are the most common causes of mortality in patients with BC [14]. Distant metastasis are commonly found in the brain, lungs, bone, and liver [14]. The estrogen receptor (ER)-α, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are the main prognostic and diagnostic biomarkers for BC [15]. Current clinical practices require assessment of tumour-core biopsies in order to accurately diagnose BC patients and choose the course of treatment [15, 16]. However, there is evidence showing that tumour-core biopsies or other needle procedures can disseminate tumour cells, potentially contributing to the development of metastasis [16]. Therefore, there is an urgent need for the development of non-invasive diagnostic tools that would allow for the monitoring of changes in tumour phenotype, without the consequence of contributing to disease progression.

It is well established that tumour cells and the surrounding stroma secrete a variety of tumour associated proteins *via* extracellular vesicles (EVs) [17-21]. Studies have shown a significant elevation of EVs in the serum of cancer patients compared to healthy subjects [17, 22]. Furthermore, elevated secretion of EVs has been correlated with cancer malignancy [23]. Therefore, it has been suggested that EVs may be an important non-invasive diagnostic and prognostic tool for cancer [18-21, 24]. To test this hypothesis, we have examined the cargo of EVs derived from the plasma of BC patients and BC cells.

Our *in vitro* studies show that ER-α is secreted *via* EVs derived from the BC MCF-7 cells. Furthermore, our mass spectrometry (MS)-based proteomic study showed that the proteomic profile of EVs from the plasma of BC patients differs from that of healthy subjects. In addition, we have also shown that vesicular abundance of proteins associated with tumour malignancy, such as tissue factor (TF), plasminogen activator inhibitor (PAI) -1, a disintegrin and metalloproteinase 12 (ADAM12) and β-Catenin is different between primary tumour and metastatic disease.

1.1 Breast Cancer Development and Characterization

The mechanism by which the progression of BC develops from a localized state to that of a metastatic state is well-established [14, 25, 26]. BC is assessed in stages, from zero to four, and each stage represents the progression of the disease state (Figure 1). The most commonly used staging system for BC is the tumour-node-metastasis (TNM)

classification system, which takes into account the tumour size, spread of cancer to the lymph nodes, and presence of distant metastasis [27, 28]. In addition, examination of tumour histology to determine tumour grade is crucial for the prediction of disease behaviour and patient outcome [29]. BC can be further classified based on the molecular profile of established BC biomarkers [1, 30]. Current diagnostic and prognostic techniques can be utilized independently, however, a better diagnosis can be achieved when such methods are used in a complementary fashion [31] .

The ductal carcinoma *in situ* (DCIS), or stage 0, is characterized as an abnormal proliferation of breast epithelium in milk ducts and lobules, respectively [32]. The DCIS is not considered invasive, however, if left untreated, it can progress to metastatic disease [25, 32]. Stage 1 BC is characterized by an increase in tumour size within the breast, with no expansion to the lymph nodes [28, 33]. In stage 2 BC, the tumour is contained within the breast tissue, but has spread to the adjacent lymph nodes [28, 33]. BC is classified as stage 3 if tumour cells have spread past the region of origin, to the muscle and lymph nodes of the chest wall, but has not spread to distant organs [28, 34]. Lastly, the hallmark of stage 4 BC is the presence of distant metastasis in the bone, liver, lungs and brain [28]. Therefore, as disease stages progress (Figure 1), the complexity of both disease and treatment become more convoluted.

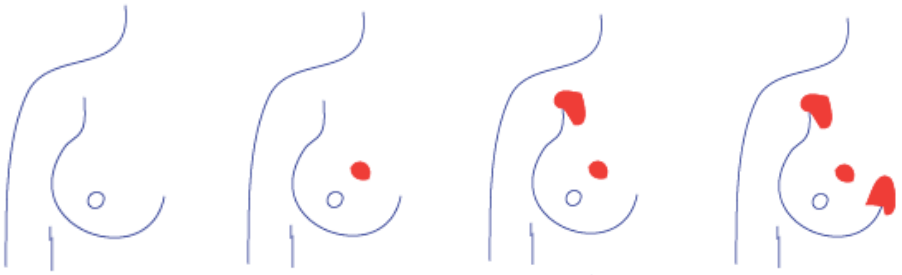
In addition to classification of the tumour with accordance to the TNM staging system, tumour tissue can also be further classified based on the tissue grade. The tumour grade system is a scoring system that is used to assess the rate of growth and the

differentiation status of tumour cells compared to normal cells in the tissue of interest [29, 35]. Based on this grading system, the tumour is then classified as low, medium or high grade, where low grade tumour cells are similar to normal cells and tend to be less aggressive than high grade tumours [29, 35]. Therefore, complimentary to the staging system, the tumour grade is predictive of patient outcome.

In addition to the TNM staging system and the tumour grade classification, BC is characterized based on the molecular profile of established BC biomarkers, including ER, PR, HER2 [14]. BC can be subdivided into four major subtypes, as well as pre-invasive subtype known as DCIS [ER±, HER2 ±] [25, 32, 36]. The subtypes of BC include: basal-like or triple negative BC (TNBC) [ER-, PR-, HER2-] [37], the HER2+ subtype [±ER+/ HER2+] [36], luminal A [ERhigh, PR+, HER2-, low Ki67] [38], luminal B [ERlow, PR+, HER2±, high Ki67] [36, 39]. Regardless of the stage and grade, these subtypes are associated with different clinical outcomes and varied responsiveness to therapy [32, 37, 38]. In addition to classification by molecular subtype, several prognostic assays have been developed based on gene expression profile of whole tumour tissues and molecular profiling of circulating tumour cells, many of which are clinically relevant [14, 40]. Furthermore, there is evidence that BC can be subdivided into ten clusters of breast-associated diseases based on copy number variation and gene polymorphisms assessed together with genomic profiles [41]. However, this technique has not yet reached clinical use. Current efforts are directed towards the development of reliable non-invasive prognostic and diagnostic tools.

Figure 1. The TNM Staging System for BC.

A simplified diagram representation of the TNM classification system for BC, as per Canadian Cancer Society recommendations. The TNM staging system takes into account the tumour size, lymph node involvement and the presence of metastasis at distant sites.

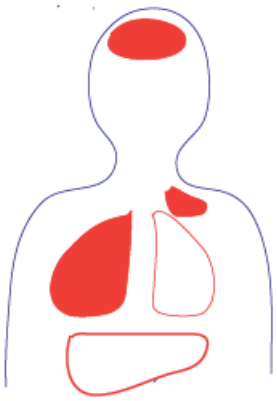


Stage 0
Abnormal noninvasive cells in the lining of the ducts or the lobules of the breast (DCIS or LCIS). Increases the risk of developing BC.

Stage I
Invasive tumour is found in the breast. The tumour is < 2 cm in diameter and has not spread to the lymph nodes or distant sites.

Stage II
The tumour is found in the breast. The tumour size is between 2-5 cm in diameter. Cancer may affect 1-3 axillary lymph nodes. No distant metastasis.

Stage III
The tumour is found in the breast. The tumour is >5 cm in diameter. Cancer spread to axillary and internal mammary lymph nodes and may affect 1-9 lymph nodes. No distant metastasis.



Stage IV
The tumour is of any size. Any degree of lymph node involvement may be observed. The tumour has spread to distant organs such as the bone, liver, lung, brain or lymph nodes far from the breast.

1.2 Breast Cancer Treatment

BC is a clinically heterogeneous disease, depending on BC stage and molecular profile of the tumour, there are many treatment options. The DCIS is a non-invasive form of BC, therefore, combination of lumpectomy (breast conserving surgery) and radiation therapy are a typical course of treatment [32]. If cancerous cells are found to spread through ducts, resulting in stage 1 BC, a combination of surgery and adjuvant molecular targeted therapy are chosen as first line of treatment by many physicians [42, 43]. For stage 1 BC, lumpectomy and mastectomy (removal of the breast) are both viable options depending on the degree that tumour cells have spread [44]. Also, both radiation therapy and hormone therapy are often recommended for patients with stage 1 BC [33, 42]. For stage 2 patients, treatment options include a combination of surgery, radiation therapy, chemotherapy, and/or hormone therapy [33, 42]. Treatment for stage 3 BC is similar to that of stage 2 BC, however, the order of interventions may differ based on the size of the tumour. For example, if the primary tumour is large, the physician may recommend chemotherapy treatment before surgery in order to reduce tumour size [34]. For metastatic BC treatment options are limited [34]. The sites and the extent of metastasis limit surgical interventions. Also, depending on the molecular profile of the tumour, stage 4 patients are predominantly treated with molecular targeted therapies as well as chemotherapy [34, 45]. Therefore, as disease progresses, the treatment options become more complex and invasive.

Molecular targeted therapies have been developed based on BC molecular subtype. Tamoxifen (TAM) is the most widely used selective ER modulator (SERM) currently used in the clinic. TAM is both an antagonist and agonist of the ER based on the tissue [46, 47]. In addition to inhibition of ER signalling by TAM, aromatase inhibitors (AIs) are also used to prevent conversion of endogenous androgens to estrogen, which further reduces estrogen signalling [48]. TAM and AIs are clinically relevant for ER+ BC, however, ineffective against HER2+ and TNBC subtypes.

The first line of therapy for HER2+ subtype entails treatment with Trastuzumab and Pertuzumab, which are both monoclonal antibodies against the HER2 receptor [49]. Trastuzumab blocks homodimerization [50] of HER2, whereas Pertuzumab blocks heterodimerization of the HER2 receptor [51]. If the first line treatment fails, the second line of intervention consists of tyrosine kinase inhibitors, such as Lapatinib. By binding to the ATP-binding pocket of HER2 and epidermal growth factor receptor (EGFR), these agents block phosphorylation, subsequently inhibiting the initiation of downstream signalling cascades [52].

TNBC is defined by lack of expression of the ER, PR and HER2 [37], consequently resulting in irresponsiveness to endocrine therapy. Therefore, there is currently no established molecular targeted therapies for TNBC, however, potential targets that are currently being studied include the phosphatidylinositide 3-kinases (PIK3) and EGFR [37, 53, 54]. Paradoxically, patients with TNBC are more sensitive to chemotherapy compared to other BC subtypes [37]. Therefore, usual treatment for TNBC

includes a combination of chemotherapy, radiation therapy and surgery [37]. The molecular profile of BC is mainly defined by the expression of ER, PR and HER2 (Table 1), however, our studies focused on characterization of the ER-α in EVs.

Table 1: Breast cancer subtypes and therapies

The summary of BC molecular subtypes, therapeutic approaches commonly used for each subtype and prevalence of each subtype expressed as a percent-value [37].

Subtype	Molecular markers	First line therapy	Second line therapy	Approximate occurence (%)
DCIS	ER±, HER2 ±	Surgery, radiation therapy, chemoteherapy	Adjuvant therapy such as Tamoxifen (ER+) or Trastuzumab (HER2+)	20
Luminal A	ERhigh, PR+, HER2-, low Ki67	Tamoxifen	Aromatase inhibitors	40
Liminal B	ERlow, PR+, HER2±, high Ki67	Tamoxifen	Aromatase inhibitors	20
HER2+	±ER+/HER2+	Trastuzumab, pertuzumab	Lapatinib	10
TNBC	ER-,PR-,HER2-	Molecular targeted therapies depending on subtype in combination with chemotherapy, radiation therapy, surgery	Chemotherapy, surgery, radiation therapy	10-20

1.3 Estrogen Receptor

The ER-α is one of the chief predictors of patient outcome and response to therapy. The ER-α is ligand-dependent transcription factor that belongs to the nuclear receptor superfamily of receptors [55, 56]. In the absence of a ligand, ER-α is sequestered to the cytoplasm within a large inhibitory heat shock protein (HSP) complex [56]. Upon binding of agonists, ER-α undergoes conformational changes, phosphorylation and dimerization, which allows the receptor to translocate to the nucleus where it modulates gene transcription [55, 56]. One such agonist is estrogen, which either up- or down-regulate the expression of their target genes *via* interaction of ER with the estrogen response element (ERE) (Figure 2) [55, 56]. Similar to other nuclear hormone receptor family members, ER-α is composed of three major functional domains. These include the N-terminus domain (NTD), the Deoxyribonucleic acid (DNA)-binding domain (DBD), and the ligand binding domain (LBD). The full transcriptional activity of ER is mediated by two activation function (AF) domains [56, 57]. The hormone-independent AF-1 is located in the NTD, and the ligand-dependent AF-2 is located in the LBD [56, 58]. The *hinge* region contains the nuclear localization signal (NLS), which serves as a region connecting the NTD and LBD [56]. The *F* region is located near the C-terminal end of the LBD and has the capacity to modulate gene transcription [56] (Figure 3).

It was believed there was only one ER, the ER-α. However, in the mid-1990s a novel ER was discovered and termed as ER-β [56, 59]. Thus, there are two independent ER receptors encoded by two independent genes, the ER-α and ER-β, encoded by *ESR1*

and *ESR2*, respectively [56]. Given that there are two distinct ERs, they can either form a homodimer or a heterodimer upon activation [60]. The ER-α and ER-β have high sequence homology in the LBD and DBD, whereas the NTD is most variable [56]. Although, the role of the ER-β isoform in the development and progression of BC is unclear, it is well established that ER-α plays a central role in this disease.

In addition to the differences mentioned above, the ER-α LBD structure consists of twelve helices, which comprise the hormone binding pocket [58]. Despite high sequence homology, research shows that ER-α and ER-β emphasize the use of different molecular forces to secure ligand binding and have different ligand binding specificity [61]. ER-α binding pocket has a very hydrophobic environment, whereas hydrogen bonding is more important for the ER-β [58]. Upon ligand binding the four helices that make up LBD rearrange and form a hydrophobic cleft with binding sites for AF-2-interacting coactivators or corepressors [58]. These include CREB-biding protein (CBP), transcriptional intermediary factor-2 (TIF2) and receptor associated activators that belong to p160 family of AF-2 coactivators, nuclear receptor corepressor (NCoR) and many others [62]. Once activated, the ER-β induces the expression of the similar genes as those regulated by the ER-α, but to a less efficient extent [56]. In addition, ER-β can also act as a transcriptional repressor, in cells that express both isoforms, by decreasing sensitivity to estrogen [56, 63]. Although, both isoforms have the same ligand and similar homology, slight differences in the ligand binding domain influence transcriptional efficiency of the target genes activated by the ERs.

ERs are found in many tissues throughout the body, including the brain, the bone, the uterine, the ovarian, the heart, the liver, prostate and the breast tissues [56, 57]. Estrogen signalling plays a central role in the normal development of female physiology, however, ER-α is also implicated in the development and progression of BC based on both clinical and animal studies [64-66]. It has been established that prolonged exposure to endogenous or exogenous estrogens can results in the development of BC [8, 67]. Furthermore, 70% of BC is initially classified as ER-positive [68]. Currently there are two hypotheses proposed to explain the mechanism by which estrogen exposure can promote the development of BC [69, 70]. The first hypothesis states that the binding of estrogen to ERs increases cellular proliferation, thereby increasing the chance of DNA mutation [69]. The second states that byproducts of estrogen metabolism result in DNA damage, resulting in point mutations [70]. Evidence exists to support both hypotheses, however, it is not clear whether these events occur exclusively or are complimentary fashion.

Due to a high prevalence of ER-positive BC cases, SERMs is one of the most frequently used forms of molecular targeted therapies [47]. However, 30-40% of BC patients develop endocrine resistance to SERMs [68]. Several mechanisms have been proposed to contribute to the development of this resistant phenotype. Acquired resistance is thought to occur as a result of loss of ER-α expression or mutation of ER-α, however, only about 20% of patients actually show loss of the receptor [71] and <1% of ER-α positive tumours have receptor mutations [68, 72, 73]. In addition, its been proposed that

the phosphorylation of serine and tyrosine residues in ER-α through induction of growth factor signalling, results in ligand-independent activation of the receptor [73]. Furthermore, increased sensitivity of the ER to lower levels of circulating estradiol is another possible mechanism [74]. More recently, it has been shown that some Caucasian women have an inactive allele for cytochrome P450 2D6 (CYP2D6), which is required to convert TAM to its active form, endoxifen, consequently reducing tamoxifen responsiveness [75]. Also, the presence of a truncated variant of ER-α, ERα-36, has been shown to play a role in the response to therapy [76]. An abundance of evidence supports the notion that multiple mechanisms contribute to the development of aggressive resistant phenotypes, however, currently there is no consensus that one mechanism is the main driver of this process [68, 73]. Furthermore, current understanding of the intricate communication between the molecular pathways contributing to the development and the progression of primary tumour to metastatic disease is limited.

Although there are many well established markers for BC, there are many promising prognostic and therapeutic targets that are currently being investigated worldwide [14]. Current research in the field of biomarker discovery aims to determine non-invasive markers of disease state and response to therapy. Our studies have focused on characterization of molecular targets involved in the regulation of tumour-associated thrombolitic/fibrinolytic events as well as extracellular matrix (ECM) remodelling. Our studies focus on the characterization of TF, PAI-1ADAM12 and β-Catenin in BC patient plasma derived EVs.

Figure 2. Canonical Estrogen Signalling

Upon estrogen binding, ER-α undergoes conformational changes resulting in phosphorylation and dimerization. ER-α then mediates estrogen effects by modulating the expression of target genes *via* estrogen response element (ERE) [55, 56].

