Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer

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Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer

A thesis submitted to the National University of Ireland as fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

By

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>αB-crystallin</td>
<td>Alpha-basic-crystallin</td>
</tr>
<tr>
<td>BCT</td>
<td>Breast Conserving Therapy</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BLBC</td>
<td>Basal-like breast cancer</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratins</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CNA</td>
<td>Copy number aberration</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Ultra-pure water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide(s)</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotritol</td>
</tr>
<tr>
<td>EC</td>
<td>Endogenous control</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial–to-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>Oestrogen receptor alpha</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>Symbol</td>
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</tr>
<tr>
<td>g</td>
<td>Gram</td>
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<td>GRB7</td>
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</tr>
<tr>
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<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPI</td>
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</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose) polymerase</td>
</tr>
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</tr>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<td>Phosphatase and tensin homolog</td>
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</tr>
<tr>
<td>RQ-PCR</td>
<td>Relative quantitative PCR</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour size, Nodal status, Metastasis</td>
</tr>
<tr>
<td>U</td>
<td>Units(s)</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
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<tr>
<td>µg</td>
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</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
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Communications arising from this work

Peer Reviewed Published Manuscripts

Prospective comparison of outcome after treatment for triple-negative and non-triple-negative breast cancer.

DP Joyce, D Murphy, AJ Lowery, C Curran, K Barry, C Malone, R McLaughlin, MJ Kerin.


Exosome-encapsulated microRNAs as circulating biomarkers for breast cancer.

DP Joyce, Kerin MJ, Dwyer R.


Published Abstracts

Exosome-Mediated Intercellular Communication in the Breast Tumour Microenvironment.

DP Joyce, S Khan, K O’Brien, MJ Kerin, RM Dwyer.


Exosome-mediated trafficking of microRNAs by breast cancer cells.
Cancer Research Volume: 75 Issue: 9 Published: May 1 2015.

British Journal of Surgery Volume: 102 Pages 21-22 Supplement 5
Published Apr 2015.

Investigation of exosome-encapsulated microRNAs as potential circulating biomarkers of breast cancer.

Secretion of Exosome-Encapsulated MicroRNAs by Breast Cancer Cell Lines.

Exosomal Transfer of Micrornas as a Potential Path for Gene Therapy.
Joyce, D.; Glynn, C.; Khan, S.; et al.

The Economic Impact of Breast Cancer Management.
D Joyce, H Heneghan, C Curran, C O’Neill, MJ Kerin.

Investigation of exosome-encapsulated microRNA secretion in breast cancer.
Source: Cancer Research October 2014.

Presentations to Learned Societies


Secretion of exosome-encapsulated microRNAs by triple negative breast cancer cell lines, ASGBI, 30th April-2nd May, Harrogate, England.

Breast cancer-derived exosomes stimulate proliferation of stromal cells in the tumour microenvironment, 2nd annual Matrix Biology Meeting, 2-4th December 2015, UCD (Runner up prize winner).


Investigation of Exosome-encapsulated microRNAs as potential circulating biomarkers of breast cancer (Plenary presentation), 23rd Sylvester O'Halloran Surgical Scientific Symposium Meeting, 6th-7th March 2015, University of Limerick.

It’s what’s inside that counts, SFI Thesisin3 final, 20/10/2014, Dublin.


Poster Presentations


Secretion of exosome-encapsulated microRNAs by basal breast cancer cells in vitro, College of Medicine, Nursing & Health Sciences Postgraduate Research Day, 28/5/2014, NUI Galway.

Grants and awards related to this research

Runner up prize for oral presentation at the 2nd annual Matrix Biology Meeting, 2-4th December 2015.

National Cancer Institute Summer Curriculum in Cancer Prevention 3rd-7th August 2015, Rockville, Maryland, Washington DC, USA. Grant awarded on competitive basis to cover travel, accommodation and subsistence while at the NCI.

Tricia McCarthy memorial scholarship, July 2013-July 2015, €25,000 per annum.


Atlantic Corridor Student Research Conference ‘Highly commendable poster prize’ awarded to a 4th year medical student for work carried out under my supervision, November 2015.
Abstract

Breast cancer is a heterogeneous group of diseases survival from which depends on stage at diagnosis. The development of blood-based biomarkers which may facilitate earlier detection and possibly subtype delineation remains the focus of international research efforts. Exosomes are membrane-derived vesicles that are actively secreted by cells and have been shown to play a role in intercellular communication in the primary breast tumour microenvironment. Exosomes contain genetic material including microRNAs (miRNA), which have the potential to function as circulating biomarkers for breast cancer. In this work, a retrospective cohort study comparing outcomes from Triple-negative and Non-Triple-negative breast cancer was used to demonstrate the inferior prognosis that is associated with the triple-negative phenotype. Exosomes were isolated from breast cancer cell lines and using Transmission Electron Microscopy and Western Blot Analysis were shown to have the characteristic shape, size (30-120nm) and associated protein (CD63). Transfer of exosomes to recipient cells, which was visualized using confocal microscopy, was shown to be capable of stimulating angiogenesis. Cell-secreted exosomal miRNAs were profiled and potentially interesting targets were investigated in matched whole blood and serum exosomal samples of patients with breast cancer and healthy controls. MiR-451a was found to be up-regulated in serum exosomes of patients with breast cancer. An in vivo model of breast cancer was also established to identify serum exosomal miRNAs that may be indicative of breast cancer. MiR-223 was identified as being potentially valuable for subtype discrimination in breast cancer. This work highlighted the need for an exosome-specific marker which would allow accurate isolation, characterisation and quantification of these microvesicles. Furthermore, the requirement for robust endogenous controls for use in serum exosomal work in addition to the importance of reproducible data normalisation was demonstrated. This exciting field offers huge potential and the clinical applicability of serum exosomal miRNAs will be investigated going forward.
Chapter 1

Introduction
1.1 Breast cancer

1.1.1 Overview

Breast cancer is a highly prevalent disease and is currently the most common female cancer in Ireland (1). Figures recently published by the National Cancer Registry, Ireland show that approximately 2,800 new cases of invasive breast cancer are diagnosed annually (1). This figure accounts for 30.2% of all invasive cancers diagnoses (1). Breast cancer is responsible for the second highest number of cancer related deaths in Irish women with figures from 2012 showing 689 mortalities (1). Superior survival rates have been identified in cases where breast cancer is detected at an early stage (2) (Table 1.1). As a result of this the National Breast Screening Programme, BreastCheck, was established with the aim of reducing deaths from breast cancer by detecting and treating the disease at an early stage. This government-funded programme provides free mammograms to women between the ages of 50-64 on an area by area basis every two years. Plans to extend BreastCheck are currently underway and by the end of 2021, all eligible women aged 50 to 69 will be invited for routine screening (3).

<table>
<thead>
<tr>
<th>Disease Stage at Diagnosis</th>
<th>5-year Relative Survival</th>
<th>95% Confidence Interval</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>96.1%</td>
<td>(91.2-98.3%)</td>
</tr>
<tr>
<td>II</td>
<td>89.5%</td>
<td>(86.7-91.7%)</td>
</tr>
<tr>
<td>III</td>
<td>66.4%</td>
<td>(61.4-71.0%)</td>
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<tr>
<td>IV</td>
<td>28.1%</td>
<td>(23.7-32.8%)</td>
</tr>
</tbody>
</table>

Table 1.1 Five-year relative survival for breast cancer by tumour stage at diagnosis (4).
Invasive breast cancer is being diagnosed more commonly than previously. The annual incidence increased by 1.7% annually between 1994 and 2012 (1). This was accompanied by an increase in breast cancer-related deaths with 652 mortalities reported in 1994 and 679 in 2009 (4). Five-year relative survival has, however, also increased from 72.1% in the period 1994-1999 to 81.4% between 2008 and 2012 (1) (Table 1.2). This decrease in mortality is multifactorial and has been attributed to both earlier detection and improved therapeutic options (5).

<table>
<thead>
<tr>
<th>Years</th>
<th>Net Survival</th>
<th>95% confidence intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994-1999</td>
<td>72.1%</td>
<td>(70.7-73.5%)</td>
</tr>
<tr>
<td>2000-2005</td>
<td>78.5%</td>
<td>(77.3-79.7%)</td>
</tr>
<tr>
<td>2006-2011</td>
<td>81.2%</td>
<td>(79.9-82.5%)</td>
</tr>
<tr>
<td>2008-2012*</td>
<td>81.4%</td>
<td>(80.2-82.6%)</td>
</tr>
</tbody>
</table>

Table 1.2 Five-year net survival from invasive female breast cancer. Table adapted from the National Cancer Registry, Ireland. Last updated March 2015. *hybrid estimate

**1.1.2 Breast cancer subtypes**

Breast cancer represents a complex heterogeneous group of tumours that display significant diversity with respect to histopathological features and therapeutic response (5, 6). Conventionally invasive breast cancer has been classified according to tumour morphology into infiltrating ductal and lobular carcinomas, tubular carcinoma, mucinous carcinoma, medullary carcinoma, invasive papillary carcinoma, metaplastic carcinoma and a small number of less common subtypes (7). The seminal work carried out by the Stanford group using gene-expression profiling allowed the identification of unique molecular portraits for breast tumours (8).
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Figure 1.1 Hierarchical clustering of 115 tumour tissues and 7 non-malignant tissues using the “intrinsic” gene set. Experimental dendrogram showing the clustering of the tumours into five subgroups. Branches corresponding to tumours with low correlation to any subtype are shown in grey (9).

Breast cancer can be broadly classified into oestrogen receptor (ER)-positive and ER-negative groups, which are then further subdivided into 5 distinct subtypes namely luminal A, luminal B, normal breast-like, human epithelial growth factor receptor-2 (HER2) over-expressing, and Basal-like breast cancer (BLBC) (9, 10) (Figure 1.1, Table 1.3).

<table>
<thead>
<tr>
<th>Epithelial Subtype</th>
<th>Receptor status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER+/PR+/HER2-</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+/PR+/HER2+</td>
</tr>
<tr>
<td>HER2</td>
<td>ER-/PR-/HER2+</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER-/PR-/HER2-</td>
</tr>
</tbody>
</table>

Table 1.3 Receptor status of breast tumours by epithelial subtype.

The breast cancer subtypes were named in accordance with the gene expression patterns of 2 epithelial cell types which are found in the normal
adult breast. The first cell type is luminal epithelial cells which line the ducts and give rise to the majority of breast cancers. These cells express Cytokeratins (CK) 8/18. The second cell type is surface or basal myoepithelial cells which form a layer that surrounds the luminal cells. These cells are juxtaposed to the basement membrane and are characterized by expression of cytokeratins 5/6 and 17 (9) (Figure 1.2). Additional cell types that are present in the breast tumour microenvironment include Mesenchymal Stem Cells and their Tumour-Associated Fibroblasts which form part of the stroma (Figure 1.2). These cells are known to give both structural and functional support to the tumour. Additionally, macrophages, endothelial cells and leucocytes are involved in cellular interactions in the tumour microenvironment (Figure 1.2).

**Figure 1.2** Schematic of normal breast architecture, and breast tumour and surrounding stroma, illustrating some of the tumour-intrinsic and microenvironmental variables contributing to disease heterogeneity. (A) Normal breast architecture; (B) Breast tumour and surrounding stroma. TAM=tumour-associated macrophage. Image by Bertos and Park (11).
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Approximately 75% of breast cancers are ER and/or PR receptor positive (12). The ER-positive tumours express ER, PR, ER-responsive genes, and other genes that encode proteins which are characteristic of luminal epithelial cells, therefore this group are referred to as the “luminal group”. Luminal tumours are further subdivided into luminal A and luminal B subtypes based on the level of expression of proliferation-related genes and/or HER2 (10, 12). The luminal A subtype is characterized by high levels of expression of ERα gene, GATA binding protein (globin transcription factor) 3 (GATA 3), B-cell CLL (chronic lymphocytic leukaemia)/lymphoma 2 (BCL2), luminal cytokeratin 8 (CK8), CK 18, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3α, oestrogen-regulated LIV-1, ERBB3 and ERBB4. The luminal B subgroup is characterized by low to moderate expression of the luminal-specific genes including ER-clusters (10, 12). ER expression is the main indicator of potential response to endocrine therapy. The use of adjuvant systemic therapy has led to a significant improvement in survival and a reduction in disease relapse, especially in women with early stage breast cancer and those with ER-positive tumours, who may receive endocrine therapy alone or in combination with cytotoxic therapy. Targeted drugs that inhibit the ER or oestrogen-activated pathways include the selective ER modulators (SERMs e.g. tamoxifen, raloxifene and lasofoxifene) and aromatase inhibitors (anastrozole, letrozole and exemestane) (13, 14). The Oncotype DX ® 21 gene assay is used in early stage ER+ breast cancer to determine the individual risk of cancer recurrence and identifies patients with minimal, if any, likelihood of benefit, and patients with substantial likelihood of benefit from chemotherapy (15).

The ER-negative group of tumours comprise approximately 20-25% of breast cancers. This group is subdivided into 3 groups: HER2-amplified, Basal-like breast cancer and normal breast-like (10, 12, 16). Tumours in the HER2-amplified group are characterized by amplification of genes located in the HER2 amplicon on 17q21 such as HER2 and growth-factor receptor bound protein 7 (GRB7). HER2-positive tumours also express a high level
of nuclear factor (NF)-κB activation and GATA4. They lack expression of ER and GATA3 (17, 18). Patients with HER2 overexpressing tumours may benefit from targeted therapy in the form of HER2 inhibitors, including trastuzumab and the dual Epidermal Growth Factor Receptor (EGFR)/HER2 inhibitor lapatinib. The humanized HER2 antibody trastuzumab targets the extracellular domain of HER2. Lapatinib inhibits the kinase activity of HER2 and EGFR (14). These agents have been shown to be effective in the adjuvant setting for patients with HER2-amplified breast cancer (14, 19).

Basal-like tumours largely lack expression of ER, PR and HER2. Cells express high levels of basal/myoepithelial markers (20). Basal-like breast cancers account for 14-20% of all cases of breast cancer (8-10, 21-23). However, Carey et al. (2006) (21) found a significantly higher incidence in premenopausal African-American women with up to 39% of patients having this subtype of breast cancer (21). These aggressive tumours are associated with a poor prognosis due to a poor response to current chemotherapeutic regimens and a lack of targeted therapies (24). As the basal-like subgroup of tumours show low expression of hormone receptor–and HER2-related genes, they are often referred to as Triple-negative breast cancers (25).

Basal subtype tumours are characterized by high levels of expression of basal/myoepithelial markers including CK 5/6/14/17, laminin, epidermal growth factor receptor (EGFR), vimentin, p-cadherin, fascin, caveolins 1 and 2 and alpha-basic-crystallin (αB-crystallin) (5, 26-31). Breast tumours with a germ-line mutation of BRCA1 (BRCA1 tumours) and Basal-like breast cancer have been shown to be associated with a high rate of TP53 mutation (32). One study found that TP53 was frequently mutated in BRCA1 (97%) and sporadic Basal-like breast cancers (92%). However, the investigators showed a higher rate of complex mutations, such as insertion/deletion, in the BRCA1 Basal-like breast cancers compared to the sporadic group of Basal-like cancers (42% and 9% respectively) (32).

Gene-expression profiling is currently considered to be the ‘gold standard’ in terms of identification of breast cancer however this technology is not
readily available in the clinical setting. It is possible, however, to employ immunohistochemical staining as a surrogate of gene-expression profiling. A number of investigators have examined the optimum immunohistochemical profile of Basal-like breast cancer. Nielsen et al. (2004) (26) identified a panel of four antibodies- ER, HER1, HER2 and cytokeratin 5/6- which can identify Basal-like breast cancer with 100% specificity and 76% sensitivity. In addition, they found that although c-KIT expression was more common in basal-like tumours it did not influence prognosis (26). Similarly, Livasy et al. (2006) (33) found that the most consistent immunophenotype seen in Basal-like breast cancer was negativity for ER and HER2, and positivity for vimentin, EGFR, cytokeratin 8/18 and cytokeratin 5/6 (33). More recently a parallel comparison of 46 proposed immunohistochemical biomarkers of Basal-like breast cancer against a gene expression profile gold standard on a tissue microarray found that Ki67 and PPH3 were the most sensitive biomarkers (both 92% sensitivity) positively expressed in the basal-like subtype. CK14, IMP3 and NGFR were the most specific (100%). Loss of INPP4B (a negative regulator of phosphatidylinositol signalling) showed the strongest association with Basal-like breast cancer (61% sensitivity and 99% specificity with the highest odds ratio (OR) at 108). The expression of nestin (a common marker of neural progenitor cells) showed the second highest OR at 29 and had a sensitivity of 54% and 96% specificity. However, as nestin is a positively expressed biomarker it possesses technical advantages over INPP4B and is therefore a more suitable candidate as a biomarker of Basal-like breast cancer. This study represents an important step in the determination of an optimized surrogate immunopanel for the definition of Basal-like breast cancer in a manner which is clinically applicable (34).

The gene expression profile of Basal-like breast cancer provides a myriad of candidate genes that might contribute to the aggressive basal-like phenotype. It also suggests a less differentiated ‘breast stem/progenitor’ cell origin for these tumours (35).
The normal breast-like subgroup is poorly understood (20). These tumours resemble normal breast tissue by high expression of many genes which are characteristic of adipose tissue and other non-epithelial cell types. In addition, they have low levels of expression of luminal markers (8). The prognosis for the aforementioned breast cancer subtypes ranges from good in the luminal A group, to poor in the HER2 and Basal-like cancers (8-10, 36, 37) (Figure 1.3).

**Figure 1.3 Survival from breast cancer by epithelial subtype. Dark blue=Luminal A; Green=Normal Like; Pink=HER2; Red=Basal-like; Turquoise=Highly-proliferating luminals.**

### 1.1.3 Inherited breast cancer

The tumour suppressor genes BRCA1 and BRCA2 play a critical role in the repair of DNA damage (38). It is well recognised that inactivation of either
of these genes significantly increases cancer risk and development (39-41). Mechanisms of allelic inactivation include germline mutations, somatic mutations, and epigenetic down-regulation (38). BRCA1-related tumours are known to be predominantly basal-like, with one study demonstrating that three quarters of cases were of the of the basal-like subtype (42). The vast majority of BRCA1-related breast cancers are triple-negative, positive for Ki67 and express basal-like markers such as CK5/6/14/, p53, EGFR and P-cadherin (42-45). In addition, BRCA1-related Basal-like breast cancers are frequently associated with X-chromosome abnormalities (46, 47). Patients with Basal-like breast cancer have poor outcomes similar to those with BRCA1-related tumours (48). The similarities between BRCA1-related breast cancer and basal-like tumours suggest that a defect in the BRCA1 pathway may be implicated in sporadic Basal-like breast cancer (49). It is not clear, however, whether BRCA1 inactivation is the cause of or a consequence of the basal-like subtype (50). Two hypotheses have been advanced in order to explain the similarities between Basal-like cancers and BRCA1-related tumours: (i) the precursors of Basal-like cancers and invasive Basal-like breast carcinomas may be more tolerant to loss of BRCA1 function than those of other breast cancer subtypes, possibly due to the phenotype of the cell of the initiating event or the concurrent inactivation of other tumour suppressor genes, such as p53; alternatively, (ii) BRCA1 may be involved in the differentiation of breast epithelial cells and, therefore, BRCA1 inactivation would lead to tumours with a stem cell-like phenotype (50). Evidence to support a BRCA1 deficiency in sporadic Basal-like cancers includes decreased BRCA1 transcript levels (51) and nuclear protein expression (52) in some Basal-like cancers. Furthermore, BRCA1 promoter hypermethylation has been reported in metaplastic breast cancers (a rare type of basal-like tumour), and overexpression of ID4, (a negative regulator of BRCA1 expression), has been demonstrated in Basal-like cancers (51).

BRCA1 and BRCA2 gene mutations only partly explain inherited predisposition to breast cancer with many families with an
apparent familial clustering of breast cancer not being diagnosed with mutations in either of these genes (53). These gene mutations are estimated to account for only 2% of breast cancer cases (53). Additional breast cancer predisposition genes include PALB2 which confers a high penetrance breast cancer risk on affected individuals (53). In addition to this PTEN, STK11, TP53, ATM, BARD1, BRIP1, CDH1, CHEK2, MRE11A, MUTYH, NBN, NF1, RAD50 and RAD51C are regularly included in breast cancer susceptibility gene panels (53, 54). Large-scale genotyping studies have identified approximately 100 independent common variants (consisting primarily of single-nucleotide polymorphisms (SNPs)) associated with breast cancer risk (54). These variants typically have minor allele frequencies higher than 1%. They confer risks that are less than 1.5 times as high as those in the general population (54).

1.1.4 Basal-like breast cancer versus Triple-negative breast cancer

The Triple-negative group of breast cancers is primarily, but not exclusively, made up of the basal-like subtype (25). Triple-negative breast cancers are negative for expression of the ER, PR and HER2 receptors (55). The majority of Basal-like breast cancers are triple-negative however there is some discordance between the triple-negative and basal-like groups (Figure 1.4).
Breast cancers that express basal markers, such as CK5 and EGFR, overlap considerably with Triple-negative breast cancers, but the overlap is not complete and not all Triple-negative tumours are positive for these markers. A subset of Triple-negative tumours has an expression profile indicating a strong immune response, presumably to breast cancer cell surface antigens.
This feature may have prognostic implications for TNBC. There are additional molecular features that characterize some but not all Triple-negative tumours including but not limited to p53 mutation (light blue), BRCA1 germ-line mutation (dark blue), and αB crystallin expression (light green). Which of these various subsets of TN cancer will have clinical differences or treatment implications is yet to be determined (25).

1.1.5 Breast cancer biomarkers

Breast cancer is a heterogeneous disease which poses both diagnostic and therapeutic challenges. The importance of classification accuracy on choice of therapy has been demonstrated. An increased understanding of the underlying disease processes through the use of molecular profiling may provide a greater range of therapeutic targets for the aggressive basal-like subtype. This may in turn improve prognosis for the subgroup of patients with this disease.

The recognition of breast cancer as a diverse group of diseases has led to the general acceptance that targeted therapy based on cancer subtype represents the optimal therapeutic strategy. On a similar vein, the diagnostic approaches to breast cancer would allow not only for the detection of disease but would also be capable of delineating tumour subtype. Traditional diagnostic methods, such as mammography, although very effective, are limited in that they require a minimum tumour size for detection. This can lead to the situation where loco-regional or possibly distant metastasis may have already taken place prior to breast cancer diagnosis. In addition to this, not all breast tumours are detectable mammographically at an early stage when they are amenable to treatment. Mammography carries a population-based sensitivity of 75-90% with this figure falling to 30-48% in dense breasts which are more frequent in young women (56, 57). Specificities of 90-95% have been reported in the literature (56). Population-based studies have shown that approximately 50% of
women screened annually for 10 years in the United States will experience a false positive. 7-17% of this cohort will proceed to biopsy. 6-46% of women with invasive cancer will have negative mammograms (false negative), especially if they are young, have dense breasts, or have mucinous, lobular, or rapidly growing cancers (58). It is as a result of these challenges, and the influence that stage at diagnosis has on survival, that multiple research groups have focused on blood-based biomarkers which will facilitate detection of breast cancer in its infancy before it has spread beyond the primary site. MicroRNAs (miRNA) have shown immense potential in this setting.

1.2 MicroRNAs (MiRNAs)

1.2.1 Definition and synthesis

MiRNAs are small non-coding RNA molecules that measure 18–24 nucleotides in length and are known to regulate gene expression post-transcriptionally (59). MiRNAs are known to play important roles in a wide range of biological processes (60). The system of miRNA nomenclature provides information on species of origin, for example ‘hsa’ denotes Homo sapiens. The presence of a lowercase or uppercase “r” in hsa-mir versus hsa-miR indicates precursor versus mature miRNA, respectively. Mir/miR is followed by a number with lower numbers indicating earlier discovery. Some miRNAs have a letter (e.g. a or b) after the number which differentiates highly similar sequences that are separately encoded. MiRNA nomenclature allows researchers to distinguish the two single stranded mature products that originate from the two strands present in the double-stranded precursor miRNA. For example, hsamIR-18b-5p and hsa-miR-18b-3p indicate the 5’ or 3’ strand origin of the mature miRNA (61).

MiRNAs are synthesized in the nucleus via the sequential actions of the endonucleases Drosha and Dicer (62) (Figure 1.5). MiRNAs are first transcribed by RNA polymerase II as longer transcripts known as pri-miRNAs. This is followed by endonuclear processing by the RNase III
nuclease known as Drosha and a protein, Pasha, into precursor miRNAs (pre-miRNAs) measuring approximately 70 nucleotides in length (62-65). Pre-miRNAs take on a stem-loop structure and contain a 5′-phosphate and a 2-nucleotide 3′-overhang (63). They are then transported out of the nucleus into the cytoplasm where Dicer acts to excise mature miRNAs from a stem of the pre-miRNA (64, 66, 67). These mature miRNAs associate with RNA-induced silencing complexes (RISC) in order to target messenger RNAs (mRNA) (62, 68). RISC-loaded miRNAs bind to target mRNAs to exert their function in the form of translational inhibition, destabilization of RNA or mRNA cleavage (68-70). A key step in this process is for miRNAs to complex with RISC-loading complex proteins Dicer, TRBP and AGO2. Dicer and TRBP are responsible for processing pre-miRNAs into miRNAs following their emergence from the nucleus (mediated by Exportin-5). The miRNAs then associate with AGO2 (59).
Figure 1.5 Schematic of miRNA biogenesis. Image by Asgari (71).
MicroRNAs exert their function via sequence-specific regulation of gene expression post-transcriptionally. The mRNA target recognition region is located in the 5′-end of mature miRNAs and is commonly referred to as the ‘seed-sequence’. MiRNAs have 2 main mechanisms of action which depend on the complementarity of the miRNA seed sequence with its target mRNA. The first of these involves the binding of miRNA to protein-coding mRNA sequences with perfect base-pairing homology which induces the RNA-mediated interference (RNAi) pathway leading to cleavage of mRNA by Argonaute in the RISC. An alternative mechanism by which miRNAs regulate target genes, is through binding to partially complementary sequences in the 3′ untranslated region (UTR) of downstream target coding mRNAs which leads to repression of protein translation (72).

1.2.2 MicroRNAs as biomarkers for breast cancer

MiRNAs have been shown to be dysregulated in a variety of cancers (73, 74), including breast cancer (75). While these changes are detectable in the malignant tissue of patients with breast cancer, dysregulated miRNA levels can also be measured in the blood of patients (75). This feature of miRNAs was demonstrated in the work by Heneghan et al., (2010) (75) which detected significant up-regulation of miR-195and let7a in the whole blood of patients with breast cancer (n=83) when compared to healthy controls (n=44), with a corresponding fold-change of 19 and 11 respectively. Circulating miR195 levels were also shown to detect patients with breast cancer with 85.5% sensitivity and 100% specificity. Sensitivity and specificity for let7a was 77.6% and 100% respectively. Furthermore, expression of both miRNAs was found to decrease significantly post-tumour resection to levels in line with that of healthy controls. In terms of tumour miRNA expression, miR195 levels were significantly higher in malignant tissue when compared to tumour-associated normal (TAN) tissue (75). This area is, however, fraught with challenges and despite immense promise, has not been successfully implemented in the clinical setting. One reason for
this is that it is unclear as to which fraction of blood is the ideal source for the detection of cancer-related miRNAs, with whole blood, serum and plasma as starting materials all being reported in the literature (76-78). The lack of a standardised approach has resulted in conflicting results, with miR10b, for example, being observed at significantly higher levels in the serum of breast cancer patients when compared to healthy controls in one study, while a separate study reported no significant difference in miR10b levels in the whole blood of breast cancer patients versus healthy individuals (76, 77). In addition to this, investigators are focusing efforts on the discovery of circulating miRNAs which may allow breast cancer subtypes to be distinguished from each other (79-81). These challenges may be addressed at least in part through the study of a specific fraction of blood known as exosomes.

1.3 Exosomes

1.3.1 Introduction

Exosomes are membrane-derived vesicles that are actively secreted by cells (82). While previously thought of as a means by which cells could dispose of unwanted biomolecules, they are now known to be capable of the transfer of genetic material including messenger RNA (mRNA), miRNA and small regulatory RNAs (sRNAs) (83-86) (Figure 1.5).
Numerous studies have shown that miRNA-containing exosomes are secreted into the circulation (83, 88). As such, exosome-encapsulated miRNAs may represent an ideal biomarker for diseases at an early stage (89). Exosomes have been shown to confer the phenotypic traits of parent cells on recipient cells (90, 91). They are released by a variety of cell types including reticulocytes, epithelial cells, B cells, T cells and cancer cells (92-96). These nanovesicles may be isolated from a variety of bodily fluids, including serum (97, 98), and their miRNA content has been shown to reflect that of parent breast cancer cells (91, 99, 100). This may allow researchers both to identify a breast tumour and to stratify the subtype to which it belongs.

**1.3.2 Definition and synthesis**

Exosomes have been defined as microscopic vesicles that have a ‘saucer-like’ morphology and are composed of a lipid bilayer (101). They arise from
intraluminal vesicles (ILV), which are formed within cellular multi-vesicular bodies (MVB, also referred to as multivesicular endosomes (MVE)) and are released into the extracellular compartment upon fusion of the MVB with the plasma membrane (82) (Figure 1.7).

Figure 1.7 Schematic of protein and RNA transfer by Exosomes (102). Membrane-associated=triangles, transmembrane proteins=rectangles and numbers refer to sequential steps described in the text.

ILVs accumulate during endosome maturation (giving a multi-vesicular appearance), during which transmembrane and peripheral membrane proteins are incorporated into their membrane (Figure 1.7). Sorting of proteins and lipids at the limiting membrane of endosomes during ILV formation leads to the encapsulation of a specific set of molecules within these nanovesicles. This cargo sorting process is thought to be mediated by ESCRT components, lipids and/or tetraspanins-enriched microdomains. Exosomes from different cellular types contain a common set of molecules but also display parent cell specific components (103). The mechanism of
MVB fusion with the plasma membrane and subsequent exosomal release, although not fully elucidated, is thought to be dependent on the Rab 11 GTPase and others (82). Once released into the extracellular milieu, ILVs are termed exosomes (82). Exosomes typically measure 50-100nm in diameter as a result of the restrictive size of the corresponding ILVs (82). The mechanism of exosome uptake in recipient cells is outlined in Figure 1.7 and may include the following steps: MVs and exosomes may dock at the plasma membrane of a target cell (Figure 1.7 (1)); bound vesicles may either fuse directly with the plasma membrane (Figure 1.7 (2)) or be endocytosed (Figure 1.7 (3)); endocytosed vesicles may then fuse with the delimiting membrane of an endocytic compartment (Figure 1.7 (4)). Both pathways result in the delivery of proteins and RNA into the membrane or cytosol of the target cell.

The definition of what constitutes an exosome has come under intense scrutiny in recent years due to a lack of consensus in terms of terminology and size. These issues were discussed at the inaugural meeting of the International Society for Extracellular Vesicles (ISEV) in Gothenberg, Sweden in 2012, relevant points from which were published by Gould and Raposo in the Journal of Extracellular Vesicles the following year (104). The first issue that was debated was that of nomenclature of extracellular vesicles, including exosomes. In terms of exosomes, three definitions of these microvesicles were identified: the first describes vesicles that are formed within endosomes and are released upon fusion of the MVB with the plasma membrane of the cell (94, 105); the second was broader, encompassing vesicles that “may serve a physiologic function”(106, 107); and the final included vesicles that are pelleted following centrifugation at approximately 70,000-100,000 x g (105). Due to this lack of consensus on the definition of an exosome, Gould and Raposo (104) advocated that authors provide a clear, consistent definition when applying the term exosomes. Furthermore, the isolation protocol should be clearly outlined in all publications. The use of the term ‘extracellular vesicle’ to describe all secreted vesicles was also encouraged (104, 107).
The matter of vesicle size and morphology was also addressed, with a broader detected size of 50-100nm agreed upon (105). The authors stated that investigators, reviewers and editors should remain cognisant of this broader size definition going forward. The defining characteristics of exosomes in terms of size, shape and membrane-associated proteins are outlined in Table 1.4.

<table>
<thead>
<tr>
<th>Defining characteristic</th>
<th>Exosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>30-120nm</td>
</tr>
<tr>
<td>Shape</td>
<td>Cup/saucer shaped vesicles</td>
</tr>
<tr>
<td>Exosome-associated proteins commonly used for characterisation (108)</td>
<td>HSPA8, CD9, GAPDH, ACTB, CD63, CD81, ANXA2, ENO1, HSP90AA1, EEF1A1, PKM2, YWHAE, SDCBP</td>
</tr>
</tbody>
</table>

Table 1.4 Defining characteristics for exosomes.

1.3.3 Isolation, characterization and quantification of breast cancer exosomes

A variety of exosome isolation protocols, which are suitable for use in the setting of human or animal serum, have been described in the literature (59, 90, 91, 109, 110). The vast majority of these report successful isolation of exosomes through the use of differential centrifugation (e.g. 800 x g for 10 minutes, then 2,000 x g for 10 minutes (59, 109)) followed by microfiltration using a 0.2-0.45µm sterile filter, and a final ultracentrifugation step (e.g. 100,000 x g for 2 hours (59, 90, 91, 109)). Successful isolation has also been reported using a combination of differential centrifugation and ultracentrifugation in the absence of microfiltration (109, 111, 112). Pelleted exosomes are typically re-suspended in phosphate buffered saline (PBS) following the final ultracentrifugation step. An alternative, although more costly, exosome
isolation method is available for use in the absence of an ultracentrifuge. This involves the use of commercially available kits that use precipitation reactions to isolate exosomes from serum. An example of this type of kit is the Total Exosome Isolation™ solution (Invitrogen) which is added to the exosome source following centrifugation at 2,000 x g (113). The exosomal source is then refrigerated overnight at 4°C and centrifuged at 10,000 x g for 1 hour in order to pellet out the exosomes which are then re-suspended in PBS (112). Similarly, the ExoQuick exosome precipitation solution™ (BioCat, Heidelberg, Germany) has been successfully used to isolate exosomes from human serum samples (98, 112, 114). This involves the addition of a specified volume of ExoQuick solution to the serum sample and refrigerating for 30 minutes. Next, the sample is centrifuged at 1500 x g in order to pellet the exosomes. The pellet is then re-suspended in sterile or nuclease-free water following the removal of supernatant. The exoEasy Maxi Kit (Qiagen) involves the addition of a buffer solution to the samples, inverting and then standing at room temperature prior to adding to an exoEasy spin column and centrifuging at 500 x g for 1 minute (115). The flow-through is then discarded and 10ml of buffer XWP is added to the column and centrifuged at 5000 x g for 5 minutes. Next, 400 μl – 1 ml of buffer XE is added to the membrane and incubated for 1 minute before centrifuging at 500 x g for 5 minutes. The final step involves the re-application of the eluate to the spin column membrane, incubating for 1 min and the centrifuging at 5000 x g for 5 minutes before collecting the exosome-containing eluate. Additional exosome isolation methods, including size-exclusion liquid chromatography (116, 117) and microfluidic devices (118), have also been reported in the literature, although they are less frequently employed.

Following successful isolation of exosomes from serum, researchers proceed to characterization of the pellet in order to verify the presence of exosomes. For the most part, a combination of morphological examination and Western Blot Analysis targeting exosome-associated proteins are used to confirm the presence of exosomes. Proteins that are commonly targeted
include CD 63 (91, 112) and CD9 (119), which are members of the tetraspanin family, FASN (120) and VCP (120). Morphological examination is typically carried out using Transmission Electron Microscopy (TEM) which allows investigators to appreciate the vesicular shape of exosomes in addition to obtaining an estimated measure of their diameter (91, 119, 121). This technique can involve the addition of an optimal concentration of fixed exosomes onto glow discharged carbon formvar 400 Mesh copper grids. The samples are allowed to absorb to the formvar before rinsing in water, staining with uranyl acetate and air drying. Exosome samples are then imaged using TEM (121). Variations on this procedure have been described by other research groups (90, 111, 122). An alternative method involves fixation of exosomes in formalin, followed by secondary fixation in osmium tetroxide. The exosomes are then dehydrated in graded ethanol before embedding in a resin, sectioning and viewing using TEM (123). In cases where Immunogold labelling is required the grids are placed in a blocking buffer and then incubated with the primary antibody of interest. Next, the grids are floated on drops of secondary antibody labelled with 10-nm gold particles. Finally, the exosomes are fixed and stained before viewing using TEM (119). Immunogold (IG) TEM was used in some cases to confirm the presence of the exosomal markers CD9, flotillin 1 and CD81 (119). In addition, IG-TEM has been used to distinguish exosomes derived from cancerous sources from those derived from non-cancerous sources through the detection of Glypican-1, which is reportedly exclusive to cancer cell exosomes (119). Fluorescence-activated cell sorting (FACS) has been utilised to detect cell surface proteoglycans, (e.g. Glypican-1) that are thought to be are enriched on exosomes from cancer cells (119). Flow cytometry methods are, however, limited by the small size of exosomes. As a result of this, exosomes must first be coupled to a larger substance e.g. aldehyde/sulphate latex beads. This has been successfully used to quantify exosomes by FACS (119).

Exosome quantification can be carried out directly or indirectly. Standardised protein assays allow indirect quantification of exosomes
through the analysis of protein content and this is the most widely reported approach used (112, 124). Alternatively, Exosome ELISA kits (System Biosciences) allow investigators to quantify the amount of exosomes that have been isolated based on the level of the exosome-associated proteins including CD 9, CD63 and CD 81 (112, 124). An alternative exosome quantification technique involves the use of Nanosight™ nanoparticle tracking analysis which uses light diffraction patterns to measure the size and the concentration of exosomes (125, 126). Similarly, direct quantification of exosomes may be performed using the qNano Gold (Izon Science) which measures nanoparticles using the Tunable Resistive Pulse Sensing (TRPS) principle, reporting concentration as a function of a defined size range (127, 128). One challenge to most current exosome quantification methods is the possibility of samples being contaminated with non-exosomal particles, which in the case of serum-derived exosomes may include albumin and Immunoglobulin G (125).

1.3.4 Exosomal microRNAs

There has been a huge surge in interest in the use of circulating exosome-encapsulated miRNAs as biomarkers for breast cancer in recent years. O’Brien et al., (2015) (129) recently reported on the role of exosomal miR-134 in Triple-negative breast cancer (91). In this study the investigators isolated extracellular vesicles from the cell-conditioned media of a triple-negative cell line, Hs578T, and an aggressive clonal variant, Hs578Ts(i)8, which they previously demonstrated to be capable of influencing the phenotype of recipient cells (90). MiRNA expression profiling was then carried out on the parent cells and their secreted exosomes using TaqMan Low-Density Assays (TLD). Differing miRNA profiles were detected in the Hs578Ts(i)8 cells and exosomes when compared to the less aggressive counterparts (Hs578T), with a greater proportion of miRNAs noted to be down-regulated in the former variant. MiR-134 demonstrated the greatest degree of down-regulation and was therefore further evaluated using the
publically-accessible GSE40525 and GSE 26659 datasets, where it was confirmed to be significantly down-regulated in breast cancer (n=138) compared to normal breast tissue (n=73). *In vitro* studies demonstrated reduced proliferation, migration and invasion in Hs578Ts(i)8 cells following transfection with miR-134, and so the functional impact of exosomes derived from these cells was investigated using Hs578Ts(i)8 parent cells. In this setting, migration and invasion were significantly reduced, with no impact on proliferation and an accompanying decreased in STAT5B and Hsp90 protein levels. Increased sensitivity to Hsp90 inhibitors which have been investigated as a potentially valuable therapy in Triple-negative breast cancer was also demonstrated. The results of this study highlighted a number of key points with respect to exosomes in this cancer subtype. Firstly, the detection of a decrease in miR-134 levels in cancer cells may be indicative of a more aggressive cancer phenotype, which may assist in cancer diagnosis and more specifically, molecular subtype identification. Furthermore, the uptake of miR-134 enriched exosomes by recipient cancer cells may represent a valuable therapeutic modality in the management of Triple-negative breast cancer.

The impact of MSC-secreted exosomes has also been investigated, with one study noting a reduction in bone marrow-metastatic breast cancer cell line proliferation in response to the addition of these exosomes (130). A similar result has also been demonstrated by Del Fattore et al., (2015) (131) where glioblastoma cell proliferation reduced following a period of 48 hours incubation in the presence of MSC exosomes.

Our understanding of exosomes is continuously evolving as result of robust laboratory based research. The elegant work performed by Melo et al., (2014) (59) underscored the fact that cancer cell-derived exosomes differ greatly in content and functional effect to exosomes derived from non-tumourigenic cell lines. The authors first demonstrated how metastatic breast cancer cell-derived (MDA-MB-231 and 4T1) exosomes were enriched in miRNAs when compared to non-metastatic breast cancer exosomes (MCF7). In addition, profiling of purified cancer cell exosomes
after 72 hours culture revealed enrichment of miRNAs compared to 24 hours incubation, an effect that was not observed in the case of control cell (MCF10A and NMuMG) exosomes (normosomes). Down-regulation of pre-miRNAs was detected in cancer exosomes over time with levels being inversely proportional to the corresponding miRNAs. No variation in normosome pre-miRNA levels were observed under the same conditions. Exosomes derived from cancer cells and from the serum of patients with breast cancer were found to contain the RNA-induced silencing complex (RISC) –loading complex (RLC) proteins, Dicer, TRBP, and AGO2, which are involved in miRNA biogenesis. The accumulation of Dicer specifically in cancer exosomes is reportedly mediated through CD43. These data demonstrated the ability of cancer cell exosomes to convert pre-miRNAs to mature miRNAs. Subsequent examination of the effect of transfer of cancer cell exosomes revealed increased survival and proliferation in a non-tumourigenic population of cells (59). This effect was examined further by injecting non-tumour cells with exosomes derived from a metastatic breast cancer cell line in a murine model, which resulted in tumour formation. A key finding in this study was that Dicer blockade inhibited tumour formation, suggesting the pivotal downstream effect that it plays in the transformation of non-tumourigenic cells to malignant cells. Similarly, serum exosomes from patients with breast cancer coinjected with MCF-10A cells resulted in tumour formation in mice while no tumours were formed when cancer exosomes were replaced with exosomes from healthy volunteers (59). Serum from patients with breast cancer was also noted to contain higher levels of exosomes than that which was isolated from healthy donors (59).

Work carried out by Tosar et al., (2015) (86) involved sequencing of the intracellular small regulatory RNAs (sRNA) content of breast epithelial cell lines (MCF-7 and MCF-10A) and comparison with extracellular fractions enriched in microvesicles, exosomes and ribonucleoprotein complexes. The results demonstrated a non-selective secretion model for the majority of microRNAs, with a few showing preferential secretion patterns. The authors
proposed that this model might explain, at least in part, some of the conflicting published data regarding miRNA secretion. In contrast, 5′ tRNA halves and 5′ RNA Y4-derived fragments of 31–33 nt showed preferential secretion patterns. These data demonstrate that different sRNA families have characteristic secretion patterns and highlight their potential role in the extracellular space.

1.3.5 Exosome-encapsulated miRNAs as Breast Cancer biomarkers

There has been a huge surge of interest in the use of circulating exosome-encapsulated miRNAs as biomarkers for breast cancer in recent years. An important factor that must be taken into consideration with any biomarker is that of the robustness of the source material, in this case, exosomes. Studies have demonstrated that exosomal-protein levels in samples derived from biofluids are well preserved when stored at -80 °C when compared to samples stored at 4 °C and processed within 1 hour (132). In addition, levels of exosomal markers were similar in samples that were stored at 4 °C for 24 hours and then at -80 °C. This finding has enhanced the clinical applicability of exosomes (97).

A variety of detection methods for circulating exosome-encapsulated miRNA detection have been described in the literature, most of which centre around the use of real-time Polymerase Chain Reaction (PCR) analysis (91, 98). Lee at al., (112) employed molecular beacon (MB), a nano-sized oligonucleotide probe to carry out in situ miRNA detection. This involved quantitative detection of miRNAs in exosomes derived from breast cancer cell lines. Permeabilization of the MCF-7-secreted exosomes using Streptolysin O bacterial toxin enhanced the delivery of MB into exosomes and increased miRNA hybridization. The investigators then detected significantly higher miR-21 levels in MCF-7 exosomes when compared to normal cell-derived exosomes thus highlighting its potential as a biomarker for breast cancer. Next, exosomes from MCF-7 cells were spiked into human serum samples in order to determine if MB had the capacity to detect
serum exosomal miRNAs without pre-treatment of the serum. Here, the investigators found that MB could be successfully used for the detection of exosome-encapsulated miRNAs spiked into serum without the need for additional treatment. This study clearly outlined a technique which may be valuable in the field of breast cancer.

A recent paper by Eichelser et al., (2014) (98) investigated the ability to detect specific miRNAs in the serum exosomes of patients with breast cancer and to determine if molecular subtype identification could be achieved using this modality. This method of subtype discrimination may be of particular value in the clinical setting given that survival from breast cancer differs significantly between subtypes: patients with Luminal A breast cancer have superior survival to those with either HER2 or basal-like subtypes (98). Molecular subtype also influences the treatment modalities that will be effective in the management of breast cancer. The researchers isolated the exosome fraction from the serum of 50 patients with breast cancer and 12 healthy controls. Cell-free and exosomal miR-101, miR-372 and miR-373 levels were compared between cohorts. Higher relative values of the aforementioned miRNAs were found in serum exosomes when compared to cell-free circulating levels (98). Furthermore, exosomal miR-101 and miR-372 were found to be significantly higher in the serum exosomes of patients with breast cancer than in healthy controls. This finding was not observed in the case of miR-373, but within the breast cancer cohort, it was found to be significantly elevated in triple-negative patients when compared to luminal cancers or healthy controls. Similarly, it was higher in ER/PR negative cases compared to receptor positive patients. The results of this study indicate that higher exosomal levels of expression of miR-373 in breast cancer are indicative of a triple-negative phenotype. These data highlight the potential for serum-specific exosomal miR-373 to serve as a biomarker for aggressive, Triple-negative and hormone receptor-negative breast cancers. A key point that must be noted in relation to this study is that a wide range of values for miR373 was detected in both ER/PR
negative cases and receptor positive patients. This level of variation may present challenges incorporating miRNAs into the clinical setting.

The use of exosome-encapsulated miRNAs as a prognostic marker for metastatic progression in breast cancer was reported on by Zhou et al., (2014) (11). In this study the authors measured serum exosomal miRNA levels in breast tumour-bearing animals compared to tumour-free controls, and found that miR-105 was significantly higher in animals in the pre- and metastatic stages. Next they detected significantly higher miR-105 levels in the serum-derived exosomes of patients who went on to develop distant metastases (n=16) compared to those who did not go on to develop metastatic disease (n=22) (mean follow up of 4.2 years). The results of this study suggest that serum-exosomal miR-105 may have the potential to predict, or diagnose at an early stage, patients who may develop or have already developed breast cancer metastasis.
1.4 Thesis aims

The molecular era of classification of breast cancer has increased our understanding of this disease, its response to therapy and outcome. This was highlighted recently through the use of Oncotype DX testing in order to identify patients with Luminal A breast cancer who may or may not benefit from chemotherapy, and the incorporation of HER2 testing into clinical practice. Additionally, Triple-negative breast cancer has been shown to be associated with adverse outcomes as a result of poor response to therapy.

The major form of breast cancer management has been centred on epithelial cell function but more recently tumour stromal cells have been found to play an important role in the primary tumour microenvironment. The influence of exosomes on cell-cell interactions may prove pivotal in this arena. Exciting studies have demonstrated the ability of these microvesicles to have a functional impact on recipient cells thereby highlighting the role that they play in intercellular communication in the tumour microenvironment (90, 133). The biomarker potential of exosomes is another facet of this vast field of research that holds huge promise and may revolutionise the manner in which we diagnose and manage breast cancer in the near future. With this in mind, the aims of this study were:

1. To assess the role of molecular classification in prediction of response to treatment and prognosis in breast cancer.

2. To determine the impact that exosomes have on intercellular communication in the primary tumour microenvironment.

3. To investigate the biomarker potential of serum exosomal microRNAs in breast cancer.
Chapter 2

Materials and Methods
2.1 Cell Culture

2.1.1 Overview

The Discipline of Surgery has three separate dedicated cell culture laboratories. One is for cell line culturing only, one for primary culture and all viral work is carried out in a separate viral culture room. All work involving non-transduced Mesenchymal Stem Cells and WI-38 cells is carried out in the primary culture rooms.

The process of cell culture involves the removal of cells from multicellular eukaryocytes, and subsequently growing them in an artificial environment (in vitro) that has been optimised for their particular requirements. Primary culture involves the propagation of cells that have been isolated from animal tissue following enzymatic digestion. Once the cells reach confluence they are passaged (or subcultured). This process involves their transfer to a new culture flask containing fresh growth medium which allows cell growth to continue. These cells can only be passaged a number of times before undergoing an event known as senescence. As such, they are termed finite. In some cases, cells become immortalised through the process of transformation. This can occur spontaneously or can be induced, either through the use of chemicals or viruses. These cells are referred to as continuous as they can divide indefinitely.

Cell lines can be cultured in a variety of formats. They are commercially available and are screened for viruses and other contaminants prior to dispatch from the supplier. The majority of cell types employed here are anchorage-dependent and are therefore grown in an adherent or monolayer culture on a solid or semi-solid substrate. The alternative culture method is suspension culture whereby the cells are grown floating in an appropriate culture medium. Monolayer culture was used in all experiments described in this thesis. The advantage of using this technique is that morphological analysis can readily be carried out using light microscopy. The presence of contamination can also be assessed using this method. Two major drawbacks are the need for cell passaging as dictated by the limited surface
area of the tissue culture flasks and the inability to assess the 3D morphology of cells. The culture flasks contained a filter which allowed efficient gas exchange and helped prevent the spread of contamination within the incubator. These flasks were purchased from Starstedt.

The benefits of the use of cell lines as models for cancer include the following: they are easy to grow and manipulate genetically; they are amenable to functional assays; and they are associated with consistency and reproducibility of results. One potential drawback to using cell lines is that multiple variants of the same cell line may be in use across various facilities. As such, results arising from experiments involving cell lines that are purported to be the same may not be directly comparable.

The cell lines that are used on our laboratory were initially purchased from the American Type Culture Collection (ATCC). Cell lines that have been present in the laboratory (either in culture or stored in liquid nitrogen), undergo Single Tandem Repeat (STR) DNA profiling (in LGC Standards, United Kingdom) every 2 years in order to verify their cell type. STR profiling allows the identification of hyper-variable regions within the genome of human cell lines derived from the tissue of a single individual, thus ensuring that misidentification or cross-contamination of cell lines has not occurred.

### 2.1.2 Asepsis in cell culture

Asepsis is defined as the absence of viable pathogenic organisms such as bacteria, viruses and parasites. This practice is promoted in the setting of cell culture in order to prevent contamination of cell lines. It includes the donning of red collared coats and gloves upon entry to the cell culture lab, the use of 70% Industrial Methylated Spirits (IMS) to spray gloves, equipment and consumables before use in the LAF hoods, and the cleaning of the LAF hoods with 70% IMS before and after use. The LAF hoods are switched on and allowed to circulate for 15 minutes before and after use, and an ultraviolet (UV) light is also enabled for 15 minutes at the end of any
period of work in the LAF. When working in the LAF hood, clean items are stored on the right hand side and waste is kept on the left. Spent media is added to a dedicated waste flask which is for use by the individual researcher only and these flasks are decontaminated using Virkon™ solution as often as possible. The Virkon solution is allowed to work for a minimum of 3 hours before being disposed of down the sink followed by copious amount of fresh water. All three cell culture laboratories are thoroughly cleaned on a weekly basis: the LAF hoods are dismantled and cleaned; the water in the water bath and incubator trays is replaced with autoclaved double distilled water; the incubators are cleaned; and consumables are replaced where necessary.

### 2.1.3 Immortalised Breast Cancer Cell Lines

Breast cancer cell lines that were purchased from the ATCC include the following: BT-20, HCC1954 and MDA-MB-231. The characteristics of these cell lines are detailed in Table 2.1. These cells were cultured in Eagle's Minimum Essential Medium (EMEM) (BT-20), RPMI-160 (HCC 1954) and Leibovitz-15 (MDA-MB-231). The culture media was supplemented with 10% Foetal Bovine Serum (FBS) and 100 IU/mL Penicillin G/100mg/mL Streptomycin sulphate (Pen/Strep). Culture media and supplements were purchased from Gibco ®. Cells were maintained at 37 °C and at 5% CO₂ in the HEPA incubators. They underwent a media change (feeding) 3 times every week and were subcultured every seven days. The receptor status of each of the cancer cell lines is outlined in Table 2.1.

### 2.1.4 Primary culture

Human MSCs employed in this work were obtained through the Regenerative Medicine Institute (REMEDI) at the National University of Ireland Galway. Bone marrow was aspirated from the iliac crests of healthy volunteers following ethical approval and informed consent. MSCs were
isolated from the bone marrow aspirates via direct plating and were cultured for 12–15 days to deplete the non-adherent haematopoietic cell fraction. The ability of MSCs to differentiate into chondrocytes, adipocytes and osteoblasts was confirmed prior to use (134). Surface receptor characterisation was performed targeting the markers CD105, CD73, CD90 (positive) and haematopoietic markers CD34, CD45 (negative). These characteristics are outlined in Table 2.1 The MSCs were cultured in α-Minimum Essential Medium (αMEM) supplemented with serotyped 10% FBS, which allows cells to remain in an undifferentiated state, and Pen/Strep.