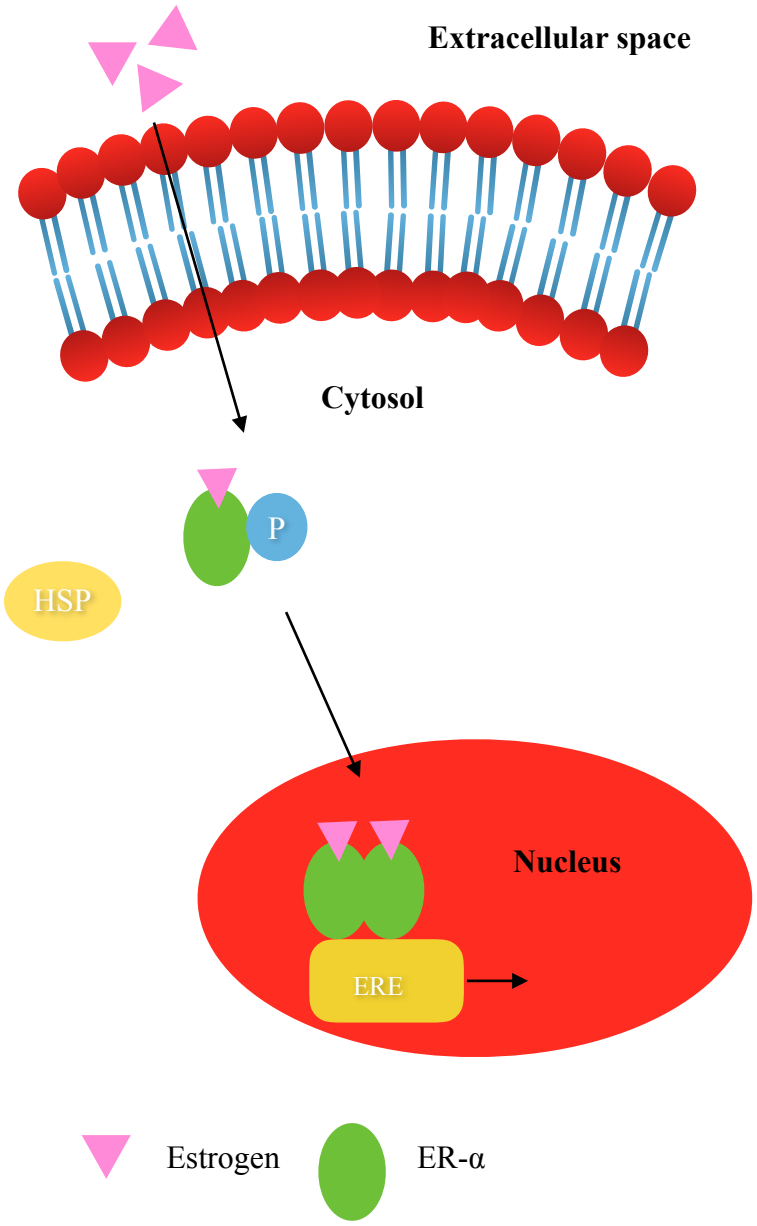
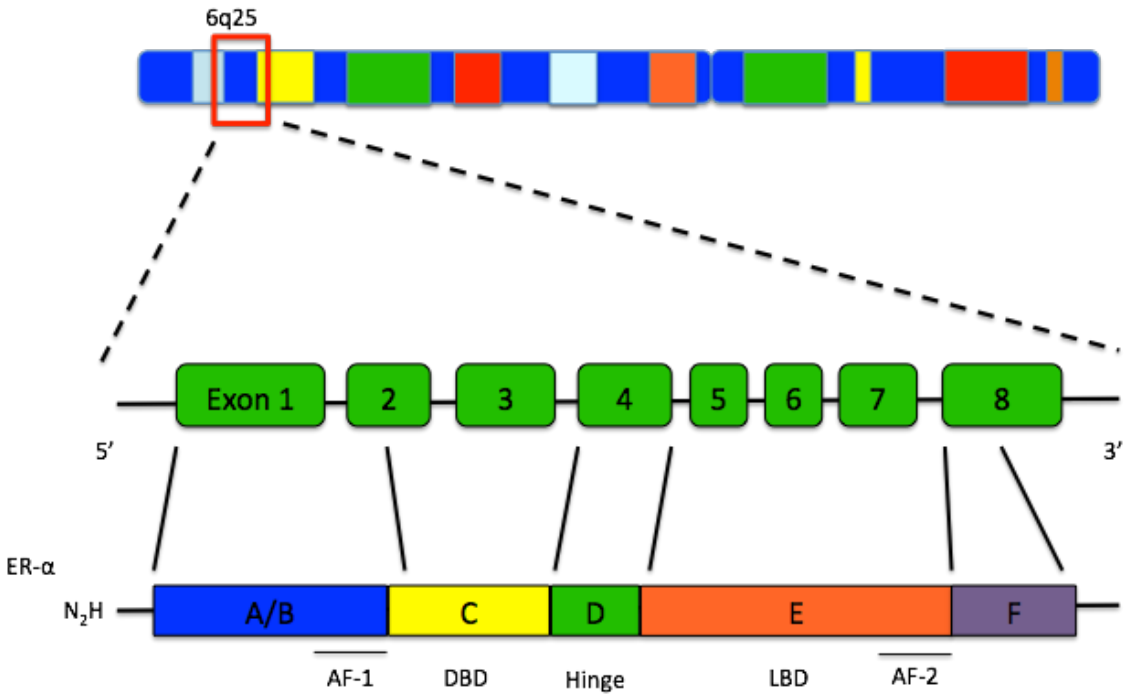

Extracellular space
Cytosol
P
HSP
Nucleus
ERE
Estrogen
ER-α

Figure 3. Structure of the ER-α

The ER-α gene is located on chromosome six. The coding sequence of the ER-α consists of eight exons. Given that full-length ER-α contains a DNA-binding domain (DBD), ligand binding domain (LBD), N-terminal domain (NTD), it is similar in structure to other nuclear receptors. In addition, ER-α also has two activation function (AF) regions, the hinge region, which contains the NLS and the "F"-region that modulates transcription [55,56].


6q25
Exon 1
2
3
4
5
6
7
8
5'
3'
ER-α
N2H
A/B
C
D
E
F
AF-1
DBD
Hinge
LBD
AF-2

1.4 Tissue Factor

The TF belongs to the cytokine receptor protein superfamily and plays a crucial role in hemostasis and clot formation [77]. Similar to other cytokine receptors, TF consists of three domains: the extracellular domain, the single-pass transmembrane domain and the short cytosolic domain [77, 78]. The N-terminal extracellular domain has a LBD composed of two fibronectin type III domains and makes up a large proportion of the receptor [79]. TF initiates clot formation upon tissue injury, and therefore it belongs to the extrinsic coagulation cascade [80, 81]. Upon activation factor VIIa binds to TF, initiating coagulation by recruiting and subsequently activating factor X, which consequently results in thrombin generation [81].

TF factor is expressed throughout the body in both healthy and malignant tissues, including the vascular adventitia cells, organ capsules, smooth muscle cells, epithelial and tumour cells [82]. In addition to regulating hemostasis in healthy individual, TF expression has been correlated to invasion and metastasis of many types of malignancies [83-89]. Therefore, the role of TF in malignant disease is an exciting new area in cancer research.

TF signalling is emerging as one of the key players involved in gene regulation and protein synthesis in cancer [88, 89]. Dysregulation in TF expression and signalling has been observed in a variety of human tumours including prostate cancer [90], pancreatic cancer (PC) [91], lung cancer [92], colorectal cancer [93, 94], glioma [95, 96] and BC [97]. In healthy tissue, TF expression is limited to the subendothelium, and is only

exposed to blood after vascular injury, however, with tumourigenesis the regulation of TF expression is lost [82, 98].

Several factors have been associated with aberrant expression of TF in malignant disease. In glioma, hypoxia and loss of the tumour suppressor gene phosphate and tension homolog (PTEN) results in induction of TF [99]. In colorectal cancer, activation of K-ras oncogene and loss of the tumour-supressor p53 regulate TF expression [100]. In addition, it has been shown that TF expression is regulated by mitogen activated protein kinase (MAPK) and PIK-3 signalling in colorectal cancer [100]. Leading to conclusion that TF expression is regulated by intricate molecular pathway(s) involved in cancer invasion and metastasis.

Hypercoagulability that is observed in many cancer patients leads to increased risk of venous thromboembolism (VTE), pulmonary embolism and hemorrhage [101]. Aberrant TF signalling has been associated with the development VTE in ovarian [102] and pancreatic [103] cancers. Furthermore, this hypercoagulable state can contribute to tumourigenesis by supporting angiogenesis [88, 104], cell migration [105, 106] and metastatic potential [107, 108]. Therefore, TF expression and protein level may be a novel reliable diagnostic and prognostic tool for malignant disease.

1.5 Plasminogen Activator Inhibitor-1

PAI-1 is a member of the serine proteinase inhibitor (SERPIN) super-family of glycoproteins, and similar to TF is essential for hemostatic regulation [109]. PAI-1 is

primarily responsible for the inhibition of dissociation of fibrin clots *via* inhibition of both the urokinase and the tissue type plasminogen activators (uPA and tPA) [109]. The tPA is mainly produced and stored in the endothelial cells [110], whereas the uPA is produced by a variety of other cell types including monocytes, T-cells, endothelial cells, epithelial cells, fibroblast, osteoblasts, smooth and striated muscle cells [111]. Both plasminogen activators cleave inactive plasminogen to generate plasmin, which then can dissociate fibrin clots [109]. In a healthy individual, PAI-1 is primarily expressed in megakaryocytes, smooth muscle cells and adipocytes [112-114]. Induction of PAI-1 expression is observed in many cell types with stress or injury [115, 116]. Furthermore, increased PAI-1 expression is indicative of poor outcome in many malignant diseases, including BC [117, 118].

Human PAI-1 is a single chain glycoprotein composed of 402 amino acids with molecular weight of 45 kDa [109]. PAI-1 is structurally similar to other SERPINs and can exist in different conformational forms. In the active form the reactive centre loop (RCL) of the molecule containing the *bait sequence* is exposed [111]. Upon reaction with the proteinase the RCL is cleaved and integrated into the β-sheet [111]. PAI-1 can also exist in latent conformation, where the RCL is integrated into the β-sheet, thus preventing inhibitory activity [119]. However, PAI-1 can spontaneously convert to latent form, and for this reason, PAI-1 has short functional half-life of one to two hours [111].

Systemic PAI-1 has a dual role in cellular processes - regulation of thrombotic/ fibrinolytic events and cellular adhesion, both of which are involved in tumourigenesis

[109]. It has been reported that PAI-1 is involved in regulation of tumour growth, angiogenesis, cell migration and ECM remodelling [109]. Clinical studies show that elevated levels of tumour tissue derived PAI-1 are associated with poor prognosis in many cancers, including lung [120], gastric [121], ovarian [122] and BC [123]. A correlation between PAI-1 expression and lymph node involvement, tumour size, and cell differentiation has been reported in BC [14, 124]. Furthermore, PAI-1 together with uPA is a clinically relevant marker for patient outcome in node-negative BC [125, 126].

Several factors have been associated with elevated PAI-1 expression in healthy and malignant cells. *In vitro* findings have shown a transforming growth factor (TGF)-β1-dependant elevation in PAI-1 expression in ovarian cancer and healthy fibroblast cells [127, 128]. Furthermore, it has been reported that MAPK activation is essential for TGF-β1mediated induction of PAI-1 expression in rat mesangial cells [129]. Lastly, it has been established that PAI-1 expression is regulated by fibroblast growth factor (FGF) [130].These findings support the notion that, similar to TF, PAI-1 expression is under tight regulation by many growth factors and that any alteration of growth factor signalling may also leads to aberrant PAI-1 expression.

1.6 A Disintegrin and Metalloprotienase-12

ADAM12 belongs to a large family of zinc-dependent transmembrane metalloportienases responsible for the regulation of ECM protein turnover [131]. Alternative splicing allows ADAM12 to exist in two isoforms: a membrane bound form

(ADAM12-L) and a secreted form (ADAM12-S). The extracellular region of both ADAM12 variants is composed of pro-metalloprotease, disintegrin-like, cysteine-rich, and epidermal growth factor (EGF)-like domains. The soluble variant of ADAM12, ADAM12-S, contains the same domains as ADAM12-L, with the exception of the cytoplasm and transmembrane domains [132]. ADAM12 is encoded by the *ADAM12* gene located on chromosome ten [133]. The membrane bound and secreted form of ADAM12 are made of 881 and 718 amino acids, respectively [134]. ADAM12 is expressed in a variety of metabolically active tissues throughout the body, such as cartilage [135], bone [135], muscle [136], liver [137], uterine [138] and brain [139] tissues.

In addition to regulating growth and repair in metabolically active tissues, ADAM12 has been implicated in the development and progression of many diseases, including cardiac hypertrophy [140], asthma [141], bladder cancer [142], lung adenocarcinoma [143], brain tumours [144] and BC [145]. Such implications are based on reports that identify a positive correlation between BC stage and urine ADAM12-S [145]. Mechanistically, it has been shown that in an estrogen-independent manner, ADAM12 increases the rate of proliferation of BC cells [146]. Studies with ADAM12 knockout PyMT mice have demonstrated that the development of tumours occurs at a slower rate compared to controls [142]. Furthermore, it has been reported that ADAM12 induces HER2 expression in human head and neck cancer cells [147].

Interestingly, evidence also shows that HER2 signalling elevates ADAM12 expression in skin cancer cells, suggesting the existence of positive feedback loop between these two proteins [147]. To lend aid to this theory, it has also been shown that direct inhibition of HER2 blocks ADAM12 transcription; in contrast, ADAM12 expression was rescued in HER2 overexpressison models [147]. Due to the positive correlation, between HER2 and ADAM12, its has been suggested that the well-established induction of HER2 in BC contributes to the induction of ADAM12 [132].

1.7 β-Catenin

β-Catenin is an evolutionary conserved protein that was first identified in *Drosophila* [148]. It is encoded by the *CTNNB1* gene and is composed of thirteen repeats, known as the armadillo repeats. Each of these repeats contains 42 amino acids arranged in an alpha helical structure [149]. β-Catenin is closely related to the cadherin proteins and plays a pivotal role cell adhesion mediated by the canonical Wnt signalling cascade [148]. In healthy tissues, inactive β-Catenin is located near the plasma membrane (PM) in association with the cytoskeleton [150]. Upon activation however, β-Catenin translocates to the nucleus where it stimulates the transcription of Wnt target genes, such as PAI-1 [151]. In addition to regulation of adhesion in healthy tissue, β-Catenin signalling is associated with fibrosis and cancer [151, 152]. Therefore, in a similar manner to TF, PAI-1 and ADAM12, the dysregulation of β-Catenin is associated with poor patient outcome.

Loss of β-Catenin expression has been observed in many primary tumoursin early stages of cancer. However, β-Catenin expression becomes incrementally rescued as cancer disease state progresses [153]. Although paradoxical, it has been shown that a β-Catenin-mediated reduction of cell adhesion contributes to metastasis during early stages of cancer [154]. In a complementary manner, the rise in expression found in later stages of disease have been shown to aid in the attachment of metastatic cancer cells to the periphery [153].

Several mechanisms have been shown to contribute to the dysregulation of β-Catenin expression in malignant cells. *In vitro* evidence has demonstrated that overexpression of miRNA-1229 in BC is associated with increased activation of Wnt/β-Catenin signalling [155]. Furthermore, a coactivator protein JRK has been shown to regulate β-Catenin transcriptional in colon cancer, ovarian cancer and BC [156]. Therefore, similar to TF, PAI-1 and ADAM12, β-Catenin signalling is subject of a highly regulated and intricate molecular mechanism(s).

1.7 Extracellular Vesicles

1.7.1 The Discovery of Extracellular Vesicles

Early studies have shown that mammalian cells shed *membrane-enclosed* vesicles, also known as EVs [157-161]. Initially it was assumed that vesicles were only released by the outward budding of PM. Recent studies however, have led to the discovery of a complex intracellular vesicle biogenesis system, known as the endocytic pathway. This

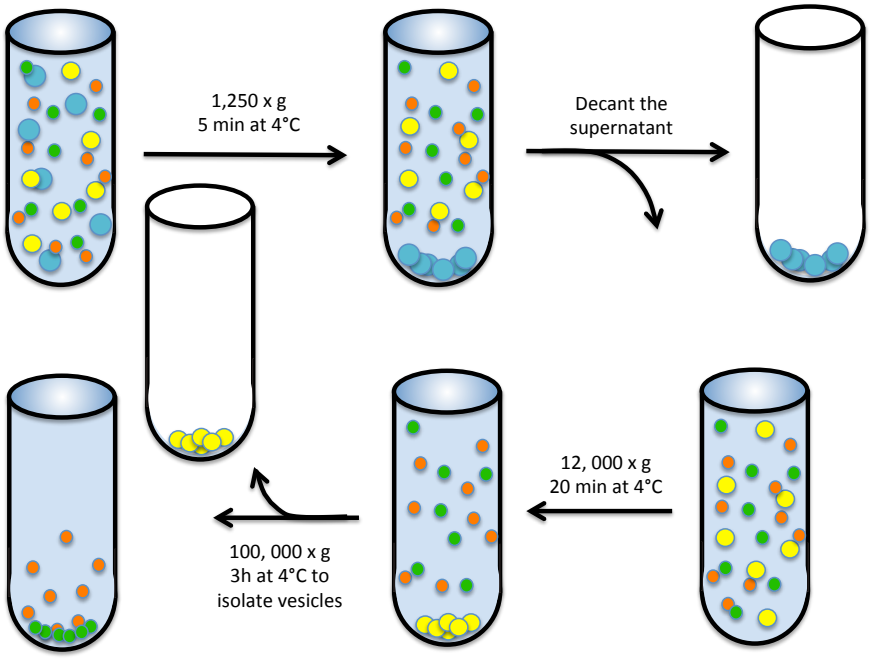
vesicle biogenesis pathway was first documented while studying the necessary loss of transferrin receptor (TfR), which naturally occurs during erythrocyte maturation. It was then observed that this loss of TfR, occurs due to increased shedding of TfR-containing vesicles. Using transferrin bound gold particle [162] or anti-TfR antibodies [163], two independent studies utilized electron microscopy (EM) to establish this process. In a consistent manner, both groups concluded that prior to shedding, the TfR is endocytosed and packaged into intracellular vesicles prior to fusion and secretion from the PM [162, 163]. These seminal studies led to the conclusion that in addition to the established PM shedding mechanism, EVs are also secreted in a manner dependent on the endocytic pathway.

1.7.2 Extracellular Vesicle Isolation Method

Currently the most widely used EV isolation method is known as the differential centrifugation [164]. In brief, the initial slow speed centrifugations remove apoptotic bodies and cell debris. The EV analyte is then collected following final ultracentrifugation at 100,000 x g (Figure 4) [165, 166]. However, ultracentrifugation only allow for the enrichment of a heterogeneous population of exosomes and microvesicles (MVs) [167, 168]. In recent years there have been a number of improvements in commercial polymer/ immunocapture based EV isolation methods, which distinguish exosomes from MVs [166-168]; these methods, however, they are not widely used [164, 168].

Figure 4. EV Isolation from Cultured Cells.

Immediately following collection, conditioned culture medium is subjected to centrifugation at 1,250 x g for five minutes at 4 °C to eliminate large cellular debris. The supernatant is then collected and subjected to a second centrifugation at 12,000 x g for 20 minutes at 4 °C to further eliminate smaller cellular debris and apoptotic bodies. The remaining supernatant is then centrifuged at 100,000 x g for three hours at 4 °C. The resulting heterogeneous population of exosomes and microvesicels are isolated following this final centrifugation.


1,250 x g
5 min at 4°C
Decant the
supernatant
12, 000 x g
20 min at 4°C
100, 000 x g
3h at 4°C to
isolate vesicles

1.7.3 The Size and Morphology of Extracellular Vesicles'

Further studies in the field allowed for the characterization of EVs. Early EM analysis of whole vesicles revealed a cup-shaped appearance, however, this morphology was later attributed to a fixation method [168]. More recently, however, cryo-EM studies revealed that EVs have a round shape [169]. EVs are derived from the endosomal compartment and from the shedding of PM, and are subsequently known as exosomes and MVs, respectively. Exosomes range from 40-150 nm in diameter [166] , whereas MVs are much larger, typically reaching sizes of ~1000 nm in diameter [170]. In addition to the differences in size and morphological features, EVs also vary in composition [168]. It is well established that EV composition reflects that of the parent cell [17, 171, 172].

1.7.4 The Composition of Extracellular Vesicles

Given the widespread use of the differential centrifugation technique, the exact composition of each subtype of EV is currently unknown [168]. Many studies have revealed that EV, including exosomes and MVs, contain a variety of proteins, lipids, and nucleic acids.

The early studies of EV composition mainly focused on proteomic content using target specific techniques, such as the western blot (WB) [163, 173]. Further advancement in scientific methods allowed for the use of MS-based proteomic analysis [174, 175], which allowed for the simultaneous identification of vast numbers number of targets. Using this method, the cellular machinery required for EV biogenesis and secretion were

quickly identified [168, 176, 177]. Furthermore, this methods also led to the identification of EV subtype-specific protein markers [167, 178]. Such markers include the endosomal sorting complex (ESCRT) [179] and Flotillin-1 [180], which contribute to EV biogenesis/ secretion, and belong to the exosome and MV subtypes, respectively.

Similar to the cell membrane, EVs are enclosed in a lipid bilayer and thus, increasing numbers of studies aim to examine EV lipid bilayer composition. These studies have successfully identified the presence of variety of lipids, including sphingomyelin, phosphatidylserine (PS), cholesterol and ceramide in the lipid bilayer of EVs [173, 181-185]. Amongst these findings, it was observed that EVs contain an elevated abundance of PS and cholesterol, the two major constituents of lipid rafts, relative to other lipid subtypes. For this reason, it was concluded that lipid rafts play a major role in the secretion of EVs [184, 186-188].

In addition to the characterization of EV protein and lipid profiles, several groups have identified genetic material, such as mRNAs and miRNAs, as a part of EV cargo [189, 190]. Furthermore, studies shown that this functional genetic material can be transferred from EV to distant cells, thereby affecting gene expression of recipient cells [190-192]. In addition, recent evidence has demonstrated that certain mRNA species are preferentially secreted *via* EVs [193]. These findings support the notion, that in a similar manner to vesicular protein and lipid, the trafficking of genetic material *via* EVs is a tightly regulated process; and therefore not a random occurrence.

Several studies show that the composition of EVs changes based on culture conditions [194] in a manner dependent of the effect of these conditions on the donor cell. For instance, inflammatory signalling has a great affect on the protein and RNA composition of EVs derived from dendritic and mesenchymal stem cells [195, 196]. Similar findings were observed in EVs isolated from epithelial and tumour cells cultured in hypoxic conditions [197, 198]. Furthermore, it has also been shown that the lipid composition of EVs changes based on culture conditions [199]. Collectively, these findings suggest that environmental changes, which affect donor cell homeostasis, in turn, change the composition of the resulting EVs.

1.7.5 The Biogenesis and Secretion of Exosomes

As mentioned above, exosomes are a subgroup of EVs with origins stemming from the endocytic pathway. The biogenesis and secretion of this type of EV can be grossly categorized into three major events, including (a) the formation of an early endosome, (b) maturation of the early endosome into a multi vesicular body (MVB), and lastly (c) exosome secretion [168, 200]. The formation of the early endosome begins with cargo assembly at clathrin-coated sites on the PM followed by the inward budding of the PM [201]. The early endosome then matures to form the late endosome or a MVB *via* accumulation of intraluminal vesicles (ILVs), the cargo of which reflects cytosolic content and PM composition of the donor cell [168, 202, 203]. Several mechanisms aiming to explain MVB biogenesis have been proposed. However, given that the role of ESCRT

(Figure 5), which plays a major role in EV biogenesis, is not well established, several of the proposed mechanisms are in a state of contradiction.

The ESCRT machinery drives the most well characterized mechanism for MVB and ILV formation. The ESCRT family is composed of thirty proteins that are assembled into five complexes, including ESCRT-0, ESCTR-I, ESCRT-II, ESCRT-III and VPS4 [168, 204]. The assembly of these complexes occurs in a sequential manner, such that ESCRT-0 recognizes and assembles monoubiquitinated proteins in the early endosome membrane. Following this, a component of the ESCRT-0 complex then recruits tumour susceptibility gene 101 (TSG101), which is a part of ESCRT-I complex. ESCRT-I then recruits ESCRT-III via ESCRT-II or ALG-2-interacting protein X (ALIX) [168, 205].

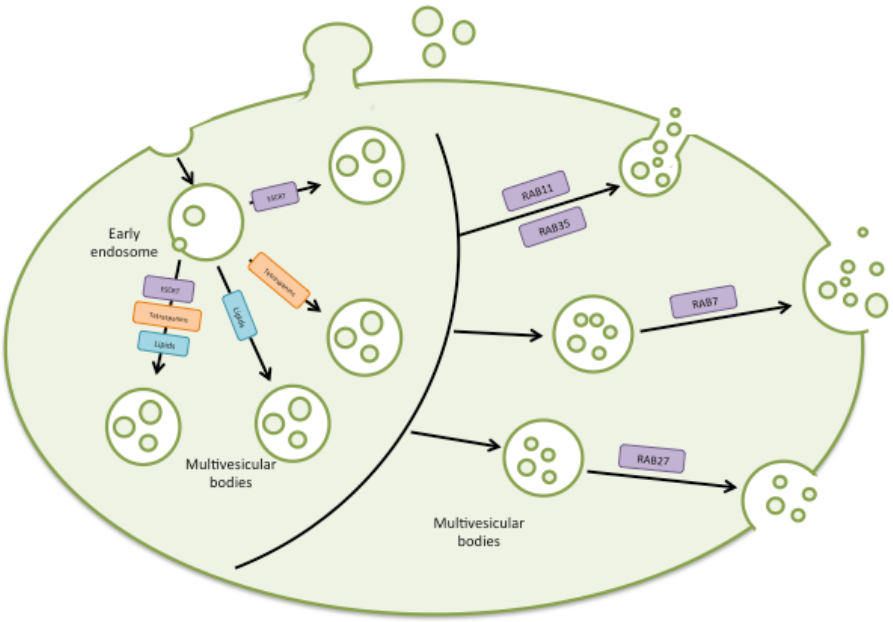
In contrast to the mechanism discussed above, several studies show that MVB formation can occur in a manner independent of the ESCRT. An alternative mechanism involving tetraspanins, such as CD63, has been shown to drive the formation of ILVs independent of ESCRT signalling [206]. Furthermore, two lipids, ceramide and phosphatic acid have been shown to initiate the inward budding of the early endosome membrane to form ILVs [183, 207]. Therefore, current evidence suggests that MVBs and ILVs can be formed in an ESCRT-dependent or -independent manner.

Intracellular vesicle trafficking between organelles is regulated by the Rab GTPases [208]. Evidence also shows that the Rab family is highly involved in MVB trafficking and secretion (Figure 5). Several Rab proteins have been shown to be involved in EV secretions, including Rab11, Rab27 and Rab35 [209-212]. Current research

suggests that Rab11 and Rab35 are associated with the early endosome, whereas Rab27 is associated with late endosome trafficking [208]. Lastly, followed by vesicular trafficking, membrane fusion allows for secretion of MVB contents into extracellular space. The soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors (SNAREs) mediate membrane fusion between two organelles [213]. The SNARE protein complexes have been shown to be involved in Ca^{2+}-dependent membrane fusion with PM in epithelial cells and neutrophils [214, 215]. Furthermore, studies show that SNARE-associated proteins are involved in the fusion of MVB with PM and consequently EV secretion PM [214, 216, 217].

Figure 5. Schematic Representation of EV Biogenesis and Secretion.

ESCRT-dependent and -independent mechanisms are associated with the formation of MVBs and molecular machinery involved in EV secretion [168].


Early
endosome
ESCRT
Tetraspanins
Lipids
Multivesicular
bodies
RAB11
RAB35
RAB7
RAB27
Multivesicular
bodies

1.7.6 The Biogenesis and Secretion of Microvesicles

The other subtype of EV, MV, are formed through the direct outward budding of the plasma membrane. Similar to exosomes, MV are characterized by diverse lipid and protein cargo composition. These vesicles have been shown to contain PS, Flotillin-1, and a variety of adhesion molecules [170]. The exact process of MV formation is unclear, however, a number of plausible mechanisms have been proposed. The majority of MVs are thought to be formed in PM regions rich in cholesterol and sphingolipids, known as lipid rafts. MV formation occurs at sites in which PS relocation to the outer leaflet of the PM is accompanied by the action of flippases, floppasees and scramblases, which in turn results in membrane asymmetry [170, 218]. The ATP-dependent induction of lipid asymmetry results in membrane curvature, and is believed to cause the formation of MVs [219, 220]. In addition to this mechanism, several other molecular mechanisms have been proposed to contribute to MV biogenesis and secretion (Figure 5). A change in polarity of actin cytoskeleton, as a result of ADP-ribosylation factor (ARF) overexpression, has been shown to be involved in formation of MVs [221]. Overall, in a manner similar to exosomes, there exist several conflicting lines of evidence supporting the mechanism by which MV are formed and secreted and therefore, additional research is required in this field. However, there exists a consensus among these studies, in that the mechanism of microvesicle biogenesis and secretion is highly regulated.

1.7.7 Extracellular Vesicles in Health and Disease

EVs are produced by a variety of healthy and malignant cells. Consequently research has shown that EV secretion and trafficking is involved in healthy and malignant processes [17, 163, 173, 222]. In cancer, EVs have been largely described as promoters of tumour progression and invasion [18]. However, due to the complexities of EVs, there also exists conflicting findings in this respect; early studies have shown EVs to have an anti-tumor effect in a manner dependent on immune response, however, it is also established that EVs can aid in immune evasion [223]. Cancer-cell extracellular vesicles are also associated with the stimulation of tumour angiogenesis and extracellular matrix remodelling [224, 225]. In addition, cancer-cell vesicles have been shown to have protumorigenic, proinvasive, and prometastatic effects, thereby further contributing to malignant cancer phenotypes. In recent years, research has focused on investigating EVs as a potential source of prognostic markers [19, 20].

Overall, given that EVs stem from donor cells, EV cargo can be representative of the tumorigenic tissue from which it was shed [21]. Its been shown in cancers such as pancreatic [19] and BC [20] that the profile of protein cargo in circulating EVs can be used as markers to determine organ specific metastasis.

OBJECTIVES

The main objectives of this study are to assess the content of EVs from BC cells for the presence of established BC biomarkers, as well as investigate potential novel markers of disease state in EVs from BC patients. The specific objectives of the study are as follows:

I. To examine the status of ER-α in extracellular vesicles from BC cells.

There is evidence indicating that EVs carry a variety of molecules, including intact receptors, as a part of their cargo [17, 168, 226]. Previous work in our laboratory has identified the androgen receptor (AR), as a part of EV cargo in PC cells (Submitted to European Journal of Cancer). The goal of this study is to investigate whether ER-α is also secreted through EVs of BC cells.

II. To assess the potential of circulating EVs in providing novel biomarkers for BC detection and prognosis.

Currently, the most common staging system for BC is the TNM system. The TNM system takes into account the tumour size, presence of cancer cells in the lymph nodes, and presence of metastasis [227-229]. In addition, the histological identification of tumour tissues biopsies is crucial for the prediction of disease behaviour and patient outcome [29]. However, it has been demonstrated that biopsies cause dislocation of malignant cells along the needle track [16, 230, 231]. This dislocation could consequently

result in dissemination of tumour cells into circulation, thereby increasing the likeliness of metastasis. For this reason, non-invasive prognostic and diagnostic techniques are urgently required to avoid potential dissemination of tumour-initiating cells. Based on our findings, regarding the AR, as well as those of others, cancer cell-derived EVs have the potential to become a powerful prognostic and diagnostic biomarker tool. Our goal is to investigate whether the proteomic profile of EVs derived from BC patient plasma reflects the disease state.

III. Validation of specific targets identified by the MS proteomic study.

Studies have shown that plasma samples of patients with advanced lung cancer, ovarian cancer, and colorectal and melanoma cancer have a higher level of EVs compared to healthy subjects [18, 232-234]. Recently, it has been shown that Glypican-1, a vesicle associated protein, can distinguish with hundred percent certainty between precancerous lesions and benign tumors in pancreatic cancer patients. This non-invasive test also has the ability to identify patients with late-stage pancreatic tumors, thereby limiting the utilization of invasive procedures [21]. Based on the outcome of the proteomic study, we are going to be focusing on identifying and validating targets that have previously been associated with cancer prognosis. For the purpose of the study, we are going to focus on protein targets that are either induced or lost in EVs from different stages from BC patients.

HYPOTHESIS

It has been reported that EVs are a source of biomarkers for the diagnosis of pancreatic cancer [21]. However, the use of this technique in the prognosis and diagnosis of BC has not yet been examined. *We hypothesize that the use of a MS-based proteomic analysis of EVs from BC patients will provide us with a rich source of biomarkers that accurately reflect disease state.*

In addition, it is well established that ER-a is critical for disease progression and that the receptor expression is a valuable prognostic biomarker [76, 235-240]. We have also shown previously that nuclear receptors, such as AR, are found within the cargo of EVs secreted from PC cells (Submitted to European Journal of Cancer). *Based on these findings, we also hypothesize that EVs derived from PC cells contain ER-α as a part of their cargo.*

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell Culture

MCF7 is a mammary epithelial cell line derived from human invasive ductal carcinoma, was purchased from the American type cell cultures (ATCC) [241]. T47-D, MDA-MB-453, and HCC 1937 cells were a generous gift from Dr. Tang's lab, at the Hamilton Kidney Research Centre (HKRC), McMaster University. T47-D and MDA-MB-453 cells have been derived from metastatic pleural effusion site [242, 243] whereas

HCC-1937 cells have been derived from ductal carcinoma [244]. The MCF7 and T47-D cells were chosen because they are known to express ER-α, as where MDA-MB-453 and HCC-1937 were chosen for lack of ER-α expression. All cells were cultured in medium listed in Table 2. For the purpose of extracellular vesicle collection, all cell lines were first cultured in complete medium: 10% fetal bovine serum (FBS) (Life Technologies) + 1% Penicillin Streptomycin (PS) (Life Technologies) until 75-80% confluence and then cultured in medium free of FBS and PS for 24 hours .

Table 2: Medium used for cell culture

All cells were cultured in medium purchased from the ATCC according to manufacturer's recommendations using appropriate aseptic techniques.

Cell Line	Medium used	Catalogue number
MCF-7	EMEM	ATCC 30-2003
HCC-937	RPMI-1640	ATCC 30-2001
MDA-MB-453	L-15	ATCC 30-2008
T47D	RPMI-1640	ATCC 30-2008

2.2 Collection of Extracellular Vesicles from Conditioned Medium

EVs from plain medium were collected as previously described [17, 164]. Briefly, all cell lines were first cultured in complete medium until 75-80% confluence. Cells were then washed with 10 mL of sterile phosphate buffered saline (PBS) (Life Technologies), and 10 mL of appropriate medium without FBS or PS was added for 24 hours. The medium was then collected and subjected to three consecutive centrifugations (Figure 4). Immediately after the collection, culture medium was centrifuged at 1250 x g for five minutes at 4°C to eliminate large cellular debris (Labnet, Hermle z400k). The supernatant was collected and transferred into a fresh tube without disrupting the pellet and subjected to second centrifugation at 12,000 x g for twenty minutes at 4°C to eliminate the remaining cellular debris (Thermo Scientific, Sorval RC 6PLUS). The supernatant collected after second centrifugation (12,000 x g) was then subjected to a final centrifugation at 100,000 x g for three hours at 4°C (Beckman Culter, Optima L-90K Ultracentrifuge). The EV pellet was then washed with a 200 µl of PBS. Vesicle protein content was then measured via modified Lawry protein assay (Bio-Rad).

2.3 Collection of Extracellular Vesicles from Patient Plasma

Plasma vesicles were purified by differential centrifugation as described above with minor modifications. Briefly, all plasma vesicle isolations were carried out using 1 mL of plasma. Serum was initially diluted in PBS at 1:1 ratio to reduce viscosity [164]. Following the dilution, plasma was subjected to differential centrifugation at 2,000 x g for

30 minutes at 4°C (Eppendorf, 5424R), 12,000 x g for 45 minutes at 4°C (Eppendorf, 5424R), and 110,000 x g for two hours at 4°C (Beckman Culter, Optima L-90k Ultracentrifuge). The pellet formed after the second centrifugation and the EVs depleted supernatant fraction (110,000 x g) were saved for further processing. Vesicles (110,000 x g pellet) were resuspended in 200 µl of PBS for further analysis.

2.4 Quantification of Total Protein

Following cell lysis with radioimmunoprecipitation assay (RIPA) buffer (50mM Tris HCl pH8, 150mM NaCl, 1%NP-40, 0.5% sodium deoxychlate, 0.1% SDS) and protease inhibitor cocktail (Thermo Scientific) mix, the total protein concentration of each sample was quantified using detergent compatible (DC) Lawry assay (Bio-Rad). Briefly, appropriate standards, controls, blanks and samples were loaded (10 µl/well) into a 96-well plate (Falcon). Next 25 µl of A+S solution was added to each well used. This solution consisted of 1 mL of reagent A and 20 µl of reagent S. Then 250 µl of reagent B was added to each well and the 96-well plate was incubated in the dark at room temperature for fifteen minutes. After the incubation period a spectrophotometer (Spectramax Plus 384 by MolecularDevices) was used to determine the optical density of each well at a wavelength of 750 nm. This data was then used to determine the standard curve and the concentration of protein in each well.