The WI-38 cell line is composed of a population of fibroblasts cells derived from lung tissue of a 3 month gestation white (Caucasian) female foetus (Table 2.1). They are considered to be a ‘normal’ stromal population and are employed as a control cell line in some experiments. These cells were purchased from the ATCC and were cultured in Eagle's Minimum Essential Medium supplemented with 10% FBS and 100 IU/mL Penicillin G/100mg/mL Streptomycin sulphate.
### Chapter 2: Materials and Methods

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Characteristics</th>
<th>Breast Cancer Subtype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-20</td>
<td>ER- PR-HER2-</td>
<td>Basal-like</td>
<td>Mammary Gland/Breast</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>ER- PR-HER2-</td>
<td>Basal-like</td>
<td>Mammary Gland/Breast; Derived From Metastatic Site: Pleural Effusion</td>
</tr>
<tr>
<td>HCC1954</td>
<td>ER- PR-HER2+</td>
<td>HER2 amplified</td>
<td>Mammary Gland; Breast/Duct</td>
</tr>
<tr>
<td>MSC</td>
<td>CD105+ CD73+ CD90+ CD34- CD45-</td>
<td>-</td>
<td>Iliac crest of healthy volunteers</td>
</tr>
<tr>
<td>WI-38</td>
<td>Fibroblast</td>
<td>-</td>
<td>Normal embryonic (3 months gestation) lung tissue</td>
</tr>
</tbody>
</table>

Table 2.1 Characteristics of cell line used in cell line and primary culture.

#### 2.1.5 Feeding cells

Cells underwent a media change 3 times per week. Spent media was decanted into the waste flask and was replaced with fresh media that had been preheated to 37 °C. The process was carried out in as efficient a manner as possible in order to avoid the cells being uncovered for extended periods of time. Additional media was added if a media change was not anticipated for 3 days for example over bank holiday weekends.
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2.1.6 Subculturing cells

Cells growing in culture must be maintained in the log phase of growth, otherwise cell growth slows or even ceases. It is therefore necessary to transfer cells to a new flask with new culture media to maintain active cell division. The subculturing process involves breaking the bonds that attach the cells to the surface of the flask and to each other. Trypsin in the presence of EDTA (Gibco ®) (0.25%, 1Mm EDTA) is used in the subculturing process. Trypsin is a proteolytic pancreatic enzyme that effectively breaks cellular bonds. It is activated by warming to 37 °C before use. EDTA is used as it enhances the action of Trypsin.

In brief, the cell monolayer was washed with Phosphate Buffered Saline (PBS) (Gibco ®) in order to remove traces of FBS which inhibits Trypsin. Trypsin/EDTA was then added for one minute before pouring off the excess and placing in a HEPA incubator for 3-5 minutes (some cells require longer periods of trypsinisation). The cells were inspected using light microscopy to ensure effective trypsinisation before re-suspending in fresh media containing FBS (which deactivates the Trypsin), and counting prior to reseeding. The PBS which was used for the purpose of subculturing was Ca²⁺/Mg²⁺ free as this reduces the concentration of divalent cations and proteins which inhibit Trypsin.

2.1.7 Counting of cells

Counting of cells was carried out using a Nucleocounter ® (Chemometec) which allows accurate estimation of cell numbers. The Nucleocounter is composed of a miniaturized low-power (1x) transmitted light fluorescence microscope which is illuminated by 8 green LEDs and a CCD camera for determining cell viability and number. Disposable cartridges (nucleocassettes, Figure 2.1) which are internally coated with propidium iodide (PI) are used in the counter.
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The operating principle for the Nucleocounter® is outlined in Figure 2.2. In brief, the total number of dead (non-viable) cells is calculated by directly loading a sample of cell suspension in a nucleocassette. The PI intercalates with the DNA of non-viable cells. In order to obtain a viable cell count, a lysis buffer (A) and a stabilisation buffer (B) are added to an equal volume of cell suspension and loaded into the counter using a cassette. The total viable cell count is calculated by subtracting the dead cell count from the total cell count and multiplying by the total volume of cell suspension. This method of cell counting is safer than the conventional method of counting using a hemacytometer and a microscope, as it does not involve exposure to trypan blue, which is carcinogenic.
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2.1.8 Cryopreservation of cells

Cryopreservation of cells was carried out periodically in order to maintain stocks and to minimize genetic change in continuous cell lines. Cells were examined using light microscopy prior to cryopreservation in order to ensure that no visible contamination was present. The cells were trypsinised in the usual manner and counted. A 5% dimethylated sulphoxide (DMSO) solution in complete media was used as a cryopreservative. This solution protects the cells from crystal formation and the resulting lysis that may result from the freeze-thaw process. DMSO (5%) was added to each 2mL cryovial (Starstedt) followed by the addition of a minimum of 1 million cells in 1.6mL of culture media. The cryovials were closed, inverted and immediately placed on ice before transferring to a ‘Mr Frosty’ (Nalgene)
container and storing in the -80 °C freezer for a minimum of 3 hours. As DMSO is toxic to cells at 37 °C it is important to have immediate cooling on ice, followed by slow cooling to reduce crystal formation. The Mr Frosty contains an isopropyl bath which allows samples to be cooled at a rate of -1 °C per minute when placed in the -80 °C freezer. Samples were then transferred to a liquid nitrogen storage unit for long-term storage.

2.1.9 Recovery of cryopreserved cells

It is critical to perform cell recovery rapidly in order to reduce exposure time to DMSO. Recovery of frozen stocks was undertaken in the following manner: pre-warmed media was placed into an appropriate sized flask and kept in the hood while the cells were thawed quickly in the water bath. The cells were then pipetted into the tissue culture flask, immediately diluting the DMSO in a large volume, and the flask was gently tilted to allow even distribution of cells. The flask was then placed in the incubator. The media was changed once the cells had adhered in order to prevent the DMSO from damaging viable cells.

2.2. Collection of patient samples

2.2.1 Ethics and consent

All blood samples collected during this work were covered by ethical approval granted by the Galway University Hospitals Research Ethics Committee (Appendix 1). Blood samples were obtained following informed consent (Appendix 2). Patients were provided with an information leaflet and the procedure was fully explained prior to signing a consent form. Of note, patients were advised that the procedure was voluntary, that they would not receive follow-up data and that the samples would not result in monetary gain for either the researcher or patient.
2.2.2 Sample collection and processing

Blood samples were collected using standard phlebotomy practice and equipment. Serum was collected into 5ml BD Vacutainer® SST™II Advance tubes. Samples were allowed to clot at room temperature for a minimum of 30 minutes, after which they were centrifuged for 10 minutes at 4 °C and 805 x g. The serum was then drawn off and aliquoted into eppendorf tubes (500µl per tube). The tubes were then labelled, assigned a Biobank reference number (R number) and stored at -80 °C until required.

Whole blood was collected into PAXgene™ Blood RNA Tubes (PreAnalytiX). These tubes contain an additive that lyses all cellular material and stabilises the RNA for storage at room temperature, 4 °C, or frozen. Samples were logged on the Biobank system (Shire) and placed in the -80 °C freezer until required.

2.3 Exosome isolation

2.3.1 Preparation of exosome-depleted media

Secreted exosomes were harvested from a variety of cell types including BT-20, HCC-1954 and MSCs. A schematic representation of the exosome isolation process is shown in Figure 2.3. The first step in this process was to ensure that cells were cultured in the presence of exosome-depleted media in order to determine the effect that cell-secreted exosomes would have on recipient cells. It was therefore necessary to deplete the foetal bovine serum (FBS), which is used as a source of growth factors in culture media, of exosomes prior to the culturing process. In order to do this, 20mL aliquots of FBS were placed in 25PC thick walled tubes (Hitachi Koki Centrifuge Ware) and ultracentrifuged for 16 hours overnight at 110,000 x g and 4 °C in order to separate out the exosome fraction. The exosome-depleted FBS was then removed and stored in 50mL aliquots at -20 °C until required. When preparing to carry out an exosome harvest, the exosome-depleted FBS was thawed and added to a fresh media bottle which was also supplemented with Pen/Strep.
2.3.2 Isolation of exosomes from cell-conditioned media

In order to isolate exosomes from a chosen cell line, a suitable number of flasks (based on visual inspection and estimation of cell density) were trypsinised in the usual manner before re-suspending in exosome-depleted media and counting using the Nucleocounter. A standardised seeding density of 2x10^6 cells was used in all exosome setups. 10mL of exosome-depleted media was pipetted into each of 10 x T175cm^2 flasks and rolled around the floor of the flask to ensure that all areas were adequately covered in media. Equal amounts of cells were added to each flask and the flask was again rolled to evenly coat the floor of the flask with cells. The cells were incubated overnight at 37 °C and 5% CO_2, allowing them to adhere to the flask. The exosome-depleted media was replaced with 10mL of fresh exosome-depleted media the following morning and placed in the incubator for 48 hours.

Following a 48 hour incubation period the conditioned media was decanted into 50mL falcons and centrifuged at 300 x g for 10 minutes (Figure 2.3).
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Figure 2.3 Schematic of exosome isolation protocol for cell conditioned media.

The supernatant was pipetted into new 50mL falcons and centrifuged at 2,000 x g for 10 minutes. In each of these steps, approximately 2.5ml of liquid was left behind above the pellet. These differential centrifugation steps remove large cells and debris. Next, the supernatant was passed through a 0.2µm sterile filter (Millipore, Billerica, MA, USA) with the purpose of removing any remaining cellular material and debris with a diameter in excess of 200nm. The filter allowed the integrity of the small
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exosomal membranes to be maintained throughout this process. The collected filtrate was placed into 25PC thick walled ultracentrifugation tubes in 20mL aliquots and ultracentrifuged at 110,000 x g for 70 minutes at 4 °C in a Hitachi Micro Ultracentrifuge CS 150FNX. If the exosomes were being isolated for the purpose of carrying out morphological analysis using Transmission Electron Microscopy, a primary fixative was added to the supernatant prior to ultracentrifugation (described in detail in Section 2.5.1). Following ultracentrifugation, the supernatant, now referred to as exosome-free conditioned media, was removed and approximately 2mL was retained for further analysis. The exosomes were scraped down from the side and bottom of the ultracentrifuge tube using 55µL of sterile PBS and a 100 µL pipette tip. The exosome-rich PBS was transferred to a taper-bottomed tube. Next, 5 µL of exosome suspension was placed in complete lysis buffer, which will be described in Section 2.2.2, for protein estimation in order to indirectly quantify the amount of exosomes that had been isolated. Both samples were then labelled and stored at -80 °C until required. If exosomes were required for Western Blot Analysis (Section 2.5.2), the entire sample was placed in complete lysis buffer (components listed in Table 2.2, Section 2.4) and stored at -80 °C.

The T175cm² flasks were trypsinised, a cell count was performed and the cells were pelleted for later analysis. The cell pellet was labelled and stored at -80 °C.

2.3.3 Isolation of exosomes from serum

Various serum exosome isolation techniques were trialled in this study. Initial attempts involved the direct ultracentrifugation of serum in 1.5mL microtubes without prior dilution. The technique which was validated involved the dilution of serum in PBS prior to the ultracentrifugation process. In brief 300-500µl of serum was thawed on ice, diluted with 12mL of PBS and inverted to mix (Figure 2.4). Next the samples were centrifuged at 800 x g for 10 minutes and then 2000 x g for 10 minutes. The supernatant
was drawn off after each differential centrifugation step leaving no more than 0.5mL of precipitant containing cellular debris behind. It was then filtered using a 0.2 µm filter into a 25PC thick walled ultracentrifugation tube. The exosome pellet was then recovered at 110,000 x g for 2 hours at 4 °C in a Hitachi Micro Ultracentrifuge CS 150FNX. The pellet was re-suspended in 55µl of PBS as before, and 5µl of this was placed into 20µl of complete lysis buffer for protein estimation. In cases where the sample was required for Western Blot, the entire re-suspended pellet was placed in complete lysis buffer (Table 2.2, Section 2.4) and stored at -80 °C. As with cell-secreted exosomes, serum-derived exosomes that were destined for visualisation using Transmission Electron Microscopy were ultracentrifuged in the presence of a primary fixative and then re-suspended in PBS.
500µl of serum thawed on ice and diluted with 12ml of PBS

Centrifuged at 800 x g, 10 mins
Supernatant drawn off

Centrifuged at 2,000 x g, 10 mins

Supernatant passed through a 0.2µm filter

Supernatant ultracentrifuged at 110,000 x g, 70 minutes

Exosome pellet re-suspended in 55µl of PBS

Figure 2.4 Schematic of exosome isolation protocol for human or animal serum samples.

### 2.4 Exosome quantification

The amount of exosomes that were isolated from conditioned media was indirectly quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific) (112, 124). This is a detergent-compatible formulation based on bicinchoninic acid (BCA) which allows the colorimetric detection and quantification of protein. This method involves the addition of 5µL of exosomes that have been re-suspended in PBS to 20µL of complete lysis buffer. The components of lysis buffer are outlined in Table 2.2. The
protease inhibitors and sodium orthovanadate were thawed and added fresh at the time of use, while the remaining stock was made up and used over a period of 6 months (stored at -20 °C). The protein assay was performed in a 96-well plate format. Albumin standard solutions were prepared according the manufacturer’s guidelines (0-2000 µg/ml). An adequate volume of working reagent, which consisted of a 50:1 ratio of Solution A: Solution B was prepared. 25 µL of standard or exosome sample were added to the 96-well plate in duplicate to which 200µL of working reagent was added. The plate was then sealed and mixed on a plate shaker for 30 seconds before incubating in the Luminoskan Ascent (Thermo Scientific) for 30 minutes at 37°C. The plate was then allowed to cool to room temperature before measuring the absorbance in the Multiscan RC Plate Reader (Labsystems) at 560nm. The protein concentrations were given in µg/ml. This information paired with knowledge of the volume of PBS in which the exosomes had been re-suspended allowed the calculation of the total amount of protein that had been isolated.

<table>
<thead>
<tr>
<th>To be added to 50ml distilled water</th>
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</thead>
<tbody>
<tr>
<td>20mM HEPES</td>
</tr>
<tr>
<td>150mM NaCl</td>
</tr>
<tr>
<td>1% Triton-X 100 (1:100 dilution in PBS)</td>
</tr>
<tr>
<td>2mM EDTA</td>
</tr>
<tr>
<td>2mM Sodium Orthovanandate</td>
</tr>
<tr>
<td>10mM Sodium Fluoride</td>
</tr>
<tr>
<td>Complete Protease Inhibitor</td>
</tr>
</tbody>
</table>

Table 2.2 Preparation of Triton-X Lysis Buffer.
2.5 Exosome characterisation

In order to ensure successful isolation of exosomes, it was necessary to characterise them using Transmission Electron Microscopy, which allowed morphological analysis and Western Blot Analysis targeting exosomal membrane proteins.

2.5.1 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was employed to carry out morphological analysis on exosomes. In order to do this, exosomes were isolated in the usual manner except for the addition of a primary fixative to the cell-conditioned media prior to the final ultracentrifugation step. The TEM protocol is outlined in Figure 2.5.
Figure 2.5 Protocol for preparation of exosome pellets for Transmission Electron Microscopy.

The primary fixative allowed the architecture of the exosomes to be protected during the ultracentrifugation process. In the case of exosome isolation from serum, the primary fixative was added following
microfiltration of the serum/PBS solution. The primary fixative was composed of 2% Gluteraldehyde (Polysciences, Warrington, PA) and 2% Paraformaldehyde in 0.1M Sodium Cacodylate / HCl buffer pH 7.2. This was added to cell-conditioned media or serum in a 1:1 ratio. The exosome pellet was re-suspended in 55µl of PBS and stored at 4°C prior to secondary fixation. The exosomes then underwent osmication using a secondary fixative composed of 1% Osmium Tetroxide (Agar Scientific, Essex, UK) in 0.1M Sodium Cacodylate / HCl buffer pH 7.2. Sufficient secondary fixative was added to cover the pellet completely. Following secondary fixation, the Osmium solution was drawn off (under fume extraction) using a Pasteur pipette into a 2% solution of Ascorbic Acid which serves to neutralise the Osmium solution toxicity. The exosome pellets were then dehydrated through a series of graded alcohols (30%, 50%, 70%, 90%, 95% and pure Ethanol). Each dehydration step was performed for 2 x 15 minutes. The pure ethanol was then drawn off and replaced by Propylene Oxide (2 x 20 min.) The Propylene Oxide (Sigma-Aldrich®) acts as a transition solvent between the final alcohol stage and the follow-up stage of impregnation of the exosomes in an Epon based resin (Ladd Industries, Burlington, VT). The resin was prepared to an exact formulation (LV Resin 16g, VH1 Hardener 5.3g, VH2 Hardener 12g, LV Accelerator 0.83g) using a commercial kit (Agar Low Viscosity Resin (LV) Kit, Agar Scientific). The exosome pellets were initially placed in a 50:50 mixture of resin and Propylene Oxide overnight. This mixture was then replaced by a 75:25 mixture of resin and Propylene Oxide for 4 hours. The exosomes were then transferred to pure resin for 2 hours. This was replaced by more pure resin and left overnight. The next morning the pure resin was again changed and the specimens were left at room temperature for 5-6 hours, before placing in a 65°C oven for 48hrs and allowing them to polymerise. All specimens were clearly labelled at each step of the protocol. Following polymerisation, the blocks were trimmed to expose the surface of the exosome pellet and sections measuring 1 micron thickness were cut onto glass slides. The sections were then stained with 1% Toluidine Blue and viewed using a light microscope. These sections are referred to as “scout sections” as they are primarily used to
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ascertain exosomal structures and components. Potentially interesting regions were then selected for subsequent trimming and ultrathin sections of a magnitude of 80nm – 100nm were cut using an ultramicrotome (Reichert-Jung Ultracut E) and lifted onto 3mm copper grids. These grids were stained for 30 min in 1.5% aqueous Uranyl Acetate and 10 min in Lead Citrate. An automated contrasting apparatus (Leica EMAC 20) was used. Sections were allowed to dry and then viewed in the TEM (Hitachi H7000) at magnification range 20,000x-60,000x.

2.5.2 Western Blot Analysis

Western Blot Analysis, targeting a variety of exosome-associated proteins was trialled out in order to confirm that exosomes had been isolated using the protocol described. The exosomal marker that is reported for this set of experiments is CD63. An important step in this process was the resuspension of isolated exosomes in complete lysis buffer instead of PBS. A BCA protein assay was carried out prior to the Western Blot which allowed quantification of the amount of protein that was present in the exosome pellet, and therefore ensured that the correct amount of protein was loaded onto the gel. The Bio-Rad Mini-Protean TGX Precast Gels (4-15%), 10-well comb, 30µl/well, were used for all Western Blots. Samples were mixed with 4X sample buffer and 10X reducing agent to a final volume of 10 µl, and were denatured in the PCR sprint machine for 10 minutes at 70ºC. The precast gels were positioned in the clamp assemblies and the chamber was filled with 10X Tris-Glycine-SDS Running Buffer (2.5mM Tris/19.2mM Glycine/0.1% SDS/H2O). Samples were loaded into the middle wells and the Molecular Weight (MW) marker was added to the 1st and 10th wells. The gel was attached to the power source and was run at 100V for 1 hour. Next, the gels were transferred onto nitrocellulose membranes to transfer the protein. The order of placement of layers in the gel sandwich was very important and was as follows: fibre pad, filter paper, equilibrated gel, nitrocellulose membrane, filter paper, fibre pad. This layering is represented in Figure 2.6.
Figure 2.6 Assembly of the Mini-Trans-Blot cassette (Adapted from Mini-PROTEAN Precast Gels Instruction Manual and Application Guide, Bio-Rad).

All components of the gel sandwich (aside from the gel) were soaked in 1x Transfer Buffer (2.5mM Tris/19.2mM Glycine/20% Methanol/H2O) prior to sandwich assembly. A cooling block was placed in the Buffer tank and the transfer was run at 100V for 30 minutes. The nitrocellulose membrane was then transferred to a 5% milk solution to block non-specific binding of proteins and placed on a shaker for 1 hour at 120 RPM. It was then transferred to a TBS-Tween washing buffer (0.5M Tris/1.5M NaCl/0.05% Tween/H2O). Again, this was placed on a shaker at 120 RPM, this time for 15 minutes. The washing step was repeated twice more for 5 minutes each. A 1:1000 dilution of primary antibody (Abcam Rabbit Polyclonal to CD63: 100µg at 1mg/ml) was prepared using Blocking Buffer (0.1% milk solution). This was added to the membrane, the dish was covered in cling film to reduce evaporation, and placed on the shaker at 120 RPM overnight at 4 °C. The following morning, the membrane underwent 3 washing steps, the first lasting 15 minutes and the latter 2 lasting 5 minutes each. Next, a
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1:3000 dilution of secondary antibody (Abcam Goat Anti-Rabbit IgG HRP, 1mg at 2mg/ml) was prepared in Blocking Buffer, and was added to the membrane. This was mixed on the shaker at 120 RPM for 90 minutes. Again, the membrane was washed in washing buffer for 15 minutes, followed by 4 x 5 minute sequential washes. Preparation of the Supersignal West Pico Chemiluminescent Substrate was carried out just before it was required. The nitrocellulose membrane was transferred onto cling film and was covered in substrate, followed by a 5 minute incubation period at room temperature. The membrane was then blotted and placed on fresh cling film and covered. Images were captured using the Gel Doc™ XR+ and ChemiDoc™ XRS+ Systems with Image Lab™ Software (Bio-Rad).

2.6 MicroRNA expression analysis

2.6.1 Asepsis

RNase A in the laboratory setting can have a negative impact on RNA quality. This can affect subsequent cDNA synthesis and the Relative Quantification Polymerase Chain Reaction (RQ-PCR) outcomes and so a number of steps were adhered to throughout the experimental process in order to eliminate RNases. These precautions aimed to prevent contamination of equipment and various solutions that were required for experimental procedures. The first step was to ensure that RNA extractions were carried out in dedicated Class II Safety Cabinets. Pieces of equipment and consumables that may be required were designated uniquely for this application. Disposable gloves, sprayed with 70% IMS were worn throughout the extraction process and were changed if they came into contact with any potential contaminants. Finally, DNase was employed where necessary in order to remove genomic DNA.
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2.6.2 RNA extraction

RNA was extracted from a variety of sources throughout this research. The priority was to obtain high quality RNA which could then be used to generate sensitive and accurate results via downstream Reverse Transcription (RT) and Polymerase Chain Reaction (PCR). Correct storage and handling of samples before, during and after RNA extraction was vital as was the choice of extraction method. Table 2.3 outlines the source material and extraction kits that were employed for various aspects of this work.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Sample Type</th>
<th>Source</th>
<th>Design Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNeasy® Mini Kit</td>
<td>Cell pellet</td>
<td>Qiagen</td>
<td>On-column method designed for the isolation of total RNA</td>
</tr>
<tr>
<td>mirVANA™ RNA Isolation Kit</td>
<td>Conditioned media, Exosomes</td>
<td>Ambion®</td>
<td>Double column method designed for the isolation of small and large RNA</td>
</tr>
<tr>
<td>PAXgene™ Blood RNA Kit</td>
<td>Whole blood</td>
<td>PreAnalytiX®</td>
<td>Manual purification of total RNA</td>
</tr>
<tr>
<td>MagNA Pure Compact Kit</td>
<td>Serum-derived exosomes</td>
<td>Roche</td>
<td>Automated RNA extraction</td>
</tr>
</tbody>
</table>

Table 2.3 RNA extraction methods utilised for cell pellets, conditioned media, exosomes and whole blood.

2.6.2.1 RNA extraction from cell line pellet

RNA was extracted from cell line pellets using the RNeasy® Mini Kit (Qiagen). Cell pellets were removed from the -80 °C freezer and were homogenized by adding 1ml of Trizol and a 2ml syringe and needle. 200µl of chloroform was then added per millilitre of Trizol and the samples were
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then vortexed for 15 seconds. They were then allowed to stand at room temperature for 5-10 minutes. Next, the samples were centrifuged at 12,000 x g for 15 minutes at 4 °C. Centrifuging allows the RNA to migrate to the upper, clear aqueous phase. The clear phase was then transferred to a new Starstedt tube taking care not to take the bottom layers (usually 600µl). Next, 3.5 volumes of 100% ethanol were added to the sample and they were vortexed until mixed thoroughly. Seven hundred microlitres of this mixture was added onto an RNeasy column at a time and centrifuged at 14,000 x g for 21 seconds at 4 °C. This step was repeated until the entire sample had passed through the column. Next, 350µl of RW1 buffer was added onto the column and centrifuged at 14,000 x g for 21 seconds at 4 °C. The flow through was discarded. DNase/Buffer RDD (80µl) was added directly onto the membrane. The sample was then incubated at room temperature for 15 minutes. Another 350µl of RW1 buffer was pipetted onto the column and centrifuged at 14,000 x g for 21 seconds at 4 °C. The flow-through was discarded and the collection tube was blotted on a paper towel. Another 500µl of RPE buffer was then added and the sample centrifuged at 14,000 x g and 4 °C for 2 minutes to dry the membrane. The column was then transferred to a 1.5ml eppendorf tube and 50µl of chilled RNase-free water was added directly onto the membrane. The water was allowed to sit on the membrane for 1 minute before a final centrifugation at 14,000 x g and 4 °C for 1 minute. The sample was then analysed using the Nanodrop Spectrophotometer and the remainder was labelled and stored at -80 °C.

**2.6.2.2 RNA extraction from conditioned media and exosomes**

The Trizol and miRVana modified method was used to extract RNA from conditioned media and exosome pellets. Trizol was added to the sample (700µl in the case of 1ml of conditioned media and 400µl of Trizol for
exosomes re-suspended in PBS). The samples were then vortexed or pipetted to mix, and placed on the bench top at room temperature for 5 minutes. Next, 140µl of chloroform was added, the tube was capped securely and shaken vigorously for 15 seconds. The samples were allowed to rest on the bench top at room temperature for 10 minutes and then centrifuged for 15 minutes at 12,000 x g at 4 °C. After centrifugation the sample was separated into 3 phases. The upper aqueous phase was transferred to a new collection tube. One third the volume of 100% ethanol was added to the aqueous phase, vortexed and inverted. A filter was placed into a tube and 700µl of the lysate was added and centrifuged for 15 seconds at 10,000 x g. The filtrate was collected in a new tube. This step was repeated until all the lysate had passed through. If the filter was required for total RNA extraction it was kept, otherwise it was discarded. Two thirds the volume of 100% ethanol was added to the filtrate. A new filter was placed into a new collection tube and 700µl of the filtrate/ethanol mix was added. The mixture was centrifuged for 15 seconds and the flow through was discarded. This step was repeated until all the sample had passed through. The collection tube was re-used for the next step which involved the application of 700µl of miRNA wash solution 1 to the filter. This was centrifuged for 5-10 seconds and the flow through was discarded. Wash solution number 2/3 (500µl) was then added, the mixture was centrifuged for 5-10 seconds and the flow through was discarded. This step was repeated and the flow through was discarded. The filter was spun for 1 minute to dry. The filter was transferred to a new collection tube and 50-60µl of pre-heated (to 95 °C) elution solution was added. This was centrifuged for 1 minute. The eluate was analysed on the Nanodrop Spectrophotometer, as described in Section 2.6.3 and then labelled and stored at -80 °C.

**2.6.2.3 RNA extraction from whole blood**

The PAXgene™ Blood RNA Kit (PreAnalytiX®) was employed to extract RNA from whole blood that had been collected into PAXgene Blood RNA
tubes. Tubes were incubated for a minimum of 2 hours at room temperature (15-25 °C) before freezing, in order to achieve complete lysis of blood cells. The first step in the RNA extraction process involved centrifuging the tube at 4500 x g for 10 min. The supernatant was removed by decanting or pipetting. Four millilitres of RNase-free water was added to the pellet and a fresh secondary Hemogard closure device was applied. The sample was then vortexed until the pellet was visibly dissolved, and then centrifuged at 4500 x g for 10 minutes. The entire supernatant was removed by decanting/pipetting. Next, 350µl of Buffer BM1 was added and the sample was vortexed again until the pellet was visibly dissolved. 1.5 mLs of sample was pipetted into a 1.5ml microcentrifuge tube, to which 300µl of Buffer BM2 and 40µl of proteinase K were added. The mixture was vortexed for 5 seconds and incubated for 10 minutes at 55 °C in a shaker-incubator at 900 RPM. The temperature of the shaker-incubator was changed to 65 °C after incubation. The sample was pipetted into a PAXgene Shredder spin column (lilac) and centrifuged at 20,000 x g for 3 min. The entire supernatant of the flow through was carefully transferred into a new microcentrifuge tube taking care not to disturb pellet. Next, 700µl of isopropanol (100%) was added and vortexed to mix. 700µl of sample was pipetted into the PAXgene RNA spin column (red) and centrifuged at 20,000 x g for 1 minute. The spin column was then placed in a fresh 2ml tube and the old processing tube containing the flow through was discarded. The remaining sample was pipetted into the spin column and centrifuged as before, discarding the flow through in the old processing tube. Next, 350µl of Buffer BM3 was added to the spin column and centrifuged for 15 seconds at 20,000 x g. The spin column was placed in a new 2ml processing tube and old processing tube containing the flow through was discarded. Ten microliters of DNase I stock solution was added to 70µl buffer RDD (per sample) in a 1.5ml tube and mixed gently by flicking. The mixture was then centrifuged briefly to collect residual liquid from the sides of the tube and 80µl was then pipetted directly onto the column membrane. It was then incubated at room temperature for 15 minutes. Next, 350µl of Buffer BM3 was added to the column and centrifuged for 15 seconds, after which the old processing tube
containing the flow though was discarded. 500µl of Buffer BM4 was then added to the spin column and centrifuged for 2 minutes. The flow through was discarded. This step was repeated, this time centrifuging for 2 minutes. The flow through was discarded in the processing tube and the spin column was placed into a new 2ml processing tube. This was then centrifuged for 1 minute to remove any residual ethanol. The spin column was then placed into a new microcentrifuge tube and 40µl of Buffer BR5 was pipetted directly onto membrane. This was centrifuged for 1 minute to elute the RNA. This step was repeated using 40µl of BM5 and the eluate was incubated at 65 °C, without shaking, for 5 minutes. It was chilled immediately on ice, analysed using the Nanodrop Spectrophotometer (using Buffer BM5 to blank), and stored at -80 °C.

2.6.3. RNA Analysis using Nanodrop Spectrophotometry

It is necessary to determine the quantity and quality of extracted RNA following each of the described extraction protocols. The Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) was used in all cases for this purpose. This method allows the concentration of RNA to be measured. Depending on the extraction method that was used, different impurities can be expected (e.g. Trizol™, humic acids, carbohydrates, Guanidine thiocyanate, nucleotides, peptides, EDTA, phenol and protein). In solution, pure RNA typically has A260/A280 ratios of 1.8 to 2.0. If the absorbance ratio is significantly less, the nucleic acid is probably contaminated with protein. Accurate quantification of nucleic acid is not reliable without prior purification, and the efficacy of this can be judged by the A260 /A280 ratio. For RNA samples, ratio values <2.0 indicate genomic DNA contamination. DNase 1 treatment during RNA purification can eliminate this. Ratio values <1.5 indicate impurities of extraction chemicals or incompletely removed constituents of cells. RNase-free water (or Buffer, depending on the preceding extraction protocol) was used to blank the instrument before beginning. A sample volume of 1.1µl was loaded onto the apparatus pedestal for each measurement and the
instrument arm was used to compress the sample to form a column held in place by surface tension. The sample type RNA-33 was selected for the analysis of miRNAs in this work. RNA concentration was calculated by the instrument using the formula below:

\[
\text{RNA concentration (ng/µl)} = \frac{(A_{260} \times e)}{b}
\]

Where:

- \(A_{260}\) = Absorbance at 260nm
- \(E\) = extinction coefficient (ng-cm/µl)
- \(B\) = pathlength (cm)

### 2.6.4 TaqMan Low Density Array (TLDA)

TaqMan Low Density Arrays were performed in order to identify potentially interesting miRNA targets in the serum-derived exosomes of nude athymic mice that had been inoculated with HCC1954 cells (Section 2.9.8). Each TaqMan Array card contains 384 wells that are pre-loaded with TaqMan miRNA Expression Assays. Card A was utilised in this work as it focuses on well-characterised miRNAs (complete list of targets shown in Appendix 3). Each TaqMan Array evaluates from eight aliquots of a cDNA sample generated in a reverse transcription step using random primers on 7900HT systems. The TaqMan Array functions as an array of reaction vessels for the PCR step. The wells of the TaqMan Array contain TaqMan miRNA expression Assays that detect real-time amplification of user-specified targets. Relative levels of miRNA expression are determined from the fluorescence data generated during PCR using the Applied Biosystems 7900 HT Fast Real-Time PCR System Relative Quantitation software. The TaqMan Array is designed for two-step RT-PCR. In the reverse-transcription (RT) step, cDNA is reverse transcribed from samples of total
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RNA using random primers from the High Capacity cDNA Archive Kit. In the PCR step, the PCR products are amplified from cDNA samples using the TaqMan® Universal PCR Master Mix and TaqMan® miRNA Expression Assays. The TaqMan Assays are pre-loaded in each reaction well of the TaqMan Array. U6 was used as a synthetic endogenous control for each plate.

One additional sample that was included in the array was an exosome isolate from HCC1954 cells which were used for comparison purposes. The quantity and quality of extracted RNA was assessed using the Nanodrop Spectrophotometer as described in Section 2.6.3. The volume of RNA for inclusion in cDNA synthesis was based on protein estimation values. cDNA synthesis was carried out by combining 4.5µl of RT reaction mix with 3µl of RNA (600ng for high yield samples and 250ng for low yield samples) in 0.2µl tubes. A No Template Control (NTC) was prepared by simply adding Nuclease-free water (NFW) to the RT Reaction Mix. The tubes were inverted six times to mix and spun briefly. They were then incubated on ice for 5 minutes, before loading into the 7900HT Thermal-cycler. Thermal-cycling conditions were as follows:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle (40 Cycles)</td>
<td>16°C</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>42°C</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>50°C</td>
<td>1 sec</td>
</tr>
<tr>
<td>Hold</td>
<td>85°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Table 2.4 Thermal-cycling conditions for Taqman Low Density Array.
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The cDNA was then stored at -20 °C until required. In order to carry out the array, cDNA samples were thawed on ice. The TaqMan Universal PCR Master Mix was mixed by swirling the bottle. The following were added to a 1.5mL microcentrifuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume for One Array (µl)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman® Universal PCR Master Mix, No AmpErase®UNG, 2X</td>
<td>450</td>
</tr>
<tr>
<td>Megaplex™ RT product</td>
<td>6</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>444</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>900</strong></td>
</tr>
</tbody>
</table>

Table 2.5 Components for RT-PCR.

*Includes 12.5% excess for volume loss from pipetting

The tubes were inverted 6 times to mix and centrifuged briefly. TaqMan® Array cards were placed flat in the hood, foil-side down. 100µl of sample-specific PCR reaction mix was aspirated using a 100µl micropipette. The micropipette was held at an angle and the tip was inserted into the fill port (Figure 2.16). The 100µl was dispensed into the fill reservoir taking care not to touch the foil below or to positively displace which may lead to the formation of air bubbles.
The same procedure was employed to fill each of the 8 reservoirs. The card was then centrifuged at for 1 minute at 1,200 RPM twice in order to draw the PCR reaction mix into the reaction wells. The cards were then sealed using a precision stylus® assembly (carriage). The top of the array card (containing the fill reservoirs) was trimmed off using a scissors. The array was then run using the 384 Wells TaqMan Low Density Array programme on the Applied Biosystems 7900HT Fast Real-Time PCR System using the following parameters:

40 cycles at:

- 50°C x 2 minutes
- 94.5 °C x 10 minutes
- 97°C x 30 seconds
- 59.7 °C x 1 minute

### 2.6.5 Reverse Transcription

Reverse Transcription (RT) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA or poly(A) RNA, a reverse transcriptase enzyme, a primer, dNTPs and an RNase inhibitor. The resulting cDNA can be used in RQ-
PCR reactions. All reverse transcription reactions were carried out using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems), in vented hoods that had been decontaminated using the UV light and 70% IMS prior to use. Reverse Transcription reaction components were thawed on ice and centrifuged prior to use. The RNase inhibitor and Multiscribe were kept in the freezer until required. Stem-loop primers were specific to the target miRNA of interest. As such, a different Premix was prepared for each target miRNA. The reaction mix for each RNA sample was prepared in 0.2µl tubes in a cooling tray and according to Table 2.6. An RT Blank was prepared for each miRNA target by using Nuclease Free water in place of RNA.

<table>
<thead>
<tr>
<th>cDNA Reaction Mix</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dDTP Mix (100mM)</td>
<td>0.17 µl</td>
</tr>
<tr>
<td>10x RT buffer</td>
<td>1.65 µl</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>4.57 µl</td>
</tr>
<tr>
<td>RNase Inhibitor (20U/uL)</td>
<td>0.21 µl</td>
</tr>
<tr>
<td>Multiscribe (500U/uL)</td>
<td>1.1 µl</td>
</tr>
<tr>
<td>Stem Loop Primer</td>
<td>3.1 µl</td>
</tr>
<tr>
<td>Premix</td>
<td>10 µl</td>
</tr>
<tr>
<td>Total miRNA</td>
<td>5.0 µl</td>
</tr>
<tr>
<td><strong>Total Reaction Volume</strong></td>
<td><strong>15µl</strong></td>
</tr>
</tbody>
</table>

Table 2.6 Volumes of reaction components for Reverse Transcription.

Samples were mixed thoroughly in a centrifuge prior to loading into the Thermal cycler, which was run at the settings outlined in Table 2.7.
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<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mins</td>
<td>16 °C</td>
</tr>
<tr>
<td>30 mins</td>
<td>42 °C</td>
</tr>
<tr>
<td>50 mins</td>
<td>85 °C</td>
</tr>
<tr>
<td>∞</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

Table 2.7 Reaction times and temperatures for Thermal Cycler.

When the reverse transcription process was complete samples were centrifuged and transferred to RNA-free polypropylene tubes, labelled and stored at -20 °C until required for RQ-PCR.

2.6.6 Relative Quantification Polymerase Chain Reaction

Relative quantification determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. RQ is performed using real-time PCR. In real-time PCR assays, the progress of the PCR is monitored as it occurs. Data are collected throughout the PCR process. In real-time PCR, reactions are characterized by the point in time during cycling when amplification of the target is first detected rather than the amount of target accumulated at the end of the PCR. The Cycle Threshold (Ct) is the point at which sufficient amplified product has accumulated to produce a detectable fluorescent signal. A lower Ct value signifies more abundant template. MiRNA pre-developed assay reagents (PDAR) were obtained from Applied Biosystems. Premix was prepared for each miRNA target, in brown eppendorf tubes in order to reduce exposure of the light-sensitive PDARs, using the components outlined in Table 2.8. An endogenous control miRNA, with stable expression across different samples, was also included for each sample in
order to facilitate relative quantification. MiR-16 and U6 were used as endogenous controls in this work.

<table>
<thead>
<tr>
<th>ABI miRNA Kit</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastermix (Fast)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>NFW (Nuclease free water)</td>
<td>3.8 µl</td>
</tr>
<tr>
<td>miRNA PDAR</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>cDNA</td>
<td>0.7 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 2.8 Reaction components for Polymerase Chain Reaction.

The premix was centrifuged before adding 9.3µl to each well of the Fast Optical 96-Well Plate using an electronic multipipette. The target-specific cDNA (0.7µl) was then added to each well bringing the final reaction volume to 10µl. A No Template Control (NTC) Blank was used for each miRNA target to ensure that no contamination was present in the Premix. This consisted of NFW in place of cDNA. All samples were loaded in triplicate. An Inter Assay Control (IAC) was used on each plate, the purpose of which was to ensure consistency across all RQ-PCR runs. The IAC consisted of cDNA for miR-744-5p that was synthesised from a cell pellet. The standard deviation between plates was required to be <0.3. Figure 2.8 illustrates a sample plate plan.
Figure 2.8 96-well plate plan for Polymerase Chain Reaction. EC= Endogenous control.

The plate was sealed and centrifuged for 1 minute at 8064 x g to ensure that any bubbles were resolved. The plate was loaded into the 7900HT Fast Real-Time PCR System (Applied Biosystems) and run using the parameters outlined below:

40 cycles at:

- 95 °C x 20 seconds
- 95 °C x 1 second
- 60 °C x 20 seconds.

2.6.7 Statistical analysis of RQ-PCR results

Relative Quantification Polymerase Chain Reaction (RQ-PCR) was used in all experiments involving miRNAs. Target miRNAs were expressed relative to endogenous controls throughout. All samples were included in triplicate, with a maximum of 0.3 standard deviations permissible among replicates.
the average of which was termed the average $C_t$ value. The $\Delta C_t$ value was calculated as follows:

$$\Delta C_t = \text{Average } C_t \text{ target - Average } C_t \text{ EC}$$

The sample that had the highest $C_t$ value (i.e. the lowest expresser) was subtracted from all $\Delta C_t$ values and was given the term $\Delta \Delta C_t$ (135). These values were converted to a linear form using the following formula:

$$RQ = 2^{-\Delta \Delta C_t}$$

Relative Quantification (RQ) refers to the fold change compared to the calibrator. The results were expressed as log values. Statistical analysis was carried out using Minitab® 16 Statistical Software. Independent two-sample $t$-tests were used to compare 2 independent groups. One-way analysis of variance (ANOVA) was used to compare more than 2 groups. Both tests allow the assessment of significance. P-values $<0.05$ were deemed statistically significant. Data were presented as boxplots where the box represents the interquartile range (75% of values) and the whiskers indicate the range of values. The horizontal line represents the median value and the circle corresponds to the mean value.

### 2.7 Profiling of exosomal microRNAs

#### 2.7.1 Global microRNA array

Prior to commencement of this project, exosomes secreted by breast cancer cell lines were isolated (as described in Section 2.3.1) and a miRNA array was performed. This encompassed greater than 2,000 annotated miRNAs. In brief, RNA was extracted from the secreted exosomes and the quantity and purity was assessed using the Nanodrop Spectrophotometer. Two micrograms of RNA per sample were sent to Exiqon Services in Denmark for analysis. Three replicates of each sample type were included for analysis. RNA quality was assessed by Exiqon using an Agilent 2100 Bioanalyzer profile. The samples were then labelled with Hy3™ and Hy5™
Materials and Methods

flourophores using the miRCURY™ LNA microRNA Hi-Power Labelling Kit. The Hy3™ labelled samples and a Hy5™ labelled reference RNA sample were mixed pair-wise and were then hybridized to the miRCURY™ LNA microRNA Array 7th Gen (Exiqon, Denmark) according to the experimental set-up employed in the laboratory. The array contained captured probes targeting all human, mouse or rat miRNAs registered in miRBASE 18.0. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). Next, they were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene 9.0 software (BioDiscovery, Inc., USA). After normalization, the data was assessed by Principal Component Analysis (PCA) and two way hierarchical clustering heat-maps. Further details of the global miRNA array are available in the PhD thesis by Glynn C, 2014. I was provided with data on exosomes from the MDA-MB-231 and BT-20 cell lines for analysis and validation. 2 highly ranking targets were selected for validation and further investigation in patient samples using a combination of online miRNA analysis tools and a review of the published literature.

2.7.2 Validation of targets from array

MiRNA targets that were deemed to be potentially interesting following analysis of the array data were validated in multiple exosome replicates using RQ-PCR to ensure that they were in fact detectable in exosomes derived from these cell lines. These targets were also investigated in RNA extracted from the whole blood of patients with breast cancer and healthy volunteers. Independent two-sample t-tests and ANOVA were carried using Minitab® 16 Statistical Software.
2.8 Functional impact of Exosomes

2.8.1 Transfer of Exosomes

Confocal microscopy was employed to visualize transfer of exosomes from Red Fluorescent Protein (RFP) labeled cells to unlabeled recipient cells. Cells were previously engineered by another group member to stably express RFP using a lentiviral construct, followed by puromycin selection. Exosomes from these cells then also fluoresced red (Figure 2.9).
Unlabelled recipient cells were seeded on coverslips in a 6-well plate. 40,000-50,000 cells were required for each well. The plate was incubated at 37 °C for approximately 1hr in order to allow the cells to adhere. The cells
were then viewed using a light microscope to ensure that they were not overly-confluent, and incubated at 37 °C overnight.

The following day RFP labeled exosomes were harvested from donor cells in accordance with the protocol described in Section 2.3.1. The exosomes were re-suspended in 30μL of sterile PBS. A strip of parafilm was placed on moist tissue paper in a humidification box. A bent needle was then used to lift the coverslips from the wells of the 6-well plate. The coverslips were placed cell side up on the parafilm. 20μL of fresh exosome-depleted media and 20μL of PBS were added to a control coverslip. 20μL of fresh exosome-depleted media and 20 μL of RFP-labelled exosomes were added to the test coverslips. A pipette was used to mix. A small square of parafilm was placed over the cells/exosomes, ensuring that the entire coverslip was covered in the liquid. The humidification box was incubated at 37 °C for 4 hours in order to allow exosomes to migrate to recipient cells.

Next, the coverslips were removed from the humidification box, placed in a 6-well plate and immersed in PBS. Cells were then fixed in 4% paraformaldehyde (PFA)/PBS for 10 minutes in the dark. The cells were rinsed in PBS followed by the addition of 0.1% TX-100/PBS to each coverslip. This step permeabilized the cells allowing them to take up the subsequent stains. A 5% Foetal Calf Serum/TX-100 solution was then added to each coverslip. They were covered and incubated at room temperature for a minimum of 1 hour. This is a blocking step i.e. it blocks non-specific binding of antibodies. The excess solution was poured off and each well was washed three times with 1ml of 0.1% TX-100/PBS. A 1% Phalloidin in FCS/TX-100 solution was prepared. Phalloidin interacts with polymeric actin. It is used to study actin structure and organization within eukaryotic cells. It was employed in this experiment as it allowed the identification of the cell membranes of recipient cells. 30 μL was added to squares of parafilm in a humidification box and the coverslip was applied cell side down onto the drop. It was then incubated at room temperature for 1hr. The coverslip was placed back into a 6-well plate and each well was washed
three times with 1ml of 0.1% TX-100/PBS. A 0.05% DAPI stain was prepared in 0.1% TX-100/PBS. 1ml was added to each well and left for exactly 2 minutes in the dark. The slides were then rinsed x3 with PBS. 20 µL of anti-fade reagent (Invitrogen) was added to a glass slide, to which 9 µL of mounting media was added and the coverslip was added cell-side down. The slide was gently blotted with blotting paper. The corners were sealed with nail varnish, labelled and stored in a dark box in the fridge until required. Immunofluorescent Z-stack images (0.1µm steps) were then obtained using an Olympus IX81 Microscope fitted with an Andor Revolution Confocal system (Andor, Belfast, Northern Ireland), 60x oil immersion lens and an EMCCD Andor iXonEM + camera. All Z stack images were processed using Andor IQ 2.3 software and images were presented as maximum intensity projections (MIP).

2.8.2 Impact of exosomal transfer on proliferation

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay was used to examine the impact of breast cancer cell-secreted exosomes on proliferation of MSCs and WI-38 cells, and MSC exosomes on breast cancer cell proliferation. This is a colorimetric method for determining the number of viable cells. The CellTiter 96® AQueous Assay is composed of solutions of a novel tetrazolium compound (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

This assay was used to determine changes in proliferation levels in MSCs and WI-38 cells in response to exosomes secreted by the BT-20 and
HCC1954 cell lines. It was also used to measure changes in proliferation in BT-20 and HCC1954 cells in response to MSC-derived exosomes.

The standard TC 96-well plates were prepared according to the plate plan in Figure 2.10.

Figure 2.10 96-well plate plan for proliferation assays. In this set-up (A) BT-20 and HCC1954-secreted exosomes (abbreviated to ‘exo.’) were added to either MSCs or WI-38 cells or (B) MSC-secreted exosomes were added to either BT-20 or HCC1954 cells.

Eight replicates of each sample were included on each plate. MSCs and WI-38 cells were seeded at a density of $1.0 \times 10^4$ in exosome-depleted media and were incubated overnight at 37 °C in 5% CO$_2$ in order to allow them to adhere to the base of the wells. BT-20 and HCC1954 cells were seeded at $1.0 \times 10^3$ cells per well and were incubated as before. The following
morning the media was changed according to the plate plan and 2µg of exosomes were added to the corresponding wells. The 96-well plate was incubated for 24 hours and the following morning 20µl of 5mg/ml MTS solution was added to each well. This step was carried out in the cell culture hood with the light extinguished as the MTS solution is light sensitive. The plate was then covered in tin foil and returned to the incubator for 3 hours. Following incubation, the plate was read on the Labsystems Multiskan RC plate reader at 490nm. Mean absorbance were calculated across the 8 replicates for each set-up. Each experiment was carried out in triplicate on 3 separate days. Mean and Standard Error of the Mean (SEM) were calculated for each experiment.

2.8.3 Impact of exosomal transfer on angiogenesis

The impact of breast cancer cell-secreted exosomes on angiogenesis was examined by measuring changes in tubule formation in Human Umbilical Vein Endothelial Cells (HUVEC). These cells are derived from the endothelium of veins from the umbilical cord. They are used as a laboratory model system for the study of the function and pathology of endothelial cells (i.e. angiogenesis). HUVEC cells were recovered into EBM® endothelial cell basal media that was supplemented with EGM™ SingleQuots® (which contain growth factors, cytokines, and supplements) 1 week in advance and allowed to reach adequate numbers. Matrigel (BD Biosciences, San Jose, CA) was thawed overnight on ice at 4 °C and then 300µl was added to each well of a standard TC 24-well plate in the cell culture hood. Pipette tips used for this process were chilled overnight at 4 °C as the Matrigel solidifies at room temperature. This was allowed to polymerize in the hood for 30 minutes. 400µl of regular media or exosome-free media were added to the wells according to the plate plan in Figure 2.11. HUVECs (1.9 x 10^4 cells) were added to each well. A positive control was used in well A1, and well A2 acted as a negative control. These consisted of: HUVECs plus regular media plus VEGF (pro-angiogenic stimulant), and HUVECs plus regular media respectively. Ten micrograms
of BT-20 or HCC1954 cell-secreted exosomes were then added to the corresponding wells. Images of each well were captured using an Olympus digital camera (Model No. C-7070 Wide Zoom) and the plate was incubated at 37 °C and 5% CO₂. The wells were imaged at 2 and 4 hours. Tubule formation was assessed visually.

Figure 2.11 Plate plan for angiogenesis assays. Positive control=HUVECs + Regular media + VEGF; Negative control=HUVECs + regular media.

2.9 Exosomal microRNAs as circulating biomarkers for breast cancer

2.9.1 Ethics and Licensing

The in vivo study was licensed under the Cruelty to Animals Act 1876 by the Department of Health and Children. Ethical approval was received from the National University of Ireland Galway Animal Care Research Ethics Committee (Appendix 4).
2.9.2 Animal Facility

The animal study was conducted in the National Centre for Biomedical Engineering Science (NCBES), which houses the animal facility. Animals were housed in individually ventilated cages, with continuous access to food and water. The animals were cared for by dedicated staff members who were responsible for cleaning cages, changing bedding material and feeding the animals regularly. Aseptic precautions were adhered to by all users of the animal facility. This included hand washing and donning of disposable gowns, gloves and hats prior to entering the facility. Seventy per cent IMS was used to clean the LAF hoods and gloves at regular intervals in order to prevent cross-contamination between animals. Instruments and consumables were autoclaved prior to use in the LAF hood.

2.9.3 In vivo Model

The in vivo study was established to identify potentially interesting serum exosome-encapsulated miRNAs which may function as biomarkers for breast cancer. BALB/c nude mice were chosen for this study. This strain of mouse is particularly useful in cancer research as they have low mammary tumour incidence. The mice were allowed to acclimatise for 7 days following delivery to the animal house prior to tumour induction. Figure 2.12 shows the timeline that was employed in the in vivo study.
Chapter 2: Materials and Methods

2.9.4 Tumour Induction

HCC1954 cells were cultured under normal growth conditions in the cell culture laboratory until adequate numbers for injection into all animals were achieved. These cells were trypsinised in the usual manner and were injected fresh into the animals. Two injection sites were chosen for tumour induction: thoracic mammary fat pad (MFP) and subcutaneous (SC) flank injection (Figure 2.13). Ten animals were assigned to the MFP group and 10 to the SC group. 3.5 x 10^6 cells in 0.2ml FBS-free HBSS media containing 50% Matrigel were injected into the 4th inguinal mammary fat pad and 4 x 10^6 HCC1954 cells in 0.2ml FBS-free HBSS media containing 50% Matrigel were injected subcutaneously. All tumour induction procedures were carried out under aseptic conditions in a LAF hood in order to prevent infection. The animals were anaesthetised using inhalation anaesthesia (Isoflurane, 5% induction of anesthesia, 1-2% for maintenance in Oxygen at 1L/min). The tumours were injected using a 24 gauge needle and 1 ml syringe. The anaesthetic source was removed following tumour induction.
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and the animals were allowed to recover spontaneously. The animals were then returned to their cage and monitored to ensure complete recovery. Animals were examined and weighed twice weekly in order to ensure general health and to monitor tumour growth. Tumour volume was monitored using a calliper measurement of tumour length (L), width (W) and depth (D), with volume (V) calculated from this equation: \( V = L \times W \times D \times 0.5 \)

Figure 2.13 Injection sites for in vivo study: (A) Subcutaneous injection site (B) Mammary fat pad injection site.