2.5 Immunobloting

Once the protein concentration was determined, 5-10 µl of 95% v/v sample buffer (Bio-Rad) and 5% v/v β-Mercaptoethanol (MP Biomedicals) solution was added to each sample. Homemade 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separating gels were used, unless otherwise specified. Each well of the gel was washed with 1x Tris/Glycine/SDS buffer (Bio-Rad) and subsequently loaded with 15-40 µg of protein, depending on the analyte and the experimental procedure. Gels were run at 170 V for one hour. During this time, filter paper (Bio-Rad) and nitrocellulose membrane (Bio-Rad) were soaked in Bjerrum and Schafer-Nielsen (BSN) transfer buffer (48mM Tris, 39mM Glycine, 0.04% SDS, 20% MeOH) in preparation for the transfer. Once completed, the 10% SDS-PAGE separating gels were removed from the glass moulds and the stacking gel was removed and discarded. While the BSN-soaked membranes and nitrocellulose were being installed into transfer apparatus, the gels were also soaked in BSN transfer buffer for five minutes. Once soaking was complete, the remaining components were added to the transfer apparatus. The order of the components from top to bottom were as follows: sponge, filter paper, nitrocellulose membrane, 10% SDS-PAGE separating gel, filter paper, sponge. The wet transfer apparatus was set to 100V for one hour.

Once the transfer was complete, nitrocellulose membranes were stained with Ponceau-S stain (Biotium) to ensure the successful transfer and examine protein loading. The membranes were destained with 1x Tris Buffered Saline with Tween-20 (TBS-T)

(50mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7,5) until Ponceau-S was fully removed. Following the removal of the stain the membrane was blocked in a 5% w/v fat-free milk or bovine serum albumin (BSA) (EDM Millipore) dissolved in 1xTBS-T solution for one hour at room temperature with gentle agitation. After blocking, primary antibodies for the target protein were added for a set amount of time (Table 3). Primary antibodies were dissolved in 5% v/w fat-free milk or BSA in 1x TBS-T solution based on manufacturers specification. Following incubation with primary antibodies, membranes were washed with 1xTBS-T for 3 x 15 minutes at room temperature. Membranes were then incubated with secondary antibody in 5% fat-free milk 1xTBS-T at room temperature with gentle agitation for one hour then washed with 1xTBS-T for an additional 3 x 15 minutes. After the washes chemiluminescence solution (Amersham) was added to each membrane and allowed to incubate for two minutes at room temperature. Membranes were then exposed and developed using Kinica Minolta Medical X-Ray Processor.

Table 3: Antibodies used for immunoblotting.

Following gel electrophoresis and blocking, all membranes were incubated with primary antibodies according to manufacturer's recommendations. Abbreviations: Rb-rabbit, MS-mouse, pAb-polyclonal antibody, mAb-monoclonal antibody, GT-goat.

Antibody	Dilution	Diluting Medium	Incubation Period
ER alpha (D8H8) Rb mAb	1:1000	5% w/v BSA, 1x TBS, 0.1% Tween-20	Overnight
β-Actin Rb mAb	1:1000	5% w/v BSA, 1xTBS, 0.1% Tween-20	Overnight
Flotilin-1 MS mAb	1:1000	5% w/v milk, 1x TBS, 0.1% Tween-20	Overnight
ADAM12 GT Ab	1:1000	5% w/v milk, 1x TBS, 0.1% Tween-20	Overnight
TF MS Ab	1:1000	5% w/v milk, 1x TBS, 0.1% Tween-20	Overnight
TSG 101 Rb pAb	1:1000	5% w/v milk, 1x TBS, 0.1% Tween-20	Overnight
PAI-1(D9C4) Rb pAb	1:1000	5% w/v milk, 1x TBS, 0.1% Tween-20	Overnight

2.6 Antibodies

Monoclonal antibodies for Flotillin-1 was purchased from BD Biosciences . The antibody for ER-α, β-Actin, and PAI-1 were purchased from Cell Signalling Technologies (CST). TF antibody was purchased from Sekisui Diagnostics. ADAM12 antibody was purchased from Novus Biological. TSG101 antibody was purchased from One World Labs. All corresponding secondary horseradish peroxidase (HRP) conjugated antibodies were also purchased from CST.

2.7 Protein Isolation from Phenol-Ethanol Supernatant

Following DNA and RNA isolation, protein fractions were isolated from the phenol-ethanol supernatant. Briefly, 1 mL of isopropanol (Caledon Laboratory Chemicals) was added to the phenol-ethanol supernatant and the mixture was incubated at room temperature for ten minutes. To pellet the protein the solution was then centrifuged at 5,000 x g for ten minutes at 4°C. The protein pellet was then re-suspended in 1.4 mL of wash solution (0.3 guanidine HCL in 95% ethanol (EtOH)) (US Biological Life Sciences) and incubated at room temperature for twenty minutes. After the incubation the suspension was centrifuged at 9,500 x g for five minutes at 4°C The wash was repeated for a total of three times. Following the third wash, 2 mL pf 100% molecular biology grade EtOH was added and the solution was incubated at room temperature for twenty minutes then centrifuged at 7,200 x g for five minutes at 4°C to pellet the protein. The

protein pellet was air-dried for five minutes and then re-suspended in 200 µl of 2% SDS. The protein suspension was stored in the -20°C before further analysis.

2.8 Sample Preparation for Proteomics

BC patient plasma was acquired from the tumour bank of Ontario Institute of Cancer Research (OICR). Upon arrival all samples were stored in liquid nitrogen until further processing. EVs were isolated from the plasma of a total of three BC patients in stage I, stage II, stage III, and three patients in stage IV as well as plasma of three age matched healthy subjects, for total of 9 samples. The excluding criteria of normal subjects were any history of cancer or benign tumours. Following the isolation as described above, vesicles were separated into total RNA, total DNA, and total protein. The total RNA and total DNA fractions were stored for further processing, while the total protein fraction from each sample was subjected to protein quantification using DC modified Lawry protein assay as described above.

Following the protein assay, 150 µg of protein from stage III, stage IV ang healthy subject preparations was run on 10% SDS-PAGE gel and stained with Coomassie brilliant blue (Bio-Rad). The bands corresponding to each sample were excised from the gel and subjected to MS-based proteomic analysis. Briefly, bands were shrunk in 50% acetonitrile (ACN) and reconstituted in 50 mM ammonium bicarbonate with 10 mM-Tris (2 carboxyethyl) phosphine (TCEP) and vortexed for one hour at 37°C. Chloroacetamide was added for alkylation to a final concentration of 55 mM. Samples were vortexed for

another hour at 37°C. 1 µl of trypsin was added and the digestion performed for eight hours at 37°C. Peptide extraction was conducted with 90% ACN. Extracted peptide samples were dried and solubilized in 5% ACN, 0.2% formic acid (FA). Samples were loaded on a homemade C18 precolumn (0.3 mm i.d. x 5 mm) connected directly to the switching valve and separated on a homemade reversed-phase column (150 µm i.d. x 150 mm) with a gradient from 10–60% ACN (0.2% FA) and a 600 nl/min flow rate on a NanoLC-2D system (Eksigent) connected to an LTQ-Orbitrap Elite (Thermo Fisher Scientific, MA, US). Label-free quantification MS experiments were performed using collision-induced dissociation in a linear ion trap.

For protein identification, MS data was converted into peak lists using PEAKS (Bioinformatics Solutions Inc) software with default parameters. Data searching and MS/MS spectra analysis were performed using PEAKS against Human1302S database (133,502 entries), assuming trypsin as digestion enzyme. The database search was set up with a parent ion mass tolerance of 10.0 PPM (Monoiscopic) and a fragment ion mass tolerance of 0.0100 Da (Monoiscopic). Deamination of asparagine and glutamine, oxidation of methionine, carbamidomethyl of cysteine and phosphorylation of serine, threonine and tyrosine were specified in PEAKS as variable modifications. Protein match probabilities were determined using expectation values and protein scores, and protein identifications were considered to be accurate when the protein of interest contained at least four unique peptide and had a score higher than identity threshold at $p<0.05$.

2.9 Immunohistology

Breast cancer tumour sections were obtained from the tumour bank of Ontario Institute of Cancer Research (OICR). The sections were deparaffinized in three changes of xylene (Fisher Scientific) for ten minutes each, and then three changes of EtOH (Commercial Alcohols) for one minute each. Deparaffinization was followed by an endogenous peroxidase block for ten minutes at room temperature (60 mL methanol, 1 mL 30% hydrogen peroxide, 4 drops of concentrated HCl). Sections were then washed with 70% EtOH followed by distilled water wash. Protease digestion antigen retrieval was then performed. Briefly, the sections were incubated with Protease (from Streptomyces griseus; 0.025 g in 50 mL PBS) (Signal Aldrich) for five minutes at room temperature.

Following the antigen retrieval, sections were washed with PBS and then with Tris buffer and moved to a humidity chamber. Sections were then blocked in 5% normal rabbit serum (NRS) for 30 minutes at room temperature in the humidity chamber. After the block, sections were incubated with primary antibody diluted in 5% NRS for one hour at room temperature in the humidity chamber. Sections were then washed with Tris buffer twice, then incubated with biotinylated secondary antibody for 30 minutes at room temperate in the humidity chamber. Following the incubation, sections were washed with Tris buffer twice and then incubated with Streptavidin-peroxidase (Vector Laboratories) for ten minutes. Following the Streptavidin-peroxidase incubation, sections were washed with Tris buffer and distilled water then incubated with Nova Red (Vector Laboratories)

until the reaction was complete. The section were washed with Tris buffer to stop the Nova Red reaction. Once the incubation was complete, slides were then washed with distilled water and counterstained with Gills hematoxylin No.3 (Sigma Aldrich) for 30 seconds. Following removal of the hematoxylin, the sections were dehydrated in two changes of 100% EtOH, two changes of xylene, the coverslips were then mounted using Permount (Fisher Scientific). The slides were allowed to dry for 24 hours before imaging with (OLYMPUS BX 41).

2.10 Film Quantification

Film quantification was completed using ChemiDoc MP System (Bio-Rad). All quantifications were done using ImageLab software. Lanes and bands were selected using the *lane and band* tool, the widths of all bands was adjusted to correspond to the width of the band of interest. The relative and absolute quantifications were done using the *quantification tool*.

2.11 Computational Analysis and Bioinformatic

3-way Venn diagram of protein identifications was generated using Venny web applications (http://bioinfogp.cnb.csic.es/tools/venny/) [245]. Gene ontological identification was performed using the PANTHER identification system (http://pantherdb.org/) [246]. Functional clustering analysis was performed using Cytoscape, an open source bioinformatics database library for network visualization and analysis [247].

2.12 Statistical Analysis

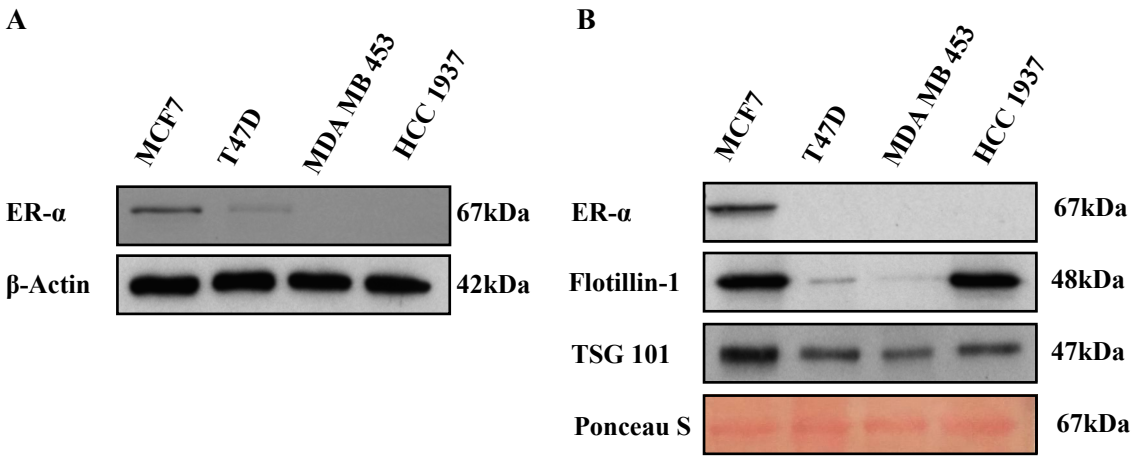
Experiments were reproduced at least three times with similar results. The quantitative data are presented as the average value of the replicates within the representative experiment. Statistical analysis for differences between groups was performed using two-tailed unpaired student's t-test. Differences between groups were considered significant a $p<0.05$ and all values are expressed as mean +/- SD.

CHAPTER 3: RESULTS

3.1 ER-α is Incorporated into Extracellular Vesicles Derived from BC Cells

The status of ER-α was determined in whole cell lysates and EVs from the following BC cell lines: MCF7, T47-D, MDA-MB-453, HCC-1937. As expected, immunoblotting confirmed that both MCF7 and T47-D cells express ER-α, (Figure 6A). However, immunoblotting of EVs from these cell lines showed that only MCF7 cells secrete ER-α (Figure 6B). Currently there are no established housekeeping genes for the determination of proper loading of EVs using immunobloting techniques, therefore, we have chosen to display a Ponceau-S stain at the molecular weight corresponding to ER-α (67 kDa). To confirm that the protein lysates utilized in our experiments were of an EV-origin, immunoblotting was also carried out on TSG 101 and Flotillin-1, which are established markers of exosomes and MVs, respectively [168, 180]. In addition to demonstrating EV-origin, the relative intensities of Flotillin-1 also show that MCF7 and HCC-1937 cells secrete more MVs than T47D and MDA MB 453 cells; TSG 101 immunoblots demonstrate that all four cell lines, with the exception of MCF7, secrete equal amounts of exosomes.

Figure 6. ER-α Protein Level in Whole Cell Lysates and Extracellular Vesicles. MCF7, T47-D, MDA-MB-453, and HCC-1937 cells were cultured in appropriate medium listed in Table 1. **A.**Whole cell lysates were lysed in RIPA buffer and subjected to immunoblot analysis examining the expression of ER-α. Equal loading was confirmed by examining β-Actin. **B**. EVs were isolated from the same cells as described previously. Immunoblot blot analysis of EVs examined presence of ER-α in vesicles from those cells. In addition, presence of Flotillin-1 and TSG 101was also examined to confirm presence of exosomes and MVs. Ponceau S. was used to confirm equal protein loading.


A
MCF7
T47D
MDA MB 453
HCC 1937
ER-α
67kDa
β-Actin
42kDa
B
MCF7
T47D
MDA MB 453
HCC 1937
ER-α
67kDa
Flotillin-1
48kDa
TSG 101
47kDa
Ponceau S
67kDa

3.2 Extracellular Vesicles Derived from BC Patient Plasma Have Unique Protein Profiles Compared to Vesicles Derived from Healthy Subjects

To determine the proteomic profile of EVs derived from BC patient plasma, the vesicle preparations were subjected to MS-based proteomic analysis. The MS analysis identified 206 protein targets with a confidence level of 95%. The vesicular abundance of protein targets found in vesicles isolated from healthy, stage III and stage IV are summarized in a heat map (Figure 7). Briefly, for analysis purposes, protein abundance was assessed in each sample group separately and subsequently compared using a matrix. The hierarchical distance clustering method was used to visualize the variations in the protein, which are shown as normalized z-score log2 LFQ values in a heat map.

Protein distribution analysis was performed using Venny web applications. Briefly, 112 of the protein targets identified using the proteomic analysis were found in the vesicles of healthy, stage III and stage IV BC patients (Figure 8). There were also targets unique to each of the three groups: 9 belonging to the healthy group, 27 belonging to the stage III group and 6 belonging to the stage IV group. Proteomic analysis also identified 20 targets present in stages III/IV, as well as 16 in stages Healthy/IV and 6 in stages Healthy/III.

Figure 7. Protein Profile of EVs from Normal Subjects, Stage III and Stage IV BC Patients.

EVs were isolated from the plasma of healthy subjects and breast cancer patients, each group includes three subjects, for a total of nine samples. EV protein content was analyzed by MS-based proteomic analysis. Proteins abundance was determined using the LFQ method. The hierarchical distance clustering method was used to visualize the variations in the protein abundance and is shown as normalized z-score log2 LFQ values in a heat map.

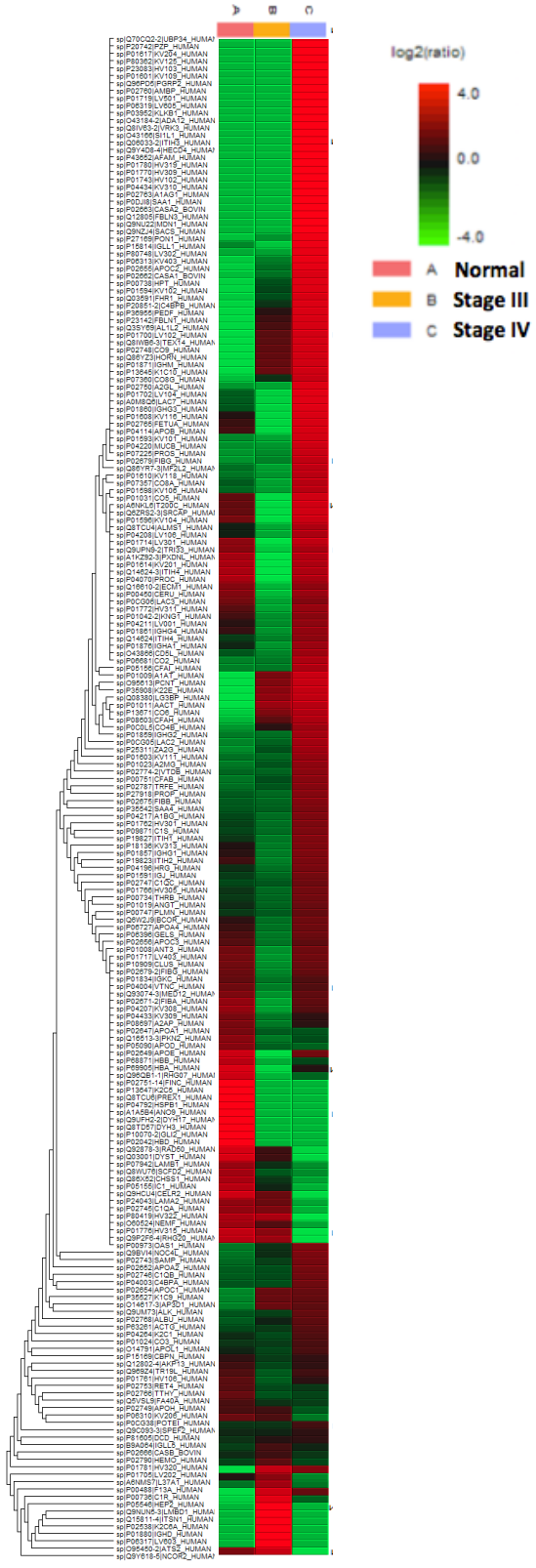
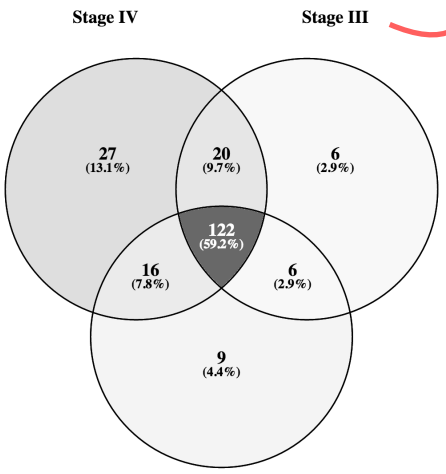

log2(ratio)
4.0
0.0
-4.0
A Normal
B Stage III
C Stage IV

Figure 8. Proteomic Analysis.

3-way Venn diagram is used to show protein distribution between healthy subjects and BC patients. The accession numbers and identification for proteins unique to each subject group are shown in the tables indicated by the arrows.

ACCESSION #	IDENTIFICATION
P01743	HV102
P20742	PZP
Q9NZJ4	SACS
Q43184-2	ADAM12
P03952	KLKB1
P80362	KV125
P02663	CASA2
Q06033-2	ITIH3
P01780	HV319
Q96PD5	PGRP2
P01601	KV109
Q12805	FBLN3
P02760	AMBP
P01770	HV309
Q8IV63-2	VRK3
P01719	LV501
P23083	HV103
P06319	LV605
P43652	AFAM
P02763	A1AG1
Q9Y4D8-4	HECD4
P04434	KV310
Q9NU22	MDN1
P01617	KV204
O43166	SI1L1
Q70CQ2-2	UBP34
P0DJI8	SAA1

ACCESSION #	IDENTIFICATION
P02538	K2C6A
P06317	LV603
P01880	IGHD
O95450-2	ATS2
Q15811-4	ITSN1
Q9NUN5-3	LMBD1



ACCESSION #	IDENTIFICATION
P10070-2	GLI2
Q8TCU6	PREX1
P13647	K2C5
A1A5B4	ANO9
Q8TD57	DYH3
P02042	HBD
P04792	HSPB1
Q92878-3	RAD50
Q9UFH2-2	DYH17

3.3 Gene Ontology of Protein Targets Identified by Proteomic Study of Extracellular Vesicles Isolated from BC Patient Plasma

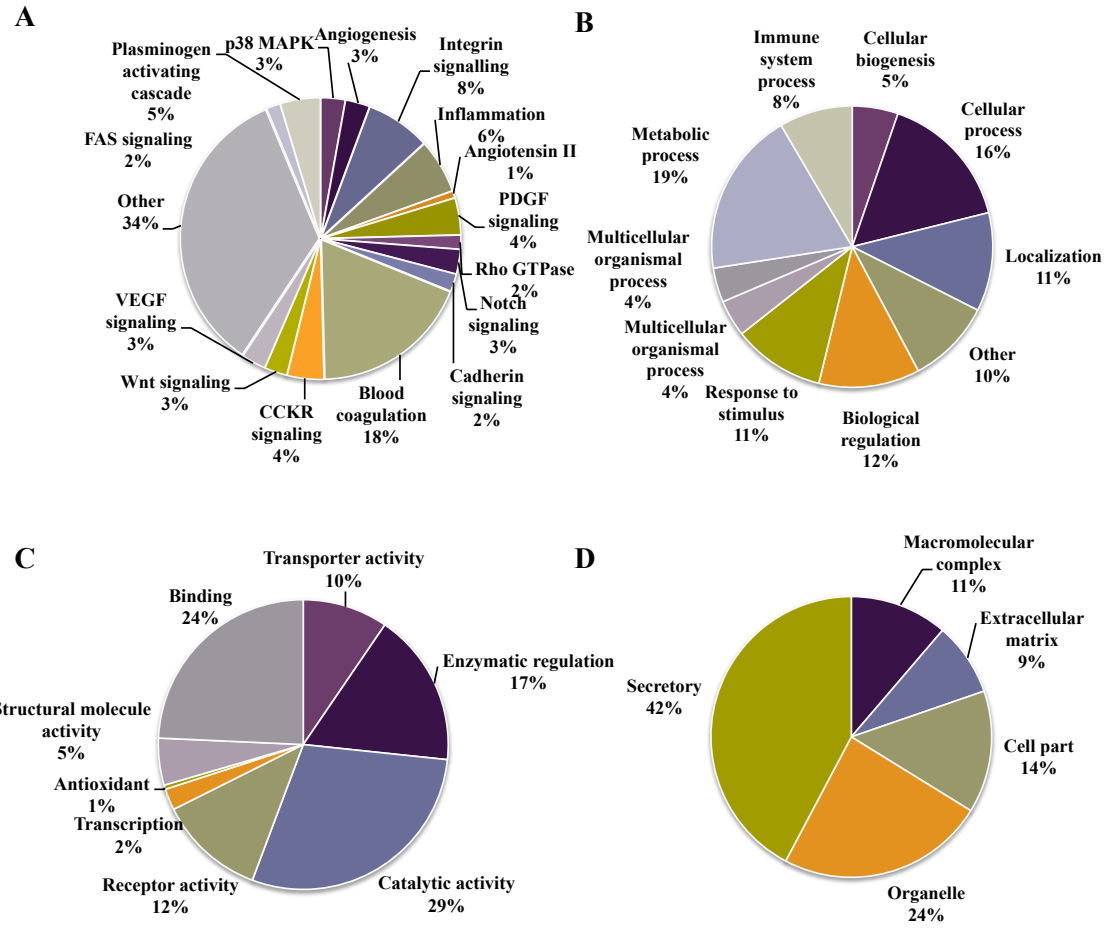
The protein targets identified via proteomic analysis were classified based on pathway activation (Figure 9A), biological processes (Figure 9B), molecular function (Figure 9C) and subcellular localization (Figure 9D). Categorization by pathway activation revealed that 3% of the targets are associated with angiogenesis, 8% in integrin signalling, 6% in inflammation, 1% in Angiotensin II signalling, 4% in platelet-derived growth factor signalling, 2% in Rho GRPase signalling, 3% in Notch signalling, 2% in Cadherin signalling, 18% in blood coagulation, 4% in cholecystokinin signalling, 3% in Wnt signalling, 3% in vascular endothelial growth factor signalling, 5% in plasminogen activator cascade, 3% in mitogen activated protein kinase cascade and 34% of targets were associated with a variety other signalling cascades unrelated to carcinogenesis.

Categorization by biological processes, demonstrated that 19% of targets are associated with metabolic function, 8% in immune response, 5% in cellular biogenesis, 16% in cellular processes, 11% in localization, 12% in biological regulation, 11% in response to stimuli, 4% in adhesion and the remaining 14% not belonging to any specific process. Our results also show that BC plasma derived EV are involved in a variety of molecular functions: 29% in catalytic activity, 24% in binding, 17% in enzyme regulatory activities, 12% in receptor activity, 10% in transporter activity, 5% in maintenance of cell structure, 1% in nucleic acid binding, 1% in transcriptional activity, 1% in antioxidant activity. The categorization of protein targets by subcellular localization revealed that the

majority of targets were associated with the extracellular compartment (42%) and 24% typically expressed in specific organelles. In addition, 14% were structural proteins associated with cell parts, mainly the cell membrane, 9% with extracellular matrix and 11% with macromolecular matrix.

Figure 9. Gene Ontology of Targets Identified by MS-based Proteomic Analysis.

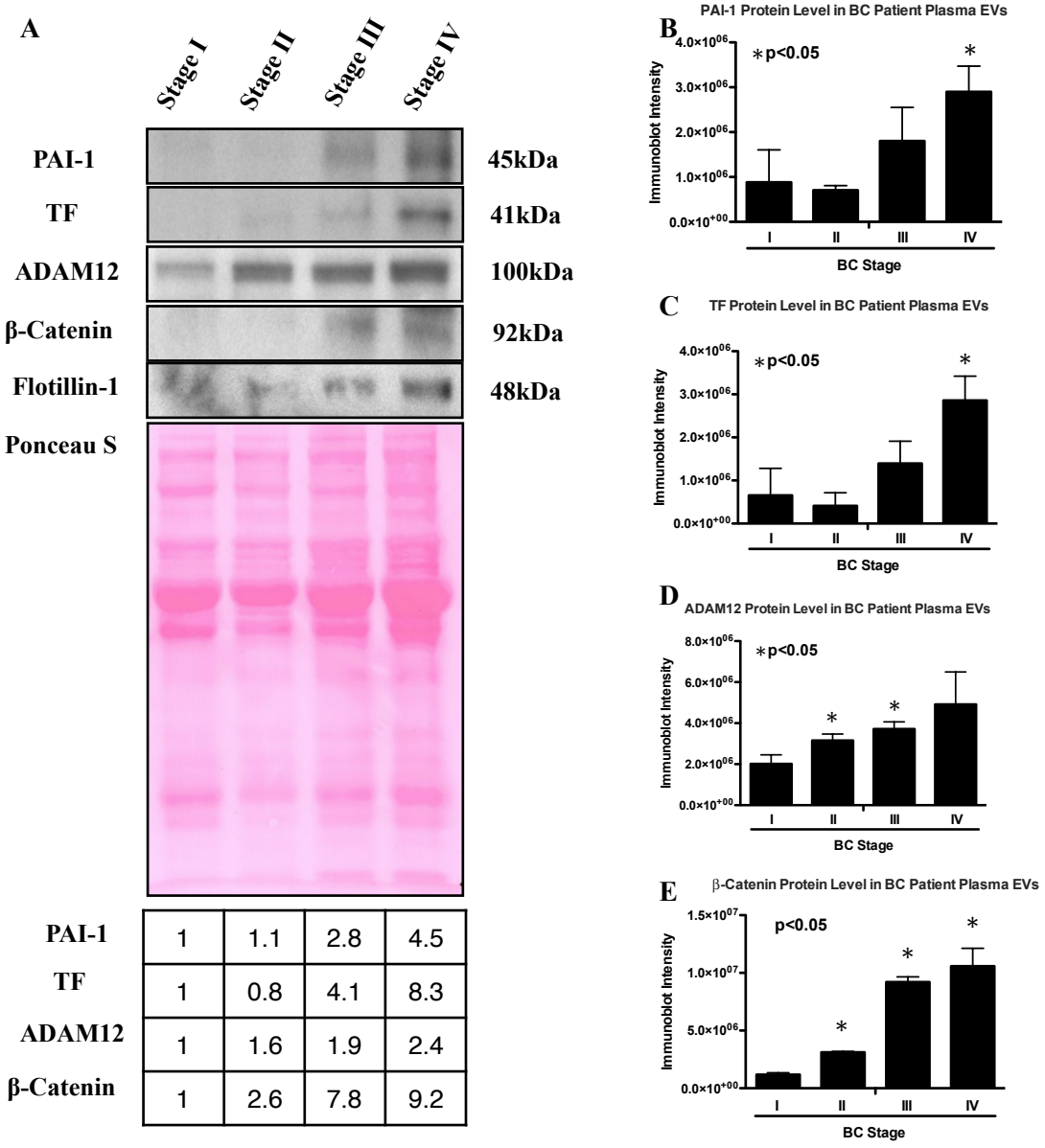
Pie charts show the PANTHER classification of targets identified by proteomic analysis based on **A,** Pathway activation. **B,** Biological processes. **C**, Molecular function. **D,** Subcellular localization.


A
Plasminogen activating cascade 5%
p38 MAPK 3%
Angiogenesis 3%
Integrin signalling 8%
Inflammation 6%
Angiotensin II 1%
PDGF signaling 4%
Rho GTPase 2%
Notch signaling 3%
Cadherin signaling 2%
Blood coagulation 18%
CCKR signaling 4%
Wnt signaling 3%
VEGF signaling 3%
FAS signaling 2%
Other 34%
B
Immune system process 8%
Cellular biogenesis 5%
Cellular process 16%
Localization 11%
Other 10%
Biological regulation 12%
Response to stimulus 11%
Multicellular organismal process 4%
Multicellular organismal process 4%
Metabolic process 19%
C
Transporter activity 10%
Enzymatic regulation 17%
Catalytic activity 29%
Receptor activity 12%
Transcription 2%
Antioxidant 1%
Structural molecule activity 5%
Binding 24%
D
Macromolecular complex 11%
Extracellular matrix 9%
Cell part 14%
Organelle 24%
Secretory 42%

3.4 EVs Derived from BC Patient Plasma Contain Targets Associated with Cancer Prognosis

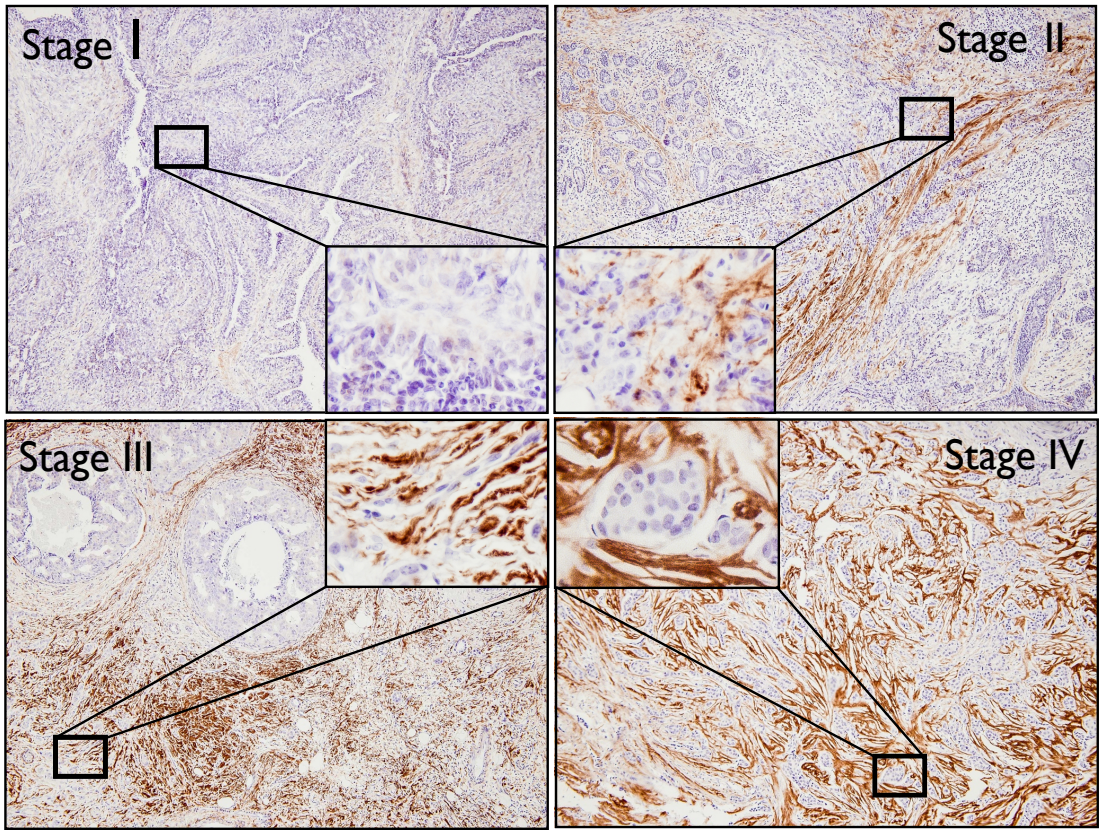
Our next aim was to determine whether the relative abundance of specific protein targets found within the extra-vesicular cargo of EVs isolated from BC patient plasma correlated with disease state. Based on the results discussed herein, as well as those of others [117, 226, 248-254], protein targets selected for assessment included PAI-1, TF, ADAM 12 and β-Catenin. Immunoblotting confirmed that EVs isolated from stage II and III BC patients contained significantly more (*,$p<0.05$) ADAM12 and β-Catenin than those isolated from stage I patients (Figure 10D/E). In addition, EVs isolated from stage IV BC patients contained significantly more PAI-1, TF and β-Catenin (*,$p<0.05$) than those isolated from stage I BC patients (Figure 10A/B/E). Immunoblot analysis also showed that increased Flotillin-1 with advanced stage of the disease (Figure 10A), further confirming increased vesicle secretion with advanced disease. In addition, histological staining of the tumour tissue confirmed increase in ADAM12 expression with advanced staging (Figure 11). ADAM12 labeling is mainly observed in the connective tissue, more specifically the fibroblast cells. The localization is consistent with that to other matrix metalloproteinases [255].

Figure 10. Immunoblot Analysis of EVs from BC Plasma. A, EVs were isolated from BC patient plasma. The immunoblot examines presence of PAI-1, TF, ADAM12, β-Catenin and Flotillin-1 in these EVs. For comparison purposes all gels were run together and membranes were exposed at the same time. Ponceau-S was used to assess protein loading. **B-E**, All film was quantified using ChemiDoc system. Statistical analysis was performed on the absolute quantification values.