2.9.5 Study Endpoint

Tumour formation was noted to be more rapid in the MFP group, and as they were located in a more obstructive location, a decision was made to sacrifice them at 30 days. Animal sacrifice was carried out in the SC group at 40 days post-tumour induction. The animals were anaesthetised as described previously and a terminal bleed was carried out through intracardiac puncture of the ventricle through the diaphragm. Blood was withdrawn slowly to prevent the heart from collapsing (Figure 2.14)
Blood samples were collected according to Figure 2.15. Briefly, blood was collected into paediatric serum tubes and allowed to clot (in the dark), for a minimum of 30 minutes. They were then centrifuged at 10,000 x g for 5 minutes. The serum was then drawn off, labelled and stored at -80 °C until required. The animals were then sacrificed using increasing concentrations of CO₂ inhalation. The tumours were then resected, weighed and placed in formalin or RNA later, depending on planned downstream analysis. A ‘Y’ incision was then made in order to allow resection of lymph nodes and solid organs, where appropriate.
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Add blood sample to brown capped paediatric serum tube

Invert sample

Keep tube out of direct sunlight in order to avoid haemolysis

Add subsequent blood samples to the serum tube until a minimum volume of 250µl has been reached

Close the lid and invert the tube after each addition

Transport tube to the Lambe Institute in a cool, dark container (e.g. coolbag if available)

Allow samples to stand at room temperature for 30 mins

Spin at 10,000xg for 5 mins

Remove serum and place into an eppendorf

Proceed directly to exosome isolation or store at -80°C until required

Figure 2.15 Schema for collection of serum from mice for exosome isolation.
2.9.6 Exosome isolation from animal serum

Exosomes were isolated from the serum of animals using the protocol previously outlined in Section 2.3.2. A protein assay was carried out in order to indirectly quantify the amount of exosomes that were present in each exosome pellet. 2 samples of serum exosomes were selected for TEM imaging, 3 were included in a TLDA, 11 were included in the validation cohort for potentially interesting miRNAs and the remaining 4 had protein yield that were deemed to be too low to proceed with reverse transcription.

<table>
<thead>
<tr>
<th>Number of serum samples (n=)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>3</td>
<td>Taqman Low Density Array (TLDA)</td>
</tr>
<tr>
<td>11</td>
<td>Validation of TLDA targets</td>
</tr>
</tbody>
</table>

Table 2.9 Fate of serum exosomes derived from *in vivo* work.

2.9.7 RNA extraction from animal serum exosomes

RNA was extracted from animal serum exosomes using the Trizol and miRVana modified method detailed in Section 2.6.2.2. Nanodrop Spectrophotometry was carried out on each sample for RNA quantification and quality control.

2.9.8 TaqMan Low Density Array (TLDA)

TaqMan Low Density Arrays were performed in order to identify potentially interesting miRNA targets in the serum-derived exosomes of nude athymic mice that had been inoculated with HCC1954 cells. One
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An additional sample that was included in the array was an exosome isolate from HCC1954 cells which were used for comparison purposes. Approximately 600ng/µl and 250ng/µl of protein were used for cDNA synthesis in the high and low yield groups respectively. The TLDA was performed according to Section 2.6.4.

2.9.9 PCR validation of targets

RQ-PCR analysis of potentially interesting miRNA targets that had been identified on the TLDA was performed using the remaining animal serum exosomes samples (n=11) in order to validate these targets. These targets were then investigated in the serum exosomes of patients with cancer and healthy controls, in addition to cell line-secreted exosomes.

2.9.10 Characterisation of animal exosomes

Exosomes isolated using the above protocol were characterised using Transmission Electron Microscopy and Western Blot as outlined in Sections 2.5.1 and 2.5.2 respectively.
Chapter 3

Prospective comparison of outcomes after treatment for Triple-negative and Non-Triple-negative breast cancer
3.1 Introduction

Breast cancer is a heterogeneous group of diseases that vary in terms of histology, patterns of metastasis, therapeutic response, and survival outcomes (137). Triple-negative breast cancers (TNBC) are characterized by a lack of expression of oestrogen (ER), progesterone (PR) and human epidermal growth factor type 2 receptors (HER 2) (138). TNBC is diagnosed more frequently in younger women, those with BRCA1 mutations and in premenopausal African and African-American women (139, 140). This breast cancer subtype is particularly aggressive, and, owing to a paucity of effective targeted therapeutic options, it is associated with a poor prognosis (138). Patients with TNBC relapse earlier than those with Non-TNBC, and have higher recurrence rates in the first 1-3 years following treatment. Additionally, they are reported to have higher rates of recurrence in visceral organs with lower rates of bony metastases compared to other breast cancer subtypes (141).

The absence of a biological target in TNBC necessitates definitive local control. The use of breast conserving surgery in this cohort of patients has been scrutinised with studies showing higher rates of locoregional recurrence in triple-negative patients who undergo BCT when compared to non-triple-negative patients (142). Abdulkarim and colleagues, however, demonstrated that locoregional recurrence was the same in triple-negative patients who underwent breast-conserving surgery versus those who were treated with mastectomy providing that they received adjuvant radiotherapy (143).

With respect to chemotherapy, it has been found that certain subtypes of TNBC are highly chemosensitive, with a pathological complete response (pCR) being indicative of a favourable prognosis (144). Conversely, patients who harbour residual disease in the resected specimen following neoadjuvant chemotherapy, have a poor prognosis (144). Ongoing research into molecular-based targets in TNBC may ultimately allow clinicians to tailor treatment to the biological tumour subtype in order to improve patient outcomes.
3.2 Aim
The aim of this study was to compare contemporary management strategies and response to therapy in a cohort of TNBC patients, to a corresponding group of age- and stage- and NPI-matched Non-TNBC patients.

3.3 Methods
Data were obtained from a prospectively maintained database that included patients who were treated for breast cancer at a tertiary referral centre. A total of 2302 new cases of breast cancer were diagnosed between January 2007 and December 2013, of which 142 patients were triple-negative. Patients were matched for International Union Against Cancer stage, Nottingham Prognostic Index (NPI) and age at diagnosis with 142 Non-TNBC patients. The primary outcome of interest was overall survival, which was defined as the interval between diagnosis and death from any cause. Disease-free survival was defined as the interval between diagnosis and recurrence of any type. The following variables were evaluated: demographic data and clinical characteristics (mean age at diagnosis and reason for presentation); tumour characteristics (type of surgery, TNM AJCC version VI classification and staging, NPI, biological tumour characterisation [ER, PR and HER2 status]); neoadjuvant therapy received (chemotherapy); adjuvant therapy received (chemotherapy, radiotherapy, hormonal therapy and trastuzumab). Statistical analysis was performed using Minitab\textsuperscript{®} 14 for Windows (Minitab, State College, Pennsylvania, USA) \textsuperscript{®}. Continuous variables were summarised using descriptive statistics, including number of patients, mean, standard deviation and median. Direct comparisons between TNBC and Non-TNBC patients were made using the unpaired t-test, Fisher’s exact test and \( \chi^2 \) test as appropriate. The statistical procedure employed for analysis of outcomes was Kaplan–Meier survival estimates in combination with the log rank test.
3.4 Results

3.4.1 Patient and tumour characteristics

A total of 2302 patients were treated between January 2007 and December 2013. One hundred and forty-two of these patients had Triple-negative breast cancer. A Non-TNBC group consisting of 142 age and stage-matched patients was selected for comparison with the TNBC group. Clinicopathological details of both groups are provided in Table 3.1.
### Clinicopathological details of patients in Triple-negative and Non-Triple-negative cohorts.

There was no significant difference in the mean age at diagnosis between the two groups (58 years (range 24-86) versus 59 years (range 21-85) years in the TNBC and Non-TNBC groups respectively, P=0.568, unpaired t-test). The

<table>
<thead>
<tr>
<th></th>
<th>TNBC n (%)</th>
<th>Non-TNBC n (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at diagnosis</td>
<td>58 years (Range 24-86)</td>
<td>59 years (Range 21-85)</td>
<td>0.568</td>
</tr>
<tr>
<td>Most common reason for referral</td>
<td>Breast lump</td>
<td>Breast lump</td>
<td></td>
</tr>
<tr>
<td>Mean tumour size (cm)</td>
<td>3.25 (+/-1.85)</td>
<td>3.46 (+/-2.23)</td>
<td>0.402</td>
</tr>
<tr>
<td>Stage at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39 (27%)</td>
<td>25 (18%)</td>
<td>0.089</td>
</tr>
<tr>
<td>2</td>
<td>68 (48%)</td>
<td>81 (57%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>25 (18%)</td>
<td>31 (21%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10 (7%)</td>
<td>5 (4%)</td>
<td></td>
</tr>
<tr>
<td>NPI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9 (6%)</td>
<td>14 (10%)</td>
<td>0.421</td>
</tr>
<tr>
<td>3</td>
<td>80 (56%)</td>
<td>82 (58%)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45 (32%)</td>
<td>38 (26%)</td>
<td></td>
</tr>
<tr>
<td>Not calculated, stage 4 at diagnosis</td>
<td>8 (6%)</td>
<td>8 (6%)</td>
<td></td>
</tr>
<tr>
<td>Primary Surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wide local excision</td>
<td>89 (63%)</td>
<td>79 (56%)</td>
<td>0.277</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>43 (30%)</td>
<td>52 (36%)</td>
<td>0.314</td>
</tr>
<tr>
<td>Axillary Surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLNB</td>
<td>64 (45%)</td>
<td>74 (52%)</td>
<td>0.285</td>
</tr>
<tr>
<td>ALND</td>
<td>69 (49%)</td>
<td>57 (40%)</td>
<td>0.189</td>
</tr>
<tr>
<td>SLNB &amp; ALND</td>
<td>18 (13%)</td>
<td>19 (13%)</td>
<td>1.0</td>
</tr>
<tr>
<td>ALND alone</td>
<td>51 (36%)</td>
<td>38 (27%)</td>
<td>0.125</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>76 (54%)</td>
<td>76 (54%)</td>
<td>0.527</td>
</tr>
<tr>
<td>N1</td>
<td>38 (27%)</td>
<td>38 (27%)</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>13 (9%)</td>
<td>13 (9%)</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>13 (9%)</td>
<td>13 (9%)</td>
<td></td>
</tr>
<tr>
<td>NX (stage 4 at diagnosis)</td>
<td>2 (1%)</td>
<td>2 (1%)</td>
<td></td>
</tr>
<tr>
<td>Epithelial subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>-</td>
<td>13 (80%)</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>-</td>
<td>19 (13%)</td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>-</td>
<td>10 (7%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1
most common reason for referral to the symptomatic breast clinic in both groups was the presence of a breast lump (68% of patients), either detected by the patients themselves or by their general practitioner. The mean size of the primary breast carcinoma was 3.25 (±1.85) cm (range 0.4-10cm) and 3.46 (±2.23) cm (range 0.3-15cm) in the TNBC and Non-TNBC groups respectively (P=0.402 unpaired t-test). There was no difference in tumour stage between the two groups (P=0.089, χ2 test). Similarly, no difference in NPI was noted between both groups (P=0.421, χ2 test). The ductal histological subtype was present in 127 (89%) and 116 (82%), TNBC and Non-TNBC tumours respectively. The lobular histological subtype was present in 4 (3%), TNBC and 18 (13%), Non-TNBC tumours. Lymph node staging was as follows for the TNBC and Non-TNBC groups respectively: pN0 in 76 patients (54%) versus 74 patients (52%); pN1 in 38 patients (27%) versus 41 patients (29%); pN2 in 13 patients (9%) versus 18 patients (13%); and pN3 in 13 patients (9%) versus 5 patients (3%).

3.4.2 Locoregional therapy

Of the 142 patients with TNBC, 89 (63%) were treated with a wide local excision (WLE), with the remainder having either a mastectomy (n=43; 30%) or no surgery (n=10; 7%). The results were similar to that of the Non-TNBC group with 79 patients (56%) undergoing a wide local excision (P=0.277 Fisher’s exact test), and 52 (36%) undergoing a mastectomy (P=0.314 Fisher’s exact test). A similar number of patients in the Non-TNBC group did not have any therapeutic breast procedure (n=11; 8%; P=1.0 Fisher’s exact test). Table 1 outlines axillary procedures that were undertaken in the triple-negative and non-triple-negative cohorts. Nine (6%) TNBC and 11 (8%) of Non-TNBC patients did not undergo any axillary staging procedure (P=0.817 Fisher’s exact test). Of the TNBC patients that did not undergo an axillary staging procedure, 6 had distant metastases at presentation and the remainder either had early stage disease or were deemed unfit for surgery due to advanced age or significant medical comorbidities. Five Non-TNBC patients were M1 at diagnosis and the
remaining patients had either age or medical factors that precluded axillary surgery. Adjuvant radiotherapy was administered to 116 (82%) of TNBC and 112 (78%) of Non-TNBC patients (P=0.655 Fisher’s exact test). A significantly higher proportion of TNBC patients who underwent WLE received adjuvant radiotherapy compared to those who were treated with mastectomy (94% (n=84) in WLE group versus 56% (n=24) in mastectomy group, P=0.0001, Fisher’s exact test). Post-mastectomy radiation therapy was administered in 56% (n=24) of TNBC and 60% (n=31) on Non-TNBC patients (P=0.835, Fisher’s exact test).

### 3.4.3 Systemic therapy

A significantly higher number of patients in the TNBC group received chemotherapy (84% (n=119) versus 61% (n=86), P=0.0001, Fisher’s exact test). Of the TNBC patients that were treated with chemotherapy, 40 (34%) were given this treatment in the neoadjuvant setting with the remainder being treated adjuvantly. In the Non-TNBC group 14 patients (16%) received neoadjuvant chemotherapy. Pathological response rates were similar between the 2 groups (P=0.398, Fisher’s exact test) (Table 3.2).

<table>
<thead>
<tr>
<th></th>
<th>TNBC n (%)</th>
<th>Non-TNBC n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete pathological response</td>
<td>10 (25%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Marked response</td>
<td>7 (18%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Partial response</td>
<td>13 (32%)</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>No response</td>
<td>10 (25%)</td>
<td>3 (21%)</td>
</tr>
</tbody>
</table>

Table 3.2 Pathological response rates in patients with TNBC and Non-TNBC cancer.
Of the 23 TNBC patients that did not receive chemotherapy, 16 patients (70%) had early stage disease (T1N0) and the remainder either had co-morbidity factors that precluded chemotherapy or elected not to undergo treatment.

### 3.4.4 Overall and disease-free survival

The difference in overall survival between the two groups was statistically significant: 77% of TNBC patients (n=109) were alive at a mean follow up of 32 months versus 92% of Non-TNBC patients (n=130) at a mean follow up of 38 months (P=0.001, Fisher’s exact test). Kaplan-Meier survival estimates in combination with the Log rank test showed a significant difference in survival between TNBC and Non-TNBC patients (P=0.0). Figure 3.1 demonstrates the inferior survival associated with the TNBC subtype.

![Figure 3.1 Overall survival in TNBC versus Non-TNBC patients (P=0.0 Log Rank Test).](image)

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This inferior survival was also noted when patients were stratified according to age (Figure 3.2, P=0.0 Log rank test).

Figure 3.2 Overall survival in TNBC versus Non-TNBC by age-group (P=0.0 Log Rank Test).
Follow-up data for TNBC and Non-TNBC patients are detailed in Table 3.3.

<table>
<thead>
<tr>
<th>Follow-up data</th>
<th>TNBC n (%)</th>
<th>Non-TNBC n (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients alive at last follow up</td>
<td>109 (76.8)</td>
<td>130 (91.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Alive without evidence of disease progression</td>
<td>89 (62.7)</td>
<td>123 (89.6)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Alive with locoregional +/- distant disease progression</td>
<td>7 (4.9)</td>
<td>-</td>
<td>0.015</td>
</tr>
<tr>
<td>Alive with distant disease</td>
<td>5 (3.5)</td>
<td>4 (2.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Alive with subsequent contralateral disease/new primary</td>
<td>1 (0.7)</td>
<td>3 (2.1)</td>
<td>0.622</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (4.9)</td>
<td>-</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Table 3.3 Follow-up data on TNBC and Non-TNBC patients.
Chapter 3: Comparison of outcomes after treatment for breast cancer

The Kaplan-Meier analysis, when repeated and stratified by NPI, showed that the survival difference between TNBC and Non-TNBC cancers was independent of NPI (P=0.0 Log rank test, Figure 3.3).

Figure 3.3 Overall survival in TNBC versus Non-TNBC by Nottingham Prognostic Index (P=0.0 Log Rank Test).
Locoregional recurrence rates were similar in TNBC patients that were treated with mastectomy compared to patients that underwent WLE (P=0.449 Log rank test, Figure 3.4).

Figure 3.4 Locoregional recurrence rates in TNBC patients undergoing wide local excision versus mastectomy (P=0.449 Log Rank Test).
No difference in survival was noted in the TNBC group of patients that received chemotherapy neoadjuvantly compared to those who were treated post-operatively (P= 0.415 Log rank test, Figure 3.5).

Figure 3.5 Overall survival in TNBC patients who received neoadjuvant chemotherapy versus those who were treated in the adjuvant setting (P=0.415 Log Rank Test).
However, a significant difference in survival was noted between TNBC patients who responded differentially to neoadjuvant chemotherapy (P=0.035 Log rank test, Figure 3.6).

![Graph showing overall survival in TNBC patients treated with neoadjuvant chemotherapy by pathological response.](image)

Figure 3.6 Overall survival in TNBC patients who were treated with neoadjuvant chemotherapy by pathological response (P=0.035 Log Rank Test).

No difference in the proportions of patients in each NPI group was noted between groups of patients who responded to varying degrees to neoadjuvant chemotherapy (P=0.7, χ² test).
3.5 Discussion

This prospective comparison study was designed to evaluate therapeutic strategies and outcomes in Triple-negative breast cancer patients attending a tertiary referral breast unit. Patients were matched for age, stage and NPI with a corresponding group of Non-TNBC patients. Overall and disease-free survival in triple-negative patients was inferior to that of their non-triple-negative counterparts, despite adherence to international best practice in the latter group. This finding reflects outcome data reported in multiple studies (138). Older patients in this study were also found to have a significantly worse prognosis. This finding may be reflective of the lower rate of chemotherapy uptake in this group or it may indicate a relative level of chemo-resistance.

Nottingham Prognostic Index correlated well with overall survival in this study however the survival curves by NPI for the triple-negative group reflected a significantly less favourable outcome for these patients. This difference in outcome, despite both cohorts being well matched in terms of age and stage, is likely a function of tumour biology, and highlights the fact that we are failing to target TNBC at a molecular level. This point emphasizes the need for the development of targeted therapeutic options in order to bring survival in line with that of Non-TNBC patients for whom effective targeted treatment is available.

The use of breast conserving therapy (BCT) in patients with TNBC has been investigated due to the lack of targeted therapeutic options available to this cohort. Locoregional recurrence rates were the same in both groups of TNBC patients regardless of the therapeutic breast procedure that was undertaken. A systematic review by Lowery et al., (2012) (142) examining locoregional recurrence rates after BCT also reported no significant difference in outcomes comparing TNBC patients treated with WLE versus mastectomy (142). The use of radiotherapy in TNBC is the subject of much debate. This study adds to the consensus that there is currently insufficient evidence to advocate post-mastectomy radiation therapy based on subtype
alone in the absence of other high-risk features (positive nodes, positive tumour margins, or tumour size greater than 5cm) (145, 146). For patients undergoing WLE, a boost to the tumour bed following whole breast radiation therapy (WBRT) is recommended as this has been shown to further reduce the risk of local recurrence beyond that of WBRT alone (147).

Subgroup analysis of TNBC patients to whom chemotherapy was administered in the neoadjuvant setting revealed that a good pathological response was indicative of a significantly improved survival when compared to those in whom no pathological response was detected. These results are in line with that of other studies where a pCR was indicative of a good prognosis (144). Consideration should therefore be given to the addition of second line chemotherapeutic agents to standard neoadjuvant regimens in order to increase the probability of achieving a pCR. The results of the I-SPY 2 trial showed promise in this regard, with a pCR rate of 52% noted when veliparib and carboplatin were administered in addition to standard therapy, versus 26% for standard therapy alone (148).

In this study, the difference in survival between TNBC patients who responded differentially to neoadjuvant chemotherapy was observed despite similar proportions of NPI group patients being present in each cohort. This highlights an important feature of the triple-negative subtype in that the use of NPI for prognostication may not be sufficient in this cohort. In order to overcome this issue, we propose that consideration should be given to the inclusion of pathological response to neoadjuvant chemotherapy in a modified NPI for use in TNBC. Future work will focus on the development and validation of such an algorithm. The development of a circulating biomarker which may facilitate prediction of response or resistance to therapy, will also be of immense value in this setting.
Chapter 4

Intercellular communication in the breast tumour microenvironment
Chapter 4: Intercellular communication in breast cancer

4.1 Introduction

Mesenchymal stem cells (MSC) are known to be present in the primary breast tumour microenvironment where they form part of the stroma, giving both functional and structural support to the tumour (149). There is conflicting evidence regarding the role that MSCs play in the tumour microenvironment, with some studies suggesting that they are essential to tumour establishment, progression and metastasis (150, 151), and others suggesting a tumour suppressor role (152, 153). Regardless of these differing opinions, MSCs are known to be present in the tumour microenvironment and to communicate with cancer cells. Studies have demonstrated that MSCs are capable of influencing the morphology and proliferation of neighbouring cells through cell-to-cell interactions and the secretion of chemoattractant cytokines and paracrine factors (114).

The ability of exosomes to shuttle genetic material, including mRNA and miRNA, between cells and to remain functional in that location, has been highlighted as a mechanism of intercellular communication (83, 154). Studies have shown that exosomes have the capacity to confer the phenotypic traits of parent cells to recipient cells (90, 129). As such they may play an important role in intercellular communication in the primary tumour microenvironment (90, 155). Work carried out by O’Brien et al., (2013) (90) demonstrated that exosomes isolated from an invasive variant of a Triple-negative breast cancer cell line were capable of significantly increasing the proliferation, migration and invasion capacity of a variety of breast cancer cell lines when compared to the parent cell exosomes. Furthermore, the invasive cell-derived exosomes increased these cancer hallmarks in the parent cell line (90). Exosomes derived from the more aggressive cell variant also stimulated significantly more endothelial tubule formation compared to parent cell exosomes (90). The development of an in depth understanding of the roles that exosomes play in intercellular communication in the primary tumour microenvironment may allow us to modify these cellular interactions for therapeutic gain.
4.2 Aims

- To isolate, quantify and characterise exosomes secreted by breast cancer cell lines and Mesenchymal Stem Cells (MSC)
- To demonstrate transfer of exosomes between cell populations
- To demonstrate the impact of exosomal transfer on proliferation and angiogenesis in recipient cells

4.3 Materials and Methods

**Isolation, quantification and characterisation of exosomes**

Exosomes were isolated from the conditioned media of breast cancer cell lines BT-20 and HCC1954 and from MSCs using the protocol described in Section 2.3.1. BCA protein assays (Section 2.4) were used to indirectly quantify the amount of exosomes that were isolated in each preparation. Exosomes were re-suspended in 55µl of PBS following ultracentrifugation, 5µl of which was then used for the protein assay. This information allowed calculation of the total amount of protein that was present in the exosome pellet. This data was used to standardise the amount of exosomes that were loaded for Western Blot Analysis, and transferred for proliferation and angiogenesis assays.

Transmission Electron Microscopy (TEM) was then used to confirm successful isolation of exosomes by facilitating morphological analysis (Section 2.5.1) and size estimation of secreted exosomes. Western Blot analysis (Section 2.5.2) of exosomes was also performed using exosome samples targeting the exosome-associated protein CD63.

**Transfer of exosomes**

Confocal microscopy was employed to visualise exosome transfer between cells. This technique is described in detail in Section 2.8.1. Briefly, exosomes were isolated from a population of breast cancer cells that had been engineered by another research group member to express red fluorescent protein (RFP). Exosomes derived from these cells then also
Chapter 4: Intercellular communication in breast cancer

contained RFP. The fluorescently labelled exosomes were transferred to recipient cells, whose nuclei were stained with DAPI, and transfer was assessed using a confocal microscope.

**Functional impact of exosomes**

In order to determine the impact of exosomes on proliferation in recipient cells, 3 different experimental set-ups were used:

- MSC-secreted exosomes were applied to BT-20 or HCC1954 cells
- BT-20 or HCC1954 exosomes were applied to MSCs
- BT-20 or HCC1954-derived exosomes were added to the control fibroblast cell population WI-38 (Section 2.8.2).

All experiments were carried out in a 96-well plate format. The impact of breast cancer exosomes on angiogenesis in HUVEC cells was also determined (Section 2.8.3).
### Chapter 4: Intercellular communication in breast cancer

#### 4.4 Results

##### 4.4.1 Isolation, quantification and characterisation of exosomes

Using differential centrifugation, microfiltration and ultracentrifugation, exosomes were isolated from cell-conditioned media. A primary fixative was added prior to ultracentrifugation, followed by secondary fixation, dehydration and embedding in a resin. The exosomes were then viewed using TEM, first at a low magnification, which allowed identification of the area of resin block in which the exosomes were contained. Higher magnification was then used to visualize exosomal size and shape, which was confirmed to be oval or round, and measuring 30-120nm in diameter (Figure 4.1). In Figure 4.1 (C), the lipid-bilayer structure of the exosomes can be appreciated.
Figure 4.1 Transmission Electron Microscopy images of BT-20 (A-D) and HCC1954 (E-H)-secreted exosomes that were isolated following 48 hours of culture in exosome-depleted media. (A), (B), (E) and (F) are taken at a magnification of 30,000x. Images (C), (D), (G) and (H) are taken at 40,000x. Exosomal measurements are present in images (B), (D), (F) and (H).
Protein was extracted from exosomes, quantified and Western Blot Analysis employed to detect the presence of the exosome-associated protein CD63. This was visualized at the predicted band size of 50-60kDa (Figure 4.2). CD63 was detectable in exosomes derived from both BT-20 and HCC1954 cells, although a stronger band was detected in the former despite equal protein loading for each sample (33µg and 35µg for BT-20 and HCC1954 exosomes respectively). A second non-specific band was also detected in the case of HCC1954 exosomes at a band size of approximately 80kDa. This data, combined with TEM images confirmed successful isolation of exosomes.

Figure 4.2 Western Blot showing detection of CD63 in exosomes derived from (A) BT-20 cells and (B) HCC-1954 cells.

4.4.2 Transfer of exosomes

Following successful isolation, confocal microscopy was employed to investigate the transfer of red fluorescent protein (RFP)-labelled exosomes to an unlabelled recipient cell population. Z-stacked confocal gave rise to a series of images that were then compressed. Maximum Intensity Projections (MIP) gave an image that is a result of the combination of the brightest part of each image. Figure 4.3 allows the visualization of recipient cells, the nuclei of which have been stained blue with DAPI. In these images, RFP-labelled exosomes (pink dots) are seen to cluster around the recipient cells. No exosomes were detectable on the areas of microscopy slide that were lacking cells.
Figure 4.3 Confocal microscopy demonstrating the transfer of fluorescently labelled exosomes to unlabelled recipient breast cancer cells. Red=Red fluorescent protein labelled exosomes. Blue=DAPI stained nuclei of recipient cells. Images taken at magnifications of (A) 100x and (B) 450x.
4.4.3 Impact of MSC derived exosomes on breast cancer cells

The functional impact of exosomes on proliferation of recipient cells was determined using the experimental set-up described in Section 2.8.2. The first experiment involved the transfer of 2µg of MSC-secreted exosomes to BT-20 cells. Proliferation was measured at 24 hours and expressed as a percentage of proliferation expressed relative to the ‘Complete Media’ reading, which was taken as 100% proliferation i.e. the expected proliferation rate in cells cultured under normal growth conditions.

No significant difference in the rate of proliferation was observed in BT-20 cells following a period of 24 hours growth in the presence of MSC-derived exosomes (P=0.601 ANOVA, Figure 4.4 (A)). Similarly, no difference in proliferation rate was detected in HCC1954 cells in response to MSC-secreted exosomes (P=1.0 ANOVA, Figure 4.4 (B)).
Figure 4.4 Impact of Mesenchymal Stem Cell-secreted exosomes on proliferation of (A) BT-20 cells and (B) HCC1954 cells. Each bar in the graphs represent the mean percentage proliferation across 3 replicates of the experiment. The Standard Error of the Mean (SEM) is shown by the Y error bars.

4.4.4 Impact of breast cancer exosomes on MSCs

The impact of breast cancer cell-derived exosomes on MSC proliferation was then assessed. No significant difference was noted in the rate of
proliferation in these cells after BT-20 exosomes were spiked into the media (P=0.378 ANOVA, Figure 4.5 (A)). Interestingly, Figure 4.5 (B) demonstrates a decrease in proliferation in MSCs cultured in exosome-free media when compared to their rate of growth under normal conditions (100% decreased to 91 ± 7%). This decrease was abrogated when breast HCC1954 cell-derived exosomes were spiked into the culture media (91 ± 7% to 100 ± 4%, P=0.462, t-test), although the variation in proliferation levels was not statistically significant (P=0.405 ANOVA).
Figure 4.5 Impact of (A) BT-20-secreted exosomes or (B) HCC1954-secreted exosomes on proliferation in MSCs. Each bar in the graphs represent the mean percentage proliferation across 3 replicates of the experiment. The Standard Error of the Mean (SEM) is shown by the Y error bars.

4.4.5 Impact of breast cancer-secreted exosomes on WI-38 cells

The impact of breast cancer exosomes on the rate of proliferation of WI-38 cells was also measured. These cells were chosen as they are a control
fibroblast population. No significant difference in the proliferation rate of WI-38 cells was found in response to BT-20 exosomes (P=0.056 ANOVA, Figure 4.6 (A)). Similarly, the proliferation rate was unchanged in WI-38 cells following the addition of HCC1954 exosomes (P=0.127 ANOVA, Figure 4.6 (B)).

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Figure 4.6 Impact of (A) BT-20-secreted exosomes or (B) HCC1954-secreted exosomes on proliferation of WI-38 cells. Each bar in the graphs represent the mean percentage proliferation across 3 replicates of the
4.4.6 Impact of breast cancer-secreted exosomes on angiogenesis

The impact of breast cancer cell-derived exosomes on angiogenesis was also determined using a tubule formation assay. Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in Matrigel in the presence of either regular media, exosome-depleted media, or exosome-depleted media to which breast cancer cell-derived exosomes were added. Standard culturing of HUVECs under normal growth conditions in complete media is shown in Figure 4.7.

Figure 4.7 Human Umbilical Vein Endothelial cells (HUVEC) viewed using light microscopy at (A) 10x and (B) 20x magnification.

Tubule formation in HUVEC cells grown in the presence of complete media in Matrigel is shown in Figure 4.8 (A). Tubule formation (quantified by counting the number of branching points) appears to increase when these cells are grown in the presence of either BT-20 or HCC1954 cell-derived exosomes as evidenced in Figure 3.8 (C), (D), (E) and (F).
Figure 4.8 Impact of breast cancer exosomes on angiogenesis in HUVEC cells cultured in (A) and (B) complete media; (C) and (D) exosome-depleted media plus BT-20-derived exosomes; or (E) and (F) exosome-depleted media plus HCC1954-derived exosomes. Images (A), (C) and (E) were taken at higher magnification to images (B), (D) and (F).
4.5 Discussion

Exosomes have been shown to be secreted by a variety of cell types, both benign and malignant (92, 93, 156). A number of exosome isolation techniques have been reported in the literature for use in the setting of cell-conditioned media (157). However, the gold standard for exosome isolation involves the use of ultracentrifugation, which is often combined with differential centrifugation and microfiltration (124, 158). A combination of these 3 techniques was employed in this study. The advantage of using ultracentrifugation over other methods is that it is cost effective, reproducible, and allows high volumes of cell-conditioned media to be processed at once.

Following successful isolation from cell-conditioned media, exosomes were characterised in order to ensure that they were present in the pellet. Transmission Electron Microscopy is commonly used to carry out morphological analysis of secreted exosomes. In this work, exosomes were isolated from BT-20 and HCC1954 cell-conditioned media, and were found to be vesicular in shape, and to measure approximately 30-120nm in diameter. These findings are consistent with that of the published literature (159, 160). Western Blot Analysis was used to detect the presence of CD63 in exosomes derived from both breast cancer cell lines. This membrane-bound protein is a member of the tetraspanin family and is a commonly reported exosome-associated protein used to confirm successful isolation from cell-conditioned media (124, 161). The combination of these features confirms successful isolation of exosomes. The identification of an exosome-specific marker would be immensely useful for isolation, characterisation and quantification purposes. Currently characterisation is a multistep process. The ideal situation would be to have a specific marker to identify exosomes from specific cell sources for example cancer-derived exosomes. Such a development may be useful in the biomarker setting. In addition to this exosomal surface markers may allow the prediction of metastatic sites for breast cancer which would allow for metastatic disease to be managed in a more timely fashion than is currently possible.
In order to investigate the role that exosomes play in intercellular communication in the primary breast tumour microenvironment it was necessary to evaluate transfer to recipient cells. This was carried out using confocal microscopy. Here, exosomes were engineered to express a red fluorescent protein and transferred to recipient cells which were detectable by DAPI stained nuclei. Confocal images showed the clustering of exosomes around recipient cells, with no exosomes detectable on areas of the slide that were deficient in cells. These findings are in line with that of the published literature whereby transfer of exosomes was visualized successfully using this imaging modality (162, 163). It must be highlighted however that confocal microscopy is a surrogate indicator of exosome uptake. Actual uptake of exosomes would result in exosomes no longer being visible as these vesicles do not remain intact on uptake. Franzen et al., (2014) (164) used an image cytometer Amnis ImageStreamX to characterise exosome uptake and internalization by human bladder cancer cells. The authors labelled bladder cancer cell-secreted exosomes with PKH-26 and analysed them on the ImageStreamX with an internal standard added to determine concentration. Labelled exosomes were co-cultured with bladder cancer cells and analysed for internalization. Exosomes associated with the cells were insensitive to trypsin, demonstrated by spot count and fluorescence intensity, suggesting that they were not attached to the surface of cells. Higher fluorescence intensity inside cells (PKH26) was detected using the IDEAS software internalization wizard compared to the fluorescence intensity of the entire cell (CAR-FITC surface staining). This finding suggested internalisation of exosomes. Deconvolution microscopy using an actin mask demonstrated that exosomes were not visible when the mask was on but became visible following its removal, also suggesting internalisation of exosomes by recipient cells (164).

Understanding the impact of exosomes on recipient cells is vital in determining the role that they play in intercellular communication in the primary tumour microenvironment. There is an increasing body of evidence demonstrating that 2 hallmarks of cancer, proliferation and angiogenesis, are mediated, at least in part by exosomes (165). The transfer of exosomes
derived from breast cancer cells has been reported to increase survival and proliferation in recipient non-tumourigenic cells (59). Furthermore, co-injection of metastatic breast cancer cell exosomes with non-tumour cells in an animal model led to the formation of a tumour (59). A similar effect has been demonstrated when serum-derived exosomes from patients with breast cancer were co-injected with non-tumourigenic cells (59). In this study, exosomes derived from breast cancer cell lines BT-20 and HCC-1954 had no impact on proliferation of WI-38 cells. In contrast, Melo et al., (2014) (59) used the unusual approach of a cell-free culture technique whereby purified cancer cell-secreted exosomes were placed in FBS-depleted culture media and incubated for 72 hours. MCF10A cells were then treated with the 72 hour cultured cancer exosomes and cultured for up to 5 days. Proliferation of MCF10A cells was found to increase in this setting. No difference in proliferation was observed when the non-tumourigenic cells were cultured in the presence of freshly isolated cancer exosomes (59). The apparent disparity between our findings and that of Melo et al., (2014) (59) may be explained by the cell-free culture technique and the length of non-tumourigenic cell incubation with exosomes that was employed in the latter study (59).

The impact of breast cancer exosomes on MSC proliferation was also investigated. No difference in the rate of proliferation was detected following 24 hours growth in the presence of BT-20 or HCC1954 exosomes. There are no published data on the impact that breast cancer exosomes have on MSC proliferation in vitro. However work performed by Cho et al., (2012) (166) found that breast tumour-derived exosomes lead to MSC differentiation into tumour-associated myofibroblasts which are known to contribute to tumour growth, progression, and metastasis in the tumour microenvironment (166, 167).

Ono et al., (2014) (130) demonstrated a decrease in proliferation in a bone marrow-metastatic breast cancer cell line in response to MSC-secreted exosomes (130). In that study bone marrow-metastatic cells were cultured in the presence of bone marrow MSC-secreted exosomes for up to 96 hours.
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and proliferation data were normalized to that of cells cultured in the absence of exosomes at 24 hours. This effect has also been observed in the setting of glioblastoma cell proliferation (131). Del Fattore et al., (2015) (131) used tunable resistive pulse sensing (TRPS) analysis for quantification of exosomes followed by FACS analysis of cells at T0 and after 48 hours of incubation with $25 \times 10^9$ EVs isolated from MSCs. The results of the current study did not show any significant alteration in the level of proliferation in BT-20 or HCC1954 cells in response to the addition of MSC-derived exosomes at 24 hours. A difference may have been observed had longer incubation periods been employed in this set of experiments as the initial 24 hours may represent a period of acclimatisation for breast cancer cells in the presence of exosomes. Attempts at longer incubation periods were, however, hindered by contamination thereby making analysis at 48 and 72 hours inaccurate. In addition, direct quantification of exosomes using a method such as TRPS may have allowed more accurate exosome quantification prior to transfer to recipient cells. Unfortunately, this technology was not available due to budgetary constraints.

Angiogenesis is the process of new capillary vessel development and is essential for tumour growth (168). Tumour size could not exceed 1-2mm$^3$ in the absence of this key process (169). In this work, exosomes isolated from the conditioned media of both BT-20 and HCC1954 cells resulted in increased tubule formation in HUVEC cells when compared to those cultured in exosome-depleted basal media. This result is remarkable considering that secreted chemokines or cytokines were not transferred to the HUVEC cells, nor were they in contact with cancer cells. Merely, tiny vesicles were transferred to endothelial cells and yet, their contents were capable of stimulating this hallmark of cancer. These findings are in line with that of the published literature where exosomes have been shown to play a pro-angiogenic role in the tumour microenvironment (170). Grange et al., (2011) (170) reported that exosomes derived from CD105+ cancer stem cells stimulated HUVEC cells to organize in vitro into capillary-like structures on Matrigel. In contrast, exosomes from CD105− cells did not induce the formation of capillary-like structures. The ability of exosomes to
influence angiogenesis in recipient cells may be manipulated for therapeutic
exosomes from the cell-conditioned media of breast cancer cells following a
period of treatment with Docosahexaenoic acid (DHA) (171). This
compound has anti-angiogenic properties and is currently under
investigation for the prevention and treatment of breast cancer (171). Exosomes from DHA treated cells were applied to endothelial cells
resulting in an increase in the expression of anti-angiogenic microRNAs in
the endothelial cells. Over-expression of these anti-angiogenic miRNAs, miR-23b and miR-320b, in endothelial cells led to a reduction in the
expression of their pro-angiogenic target genes. A significant reduction in
tubule formation was also noted (171). This avenue of research may
represent a promising method of exosome-based targeted therapy for
aggressive breast cancer subtypes.
Chapter 5

MicroRNAs as circulating biomarkers for Breast Cancer
5.1 Introduction

Survival from breast cancer depends on the stage at which it is diagnosed, with patients who are detected at an earlier stage, with a lower disease burden having superior outcomes (172). It follows, therefore, that global research efforts have endeavoured to identify a biomarker which will facilitate earlier detection of breast cancer. As previously outlined, circulating miRNAs have been shown to be dysregulated in many cancers, including breast cancer (75, 173, 174). They have tremendous potential in the setting of circulating breast cancer biomarkers and have been the subject of multiple research studies over the last two decades (75, 175). There is, however, a lack of consensus as to which fraction of blood (whole blood, serum or plasma) represents the highest level of validity in the clinical setting (76-78). One possible solution to this challenge may lie in the study of exosomes, which are known to actively and selectively package miRNAs for release from cells (158). These extracellular vesicles have been shown to be involved in intercellular communication in the primary tumour microenvironment (90). The miRNA content of exosomes has been shown to reflect that of parent breast cancer cells (99, 100). Exosomes have been isolated from a variety of body fluids, including serum (98, 176). Consequently, these nano-vesicles have the potential to function as circulating biomarkers for breast cancer, which may be of value in the detection of a breast tumour and in identifying the epithelial subtype to which the tumour belongs. Furthermore, they may represent a method by which clinicians can monitor a patient’s response to neoadjuvant and adjuvant therapies, and may possibly facilitate the detection of disease recurrence (177, 178).
5.2 Aims

- To identify potentially interesting microRNAs (miRNA) from a global miRNA array performed on exosomes derived from cell-secreted exosomes
- To investigate the presence and potential biomarker role of these miRNAs in the circulation of patients with breast cancer and healthy controls
- To determine whether there is a relationship between serum exosomal miRNA levels and miRNA levels in whole blood
5.3 Materials and Methods

MicroRNA array

A global miRNA array (Section 2.7.1) was carried out externally in Exiqon (Denmark) on exosomes that had previously been isolated from MDA-MB-231 and BT-20 breast cancer cells. Analysis of that array data allowed the identification of potentially interesting miRNA targets that had been packaged into exosomes and secreted by the respective cell populations for further evaluation. Multiple exosome isolates were used to confirm the presence of selected targets as described in Section 2.7.2.

Investigation of miRNA targets in whole blood from breast cancer patients and healthy volunteers

The presence of the chosen miRNA targets was then investigated, and their potential as biomarkers determined in the whole blood of patients with breast cancer (n=50) and healthy volunteers (n=45). RNA was extracted from whole blood using the PAXgene™ Blood RNA Kit (PreAnalytiX®) (Section 2.6.2.3). Nanodrop Spectrophotometry was used to assess the quantity and quality of extracted RNA (Section 2.6.3). 5 µl of RNA was reverse transcribed into cDNA targeting miR-16, miR-451a and miR-744-5p as described in Section 2.6.5, and RQ-PCR was performed as per Section 2.6.6.

Isolation of serum exosomes from breast cancer patients and healthy volunteers

Exosomes were isolated from the serum of patients with breast cancer (n=29) and healthy controls (n=16). Successful isolation was confirmed using TEM (Section 2.5.1) and Western Blot Analysis (Section 2.5.2). The profile of circulating (PAXgene stabilised whole blood) versus serum exosomal miR-16, miR-451a and miR-744-5p were compared for all matched samples by RQ-PCR (Section 2.6.6).
5.4 Results

5.4.1 MicroRNA array

A global miRNA array targeting 2083 miRNAs was carried out in Exiqon (Denmark) on exosomes that were isolated by another research group member. Exosomes from MDA-MB-231 and BT-20 cell lines were chosen for inclusion in this work. Hierarchical clustering was carried out by biostatisticians in the National University of Ireland Galway, and analysis of this data allowed the identification of potentially interesting miRNA targets for further investigation. The dendrogram in Figure 5.1 shows relative expression levels of miRNAs in exosome pellets from both cell lines. 394 and 382 miRNAs were detectable in exosomes derived from the MDA-MB-231 and BT-20 cell lines respectively. In total 329 miRNAs were common to exosomes derived from both cell lines and a small selection were detected in exosomes secreted by 1 cell line and not the other.
Figure 5.1 Dendrogram showing relative levels of expression of miRNAs in exosomes pellets derived from BT-20 and MDA-MB-231 cell lines. Each line corresponds to a different miRNA target. The green and yellow lines indicate relatively high levels of expression of particular miRNAs. A and B indicate areas of relatively high expression of miRNAs.
Online miRNA analysis tools, including DIANA TOOLS and miRTarBase were used for pathway analysis of highly ranked miRNAs (179, 180). This work, in combination with a review of published literature led to the selection of 2 miRNAs for further investigation. The first of these was miR-451a which was detected in exosomes derived from both cell lines. This miRNA was ranked in the top 30 most highly expressed in exosomes from both MDA-MB-231s and BT-20s. It has also been reported as a potentially relevant biomarker for breast cancer in the literature (181). The characteristics of this miRNA and miR-744-5p, which was detected in BT-20 exosomes only, are outlined in Table 5.1.

<table>
<thead>
<tr>
<th>Target</th>
<th>Reasons for selection for further investigation</th>
</tr>
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<tr>
<td>miR-451a</td>
<td>• Common to exosomes from both cell lines</td>
</tr>
<tr>
<td></td>
<td>• Identified as a potentially promising biomarker for breast cancer in the literature</td>
</tr>
<tr>
<td>miR-744-5p</td>
<td>• Detected in BT-20-derived exosomes</td>
</tr>
<tr>
<td></td>
<td>• Not previously studied in patients with breast cancer</td>
</tr>
<tr>
<td></td>
<td>• Potentially interesting on pathway analysis</td>
</tr>
<tr>
<td></td>
<td>• Work carried out previously in our laboratory showed that miR-744-5p was dysregulated in a murine model of breast cancer during disease progression (81)</td>
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</tbody>
</table>

Table 5.1 MiRNAs selected from global miRNA array for further investigation as potential biomarkers for breast cancer.

MiR-744-5p was considered to be potentially interesting as work previously carried out in our laboratory demonstrated that it was dysregulated in the whole blood of a murine model of breast cancer during disease progression.
These targets were then validated in multiple exosome isolates in order to ensure that they were in fact routinely packaged into exosomes and secreted by the cells. Table 5.2 demonstrates that miR-451a was detected in 3 out of 3 exosome pellets derived from MDA-MB-231 cell-conditioned media. MiR-744-5p was not detectable in any of these samples, in keeping with the array data. The purpose of this validation was to ensure that the chosen miRNAs were in fact detectable in multiple exosome isolates as the array data was a point in time measurement.

<table>
<thead>
<tr>
<th>Exosome pellet</th>
<th>MiR-451a detected</th>
<th>Mean (C_t) value</th>
<th>MiR-744-5p detected</th>
<th>Mean (C_t) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>32.40</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>35.06</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>35.33</td>
<td>-</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 5.2 Expression of miR-451a in exosomes derived from the MDA-MB-231 cell line. MiR-744-5p was not detectable in exosomes derived from this cell line in accordance with the global miRNA array data.

Exosomes were then isolated from the BT-20 cell line. MiR-451a and miR-744-5p were detectable at low levels in 2 out of 3 exosome isolates (Table 5.3). The high \(C_t\) values that were obtained in the above validation work (greater than 35 in the majority of samples) may be reflective of the relatively small amount of miRNA that is contained within exosomes. Interestingly, in the case of BT-20 exosomes, both miRNAs were detected in the same 2 exosome pellets and were absent from the remaining 1 pellet. The latter result may have been due to an error in reverse transcription.
<table>
<thead>
<tr>
<th>Exosome pellet</th>
<th>MiR-451a detected</th>
<th>Mean C_t value</th>
<th>MiR-744-5p detected</th>
<th>Mean C_t value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>37.73</td>
<td>+</td>
<td>36.93</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>N/A</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>37.32</td>
<td>+</td>
<td>34.67</td>
</tr>
</tbody>
</table>

Table 5.3 Expression of miR-451a and miR-744-5p in exosomes derived from BT-20 cell-conditioned media.

5.4.2 Investigation of miRNA targets in whole blood from patients with breast cancer and healthy volunteers

The potential for miR-451a and miR-744-5p to function as circulating biomarkers for breast cancer was then investigated. In this study whole blood samples were obtained from patients with breast cancer and healthy volunteers. Whole blood was collected directly into PAXgene tubes, which contain an additive that lysed the entire sample and stabilises the RNA within. RNA was extracted from PAXgene stabilised samples using the PAXgene™ Blood RNA Kit (Section 2.6.2.3). The amount of RNA that had been isolated was quantified using the Nanodrop Spectrophotometer (Section 2.6.3). This facilitated calculation of the amount of RNA that was to be loaded for reverse transcription. The clinicopathological details of patients and healthy volunteers included in this study are outlined in Table 5.4. Participants in the control cohort were younger than patients in the cancer group. In addition to this the majority were pre-menopausal. This difference was due to difficulty in recruiting volunteers for the control cohort and so, a large number of samples were obtained from students and doctors following the informed consent process.
Table 5.4 Clinicopathological details of patients and controls included in the investigation of the potential role of miR-451a and miR-744-5p to function as circulating biomarkers for breast cancer.
It was first necessary to determine if miR-451a was detectable in the whole blood of patients with breast cancer and healthy controls and so its levels were quantified using RQ-PCR. MiR-451a was successfully detected in all samples. Expression levels in patient samples were then examined. MiR-16 was used as an endogenous control in this set of experiments. Figure 5.2 shows the mean C\textsubscript{T} values for miR-16 in 50 cancers samples and 37 controls. The maximum C\textsubscript{T} value for cancers was 21.04 and the minimum was 14.30. 60\% of samples fell within 2 C\textsubscript{T} values of each other. In terms of controls, the maximum and minimum C\textsubscript{T} values were 18.72 and 14.04 respectively, with 78\% of samples falling within 2 C\textsubscript{T} values.

Figure 5.2 MiR-16 expression levels in cancers and controls. Mean C\textsubscript{T} values are shown for each sample.

The boxplot in Figure 5.3 (A) demonstrates that no difference was found between mi451a levels in controls versus cancers. In this graph the mean value is shown by a circle containing a dot and the median value is shown by the horizontal line. The range of values for samples is shown by the extending lines with the interquartile range shown by the box which
contains 75% of samples. Similarly, no difference was noted in miR-451a expression across epithelial subtypes using analysis of variance (ANOVA). MiR-451a expression was then analysed across cancer samples based on tumour grade (Figure 5.3 (C)). Again, no significant difference was noted using this approach. Finally samples were subdivided by stage at diagnosis. This classification, known as the TNM system, takes tumour size (T), lymph node burden (N) and metastatic status into account (182). Figure 5.3 (D) demonstrates no difference in miR-451a in the circulation of patients with disease of difference stages.
Figure 5.3 MiR-451a expression in the whole blood of (A) healthy controls and patients with breast cancer; (B) cancers by epithelial subtype; (C) cancers by tumour grade; and (D) cancers by TNM stage.
As with miR-451a, it was necessary to investigate if miR-744-5p was actually detectable in the whole blood of patients with breast cancer and healthy controls. Following the detection of this miRNA in all samples, the potential role of miR-744-5p to function as a circulating biomarker for breast cancer was investigated using miR-16 as an endogenous control. The same samples outlined in Table 5.4 were included in this arm of the study. MiR-744-5p expression was first quantified in healthy controls versus cancers, with no difference detected between groups (Figure 5.4 (A)). Relative expression of miR-744-5p across cancers subdivided according to epithelial subtype showed no difference in expression levels (Figure 5.4 (B)). Tumours were then divided according to histological grade (Figure 5.4 (C)), and tumour stage at diagnosis (Figure 5.4 (D)), with no significant difference in expression levels noted between groups.
Figure 5.4 MiR-744-5p expression in the whole blood of (A) healthy controls and patients with breast cancer; (B) cancers by epithelial subtype; (C) cancers by tumour grade; and (D) cancers by TNM stage. * indicates outliers.
5.4.3 Isolation of serum exosomes from breast cancer patients and healthy volunteers

Following the confirmation of a detectable miRNA signal in the whole blood of patients and healthy volunteers, the potential role of serum-specific exosomal miRNAs to serve as circulating biomarkers for breast cancer was then examined. The first step was to isolate and characterise exosomes from multiple test serum samples. These were obtained from healthy volunteers following informed consent. Multiple attempts at exosome isolation and visualisation using TEM were trialled without success. This included differential centrifugation, microfiltration and ultracentrifugation of serum directly in 1.5 ml microtubes without prior dilution in PBS. Figure 5.5 (A) shows an example of the images obtained following TEM of these samples. Subsequently, the protocol was modified to include dilution of serum in PBS. Exosomes were then successfully isolated using this protocol which is described in Section 2.3.2. Transmission Electron Microscopy (TEM) was performed on exosome isolates following fixation, embedding in resin and sectioning. Figure 5.5 (B) and (C) show examples of exosomes isolated using the modified protocol and visualized using TEM at low and then higher magnification respectively. Exosomes were shown to be vesicular in shape and to measure between 30 and 120nm in diameter as expected (Figure 5.5 (B) and (C)).
Figure 5.5 TEM images obtained following (A) unsuccessful serum exosome isolation; or successful serum exosome isolation viewed at a magnification of (B) 40,000 x; and (C) 70,000 x.
Western Blot analysis was performed targeting the exosome-associated protein CD63. Two serum exosomal samples were included for analysis. Figure 5.6 shows the detection of CD63 at the appropriate band size of 50-60kDa in both exosomal samples. This data coupled with the TEM images confirmed successful isolation of exosomes from serum.

Figure 5.6 Western Blot showing detection of CD63 in exosomes derived from human serum.