PAI-1	1	1.1	2.8	4.5
TF	1	0.8	4.1	8.3
ADAM12	1	1.6	1.9	2.4
β-Catenin	1	2.6	7.8	9.2

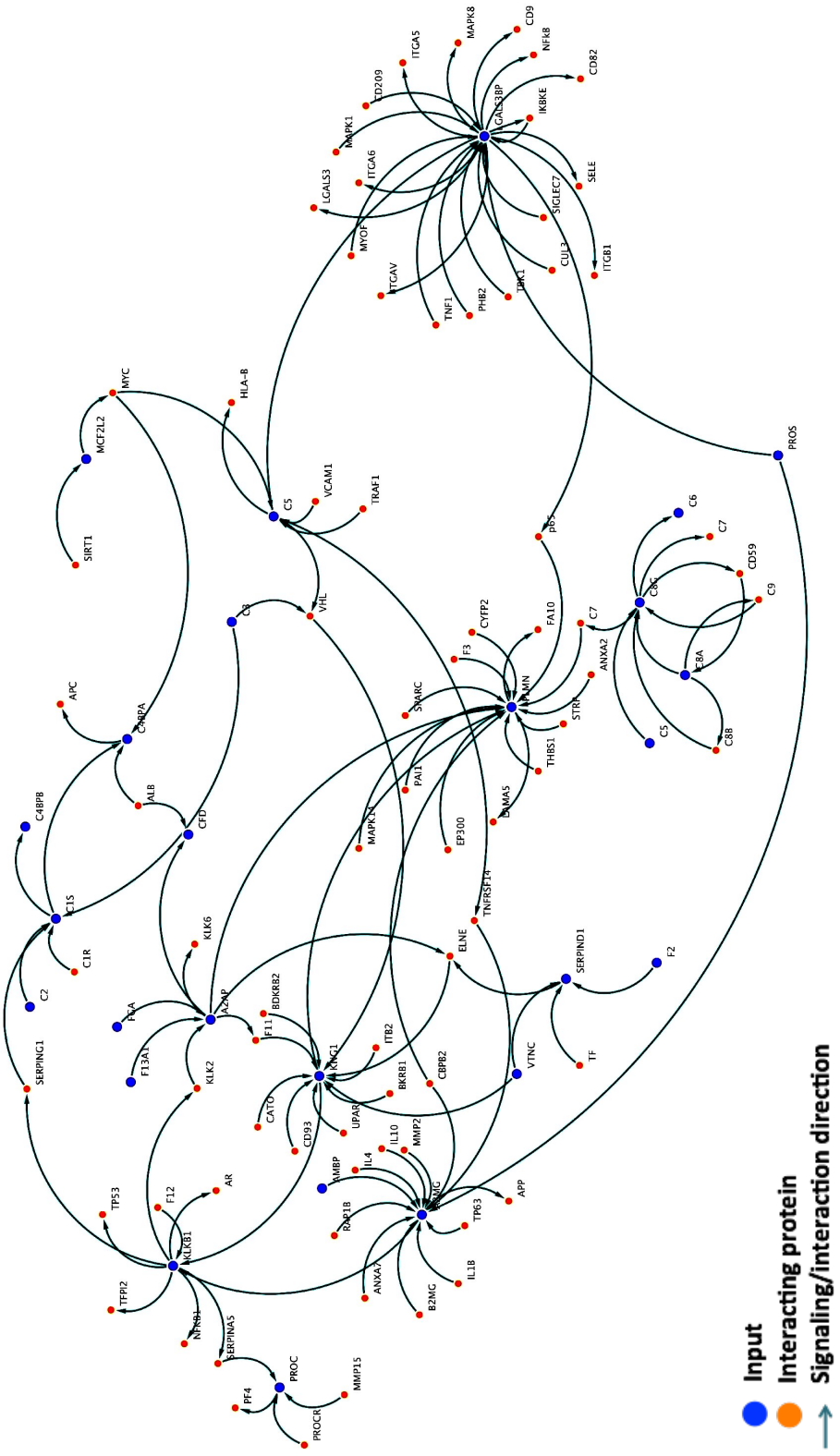
Figure 11. Localization of ADAM12 in Human Specimens of Breast Carcinoma. BC tumour sections were obtained from tumour bank of OICR. The sections were stained with ADAM12. Histological analysis of BC tumor tissue reveals an increase in ADAM12 expression with advanced disease. The labelling is mainly observed in the connective tissue.


Stage I
Stage II
Stage III
Stage IV

3.6 Biological Network Integration of Targets Identified by Proteomic Analysis

In order to assess downstream signalling initiated by proteomic cargo of EVs from BC patient plasma, we constructed a network interaction analysis. The majority of the targets identified by the proteomic analysis are involved in inflammatory processes, coagulation, and immune response. Importantly, it is well established that these processes contribute to tumour growth and metastasis [14]. Cytoscape, which is an open source bioinformatics database, was used for network visualization and analysis. A total of 30 targets identified by proteomic analysis were chosen for additional interaction analysis. The resulting network produced by the Cytoscape is a gross representation of signalling interactions between targets of interest and proteins involved in the immediate up or downstream signalling as observed in healthy individuals (Figure 12). It is noteworthy that this network does not reflect any differences in the amount of target proteins in vesicles of different BC patient groups. However, the network does reflect the signalling cascades that have been shown to be induced or suppressed by proteins secreted as part of vesicle cargo.

Figure 13. Integration of Biological Networks of Protein Targets Identified by Proteomic Analysis. The signalling network produced by the Cytoscape software is a gross representation of signalling interaction between targets identified by proteomic analysis (represented by blue circles), and proteins involved in the immediate up or downstream signalling (represented by orange circles). The main purpose of the network analysis is to review signalling interactions that have the potential to be initiated by proteins secreted as a part of EV cargo from BC patients.



Input
Interacting protein
Signaling/interaction direction

CHAPTER 4: DISCUSSION

This study aims to characterize ER-α in EVs from BC cells and examine the proteomic profile of EVs derived from BC patient plasma. Our studies show that ER-α is secreted *via* EVs of MCF7 cells (Figure 6B). Mass spectrometry analysis of the BC patient derived EVs shows that vesicles from metastatic cancer patients have a unique proteomic profile. The unique protein cargo from different stages of the disease, leads us to believe that EVs reflect the disease state, and therefore, are a rich source of novel non-invasive diagnostic markers. Further analysis of the proteomic data allowed us to identify four targets for further validation. We chose to focus on the validation of PAI-1, TF, ADAM12, and β-Catenin.

4.1 ER-α is Incorporated into Extracellular Vesicles Derived from BC Cells

Our study reports novel findings that ER-α is secreted *via* EVs from MCF7 cells (Figure 6B). Although, we failed to detect ER-α in EV from T47D cells, there are a couple possible explanations for this result. T47D cells have less endogenous expression of ER-α than MCF7 cells [256-261] (Figure 6A), therefore, it is reasonable to assume that less ER-α is secreted *via* EVs. Consistent with this, an experiment in which twice the amount vesicles from T47D cells were collected, still failed to yield EV ER-α content (data not shown). Comparison of vesicular protein shows that T47D cells secrete almost three times the amount of protein cargo than MCF7 cells, thus it is also possible that ER-α

is specifically not being secreted as a part of EV cargo in the T47D cells due to some unknown mechanism(s).

It is well established that ER-α expression is lost in advanced/aggressive stages of BC [262-265]. Moreover, it is also established that intact, functional, receptors and other signalling molecules can be secreted *via* EVs [17, 266, 267]. Given that we provide evidence that ER-α is found within the protein cargo of EVs secreted from BC cells, our findings suggest that EV secretion may contribute to the loss of ER-α during the progression of BC disease state. Consistent with this, Gabriel et al. indicated that EV secretion contributed to the loss of tumour suppressor PTEN in PC cells [268]. To lend aid to this model, EV secreted from chemotherapy-treated cancer cells have also been found to contain chemotherapeutic agents within their cargo, thereby further contributing to cancer progression *via* resistance to therapy [269].

Reports show that EVs shuttle their cargo from donor cells to cells of the periphery resulting in the induction of distant signalling cascades in recipient cells [17]. Kaplan et al. reported that EVs contribute to the formation of a pre-metastatic niche microenvironment in lung cancer [270]. Furthermore, Hoshino et al. showed that tumour derived EVs dictate the site of organ specific metastasis [20]. Taken together, these findings suggest that EVs have the potential to translocate functional ER-α to distant cells, where it can stimulate downstream signalling. Activation of the ER-α signalling results in proliferation and migration of cells, thereby contributing to cancer metastasis

[250, 271, 272]. Therefore, the model that we propose is that secreted EVs contribute to disease onset and progression *via* ER-α.

4.2 Validation of PAI-1, TF, ADAM12 and β-Catenin

Our study reports a novel finding that PAI-1, TF, ADAM12 and β-Catenin are present in EV from BC patient plasma. Furthermore, our results show that there is an increase in abundance of these targets in vesicles in advanced stages of the disease compared to primary tumors. Our results correlated with *in vivo* and *in vitro* studies conducted previously [117, 250, 252, 253, 273-275] that show a similar increase in cells and tumour tissues. The consistency between our results and the findings of other further proves our hypothesis that EVs are a source of markers of disease, and a potential diagnostic tool.

Presence of Flotillin-1 in EVs from BC patient plasma indicate secretion of MVs [180]. We report an increase of Flotillin-1 protein in EVs from BC patient plasma with advanced stages of the disease, confirming increased microvesicle secretion, which is consistent with previous studies [276].

Our results show increase EVs secretion and the amount of total target protein (PAI-1, TF, ADAM12, β-Catenin). Which brings to question of whether our results show an increase in EV secretion that result in increase total target protein, or there is an increased induction of target proteins into EVs. The other possibility is that accompanied with increased protein secretion there also an increased induction of certain proteins into

EV cargo. The latter is possible due to our results showing that the increase in EVs secretion with advanced staging is not as robust as the increase in the target we examined (Figure 10A). Therefore, these results further prove our hypothesis that EV cargo can be used as a diagnostic tool.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

Consistent with other studies, the results of the MS-based proteomic study show that EVs are a rich source of proteins that can be used as prognostic and diagnostic tools in BC [21, 277]. Characterization of EV protein cargo from stage I and stage II BC patient plasma is of interest because it would provide insight into changes of EV cargo with disease progression. Our studies examined EV protein cargo in a total of three patient samples in each group, for a total of nine, thus assessment of additional samples is of interest to strengthen our results.

Based on our studies and the work of others we know that characterization of EV cargo can lead to the development of novel biomarkers of the disease state. Assessment of genetic material present in EVs is of special interest due to previous reports showing that EV derived miRNA can travel to distance cells where they change gene expression [190-192]. Evidence shows that miRNA selection into EVs is not random [191, 193], thus based on the results of the proteomic study it can be concluded that miRNA content of EV

from plasma of BC patients may also differ between stages BC, possibly reflecting disease state.

Our novel findings show that ER-α is incorporated as a part of EV cargo in the MCF7 cells, which may be a potential mechanism for development of aggressive tumour phenotype. Future studies are required to examine ER-α can be transferred to distant cells that do not express the receptor and result in activation of ER-α downstream signalling. In conclusion, the results of our studies raise many questions which merit attention to further studies.

REFERENCES

1. Maxmen, A., *The hard facts.* Nature, 2012. **485**(7400): p. S50-1.

2. Statistics, C.C.S.s.A.C.o.C. *Canadian Cancer Statistics 2015*. 2015.

3. Drooger, J.C., et al., Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for BRCA1 and BRCA2 mutation carriers: a critical review of the literature. Cancer Treat Rev, 2015. **41**(2): p. 187-96.

4. Scully, R., Role of BRCA gene dysfunction in breast and ovarian cancer predisposition. Breast Cancer Res, 2000. **2**(5): p. 324-30.

5. King, M.C., et al., Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. Science, 2003. **302**(5645): p. 643-6.

6. Larsen, M.J., et al., Hereditary breast cancer: clinical, pathological and molecular characteristics. Breast Cancer (Auckl), 2014. **8**: p. 145-55.

7. Pettersson, A., et al., Mammographic density phenotypes and risk of breast cancer: a meta-analysis. J Natl Cancer Inst, 2014. **106**(5).

8. Romieu, I., J.A. Berlin, and G. Colditz, Oral contraceptives and breast cancer. Review and meta-analysis. Cancer, 1990. **66**(11): p. 2253-63.

9. Schernhammer, E.S., et al., Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. J Natl Cancer Inst, 2001. **93**(20): p. 1563-8.

10. Singletary, K.W. and S.M. Gapstur, Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. JAMA, 2001. **286**(17): p. 2143-51.

11. Terry, P.D. and T.E. Rohan, Cigarette smoking and the risk of breast cancer in women: a review of the literature. Cancer Epidemiol Biomarkers Prev, 2002. **11**(10 Pt 1): p. 953-71.

12. Protani, M., M. Coory, and J.H. Martin, Effect of obesity on survival of women with breast cancer: systematic review and meta-analysis. Breast Cancer Res Treat, 2010. **123**(3): p. 627-35.

13. Hou, N., et al., Hormone replacement therapy and breast cancer: heterogeneous risks by race, weight, and breast density. J Natl Cancer Inst, 2013. **105**(18): p. 1365-72.

14. Weigelt, B., J.L. Peterse, and L.J. van 't Veer, *Breast cancer metastasis: markers and models.* Nat Rev Cancer, 2005. **5**(8): p. 591-602.

15. Brennan M, L.B., The Actual Role of Receptors as Cancer Markers, Biochemical and Clinical Aspects: Receptors in Breast Cancer., ed. R. Scatena. Vol. 867. 2015, Advances in Cancer Biomarkers: Springer Netherlands.

16. Youngson, B.J., M. Cranor, and P.P. Rosen, Epithelial displacement in surgical breast specimens following needling procedures. Am J Surg Pathol, 1994. **18**(9): p. 896-903.

17. Al-Nedawi, K., et al., Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol, 2008. **10**(5): p. 619-24.

18. Peinado, H., et al., Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med, 2012. **18**(6): p. 883-91.

19. Costa-Silva, B., et al., Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol, 2015. **17**(6): p. 816-26.

20. Hoshino, A., et al., Tumour exosome integrins determine organotropic metastasis. Nature, 2015. **527**(7578): p. 329-35.

21. Melo, S.A., et al., Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature, 2015. **523**(7559): p. 177-82.

22. Ginestra, A., et al., Membrane vesicles in ovarian cancer fluids: a new potential marker. Anticancer Res, 1999. **19**(4C): p. 3439-45.

23. Ginestra, A., et al., The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. Anticancer Res, 1998. **18**(5A): p. 3433-7.

24. Skog, J., et al., Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol, 2008. **10**(12): p. 1470-6.

25. Wiechmann, L. and H.M. Kuerer, The molecular journey from ductal carcinoma in situ to invasive breast cancer. Cancer, 2008. **112**(10): p. 2130-42.

26. Zhang, Y., B. Ma, and Q. Fan, Mechanisms of breast cancer bone metastasis. Cancer Lett, 2010. **292**(1): p. 1-7.

27. Benson, J.R., et al., The TNM staging system and breast cancer. Lancet Oncol, 2003. **4**(1): p. 56-60.

28. IUAC, *TNM Classification of Malignant Tumours.*, ed. 7. Vol. 2016. 2009: Wiley Blackwell. 336.

29. Elston, C.W. and I.O. Ellis, Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology, 1991. **19**(5): p. 403-10.

30. Martin, M., Molecular biology of breast cancer. Clin Transl Oncol, 2006. **8**(1): p. 7-14.

31. Duffy, M.J., Biochemical markers as prognostic indices in breast cancer. Clin Chem, 1990. **36**(2): p. 188-91.

32. Leonard, G.D. and S.M. Swain, Ductal carcinoma in situ, complexities and challenges. J Natl Cancer Inst, 2004. **96**(12): p. 906-20.

33. Van Zyl, J.A. and A.G. Muller, Breast-conserving treatment for stage I and II cancer. Tumour excision, axillary dissection, peri-operative interstitial irradiation, with or without peri-operative chemotherapy, followed by breast irradiation--the Tygerberg Hospital experience. S Afr Med J, 1989. **75**(11): p. 519-23.

34. Shenkier, T., et al., Clinical practice guidelines for the care and treatment of breast cancer: 15. Treatment for women with stage III or locally advanced breast cancer. CMAJ, 2004. **170**(6): p. 983-94.

35. Eichner, W.J., H.M. Lemon, and G. Friedell, *Tumor grade in the prognosis of breast cancer.* Nebr State Med J, 1970. **55**(7): p. 405-9.

36. Baker, A.T., A. Zlobin, and C. Osipo, Notch-EGFR/HER2 Bidirectional Crosstalk in Breast Cancer. Front Oncol, 2014. **4**: p. 360.

37. Bianchini, G., et al., Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol, 2016.

38. Ignatiadis, M. and C. Sotiriou, Luminal breast cancer: from biology to treatment. Nat Rev Clin Oncol, 2013. **10**(9): p. 494-506.

39. Ades, F., et al., Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. J Clin Oncol, 2014. **32**(25): p. 2794-803.

40. Karagiannis, G.S., et al., Signatures of breast cancer metastasis at a glance. J Cell Sci, 2016. **129**(9): p. 1751-8.

41. Curtis, C., et al., The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature, 2012. **486**(7403): p. 346-52.

42. Yarnold, J.R., Early stage breast cancer: treatment options and results. Br Med Bull, 1991. **47**(2): p. 372-87.

43. Richards, P., et al., The use of surgery in the treatment of ER+ early stage breast cancer in England: Variation by time, age and patient characteristics. Eur J Surg Oncol, 2016. **42**(4): p. 489-96.

44. Albornoz, C.R., et al., Bilateral Mastectomy versus Breast-Conserving Surgery for Early-Stage Breast Cancer: The Role of Breast Reconstruction. Plast Reconstr Surg, 2015. **135**(6): p. 1518-26.

45. Lang, K., et al., Treatment patterns among elderly patients with stage IV breast cancer treated with HER-2-targeted therapy. J Comp Eff Res, 2014. **3**(5): p. 481-90.

46. Gallo, M.A. and D. Kaufman, Antagonistic and agonistic effects of tamoxifen: significance in human cancer. Semin Oncol, 1997. **24**(1 Suppl 1): p. S1-71-S1-80.

47. Riggs, B.L. and L.C. Hartmann, Selective estrogen-receptor modulators -- mechanisms of action and application to clinical practice. N Engl J Med, 2003. **348**(7): p. 618-29.

48. Miller, W.R., Aromatase inhibitors: mechanism of action and role in the treatment of breast cancer. Semin Oncol, 2003. **30**(4 Suppl 14): p. 3-11.

49. Nahta, R., M.C. Hung, and F.J. Esteva, The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. Cancer Res, 2004. **64**(7): p. 2343-6.

50. Hudis, C.A., Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med, 2007. **357**(1): p. 39-51.

51. Harbeck, N., et al., HER2 Dimerization Inhibitor Pertuzumab - Mode of Action and Clinical Data in Breast Cancer. Breast Care (Basel), 2013. **8**(1): p. 49-55.

52. Segovia-Mendoza, M., et al., Efficacy and mechanism of action of the tyrosine kinase inhibitors gefitinib, lapatinib and neratinib in the treatment of HER2-positive breast cancer: preclinical and clinical evidence. Am J Cancer Res, 2015. **5**(9): p. 2531-61.

53. Corkery B, C.J., Clynes M, O'Donovan N., Epidermal growth factor receptor as a potential therapeutic target in triple-negative breast cancer. Ann Oncol, 2009. **20**(5): p. 862-867.

54. Gordon, V. and S. Banerji, Molecular pathways: PI3K pathway targets in triple-negative breast cancers. Clin Cancer Res, 2013. **19**(14): p. 3738-44.

55. Horwitz, K.B. and W.L. McGuire, Nuclear mechanisms of estrogen action. Effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing. J Biol Chem, 1978. **253**(22): p. 8185-91.

56. Heldring, N., et al., Estrogen receptors: how do they signal and what are their targets. Physiol Rev, 2007. **87**(3): p. 905-31.

57. Dahlman-Wright, K., et al., International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev, 2006. **58**(4): p. 773-81.

58. Brzozowski, A.M., et al., Molecular basis of agonism and antagonism in the oestrogen receptor. Nature, 1997. **389**(6652): p. 753-8.

59. Tremblay, G.B., et al., Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. Mol Endocrinol, 1997. **11**(3): p. 353-65.

60. Xiaodong Li, J.H., Ping Yi,Robert A. Bambara, Russell Hilf, and Mesut Muyan, Single-Chain Estrogen Receptors (ERs) Reveal that the ERα/β Heterodimer Emulates Functions of the ERα Dimer in Genomic Estrogen Signaling Pathways. Mol Cell Biol., 2004. **24**(17): p. 7681-7694.

61. Kuiper, G.G., et al., Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology, 1997. **138**(3): p. 863-70.

62. Hall, J.M. and D.P. McDonnell, Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. Mol Interv, 2005. **5**(6): p. 343-57.

63. Hall, J.M. and D.P. McDonnell, The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology, 1999. **140**(12): p. 5566-78.

64. Yue, W., et al., Effects of estrogen on breast cancer development: Role of estrogen receptor independent mechanisms. Int J Cancer, 2010. **127**(8): p. 1748-57.

65. Secreto, G. and B. Zumoff, Role of androgen excess in the development of estrogen receptor-positive and estrogen receptor-negative breast cancer. Anticancer Res, 2012. **32**(8): p. 3223-8.

66. Zambrano, E., et al., Fetal programming of sexual development and reproductive function. Mol Cell Endocrinol, 2014. **382**(1): p. 538-49.

67. Collaborative Group on Hormonal Factors in Breast, C., Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. Lancet Oncol, 2012. **13**(11): p. 1141-51.

68. Riggins, R.B., et al., Pathways to tamoxifen resistance. Cancer Lett, 2007. **256**(1): p. 1-24.

69. Preston-Martin, S., et al., Increased cell division as a cause of human cancer. Cancer Res, 1990. **50**(23): p. 7415-21.

70. Fernandez, S.V., I.H. Russo, and J. Russo, Estradiol and its metabolites 4-hydroxyestradiol and 2-hydroxyestradiol induce mutations in human breast epithelial cells. Int J Cancer, 2006. **118**(8): p. 1862-8.

71. Gutierrez, M.C., et al., Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J Clin Oncol, 2005. **23**(11): p. 2469-76.

72. Herynk, M.H. and S.A. Fuqua, Estrogen receptor mutations in human disease. Endocr Rev, 2004. **25**(6): p. 869-98.

73. Musgrove, E.A. and R.L. Sutherland, Biological determinants of endocrine resistance in breast cancer. Nat Rev Cancer, 2009. **9**(9): p. 631-43.

74. Ring, A. and M. Dowsett, Mechanisms of tamoxifen resistance. Endocr Relat Cancer, 2004. **11**(4): p. 643-58.

75. Hoskins, J.M., L.A. Carey, and H.L. McLeod, CYP2D6 and tamoxifen: DNA matters in breast cancer. Nat Rev Cancer, 2009. **9**(8): p. 576-86.

76. Shi, L., et al., Expression of ER-{alpha}36, a novel variant of estrogen receptor {alpha}, and resistance to tamoxifen treatment in breast cancer. J Clin Oncol, 2009. **27**(21): p. 3423-9.

77. Konigsberg, W., et al., The TF:VIIa complex: clinical significance, structure-function relationships and its role in signaling and metastasis. Thromb Haemost, 2001. **86**(3): p. 757-71.

78. Martin, D.M., C.W. Boys, and W. Ruf, Tissue factor: molecular recognition and cofactor function. FASEB J, 1995. **9**(10): p. 852-9.

79. Harlos, K., et al., Crystal structure of the extracellular region of human tissue factor. Nature, 1994. **370**(6491): p. 662-6.

80. Shoji, M., et al., Activation of coagulation and angiogenesis in cancer: immunohistochemical localization in situ of clotting proteins and vascular endothelial growth factor in human cancer. Am J Pathol, 1998. **152**(2): p. 399-411.

81. Adams, R.L. and R.J. Bird, Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. Nephrology (Carlton), 2009. **14**(5): p. 462-70.

82. Drake, T.A., J.H. Morrissey, and T.S. Edgington, Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. Am J Pathol, 1989. **134**(5): p. 1087-97.

83. Fidler, I.J., Tumor heterogeneity and the biology of cancer invasion and metastasis. Cancer Res, 1978. **38**(9): p. 2651-60.

84. Dvorak, H.F., D.R. Senger, and A.M. Dvorak, Fibrin as a component of the tumor stroma: origins and biological significance. Cancer Metastasis Rev, 1983. **2**(1): p. 41-73.

85. Rickles, F.R. and R.L. Edwards, Activation of blood coagulation in cancer: Trousseau's syndrome revisited. Blood, 1983. **62**(1): p. 14-31.

86. Rickles, F.R., et al., Abnormalities of blood coagulation in patients with cancer. Fibrinopeptide A generation and tumor growth. Cancer, 1983. **51**(2): p. 301-7.

87. Hu, T., et al., Procoagulant activity in cancer cells is dependent on tissue factor expression. Oncol Res, 1994. **6**(7): p. 321-7.

88. Rak, J., et al., Tissue factor in cancer and angiogenesis: the molecular link between genetic tumor progression, tumor neovascularization, and cancer coagulopathy. Semin Thromb Hemost, 2006. **32**(1): p. 54-70.

89. Milsom, C. and J. Rak, *Tissue factor and cancer.* Pathophysiol Haemost Thromb, 2008. **36**(3-4): p. 160-76.

90. Abdulkadir, S.A., et al., Tissue factor expression and angiogenesis in human prostate carcinoma. Hum Pathol, 2000. **31**(4): p. 443-7.

91. Kakkar, A.K., et al., Tissue factor expression correlates with histological grade in human pancreatic cancer. Br J Surg, 1995. **82**(8): p. 1101-4.

92. Koomagi, R. and M. Volm, Tissue-factor expression in human non-small-cell lung carcinoma measured by immunohistochemistry: correlation between tissue factor and angiogenesis. Int J Cancer, 1998. **79**(1): p. 19-22.

93. Lykke, J. and H.J. Nielsen, The role of tissue factor in colorectal cancer. Eur J Surg Oncol, 2003. **29**(5): p. 417-22.

94. Wan, Y.L., et al., [The role of tissue factor in the invasion and metastasis of colorectal carcinoma cells]. Zhonghua Wai Ke Za Zhi, 2005. **43**(19): p. 1265-7.

95. Hamada, K., et al., Expression of tissue factor correlates with grade of malignancy in human glioma. Cancer, 1996. **77**(9): p. 1877-83.

96. Hamada, K., et al., Expression of tissue factor in glioma. Noshuyo Byori, 1996. **13**(2): p. 115-8.

97. Vrana, J.A., et al., Expression of tissue factor in tumor stroma correlates with progression to invasive human breast cancer: paracrine regulation by carcinoma cell-derived members of the transforming growth factor beta family. Cancer Res, 1996. **56**(21): p. 5063-70.

98. Anand, M. and D.J. Brat, Oncogenic regulation of tissue factor and thrombosis in cancer. Thromb Res, 2012. **129 Suppl 1**: p. S46-9.

99. Rong, Y., et al., PTEN and hypoxia regulate tissue factor expression and plasma coagulation by glioblastoma. Cancer Res, 2005. **65**(4): p. 1406-13.

100. Yu, J.L., et al., Oncogenic events regulate tissue factor expression in colorectal cancer cells: implications for tumor progression and angiogenesis. Blood, 2005. **105**(4): p. 1734-41.

101. Prandoni, P., et al., Recurrent venous thromboembolism and bleeding complications during anticoagulant treatment in patients with cancer and venous thrombosis. Blood, 2002. **100**(10): p. 3484-8.

102. Uno, K., et al., Tissue factor expression as a possible determinant of thromboembolism in ovarian cancer. Br J Cancer, 2007. **96**(2): p. 290-5.

103. Khorana, A.A., et al., Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. Clin Cancer Res, 2007. **13**(10): p. 2870-5.

104. Zhang, Y., et al., Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. J Clin Invest, 1994. **94**(3): p. 1320-7.

105. Ott, I., et al., Tissue factor cytoplasmic domain stimulates migration by activation of the GTPase Rac1 and the mitogen-activated protein kinase p38. Circulation, 2005. **111**(3): p. 349-55.

106. Siegbahn, A., et al., Regulation of chemotaxis by the cytoplasmic domain of tissue factor. Thromb Haemost, 2005. **93**(1): p. 27-34.

107. Bromberg, M.E., et al., Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc Natl Acad Sci U S A, 1995. **92**(18): p. 8205-9.

108. Ngo, C.V., et al., CNTO 859, a humanized anti-tissue factor monoclonal antibody, is a potent inhibitor of breast cancer metastasis and tumor growth in xenograft models. Int J Cancer, 2007. **120**(6): p. 1261-7.

109. Chorostowska-Wynimko, J., E. Skrzypczak-Jankun, and J. Jankun, Plasminogen activator inhibitor type-1: its structure, biological activity and role in tumorigenesis (Review). Int J Mol Med, 2004. **13**(6): p. 759-66.

110. Emeis, J.J., et al., An endothelial storage granule for tissue-type plasminogen activator. J Cell Biol, 1997. **139**(1): p. 245-56.

111. Stefansson, S., et al., Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. Curr Pharm Des, 2003. **9**(19): p. 1545-64.

112. Alessi, M.C., et al., Detection of plasminogen activator inhibitor-1 (PAI-1) mRNA in human megakaryocytes by in situ hybridization. Thromb Haemost, 1994. **72**(6): p. 931-6.

113. Gallicchio, M., et al., Stimulation of PAI-1 expression in endothelial cells by cultured vascular smooth muscle cells. Arterioscler Thromb, 1994. **14**(5): p. 815-23.

114. Crandall, D.L., et al., Release of PAI-1 by human preadipocytes and adipocytes independent of insulin and IGF-1. Biochem Biophys Res Commun, 2000. **279**(3): p. 984-8.

115. Providence, K.M., et al., PAI-1 gene expression is regionally induced in wounded epithelial cell monolayers and required for injury repair. J Cell Physiol, 2000. **182**(2): p. 269-80.

116. Oszajca, K., et al., Effect of oxidative stress on the expression of t-PA, u-PA, u-PAR, and PAI-1 in endothelial cells. Biochem Cell Biol, 2008. **86**(6): p. 477-86.

117. Chappuis, P.O., et al., Functional evaluation of plasmin formation in primary breast cancer. J Clin Oncol, 2001. **19**(10): p. 2731-8.

118. Chorostowska-Wynimko, J., E. Skrzypczak-Jankun, and J. Jankun, Plasminogen activator inhibitor type-1 controls the process of the in vitro sprout formation. J Physiol Pharmacol, 2004. **55 Suppl 3**: p. 49-56.

119. Mottonen, J., Arne Strand, Jindrich Symersky, Robert M. Sweet, Dennis E. Danley, Kieran F. Geoghegan, Robert D. Gerard, and Elizabeth J. Goldsmith, *Structural basis of latency in plasminogen activator inhibitor-1*. Nature, 1992. **355**(6357): p. 270-173.

120. Werle, B., et al., Cathepsin B, plasminogenactivator-inhibitor (PAI-1) and plasminogenactivator-receptor (uPAR) are prognostic factors for patients with non-small cell lung cancer. Anticancer Res, 2004. **24**(6): p. 4147-61.

121. Nekarda, H., et al., Prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in completely resected gastric cancer. Cancer Res, 1994. **54**(11): p. 2900-7.

122. Kuhn, W., et al., Urokinase (uPA) and PAI-1 predict survival in advanced ovarian cancer patients (FIGO III) after radical surgery and platinum-based chemotherapy. Gynecol Oncol, 1994. **55**(3 Pt 1): p. 401-9.

123. Schmitt, M., et al., Cancer therapy trials employing level-of-evidence-1 disease forecast cancer biomarkers uPA and its inhibitor PAI-1. Expert Rev Mol Diagn, 2011. **11**(6): p. 617-34.

124. Eljuga, D., et al., Prognostic importance of PAI-1 in node negative breast cancer patients--results after 10 years of follow up. Pathol Res Pract, 2011. **207**(5): p. 290-4.

125. Duffy, M.J. and C. Duggan, The urokinase plasminogen activator system: a rich source of tumour markers for the individualised management of patients with cancer. Clin Biochem, 2004. **37**(7): p. 541-8.

126. Duffy, M.J., et al., uPA and PAI-1 as biomarkers in breast cancer: validated for clinical use in level-of-evidence-1 studies. Breast Cancer Res, 2014. **16**(4): p. 428.

127. Overall, C.M., J.L. Wrana, and J. Sodek, Transforming growth factor-beta regulation of collagenase, 72 kDa-progelatinase, TIMP and PAI-1 expression in rat bone cell populations and human fibroblasts. Connect Tissue Res, 1989. **20**(1-4): p. 289-94.

128. Wakahara, K., et al., Transforming growth factor-beta1-dependent activation of Smad2/3 and up-regulation of PAI-1 expression is negatively regulated by Src in SKOV-3 human ovarian cancer cells. J Cell Biochem, 2004. **93**(3): p. 437-53.

129. Guo, B., et al., MAPK/AP-1-dependent regulation of PAI-1 gene expression by TGF-beta in rat mesangial cells. Kidney Int, 2005. **68**(3): p. 972-84.

130. Escaffit, F., et al., FGF-2 isoforms of 18 and 22.5 kDa differentially modulate t-PA and PAI-1 expressions on the pancreatic carcinoma cells AR4-2J: consequences on cell spreading and invasion. Int J Cancer, 2000. **85**(4): p. 555-62.

131. Tortorella, M.D., et al., A review of the ADAMTS family, pharmaceutical targets of the future. Curr Pharm Des, 2009. **15**(20): p. 2359-74.

132. Nyren-Erickson, E.K., et al., A disintegrin and metalloproteinase-12 (ADAM12): function, roles in disease progression, and clinical implications. Biochim Biophys Acta, 2013. **1830**(10): p. 4445-55.

133. Gilpin, B.J., et al., A novel, secreted form of human ADAM 12 (meltrin alpha) provokes myogenesis in vivo. J Biol Chem, 1998. **273**(1): p. 157-66.

134. Rocks, N., Cataldo, D, ADAM12 (ADAM metallopeptidase domain 12 (meltrin alpha)). Atlas Genet Cytogenet Oncol Haematol, 2009. **13**(4): p. 249-252.

135. Kveiborg, M., et al., ADAM12-S stimulates bone growth in transgenic mice by modulating chondrocyte proliferation and maturation. J Bone Miner Res, 2006. **21**(8): p. 1288-96.

136. Yagami-Hiromasa, T., et al., A metalloprotease-disintegrin participating in myoblast fusion. Nature, 1995. **377**(6550): p. 652-6.

137. Le Pabic, H., et al., ADAM12 in human liver cancers: TGF-beta-regulated expression in stellate cells is associated with matrix remodeling. Hepatology, 2003. **37**(5): p. 1056-66.

138. Zhang, L., et al., Adam12 plays a role during uterine decidualization in mice. Cell Tissue Res, 2009. **338**(3): p. 413-21.

139. Bernstein, H.G., et al., ADAM (a disintegrin and metalloprotease) 12 is expressed in rat and human brain and localized to oligodendrocytes. J Neurosci Res, 2004. **75**(3): p. 353-60.

140. Asakura, M., et al., Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. Nat Med, 2002. **8**(1): p. 35-40.

141. Estrella, C., et al., Role of A disintegrin and metalloprotease-12 in neutrophil recruitment induced by airway epithelium. Am J Respir Cell Mol Biol, 2009. **41**(4): p. 449-58.

142. Frohlich, C., et al., ADAM12 produced by tumor cells rather than stromal cells accelerates breast tumor progression. Mol Cancer Res, 2011. **9**(11): p. 1449-61.

143. Rocks, N., et al., The metalloproteinase ADAM-12 regulates bronchial epithelial cell proliferation and apoptosis. Cell Prolif, 2008. **41**(6): p. 988-1001.

144. Kanakis, D., et al., ADAM 12: a putative marker of oligodendrogliomas? Dis Markers, 2013. **34**(2): p. 81-91.

145. Roy, R., et al., ADAM 12 cleaves extracellular matrix proteins and correlates with cancer status and stage. J Biol Chem, 2004. **279**(49): p. 51323-30.

146. Roy, R. and M.A. Moses, ADAM12 induces estrogen-independence in breast cancer cells. Breast Cancer Res Treat, 2012. **131**(3): p. 731-41.

147. Rao, V.H., et al., A positive feedback loop between HER2 and ADAM12 in human head and neck cancer cells increases migration and invasion. Oncogene, 2012. **31**(23): p. 2888-98.

148. Widelitz, R., Wnt signaling through canonical and non-canonical pathways: recent progress. Growth Factors, 2005. **23**(2): p. 111-6.

149. Huber, A.H., W.J. Nelson, and W.I. Weis, Three-dimensional structure of the armadillo repeat region of beta-catenin. Cell, 1997. **90**(5): p. 871-82.

150. Ozawa, M. and R. Kemler, Altered cell adhesion activity by pervanadate due to the dissociation of alpha-catenin from the E-cadherin.catenin complex. J Biol Chem, 1998. **273**(11): p. 6166-70.

151. Guo, Y., et al., Wnt/beta-catenin signaling: a promising new target for fibrosis diseases. Physiol Res, 2012. **61**(4): p. 337-46.

152. Wang, Z., et al., Clinical implications of beta-catenin protein expression in breast cancer. Int J Clin Exp Pathol, 2015. **8**(11): p. 14989-94.

153. Bukholm, I.K., J.M. Nesland, and A.L. Borresen-Dale, Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients [seecomments]. J Pathol, 2000. **190**(1): p. 15-9.

154. Kawanishi, J., et al., Loss of E-cadherin-dependent cell-cell adhesion due to mutation of the beta-catenin gene in a human cancer cell line, HSC-39. Mol Cell Biol, 1995. **15**(3): p. 1175-81.

155. Tan, Z., et al., MicroRNA-1229 overexpression promotes cell proliferation and tumorigenicity and activates Wnt/beta-catenin signaling in breast cancer. Oncotarget, 2016.

156. Pangon, L., et al., JRK is a positive regulator of beta-catenin transcriptional activity commonly overexpressed in colon, breast and ovarian cancer. Oncogene, 2016. **35**(22): p. 2834-41.

157. Anderson, H.C., Vesicles associated with calcification in the matrix of epiphyseal cartilage. J Cell Biol, 1969. **41**(1): p. 59-72.

158. Crawford, N., The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma. Br J Haematol, 1971. **21**(1): p. 53-69.

159. Dvorak, H.F., et al., Tumor shedding and coagulation. Science, 1981. **212**(4497): p. 923-4.

160. Trams, E.G., et al., Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta, 1981. **645**(1): p. 63-70.

161. George, J.N., et al., Isolation of human platelet membrane microparticles from plasma and serum. Blood, 1982. **60**(4): p. 834-40.