Following successful isolation of exosomes, it was necessary to extract the RNA from within. As the MagNA Pure Compact automated RNA extraction platform had not previously been used for this purpose in our laboratory, it was first necessary to validate it for use in exosomal research. This was performed using 2 samples of exosomes derived from control serum samples and 1 exosome pellet isolated from cell-conditioned media. Each sample was divided into 2 equal aliquots, 1 of which was destined for RNA extraction using the mirVANA™ RNA Isolation Kit, and the other using the MagNA Pure Compact. Table 5.5 demonstrates the mean Ct values at which miR-16 was detectable in each sample for the mirVANA™ RNA Isolation Kit versus MagNA Pure Compact.
Chapter 5: MicroRNAs as circulating biomarkers for breast cancer

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean $C_t$ value for miR-16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mirVANA™ RNA Isolation Kit</td>
</tr>
<tr>
<td>Serum exosomes 1</td>
<td>30.56</td>
</tr>
<tr>
<td>Serum exosomes 2</td>
<td>29.15</td>
</tr>
<tr>
<td>Cell-secreted exosomes</td>
<td>30.14</td>
</tr>
</tbody>
</table>

Table 5.5 Comparison of Mean $C_t$ values for miR-16 in samples extracted using the mirVANA™ RNA Isolation Kit versus the MagNA Pure Compact.

As both platforms were shown to give comparable results, the automated MagNA Pure Compact was used for RNA extractions in all subsequent work.

In order to investigate the potential for miR-451a and miR-744-5p to function as serum exosome-encapsulated circulating biomarkers for breast cancer, it was first necessary to determine if they were detectable in exosomes isolated from the serum of both healthy volunteers and patients with breast cancer. A cohort consisting of 40 cancers and 20 controls was identified. The clinicopathological details of patients and controls are outlined in Table 5.6. The cohort of patients with breast cancer was enriched for Basal-like breast cancer as miR-451a and miR-744-5p were originally identified in exosomes isolated from triple-negative cell lines.
Table 5.6 Clinicopathological details of patients and controls included in the investigation of the potential role for miR-451a and miR-744-5p as serum-exosomal circulating biomarkers for breast cancer.

<table>
<thead>
<tr>
<th></th>
<th>Cancers</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of patients</strong></td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td><strong>Mean age (range)</strong></td>
<td>58 (37-82)</td>
<td>50 (30-72)</td>
</tr>
<tr>
<td><strong>Menopausal status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>22</td>
<td>11</td>
</tr>
<tr>
<td><strong>Epithelial subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>HER2+</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Basal-like</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
Both PAXgene stabilised whole blood samples and serum were available for all participants in the study. RNA was extracted directly from whole blood. Exosomes were isolated from the matched serum samples (volume of serum used was 500µl for each sample) and protein assays were used to indirectly quantify the amount of exosomes that had been isolated. The mean protein yield for serum exosomes derived from cancers and controls are shown in Table 5.7, where no significant difference was detected between the 2 groups (P=0.86, t-test).

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Mean protein yield (ng/µl)</th>
<th>Protein yield range (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>791</td>
<td>421-1293</td>
</tr>
<tr>
<td>Cancers</td>
<td>806</td>
<td>366-1820</td>
</tr>
</tbody>
</table>

Table 5.7 Mean protein yield from serum exosomes derived from patients with breast cancer compared to healthy controls.

RNA was extracted from the remaining exosome pellet using the MagNA Pure Compact. Protein yield was used to determine the amount of RNA that was to be loaded for cDNA synthesis with a threshold protein level of 3.5µg selected for normalisation of samples. This was based on the available average yield per sample. The relative expression levels of miR-451a were determined for both sets of samples (serum exosomes and whole blood) using miR-16 as an endogenous control. Both miR-451a and miR-16 were detectable in all samples. Table 5.8 shows the mean and range of C\textsubscript{i} values that were detectable for miR-451a and miR-16.
<table>
<thead>
<tr>
<th></th>
<th>MiR-451a</th>
<th>MiR-16</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Ct value</strong></td>
<td>30.07</td>
<td>32.08</td>
</tr>
<tr>
<td><strong>Range of Ct values</strong></td>
<td>24.83-35.59</td>
<td>26.87-39.56</td>
</tr>
</tbody>
</table>

Table 5.8 Mean and range of Ct values detectable for miR-451a and miR-16 in serum exosomes of cancers and controls.

44% of samples had miR-451a levels that fell within 2 Ct values, and 33% of samples showed miR-16 expression levels within a range of 2 Ct values. There was no correlation between the amount of RNA that was reverse transcribed and the Ct value that was obtained using PCR for either miR-451a (P=0.703, Pearson correlation=-0.058) or miR-16 (P=0.239, Pearson correlation=0.179).

No difference in miR-451a expression levels was detected between cancers and controls when whole blood was examined as a starting material (Figure 5.7 (A)). However, when the matched serum exosomal samples were analysed, significantly higher levels of expression of miR-451a were detected in cancers compared to controls (Mean ± SEM for controls: 16 ± 0.4 Log₁₀ Relative Quantity (RQ), and cancers: 28 ± 1.0 Log₁₀ RQ, P <0.05, Figure 5.7 (B)).
Figure 5.7 MiR-451a expression in healthy controls and patients with breast cancer (A) whole blood levels and (B) serum exosomal levels.
MiR-16 expression was found to be highly variable in whole blood (Mean=17.51, Range=14.74-24.82, Figure 5.8 (A)) with 49% of samples having values within a 2 C_t range. Similarly, miR-16 expression in serum exosomes was variable in samples that were normalised according to protein levels (Mean=32.18, Range=26.92-37.48, 54% of samples within a 2 C_t range, Figure 5.8 (B)). MiR-16 expression was also variable in samples that were not normalised based on protein (Mean=31.90, Range=26.87-39.56, 35% of samples fell within a 2 C_t range, Figure 5.8 (B)).
Figure 5.8 MiR-16 expression levels in (A) whole blood samples and (B) serum exosomal samples.
4 whole blood samples (3 cancers and 1 control) were noted to have particularly high $C_t$ values for miR-16 and given the potential for these samples to skew the data the analysis was repeated excluding these samples. Again, no difference was detected between cancers and controls for miR-451a (Figure 5.9).
Figure 5.9 MiR-16 expression levels in (A) whole blood of healthy controls and patients with breast cancer (red circles indicate outliers) and (B) healthy controls and patients with breast cancer in the absence of samples with outlying results for miR-16.
As no difference in miR-451a levels was detected in the whole blood of patients with breast cancer versus that of controls, an analysis of miR-451a expression by epithelial subtype was performed. Here no difference was seen between tumour subtypes (Figure 5.10).

Given the high variability in miR-16 expression in serum exosome samples (Figure 5.8 (B)), data were normalised according to protein loading levels. This technique was employed in an effort to standardise data in the absence of a validated endogenous control. A threshold protein loading value of 3.5µg was selected in order to standardise the amount of RNA that was loaded for reverse transcription for each sample. Although a significant change in miR-451a expression was noted when all serum exosome samples were analysed, no significant difference was noted between cancers and controls when samples were normalised by protein loading and miR-16 was used as an endogenous control (Figure 5.10 (A)), or when a comparison of mean C_t values for miR-451a was performed for these groups (Figure 5.10 (B)). However, the range of values in exosomes isolated from the serum of...
cancer patients remained much broader than that detected in healthy controls.
Figure 5.11 MiR-451a expression levels in (A) serum exosomal samples normalised by protein loading with miR-16 as an endogenous control and (B) serum exosomal samples by protein loading with levels shown as mean C_t values.
MiR-451a expression was determined in cancers divided by subtype. No statistically significant difference was noted in expression of miR-451a in serum exosomes (Figure 5.11 (A)), serum exosomes normalised by protein (Figure 5.11 (B)) or serum exosomes normalised by protein and expressed as mean $C_t$ values (Figure 5.11 (C)). A trend towards lower expression of miR-451a in Basal-like cancers compared to Luminal A cancers was noted in the latter group ($P=0.074$). Given that only 4 samples were present in each group it may potentially be worth investigating this relationship further in a larger cohort.
Figure 5.12 MiR-451a expression levels in cancers subdivided by epithelial subtype in (A) serum exosomes; (B) serum exosomes normalised by protein; and (C) serum exosomes normalised by protein and expressed as mean Ct values.
MiR-451a expression by tumour grade was also examined. No significant difference was noted between groups when serum exosomes, serum exosomes normalised according to protein or serum exosomes normalised by protein and expressed as C_t values were examined (boxplots not shown). Similarly when tumours were categorised according to TNM stage at diagnosis no statistically significant difference was noted in miR-451a expression in serum exosomes or serum exosomes normalised by protein (boxplots not shown).

MiR-744-5p was expressed in a low number of serum exosomal samples only (detectable in 4 out of 38 samples, 11%, range of C_t values=36.49-38.66) despite being detectable in all whole blood samples. A comparison of serum exosomal to PAXgene levels was therefore not performed for this target.
5.5 Discussion

This study was designed to identify potentially interesting miRNAs that were present in breast cancer cell-secreted exosomes and to investigate the potential of these miRNAs to function as circulating biomarkers for breast cancer. A global miRNA array was used to identify a panel of miRNAs that were present in MDA-MB-231 and BT-20 cell-secreted exosomes. A minimum of 382 out of a possible 2089 miRNAs were detected in exosomes derived from each cell line indicating a degree of selectivity in miRNA packaging into exosomes. Significant overlap was noted between exosome-encapsulated miRNAs that were detected in both cell lines. This may be reflective of the triple-negative phenotype of both cell types and highly expressed miRNAs may confer the ‘aggressive’ features that are typical of this breast cancer subtype. MiR-451a and miR-744-5p were identified as being potentially interesting following statistical analysis of the array data and an extensive review of the published literature. MiR-451a has previously been implicated in the suppression of resistance to Tamoxifen through the regulation of autophagy, and 14-3-3zeta and ERα expression. These data suggest miR-451a to be a potential target for reversing Tamoxifen resistance (183). On a similar vein, the authors demonstrated that down-regulation of miR-451a led to the up-regulation of migration inhibitory factor (MIF) expression and increased breast cancer cell growth, invasion, and tamoxifen sensitivity in vitro (184). MiR-744 has also previously been studied in this laboratory and was found to be in the top ten miRNAs that showed the greatest changes in whole blood levels in a murine model of breast cancer during weeks 1 to 6 of tumour growth (81).

The presence of miR-451a and miR-744-5p was confirmed in multiple exosome isolates in order to validate their role as potential targets prior to investigation in patient samples. Earlier validation work isolated exosomes from the conditioned media of 2 flasks of cells. Later work involved exosome isolation from 10 flasks per cell line as we were able to extract a greater amount of RNA from a higher number of cells and therefore gain a more accurate reflection of miRNA presence-absence.
The ability to detect miR-451a in the whole blood of patients with breast cancer and healthy controls was first demonstrated. MiR-451a was then investigated as a circulating biomarker for breast cancer in the PAXgene stabilised blood of patients with breast cancer and healthy controls. These tubes contain an additive that lyses all cellular material and stabilises the RNA. Stability is maintained for 3 days when stored at room temperature, and up to 8 years when stored at -80 °C (185). This allows for accurate miRNA quantification. This use of PAXgene stabilised samples was vital in this study as published work has demonstrated less variability and increased detection rates for miRNAs in PAXgene stabilised samples when compared with either plasma or serum (186).

No difference in the expression of miR-451a was noted between cancers and controls or when cancers were subdivided according to epithelial subtype, stage or grade (Figure 5.3). Chang et al., (2015) reported that miR-451a was up-regulated in peripheral blood mononuclear cells of patients who went on to develop breast cancer compared to healthy controls (187). The authors failed to validate this finding in further case/control pairs stating that the longer interval between blood collection and diagnosis in the validation set may have led to the disparity in results (187). Ouyang et al., (2014) found a reduction in tissue miR-451a levels in Triple-negative breast cancers (181). In vitro assays demonstrated an increase in MDA-MB-231 sensitivity to the chemotherapeutic agent Doxorubicin following the up-regulation of miR-451a. The authors proposed that this finding suggests that aberrant expression if miR-451a in breast cancer tissue may be responsible for TNBC evading chemotherapeutic control and early relapse and death (181). Circulating miR-451a levels were not reported in that study.

The second miRNA of interest, miR-744-5p, was found to be consistently detectable in whole blood but was not dysregulated in cancers versus controls in this study or among the various cancer subdivisions (Figure 5.4). In addition to the published work from our laboratory that was discussed earlier, miR-744-5p has also been demonstrated to be significantly down-regulated in HER2-positive breast tumours compared to HER2-negative
tumours in patients with breast cancer (188). The differing results between this study and published data, for both miR-451a and miR-744-5p may be explained by the different starting materials that were used across the various studies. In this work, whole blood was used throughout while the studies reported above examined tissue or peripheral blood mononuclear cell levels. Published work has highlighted that different starting materials are not suitable for comparison of miRNA expression levels and this may account for the apparently disparate results (186).

The second part of this study aimed to determine whether there is a relationship between serum exosomal miRNA levels and miRNA levels in the whole blood of patients with breast cancer and healthy controls. As indicated by in vitro results, miRNA packaging into exosomes is a selective process, and so the latter may represent an ideal source of miRNAs that are reflective of the health status of a patient. It was first necessary to demonstrate successful isolation of exosomes from serum. To this end, a number of isolation techniques were employed following their identification from the literature (189-191). The technique described by Melo et al., (2014), which involves the dilution of serum in PBS, followed by differential centrifugation, microfiltration and ultracentrifugation, was found to successfully isolate exosomes in a number of test samples (59). Successful isolation of serum exosomes was confirmed using TEM and Western Blot. TEM allowed the visualisation of vesicles measuring approximately 30-120nm in diameter, a finding which is in keeping with that of the published literature (90). Western blot analysis confirmed the detection of the tetraspanin CD63 which is a widely reported exosome-associated protein (112, 124, 129). Both of these results taken together confirm successful isolation of exosomes from serum.

RNA extraction from serum exosomes was performed using the MagNA Pure Compact. This automated platform was developed as a bench top solution for versatile nucleic acid purification. Benefits outlined by the manufacturers include the compact size of the instrument, the ability to process up to 8 samples in 1 run and the isolation of high quality nucleic
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acids(192). This new platform was compared to that of the mirVANA™ RNA Isolation Kit in 3 samples (2 serum exosomal samples and 1 cell-secreted exosome pellet) in order to determine if it yielded comparable results. MiR-16 expression was equivalent across all samples and therefore the MagNA Pure Compact was used for subsequent patient samples.

Protein assays were used in this set of experiments to indirectly quantify the amount of exosomes that had been isolated from serum. This method of quantification is well documented in the literature (112, 124). The mean protein yield did not differ significantly between cancers and controls. This finding is at odds with published data whereby a greater number of exosomes (quantified using protein assays) were isolated from the serum of patients with breast cancer compared to healthy controls (59). These differing results may be a result of greater patient numbers in our study (cancers n=29, controls n=16) versus that of Melo et al., (2014) (cancers n=8, controls n=11), and therefore our data of be a more accurate reflection of serum exosomal levels.

The inclusion of an endogenous control to allow for robust data normalisation is a prerequisite for accurate interpretation of RQ-PCR data (193). Endogenous controls should critically be expressed at a constant level in all samples, be unaffected by health status, and should be of similar length to the target miRNAs (194). MiR-16 has been widely used as an endogenous control when whole blood is used as a starting material however it has not been validated for use in exosome-centred research (195, 196). In this study, whole blood and serum exosomal miR-451a and miR-16 levels were quantified in matched patient samples in order to investigate the utility of the former to function as a biomarker for breast cancer and the latter to serve as an endogenous control.

No difference in the expression of miR-451a in whole blood was noted between cancers and controls, however miR-451a was significantly up-regulated in serum exosomes derived from patients with breast cancer. MiR-16 expression levels were highly variable in whole blood and in serum exosomes, both for samples that were loaded according to protein and those
that were not. Additionally, the mean $C_t$ value for miR-16 in serum exosomes was greater than 31 and therefore may not be reliably detected in all samples in a given cohort. Both of these findings suggest that miR-16 is not a suitable endogenous control for serum exosomal work. Published work has also reflected this finding (196). This challenge may potentially be overcome through the use of protein estimation data for normalisation of samples until a validated endogenous control is identified and so this possibility was explored. In this analysis, no difference in miR-451a levels was noted between cancers and controls when samples were selected according to protein levels. The discrepancy between this finding and that of the previous experiment may be a result of the smaller sample size in the latter cohort. While reverse transcription based on protein estimation data for exosomes may represent a valuable method of data normalisation in the absence of a validated endogenous control, this technique is not without its drawbacks. Potential challenges to this method of data normalisation include variable protein content of exosomes and the presence exosomal surface proteins which may impact on protein estimation data, and therefore may lead to inaccurate quantification using this technique. Based on these observations, the potential of miR-451a as a serum exosomal biomarker has yet to be fully elucidated. This should be performed following the identification of a robust endogenous control.

No significant difference in miR-451a levels was detected between cancer epithelial subtypes in whole blood, serum exosomes or serum exosomes following normalisation based on protein. Mean $C_t$ values for miR-451a were significantly higher in Basal-like cancers (indicating lower levels of expression in this subgroup). No difference in serum exosomal miR-451a levels were noted when tumours were divided according to grade and stage. There are no published data regarding serum exosomal miR-451a levels in patients with breast cancer versus controls. However Guduric-Fuchs et al., (2012) (197) found that miR-451 was consistently the most highly exported miRNA into exosomes derived from many different cell types (197). Our findings coupled with reportedly consistent packaging of miR-451a into exosomes by a variety of cell types regardless of origin, suggest that this
miRNA may represent a promising serum-exosomal biomarker for breast cancer. This possibility warrants further investigation in future work.

MiR-744-5p was undetectable in almost 90% of the initial 38 serum exosomal samples and so it was not analysed for the remaining samples. This finding illustrates an important point that has been documented in published literature in that packaging of miRNAs into exosomes by cells is a selective process (198, 199). In other words, not all miRNAs that are present in the whole blood are detectable in serum exosomes and therefore this fraction of blood may represent a ‘cleaner’ source for the detection of breast cancer-related miRNAs.

When considering the relative merits and demerits of a biomarker source it is necessary to take into account the degree of processing that is required prior to miRNA quantification. Whole blood collected in PAXgene tubes, while relatively expensive, offers a more efficient method of miRNA detection. In contrast to this, serum exosomes require the additional steps of exosome isolation and protein estimation, which makes for a more labour intensive process. On the other hand, whole blood contains many additional components (red blood cells for example) which may act as confounding factors leading to difficulty in the detection of a miRNA signal above background levels. Serum exosomes, however, do not contain all miRNAs that are present in whole blood and those that are secreted by cancer cells may encapsulate a more ‘cancer-specific’ miRNA panel, thereby making them more relevant in the biomarker setting.
Chapter 6

Serum exosomal microRNAs as circulating biomarkers for breast cancer
6.1 Introduction

Biomarker discovery and validation has been at the forefront of breast cancer research in recent decades with much focus being on the potential utility of miRNAs in this setting (81, 200). The limitations that current gold standard cancer detection methods, such as mammography, place on early diagnosis may be addressed through the development of blood-based or ‘liquid’ biopsy-based biomarkers (201). Such a test may facilitate rapid, cost-effective and minimally invasive cancer detection and has the potential to serve as a point of care test for breast malignancy (201). Ideally, miRNAs would serve to compliment traditional breast imaging modalities and allow clinicians to detect at an early stage patients who warrant further investigation in the form of triple-assessment in a symptomatic breast unit (201).

Blood-based biomarkers may also have the potential to allow clinicians to monitor a patient’s response to neo-adjuvant and adjuvant cancer therapies, with unchanging miRNAs levels for example indicating failure of the treatment to gain an adequate disease control (202). In addition to this, miRNA profiles may allow the prediction of a patient’s response to therapy (203). This tailored approach to cancer management would ensure that the appropriate therapy is administered, or altered in the case of poor response, in a timely fashion. Similarly circulating miRNAs may be used to detect cancer relapse at an earlier stage than is currently possible using clinical examination and imaging techniques (204).

The discovery of exosome-encapsulated miRNA secretion by breast cancer cells represents a novel avenue of biomarker research. Serum-derived exosomes may contain a miRNA profile that is reflective of the parent cancer cell and therefore may represent a more refined fraction of blood for biomarker discovery.
Chapter 6: Exosomal microRNAs as biomarkers for breast cancer

6.2 Aims

- To isolate serum exosomes from animals in a murine model of breast cancer
- To carry out a low density array of miRNAs present in the serum exosomes of these animals and identify potentially interesting targets for further evaluation
- To validate these miRNA targets in further animal samples
- To investigate the role of potentially interesting miRNAs to function as biomarkers in patients with breast cancer and healthy controls

6.3 Materials and Methods

**In vivo model**

This study was licensed under the Cruelty to Animals Act 1876 by the Department of Health and Children. Ethical approval was received from the National University of Ireland Galway Animal Care Research Ethics Committee (Appendix 4). BALB/c nude mice were inoculated with HCC1954 in this study. 10 animals received mammary fat pad injection (MFP) and 10 were inoculated subcutaneously (SC) as described in Section 2.9.4. The animals were sacrificed at day 30 and day 40 in the MFP and SC groups respectively following the collection of a terminal bleed (Section 2.9.5).

**Taqman Low Density Array (TLDA) and target validation**

Exosomes were isolated from the serum of 20 animals. 3 samples were selected for inclusion in a TLDA targeting 36 miRNAs, along with HCC1954 cell-derived exosomes (Sections 2.6.4 and 2.9.8). The TLDA data were analysed and potentially interesting targets were selected for further investigation. U6, miR-223, miR-106a and miR-485-3p were investigated in the remaining 11 animal serum exosome isolates (Section 2.6.6 and 2.9.9).
Investigation of targets in patients with breast cancer and healthy controls

The potential biomarker roles of miR-223 and miR-106a were investigated by RQ-PCR in patients with breast cancer (n=52) and healthy controls (n=21) as described in Sections 2.6.6 and 2.9.9.

6.4 Results

6.4.1 In vivo model

The in vivo study timeline is shown in Figure 6.1. The day on which the animals arrived is referred to as Day-7. Tumour inoculation was performed on Day 0. Weekly measurements of tumour volume and general animal health were performed as shown. Tumour growth was noted to be more rapid in the MFP group, and given that the tumours were in a more cumbersome location, a decision was made to sacrifice these animals on Day 30. Animals in the SC group were sacrificed 10 days later (Day 40).
Figure 6.1 *In vivo* miRNA study timeline.
Animals were anaesthetised using inhalation anaesthesia (Isoflurane, 5% induction of anaesthesia, 1-2% for maintenance in Oxygen at 1L/min). This was followed by an intracardiac puncture and collection of blood into a paediatric serum tube. Animals were sacrificed by CO₂ inhalation. The serum was processed according to the protocol outlined in Figure 2.15. The volume of serum obtained from animals in the SC and MFP groups are outlined in Table 6.1. Details of tumour injection site, tumour characteristics and lymph node status of each of the 20 animals are also shown in the tables. Exosomes were isolated from the serum of each animal and protein assays were carried out in order to indirectly quantify the amount of exosomes that had been isolated.
### Subcutaneous Injection Site

<table>
<thead>
<tr>
<th>Animal Identifier</th>
<th>Serum Volume (µl)</th>
<th>Tumour present/absent (weight)</th>
<th>Lymph node status (positive/negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>520</td>
<td>+ (78mg)</td>
<td>+</td>
</tr>
<tr>
<td>2*</td>
<td>570</td>
<td>+ (100mg)</td>
<td>+</td>
</tr>
<tr>
<td>3*</td>
<td>540</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>550</td>
<td>+ (21mg)</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>530</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>600</td>
<td>+ (76mg)</td>
<td>+</td>
</tr>
<tr>
<td>7***</td>
<td>150</td>
<td>+ (93mg)</td>
<td>+</td>
</tr>
<tr>
<td>8**</td>
<td>150</td>
<td>+ (73mg)</td>
<td>+</td>
</tr>
<tr>
<td>9***</td>
<td>300</td>
<td>+ (200mg)</td>
<td>-</td>
</tr>
<tr>
<td>10***</td>
<td>400</td>
<td>+ (10mg)</td>
<td>+</td>
</tr>
</tbody>
</table>

### Mammary Fat Pad Injection Site

<table>
<thead>
<tr>
<th>Animal Identifier</th>
<th>Serum Volume (µl)</th>
<th>Tumour present/absent (weight)</th>
<th>Lymph node status (positive/negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>400</td>
<td>+ (159mg)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>450</td>
<td>+ (64mg)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>400</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>550</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>400</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>550</td>
<td>+ (80mg)</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>490</td>
<td>+ (30mg)</td>
<td>-</td>
</tr>
<tr>
<td>9**</td>
<td>150</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10***</td>
<td>300</td>
<td>+ (180mg)</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 6.1 Serum volume, tumour burden and lymph node status of animals in the SC and MFP Injection Site groups. *Indicates animals that were selected for inclusion in the TLDA. **Indicates animal samples that were exhausted for Transmission Electron Microscopy. ***Indicates animals where protein yield was too low to proceed with reverse transcription.
6.4.2 Taqman Low Density Array (TLDA) and target validation

Three animals from the SC inoculation site group were chosen for inclusion in the Taqman Low Density Array (TLDA). This animal group had protein estimation readings of between 564-759ng/µl following exosome isolation. A sample of exosomes that were isolated from the cell-conditioned media of HCC1954 cells was also included on a fourth array card for comparison purposes. Table 6.2 outlines the miRNAs that were detected within each sample on the array.
### Table 6.2 Low density array of serum exosomes from 3 individual tumour bearing animals and HCC1954 cell-secreted exosomes.