162. Harding, C., J. Heuser, and P. Stahl, Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol, 1983. **97**(2): p. 329-39.

163. Pan, B.T. and R.M. Johnstone, Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell, 1983. **33**(3): p. 967-78.

164. Thery, C., et al., Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.

165. Taylor, D.D., W. Zacharias, and C. Gercel-Taylor, Exosome isolation for proteomic analyses and RNA profiling. Methods Mol Biol, 2011. **728**: p. 235-46.

166. Greening, D.W., et al., A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. Methods Mol Biol, 2015. **1295**: p. 179-209.

167. Tauro, B.J., et al., Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. Methods, 2012. **56**(2): p. 293-304.

168. Colombo, M., G. Raposo, and C. Thery, Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol, 2014. **30**: p. 255-89.

169. Raposo, G. and W. Stoorvogel, Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol, 2013. **200**(4): p. 373-83.

170. Lee, T.H., et al., Microvesicles as mediators of intercellular communication in cancer--the emerging science of cellular 'debris'. Semin Immunopathol, 2011. **33**(5): p. 455-67.

171. Baj-Krzyworzeka, M., et al., Platelet-derived microparticles stimulate proliferation, survival, adhesion, and chemotaxis of hematopoietic cells. Exp Hematol, 2002. **30**(5): p. 450-9.

172. Baj-Krzyworzeka, M., et al., Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunol Immunother, 2006. **55**(7): p. 808-18.

173. Johnstone, R.M., et al., Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). J Biol Chem, 1987. **262**(19): p. 9412-20.

174. Thery, C., et al., Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. J Cell Biol, 1999. **147**(3): p. 599-610.

175. Thery, C., et al., Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol, 2001. **166**(12): p. 7309-18.

176. Thery, C., M. Ostrowski, and E. Segura, Membrane vesicles as conveyors of immune responses. Nat Rev Immunol, 2009. **9**(8): p. 581-93.

177. Chaput, N. and C. Thery, Exosomes: immune properties and potential clinical implementations. Semin Immunopathol, 2011. **33**(5): p. 419-40.

178. Tauro, B.J., et al., Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-derived organoids. Mol Cell Proteomics, 2013. **12**(3): p. 587-98.

179. Luyet, P.P., et al., The ESCRT-I subunit TSG101 controls endosome-to-cytosol release of viral RNA. Traffic, 2008. **9**(12): p. 2279-90.

180. Glebov, O.O., N.A. Bright, and B.J. Nichols, Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol, 2006. **8**(1): p. 46-54.

181. Laulagnier, K., et al., PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. FEBS Lett, 2004. **572**(1-3): p. 11-4.

182. Laulagnier, K., et al., Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization. Biochem J, 2004. **380**(Pt 1): p. 161-71.

183. Trajkovic, K., et al., Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science, 2008. **319**(5867): p. 1244-7.

184. Brouwers, J.F., et al., Distinct lipid compositions of two types of human prostasomes. Proteomics, 2013. **13**(10-11): p. 1660-6.

185. Llorente, A., et al., Molecular lipidomics of exosomes released by PC-3 prostate cancer cells. Biochim Biophys Acta, 2013. **1831**(7): p. 1302-9.

186. Ikonen, E., Roles of lipid rafts in membrane transport. Curr Opin Cell Biol, 2001. **13**(4): p. 470-7.

187. Del Conde, I., et al., Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood, 2005. **106**(5): p. 1604-11.

188. Staubach, S., H. Razawi, and F.G. Hanisch, Proteomics of MUC1-containing lipid rafts from plasma membranes and exosomes of human breast carcinoma cells MCF-7. Proteomics, 2009. **9**(10): p. 2820-35.

189. Lotvall, J. and H. Valadi, Cell to cell signalling via exosomes through esRNA. Cell Adh Migr, 2007. **1**(3): p. 156-8.

190. Pegtel, D.M., et al., Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A, 2010. **107**(14): p. 6328-33.

191. Montecalvo A, L.A., Shufesky WJ, Stolz DB, Sullivan ML,, Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood, 2012(119): p. 756–66.

192. Ismail, N., et al., Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. Blood, 2013. **121**(6): p. 984-95.

193. Batagov, A.O., V.A. Kuznetsov, and I.V. Kurochkin, Identification of nucleotide patterns enriched in secreted RNAs as putative cis-acting elements targeting them to exosome nano-vesicles. BMC Genomics, 2011. **12 Suppl 3**: p. S18.

194. Kucharzewska, P. and M. Belting, Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress. J Extracell Vesicles, 2013. **2**.

195. Segura, E., et al., ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming. Blood, 2005. **106**(1): p. 216-23.

196. Kilpinen, L., et al., Extracellular membrane vesicles from umbilical cord blood-derived MSC protect against ischemic acute kidney injury, a feature that is lost after inflammatory conditioning. J Extracell Vesicles, 2013. **2**.

197. de Jong OG, V.M., Chen Y, Vader P, Gremmels H, Posthuma G, Schiffelers RM, Gucek M, van Balkom BW., Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. J Extracell Vesicles, 2012. **16**(1).

198. Kucharzewska, P., et al., Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. Proc Natl Acad Sci U S A, 2013. **110**(18): p. 7312-7.

199. Parolini, I., et al., Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem, 2009. **284**(49): p. 34211-22.

200. Kharaziha, P., et al., Tumor cell-derived exosomes: a message in a bottle. Biochim Biophys Acta, 2012. **1826**(1): p. 103-11.

201. Bennett, E.M., et al., Clathrin hub expression affects early endosome distribution with minimal impact on receptor sorting and recycling. Mol Biol Cell, 2001. **12**(9): p. 2790-9.

202. Stoorvogel, W., et al., Late endosomes derive from early endosomes by maturation. Cell, 1991. **65**(3): p. 417-27.

203. Yellon, D.M. and S.M. Davidson, Exosomes: nanoparticles involved in cardioprotection? Circ Res, 2014. **114**(2): p. 325-32.

204. Mageswaran, S.K., et al., Binding to any ESCRT can mediate ubiquitin-independent cargo sorting. Traffic, 2014. **15**(2): p. 212-29.

205. Colombo, M., et al., Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci, 2013. **126**(Pt 24): p. 5553-65.

206. van Niel, G., et al., The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev Cell, 2011. **21**(4): p. 708-21.

207. Ghossoub, R., et al., Syntenin-ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. Nat Commun, 2014. **5**: p. 3477.

208. Stenmark, H., Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol, 2009. **10**(8): p. 513-25.

209. Savina, A., M. Vidal, and M.I. Colombo, The exosome pathway in K562 cells is regulated by Rab11. J Cell Sci, 2002. **115**(Pt 12): p. 2505-15.

210. Hsu, C., et al., Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. J Cell Biol, 2010. **189**(2): p. 223-32.

211. Zheng, Y., et al., Monitoring the Rab27 associated exosome pathway using nanoparticle tracking analysis. Exp Cell Res, 2013. **319**(12): p. 1706-13.

212. Ostenfeld, M.S., et al., Cellular disposal of miR23b by RAB27-dependent exosome release is linked to acquisition of metastatic properties. Cancer Res, 2014. **74**(20): p. 5758-71.

213. Zylbersztejn, K. and T. Galli, Vesicular traffic in cell navigation. FEBS J, 2011. **278**(23): p. 4497-505.

214. Rao, S.K., et al., Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. J Biol Chem, 2004. **279**(19): p. 20471-9.

215. Logan, M.R., et al., A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. Allergy, 2006. **61**(6): p. 777-84.

216. Proux-Gillardeaux, V., et al., Expression of the Longin domain of TI-VAMP impairs lysosomal secretion and epithelial cell migration. Biol Cell, 2007. **99**(5): p. 261-71.

217. Fader, C.M., et al., TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim Biophys Acta, 2009. **1793**(12): p. 1901-16.

218. Camussi, G., et al., Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int, 2010. **78**(9): p. 838-48.

219. Graham, T.R., Flippases and vesicle-mediated protein transport. Trends Cell Biol, 2004. **14**(12): p. 670-7.

220. Muralidharan-Chari, V., et al., Microvesicles: mediators of extracellular communication during cancer progression. J Cell Sci, 2010. **123**(Pt 10): p. 1603-11.

221. Muralidharan-Chari, V., et al., ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol, 2009. **19**(22): p. 1875-85.

222. Inal, J.M., et al., *Microvesicles in health and disease.* Arch Immunol Ther Exp (Warsz), 2012. **60**(2): p. 107-21.

223. Taylor, D.D. and C. Gercel-Taylor, Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. Semin Immunopathol, 2011. **33**(5): p. 441-54.

224. Antonyak, M.A., et al., Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells. Proc Natl Acad Sci U S A, 2011. **108**(12): p. 4852-7.

225. Luga, V., et al., Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell, 2012. **151**(7): p. 1542-56.

226. Langer, F., et al., Tissue factor procoagulant activity of plasma microparticles in patients with cancer-associated disseminated intravascular coagulation. Ann Hematol, 2008. **87**(6): p. 451-7.

227. Escobar, P.F., et al., The 2003 revised TNM staging system for breast cancer: results of stage re-classification on survival and future comparisons among stage groups. Ann Surg Oncol, 2007. **14**(1): p. 143-7.

228. Park, Y.H., et al., Clinical relevance of TNM staging system according to breast cancer subtypes. Ann Oncol, 2011. **22**(7): p. 1554-60.

229. Orucevic, A., et al., Is the TNM staging system for breast cancer still relevant in the era of biomarkers and emerging personalized medicine for breast cancer - an institution's 10-year experience. Breast J, 2015. **21**(2): p. 147-54.

230. Liberman, L., et al., Epithelial displacement after stereotactic 11-gauge directional vacuum-assisted breast biopsy. AJR Am J Roentgenol, 1999. **172**(3): p. 677-81.

231. Tardivon, A.A., et al., Histological findings in surgical specimens after core biopsy of the breast. Eur J Radiol, 2002. **42**(1): p. 40-51.

232. Taylor, D.D. and C. Gercel-Taylor, MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol, 2008. **110**(1): p. 13-21.

233. Rabinowits, G., et al., Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer, 2009. **10**(1): p. 42-6.

234. Silva, J., et al., Analysis of exosome release and its prognostic value in human colorectal cancer. Genes Chromosomes Cancer, 2012. **51**(4): p. 409-18.

235. Bieche, I., et al., Quantification of estrogen receptor alpha and beta expression in sporadic breast cancer. Oncogene, 2001. **20**(56): p. 8109-15.

236. Lagiou, P., et al., Estrogen alpha and progesterone receptor expression in the normal mammary epithelium in relation to breast cancer risk. Int J Cancer, 2009. **124**(2): p. 440-2.

237. Klinge, C.M., et al., Estrogen receptor alpha 46 is reduced in tamoxifen resistant breast cancer cells and re-expression inhibits cell proliferation and estrogen receptor alpha 66-regulated target gene transcription. Mol Cell Endocrinol, 2010. **323**(2): p. 268-76.

238. Kerdivel, G., G. Flouriot, and F. Pakdel, Modulation of estrogen receptor alpha activity and expression during breast cancer progression. Vitam Horm, 2013. **93**: p. 135-60.

239. Yan, Y., et al., Expression of both estrogen receptor-beta 1 (ER-beta1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with estrogen receptor-alpha (ER-alpha)-negative early breast cancer (EBC). Ann Oncol, 2013. **24**(8): p. 1986-93.

240. Hosseini, A., et al., Estrogen receptor alpha gene expression in breast cancer tissues from the Iranian population--a pilot study. Asian Pac J Cancer Prev, 2014. **15**(20): p. 8789-91.

241. Soule, H.D., et al., A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst, 1973. **51**(5): p. 1409-16.

242. Cailleau, R., M. Olive, and Q.V. Cruciger, Long-term human breast carcinoma cell lines of metastatic origin: preliminary characterization. In Vitro, 1978. **14**(11): p. 911-5.

243. Keydar, I., et al., Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer, 1979. **15**(5): p. 659-70.

244. Tomlinson, G.E., et al., Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. Cancer Res, 1998. **58**(15): p. 3237-42.

245. Oliveros, J.C., Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html, 2007-2015.

246. Paul D. Thomas, M.J.C., Anish Kejariwal, Huaiyu Mi, Brian Karlak, Robin Daverman, Karen Diemer, Anushya Muruganujan, Apurva Narechania., *PANTHER: a library of protein families and subfamilies indexed by function.* Genome Res, 2003. **13**(2129-214).

247. Cline, M.S., et al., Integration of biological networks and gene expression data using Cytoscape. Nat Protoc, 2007. **2**(10): p. 2366-82.

248. Lindahl, A.K., M.C. Boffa, and U. Abildgaard, *Increased plasma thrombomodulin in cancer patients.* Thromb Haemost, 1993. **69**(2): p. 112-4.

249. Koizume, S., et al., Activation of cancer cell migration and invasion by ectopic synthesis of coagulation factor VII. Cancer Res, 2006. **66**(19): p. 9453-60.

250. Dwyer, M.A., et al., WNT11 expression is induced by estrogen-related receptor alpha and beta-catenin and acts in an autocrine manner to increase cancer cell migration. Cancer Res, 2010. **70**(22): p. 9298-308.

251. Ravindranath, A., et al., Wnt-beta-catenin-Tcf-4 signalling-modulated invasiveness is dependent on osteopontin expression in breast cancer. Br J Cancer, 2011. **105**(4): p. 542-51.

252. Ferroni, P., et al., Plasma plasminogen activator inhibitor-1 (PAI-1) levels in breast cancer - relationship with clinical outcome. Anticancer Res, 2014. **34**(3): p. 1153-61.

253. Ma, B., et al., ADAM12 expression predicts clinical outcome in estrogen receptor-positive breast cancer. Int J Clin Exp Pathol, 2015. **8**(10): p. 13279-83.

254. Ruff, M., et al., The Disintegrin and Metalloprotease ADAM12 Is Associated with TGF-beta-Induced Epithelial to Mesenchymal Transition. PLoS One, 2015. **10**(9): p. e0139179.

255. Lhotak, S., et al., Immunolocalization of matrix metalloproteinases and their inhibitors in clinical specimens of bone metastasis from breast carcinoma. Clin Exp Metastasis, 2000. **18**(6): p. 463-70.

256. Coezy, E., J.L. Borgna, and H. Rochefort, Tamoxifen and metabolites in MCF7 cells: correlation between binding to estrogen receptor and inhibition of cell growth. Cancer Res, 1982. **42**(1): p. 317-23.

257. Horwitz, K.B., M.B. Mockus, and B.A. Lessey, Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. Cell, 1982. **28**(3): p. 633-42.

258. Powell, C.E., A.M. Soto, and C. Sonnenschein, Identification and characterization of membrane estrogen receptor from MCF7 estrogen-target cells. J Steroid Biochem Mol Biol, 2001. **77**(2-3): p. 97-108.

259. Cassoni, P., et al., Oxytocin modulates estrogen receptor alpha expression and function in MCF7 human breast cancer cells. Int J Oncol, 2002. **21**(2): p. 375-8.

260. Johnson, A.E., et al., Estrogen-dependent growth and estrogen receptor (ER)-alpha concentration in T47D breast cancer cells are inhibited by VACM-1, a cul 5 gene. Mol Cell Biochem, 2007. **301**(1-2): p. 13-20.

261. Sotoca, A.M., et al., Quantitative proteomics and transcriptomics addressing the estrogen receptor subtype-mediated effects in T47D breast cancer cells exposed to the phytoestrogen genistein. Mol Cell Proteomics, 2011. **10**(1): p. M110 002170.

262. Erenburg, I., et al., Loss of an estrogen receptor isoform (ER alpha delta 3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. Mol Endocrinol, 1997. **11**(13): p. 2004-15.

263. Yoshida, T., et al., Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. Carcinogenesis, 2000. **21**(12): p. 2193-201.

264. Naughton, C., et al., Progressive loss of estrogen receptor alpha cofactor recruitment in endocrine resistance. Mol Endocrinol, 2007. **21**(11): p. 2615-26.

265. Stossi, F., Z. Madak-Erdogan, and B.S. Katzenellenbogen, Macrophage-elicited loss of estrogen receptor-alpha in breast cancer cells via involvement of MAPK and c-Jun at the ESR1 genomic locus. Oncogene, 2012. **31**(14): p. 1825-34.

266. Angelucci, A., et al., Vesicle-associated urokinase plasminogen activator promotes invasion in prostate cancer cell lines. Clin Exp Metastasis, 2000. **18**(2): p. 163-70.

267. Ian del Conde, C.N.S., Perumal Thiagarajan, and José A. López, Tissue-factor–bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood, 2005. **106**(5): p. 1604-1411.

268. Gabriel, K., et al., Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer. PLoS One, 2013. **8**(7): p. e70047.

269. Shedden, K., et al., Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. Cancer Res, 2003. **63**(15): p. 4331-7.

270. Kaplan, R.N., et al., VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature, 2005. **438**(7069): p. 820-7.

271. Han, W.D., et al., Up-regulation of LRP16 mRNA by 17beta-estradiol through activation of estrogen receptor alpha (ERalpha), but not ERbeta, and promotion of human breast cancer MCF-7 cell proliferation: a preliminary report. Endocr Relat Cancer, 2003. **10**(2): p. 217-24.

272. Kang, H.J., et al., Differential regulation of estrogen receptor alpha expression in breast cancer cells by metastasis-associated protein 1. Cancer Res, 2014. **74**(5): p. 1484-94.

273. Kocaturk, B. and H.H. Versteeg, Tissue factor isoforms in cancer and coagulation: may the best isoform win. Thromb Res, 2012. **129 Suppl 1**: p. S69-75.

274. Cuello-Carrion, F.D., et al., HER2 and beta-catenin protein location: importance in the prognosis of breast cancer patients and their correlation when breast cancer cells suffer stressful situations. Clin Exp Metastasis, 2015. **32**(2): p. 151-68.

275. Koizume, S., et al., High-level secretion of tissue factor-rich extracellular vesicles from ovarian cancer cells mediated by filamin-A and protease-activated receptors. Thromb Haemost, 2016. **115**(2): p. 299-310.

276. Galindo-Hernandez, O., et al., Elevated concentration of microvesicles isolated from peripheral blood in breast cancer patients. Arch Med Res, 2013. **44**(3): p. 208-14.

277. Cohen, A., et al., A mass spectrometry-based plasma protein panel targeting the tumor microenvironment in patients with breast cancer. J Proteomics, 2013. **81**: p. 135-47.