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Cell exosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7e</td>
<td>miR159a</td>
<td>miR-16</td>
<td><strong>miR-106a</strong>*</td>
</tr>
<tr>
<td>let-7f</td>
<td>let-7f</td>
<td>miR-17**</td>
<td>miR-194</td>
</tr>
<tr>
<td>miR-10b</td>
<td>let-7f</td>
<td>miR-194</td>
<td>miR-323-3p</td>
</tr>
<tr>
<td>miR-139-3p</td>
<td><strong>miR-106a</strong>*</td>
<td><strong>miR-223</strong></td>
<td>miR-425-5p</td>
</tr>
<tr>
<td>miR-142-3p</td>
<td>miR-136</td>
<td>miR-24</td>
<td><strong>miR-485-3p</strong>*</td>
</tr>
<tr>
<td>miR-15a</td>
<td>miR-140-3p</td>
<td>miR-28-3p</td>
<td>miR-509-5p</td>
</tr>
<tr>
<td>miR-16</td>
<td>miR-146a</td>
<td>miR-29a</td>
<td><strong>U6 snRNA</strong>*</td>
</tr>
<tr>
<td><strong>miR-17</strong>**</td>
<td>miR-16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-191</td>
<td><strong>miR-17</strong>**</td>
<td>miR-302b</td>
<td></td>
</tr>
<tr>
<td>miR-194</td>
<td></td>
<td>miR-323-3p</td>
<td></td>
</tr>
<tr>
<td>miR-19b</td>
<td>miR-191</td>
<td>miR-425-5p</td>
<td></td>
</tr>
<tr>
<td>miR-200b</td>
<td>miR-19a</td>
<td>miR-484</td>
<td></td>
</tr>
<tr>
<td>miR-208b</td>
<td>miR-19</td>
<td><strong>miR-485-3p</strong>*</td>
<td></td>
</tr>
<tr>
<td>miR-20a</td>
<td>miR-20a</td>
<td>miR-486</td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>miR-222</td>
<td>miR-509-5p</td>
<td></td>
</tr>
<tr>
<td><strong>miR-222</strong></td>
<td></td>
<td></td>
<td><strong>miR-597</strong></td>
</tr>
<tr>
<td><strong>miR-223</strong>**</td>
<td>miR-24</td>
<td>miR-652</td>
<td></td>
</tr>
<tr>
<td>miR-25</td>
<td>miR-299-5p</td>
<td>miR-708</td>
<td></td>
</tr>
<tr>
<td>miR-29b</td>
<td>miR-30c</td>
<td>miR-885-3p</td>
<td></td>
</tr>
<tr>
<td>miR-31</td>
<td>miR-323-3p</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
</tr>
<tr>
<td>miR-330</td>
<td>miR-422a</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
</tr>
<tr>
<td><strong>miR-338-3p</strong></td>
<td></td>
<td></td>
<td><strong>miR-485-3p</strong>*</td>
</tr>
<tr>
<td>miR-371-3p</td>
<td>miR-486</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-425-5p</td>
<td>miR-509-5p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-484</td>
<td>miR-517a</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>miR-485-3p</strong>*</td>
<td>miR-520b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-597</td>
<td>miR-598</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-652</td>
<td>miR-654-3p</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-98</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-99a</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-129-3p</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-491</td>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>U6 snRNA</strong>*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Green** = common to all 4 arrays  
**Red**** = present in the 3 animal arrays but not the cell exosomes array  
**Blue*** = detected in 1 animal sample array and the cell exosomes array
U6 and MiR-485-3p were common to all 4 arrays. MiR-17 and MiR-223 were present in the 3 animal serum exosome arrays. MiR-106a was detected in 1 animal sample and also the HCC1954 cell-derived exosomes. It was also found to be dysregulated in a murine model of breast cancer during disease progression in a previous study carried out in this laboratory and for this reason it was deemed to be of interest (81).

The next step was to validate these targets using exosome samples isolated from the remaining animals (n=11) to ensure that they were in fact detectable in animal serum exosomes. MiR-17, although detectable in all animal samples on the array, was not deemed to be of interest as it has been extensively reported on in the literature and so it was not validated in the remaining animal samples (205-208). Table 6.3 shows the mean Cycle Threshold (C\text{t}) values for U6, miR-223, miR-106a and miR-485-3p detectable by RQ-PCR. Higher C\text{t} values, by virtue of the fact that they detected later, correspond to lower levels of miRNA expression. All miRNAs were reproducibly detectable, except for miR-485-3p which was undetermined in all cases (Table 6.3). MiR-485-3p was not analysed any further.
### Table 6.3 Expression levels of U6, miR-223, miR-106a and miR-485-3p in 11 serum exosome samples.

*MiR-106a not investigated for sample number 2 as there was not enough RNA remaining following the investigation of the other 3 miRNA targets. T+ = tumour present; T- = Tumour absent; LN+ = clinically positive lymph node; LN- = clinically negative lymph node.

### 6.4.3 Investigation of targets in patients with breast cancer and healthy controls

Following on from this validation work, the next step was to investigate the presence and potential for the chosen miRNAs as exosome-encapsulated circulating biomarkers for breast cancer. In order to do this, serum samples were obtained from patients with breast cancer and healthy volunteers.
following the informed consent process. A total of 52 patients with breast cancer and 26 healthy controls, with no personal or family history of cancer, no significant medical co-morbidities and no surgery in the previous 12 months, were included in this work. Table 6.4 outlines the clinicopathological data for each patient/volunteer that participated in the study. Tumour stage was unknown for 15 patients with breast cancer as one or more components of the TNM classification system were undetermined.
### Table 6.4 Clinicopathological details of patients and healthy volunteers included for the investigation of serum exosome-encapsulated miRNAs to function as circulating biomarkers for breast cancer.

<table>
<thead>
<tr>
<th></th>
<th>Cancers</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of patients</strong></td>
<td>52</td>
<td>26</td>
</tr>
<tr>
<td><strong>Mean age (range)</strong></td>
<td>59 (38-82)</td>
<td>58 (33-81)</td>
</tr>
<tr>
<td><strong>Menopausal status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>Premenopausal</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>Epithelial subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>15</td>
<td>N/A</td>
</tr>
<tr>
<td>Luminal B</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>HER2+</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Basal-like</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>
The first target that was investigated was U6, which has been suggested in the literature as a potential endogenous control for use in exosome-related research (209). This was quantified in exosomes from 47 cancers and 16 controls. Figure 6.2 shows the levels of expression of U6 in 19 cancer and control samples.

![U6 expression in cancers and controls](image)

Figure 6.2 Expression levels of U6 in serum-derived exosomes of patients with breast cancer and healthy controls.

All samples included in this graph reached a threshold protein reading to ensure equal loading of RNA for reverse transcription. $C_t$ values for this target ranged from 28.51 to 39.87, with a mean $C_t$ value of 34.82. U6 was undetectable in a further 12 samples for which adequate protein levels were reached. Given the low rate of U6 detection (undetectable in 17 out of 63 samples in total, 27%), combined with the broad range of values at which it was detected, a decision was made not to proceed with this target when evaluating additional samples. In addition to this, the high variability in target detection and levels also indicated that it is not a suitable endogenous control for exosomal work.
MiR-223 was detectable in 47 serum exosomal samples from patients with breast cancer and 24 healthy controls. The mean and range of values for miR-223 and miR-106a are shown in Table 6.5.

<table>
<thead>
<tr>
<th></th>
<th>MiR-223</th>
<th>MiR-106a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean C&lt;sub&gt;t&lt;/sub&gt; value</td>
<td>32.84</td>
<td>34.13</td>
</tr>
<tr>
<td>Range of C&lt;sub&gt;t&lt;/sub&gt; values</td>
<td>27.16-36.68</td>
<td>29.79-38.54</td>
</tr>
<tr>
<td>Undetectable</td>
<td>n=5 (9%)</td>
<td>n=2 (4%)</td>
</tr>
</tbody>
</table>

Table 6.5 Mean and range of C<sub>t</sub> values detected for miR-223 and miR-106a.

Expression levels of miR-223 in cancers versus controls are shown in Figure 6.3. In the absence of a suitable endogenous control levels are expressed as mean C<sub>t</sub> values and boxplots are shown for all samples. No difference was noted in miR-223 expression in cancers versus controls (Figure 6.3 (A)), however when cancers were subdivided according to epithelial subtype, exosomes isolated from patients with HER2 cancers showed higher levels of expression compared to luminal A tumours (P<0.005 ANOVA, Figure 6.3 (B)). No difference in miR-223 levels was seen when tumours were categorised by grade or stage at diagnosis (Figure 6.3 (C) and (D)).
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Figure 6.3 Serum exosomal miR-223 levels (A) in healthy controls and patients with breast cancer; (B) by epithelial subtype; (C) by tumour grade; and (D) by tumour stage at diagnosis.
When samples were normalised according to protein loading no significant difference in expression levels was detected either between cancers and controls, or when tumours were analysed by epithelial subtype, grade or TNM stage at diagnosis. MiR-223 levels were, however, found to be significantly decreased in Luminal A cancers compared to controls (P<0.005).

MiR-106a was detectable in 50 serum exosomal samples from patients with breast cancer and 24 samples from healthy controls. In terms of miR-106a expression levels, no significant difference was measured when all cancers were compared to all controls (Figure 6.4 (A)). In addition to this, no difference was found when tumours were categorised according to epithelial subtype (Figure 6.4 (B)), grade (Figure 6.4 (C)), or stage (Figure 6.4 (D)).
Figure 6.4 Serum exosomal miR-106a levels (A) in healthy controls and patients with breast cancer; (B) by epithelial subtype; (C) by tumour grade; and (D) by tumour stage at diagnosis.
When only samples that were normalised based on protein level were analysed, no difference in miR-106a expression was noted between cancers and controls or in cancers categorised by subtype, grade or stage.
Chapter 6: Exosomal microRNAs as biomarkers for breast cancer

6.5 Discussion

This study was undertaken in an effort to identify miRNAs that may represent circulating biomarkers for breast cancer. An in vivo murine model of breast cancer was established for this purpose as nude athymic animals may represent a ‘cleaner’ platform for biomarker discovery. 3 animals were chosen from the subcutaneous injection site group for inclusion on a TLDA along with HCC1954 cell-secreted exosomes. This was carried out in order to identify the range of targets that were both common and unique between samples. The array, which targeted 365 miRNAs, yielded 4 potentially interesting targets based on their differential detection across the various arrays: U6, miR-223, miR-106a and miR-485-3p. The latter has not been studied in the circulation but was examined in vitro in the setting of a prostate cancer cell line where it was found to be linked to sensitivity to fludarabine, with up-regulation of miR-485-3p being associated with resistance to this chemotherapeutic agent in vitro (210). It has also been shown to have decreased expression in breast cancer tissues, with overexpression inhibiting spontaneous metastasis of breast cancer cells in vivo (211). MiR-485-3p was undetectable in validation work that was carried out on the remaining animal samples and so was excluded from further analysis at this point. Validation of the array data was crucial prior to the commencement of a patient-based study as the TLDA represents a point in time measurement and therefore targets must be shown to be reproducibly detectable in additional samples. U6 was detected in all additional animal samples (range 30.79-35.02) and has been identified in the literature as a potential endogenous control for serum exosomal research (209).

Expression levels of selected miRNAs were investigated in exosomes that were isolated from the serum of 52 patients with breast cancer and 26 healthy controls. Patients were matched for age and menopausal status in order to allow for an accurate comparison whereby any dysregulation in miRNA levels are likely to be a result of cancer or control status as opposed to being a function of age or hormonal variations. U6 levels were measured in 47 cancers and 16 controls. This target was undetectable in 27% of samples and when present demonstrated highly variable expression levels.
In addition to this, the mean cycle threshold at which it was detectable was in excess of that which is desirable in an endogenous control. There are conflicting data in the literature regarding U6 expression in exosomes with some groups suggesting that it may be suitable endogenous controls and others reporting highly variable levels (196, 209, 212). Our findings corroborate that of the latter whereby U6 was deemed to be an unsuitable candidate endogenous control due large fluctuations in expression levels (196, 212). U6 expression has also been reported to gradually decrease after repeated freeze-thaw cycles (212). It is as a result of these findings, in conjunction with that of the work reported here, that U6 expression levels were not quantified for the remaining 15 patient serum exosome samples.

MiR-223 was also expressed in all animal serum exosome samples in the validation cohort. This miRNA was found to be detectable in the majority of patient and control samples, with a wide range of values across samples. Expression levels for this miRNA were not significantly different between cancers and controls, however, when the cancers were classified by epithelial subtype levels were significantly higher in HER2 cancers compared to Luminal A cancers. Although small numbers were present in each group this finding is interesting as this particular miRNA was initially identified in serum exosomes of animals bearing HER2 amplified tumours. MiR-223 has been found to exhibit significantly lower levels in post-operative serum samples compared to pre-operative samples from 24 patients with ER-positive post-menopausal breast cancer, suggesting that it may serve as a biomarker for this disease (213). In the same study, Kodahl et al., (2014) (213) did not compare the cancer samples to healthy controls or to other breast cancer subtypes. Additionally, whole serum samples were analysed without an evaluation of the exosomal fraction. In contrast to this, a separate study found that whole blood miR-223 levels showed significantly reduced expression in patients with Luminal A breast cancer compared to healthy controls, a finding that was reflected in this work (200). This finding adds further evidence for the potential role of this miRNA to function as a breast cancer biomarker allowing accurate subtype
Chapter 6: Exosomal microRNAs as biomarkers for breast cancer discrimination, although increased numbers in each subtype would be required for further validation work.

No association with other clinicopathological characteristics was noted for miR-223. Levels of this miRNA were also measured for the samples in which adequate protein levels were detected on protein assay in order to allow normalisation of data in the absence of a robust endogenous control. Here no difference was noted in the results obtained when samples were analysed except for in the epithelial subtype analysis where the difference between HER2 and Luminal A cancers was no longer significant. These differing findings may be a result of smaller sample numbers in each group. Alternatively, they may indicate that normalisation based on protein is not a viable substitute for an endogenous control due to the variable protein content of exosomes and the presence of surface proteins. It is worth noting that exosomes were isolated from equal volumes of serum for each patient/control and this may serve as a valid method of data normalisation in the interim. Due to the lack of definitive data regarding the clinical relevance of miR-223, further investigation using a validated endogenous control is warranted.

MiR-106a was detectable in 8 out of 10 animal samples in the validation cohort, again with a wide range of C\textsubscript{T} values detected across samples. No difference was detected in miR-106a expression in serum exosomes of patients with cancer compared to healthy controls in this study. MiR-106a expression has been identified as a potentially promising biomarker in the literature with up-regulation in plasma levels being indicative of metastatic breast cancer (214). In a small study by Zhao et al., (2016) (214) the authors used QRT-PCR based miRNA detection panels to identify the circulating miRNA profile in six cancer specimens and four normal controls. Up-regulation of miR-106a was confirmed in plasma samples from 10 patients with metastatic breast cancer compared to 10 healthy controls (214). Wang et al., (2010) demonstrated increased expression of miR-106a in the serum of patients with breast cancer (n=68) compared to healthy controls (n=40) (215). Furthermore, elevated expression was noted in higher grade tumours
Chapter 6: Exosomal microRNAs as biomarkers for breast cancer and ER and PR negative tumours (215). Published work carried out in this department found decreased expression of miR-106a in the whole blood of animals in a murine model of breast cancer during disease progression (81). Levels were not significantly dysregulated in patients with breast cancer compared to controls but were significantly decreased in patients with Basal-like tumours (81). The findings of the current study reflect that of Waters et al., (2014) (81) whereby no significant difference was seen between miR-106a expression in patients with breast cancer compared to controls. Changing to the serum exosomal fraction of blood did not change the expression levels of miR-106a. This may indicate the serum exosomal miR-106a may not be a valuable breast cancer biomarker.
Chapter 7

Final Discussion and Future Directions
7.1 Core findings and clinical relevance

The field of exosome-encapsulated miRNAs as serum-based biomarkers is evolving rapidly and this facet of cancer research offers great potential. Firstly, our understanding of exosomes in terms of nomenclature and size is becoming more refined as a result of international collaboration (104). Furthermore, exosomes have been shown to be robust and can be stored for extended periods without significantly affecting the integrity of encapsulated miRNAs (97, 132). This feature of exosomes increases their potential applicability in the laboratory/clinical interface. In this study cell-secreted exosomes were successfully isolated from breast cancer cell lines and MSCs. Successful isolation was demonstrated using TEM and Western Blot, which is currently the gold standard for confirmation of successful isolation. The exosome-associated protein, CD63, which was used in this work, is not specific to exosomes and therefore the identification of an exosome-specific marker which could be used for characterisation, isolation and quantification purposes would be valuable in this field. Such a marker may also allow the identification of the type of cells from which exosomes are secreted and may enhance the biomarker potential of these nanovesicles.

The role that exosomes play in the primary tumour microenvironment was investigated. The ability of exosomes to transfer to recipient cells was demonstrated using confocal microscopy. The impact of transferred exosomes on recipient cells was investigated using proliferation and angiogenesis assays. Breast cancer cell exosomes did not impact on the rate of proliferation of MSCs or stromal WI-38 cells. Similarly, MSC-secreted exosomes had no impact on breast cancer cell proliferation. Although these results differ from that of the published literature this is likely a function of the duration of incubation of cells with exosomes. In this work, breast cancer cell-secreted exosomes were demonstrated to stimulate angiogenesis, a finding which is remarkable given the minute size of transferred exosomes.

The miRNAs that are contained within breast cancer cell-derived exosomes are reflective of their parent cells and therefore may offer a tumour-related
Chapter 7: Final discussion and future directions

profile that is more specific than the miRNA profile of whole blood or even serum (99, 100). The potential applications of serum exosome-encapsulated miRNAs are vast: they have the potential to be used for breast cancer diagnosis, subtype specification, and for prediction and monitoring of response to therapy. Recent exciting work demonstrated how tumour-derived exosomes that are taken up by organ-specific cells prime the pre-metastatic niche through the expression of differing integrin profiles (133). Such data on exosomal integrins may allow researchers and clinicians to predict the metastatic site of breast tumours (133) and may compliment exosomal-miRNA research in order to give a patient specific tumour profile and prognostication information. As plasma/serum is routinely collected in multiple cancer centres worldwide there exists a wealth of samples from patients with breast cancer on which validation of findings from smaller studies may be carried out in a relatively short period of time. Such collaboration is the key to moving this field of research forward and examining its true applicability in the clinical setting.

The potential for exosome-encapsulated miRNAs to function as biomarkers for breast cancer was investigated with multiple potentially interesting targets examined. MiR-451a was found to be packaged into exosomes and secreted by breast cancer cell lines in vitro. Further analysis revealed the presence of this miRNA in whole blood, although not significantly altered between patients with breast cancer and healthy controls. Given that this miRNA was initially identified in exosomes secreted by cell lines this miRNA was next investigated in serum exosomes. MiR-451a expression was significantly higher in the serum-derived exosomes of patients with breast cancer compared to healthy controls. However, when samples were evaluated based on protein normalisation data no difference was seen. Protein estimation data is, however, a poor surrogate for exosome quantification, with levels being influenced by exosomal content as well as membrane-associated proteins. Direct quantification such as that which may be possible with an exosome-specific marker may be a valuable method of data normalisation. Although this data highlights the need for robust
normalisation of serum exosomal miRNAs, it does suggest that miR-451a is worthy of further evaluation as a potential biomarker for breast cancer.

MiR-744-5p was detected in the BT-20 cell-secreted exosomes and not in MDA-MB-231 exosomes indicating some degree of selectivity of miRNA packaging into exosomes. MiR-744-5p was not altered (although detected) in the whole blood of patients with breast cancer compared to controls. When this miRNA was further examined in the circulation it was undetectable in the majority of serum exosomal samples. Again, this finding demonstrates selectivity of packaging of exosomal miRNAs.

This work highlighted the need for a robust endogenous control for serum exosomal miRNA work. MiR-16 levels were found to be highly variable across samples regardless of normalisation based on protein levels. U6 was also investigated as a potential endogenous control for exosomal work. Levels were found to be highly variable or even undetectable and so it was not deemed to be a promising endogenous control going forward.

An *in vivo* model of breast cancer was used to identify potentially interesting serum exosomal miRNAs that may function as biomarkers for breast cancer. A nude athymic mouse model was employed as it represents a clean control setting, whereby differences in miRNA expression levels are likely to be a result of breast cancer as opposed to other disease processes which may act as confounders in humans. 2 potentially interesting miRNA targets were identified from a TLDA which analysed animal serum exosomes. These targets, miR-223 and miR-106a, were then investigated in the human serum exosomes. MiR-223 expression was significantly higher in serum exosomes of patients with HER2 cancers compared to Luminal A cancers, a finding which is of interest as this miRNA was initially identified in serum-derived exosomes of animals bearing HER2 amplified tumours. Normalisation of samples based on protein led to loss of this difference in expression levels. This finding may indicate that normalisation of data based on protein may not be an ideal method of standardisation across samples. A marker which is specific to exosomes that would allow accurate quantification, coupled with a robust endogenous control, would add
immense value in this field. The isolation of exosomes from an equal volume of serum for every sample as in this study, may represent a method of data normalisation in the interim. Although published studies have suggested the presence of greater numbers of exosomes in patients with breast cancer compared to healthy controls (59), the findings of this study did not support this suggestion. This work reinforces the need for reliable data normalisation. It also suggests the potential role for miR-223 to serve as a biomarker which may facilitate accurate breast cancer subtype discrimination. Further investigation of this miRNA in the presence of a validated endogenous control would be pivotal in the determination of its utility.

No difference in miR-106a expression was noted between patients with breast cancer and healthy controls, or among cancer subtypes regardless of the subset of samples that were examined (serum exosomes or serum exosomes normalised according to protein), and so this was not deemed to be an interesting biomarker for breast cancer going forward.

One challenge that exosome-based research currently faces is that of the multistep process that is required from serum collection to miRNA quantification. This represents a disadvantage when compared to developing breast cancer biomarker detection methods, some of which entail the detection of miRNAs or other markers directly in whole blood or serum. The use of such starting materials which do not require extensive processing may potentially be more readily integrated into the clinical setting. A plethora of kits are currently available, however, which allow rapid exosome isolation and RNA extraction. As this field of research continues to grow, platforms for analysis, with high throughput capabilities will undoubtedly be developed. In addition to this, serum exosomal miRNAs may offer a cleaner fraction of blood which reflects more accurately the characteristics of tumour cells. Therefore, despite the additional processing which they require, serum exosomes may offer a robust source of miRNAs which may function as circulating breast cancer biomarkers.
An additional potential limitation to the use of exosomal miRNAs as biomarkers for breast cancer is the strong variation that has been noted in exosomal miRNA levels as evidenced by wide ranging cycle threshold values on RQ-PCR (98, 111). Such variation may impact on the clinical applicability of exosomal miRNAs as breast cancer biomarkers. Furthermore, patient co-morbidities may also impact on miRNA levels, and may lead to a lack of specificity for breast cancer. This difficulty may be addressed to some degree by ensuring that appropriate statistical tests are employed when analysing exosomal miRNA data. Additionally, when exosomes are isolated from a small volume of serum/plasma (for example 250µl) the resulting RNA yields may be low (216). This may lead to difficulty in the downstream detection of miRNAs using RQ-PCR. Understanding exosome source, factors impacting their abundance and content, and the identification of endogenous controls that are not impacted by disease status will be key to this.

7.2 Future Directions

This work highlights a number of key issues in the field of exosomal miRNAs as circulating biomarkers for breast cancer. Firstly, the need for a marker that is specific to exosomes and would facilitate accurate isolation, characterisation and quantification of exosomes has been demonstrated. 2 potentially interesting serum exosomal miRNAs, miR-451a and miR-223, which may function as circulating biomarkers for breast cancer, were also identified using a combination of in vitro, in vivo and patient-based models. However, their relevance in the clinical setting remains uncertain due to the lack of an endogenous control for use in serum exosomal work. The aforementioned miRNAs warrant further investigation in greater sample numbers using a validated endogenous control, in order to determine their clinical utility. The most significant finding from this body of work is the lack an endogenous control for use in serum exosomal research, which is a rate limiting factor in the generation of robust exosomal miRNA data. This specific area merits close attention in the short-term. It is imperative that
Chapter 7: Final discussion and future directions

researchers remain cognisant of these challenges and do not publish data that has not been adequately normalised. We must strive to publish only robust, transparent and reproducible data in order to determine the clinical utility of exosomal miRNAs as breast cancer biomarkers, so that they may have a meaningful impact on patient outcomes in the near future.
Chapter 8

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Chapter 9

Appendices
9.1 Biobank ethical approval and patient consent form

Research Ethics Committee
Unit 4
Merlin Park Hospital
Galway.


Professor Michael Kerin
Department of Surgery
Clinical Science
University College Hospital
Galway.

Ref: 45/05 - The Provision of a Breast Cancer BioBank research resource for use in Molecular and Cellular Studies and Clinical Trials

Dear Michael,

The informed consent form for participation in the BioBank was approved by the CREC subject to a single amendment. It was felt that a stronger statement should be included to ensure that participants were aware their histological details would be linked to their clinical data and to their overall health outcome.

Yours sincerely,

Dr. S. T O’Keeffe
Chairman Research Ethics Committee.
Chapter 9: Appendices

GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

Patient Information

Introduction

We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to establish a BioBank. The purpose of the BioBank is to set up a resource that can support a diverse range of research programmes intended to improve the prevention, diagnosis and treatment of cancer. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research. Galway University Hospitals are actively involved in research that aims to identify markers that will predict how a cancer develops, progresses and responds to a variety of treatments. This type of work requires the use of tissue and blood samples. It is hoped that it will eventually lead to improvements in the diagnosis, treatment and outcome for those who have cancer. Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future.

Your Involvement

If you volunteer to participate in our BioBank, there will be no additional risks to you outside those of your standard investigation and treatment. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Procedure

We invite all patients who are undergoing treatment and/or investigation to participate. All samples for research will be taken at the time you are attending the hospital for routine diagnostic tests.

(i) Tissue Samples

By participating, you give us consent to retain small pieces of your tissue obtained at the time of surgery. These samples will be stored and used in
the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland. This will not affect your diagnosis in any way.

(ii) **Blood Samples**

By participating, you give us consent to take an extra blood sample (equivalent of 4 teaspoonfuls) at the same time that your blood is being taken for routine tests. These samples will be stored and used in the future for research. They may be analysed in the surgical laboratory at GUH, or may be transferred to another laboratory for additional analysis using specialised equipment which is not yet available in Ireland.

(iii) **Clinical Information**

By participating, you give us consent to store information relating to your diagnosis and treatment on a database. This information is only accessed by personnel directly involved in research within the Surgical Research Unit.

**Further Information**

If you would like further information about our BioBank, your participation and your rights, please contact the Surgical Research Unit (Tel: 091 524390).

If you would like further information about research projects that may be conducted, please contact your Consultant.

Thank you in anticipation of your assistance. Please read and sign the Consent section.

I have read the attached information sheet on the above project, dated

Please Initial Box
GALWAY UNIVERSITY HOSPITALS - BIOBANK INFORMED CONSENT

PARTICIPANT DECLARATION

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. I understand that I may withdraw from the study at any time.

(Name of sponsor): …………………………………………………

PARTICIPANT'S NAME:……………………………………………………

CONTACT DETAILS:……………………………………………………

PARTICIPANT'S Signature:………………………………………………

DATE: ……………………………………………………………

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the participant is a minor (under 18 years old) the signature of parent or guardian must be obtained:
NAME OF CONSENDER, PARENT, OR GUARDIAN: ..............................................................

SIGNATURE: ...........................................................................................

RELATION TO PARTICIPANT: ....................................................................... 

DECLARATION OF INVESTIGATOR’S RESPONSIBILITY

I have explained the nature and purpose of this research study, the procedures to be undertaken and any risks that may be involved. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

NAME OF RESEARCH NURSE OR INVESTIGATOR: ..............................................................

SIGNATURE: ..............................................................................................

DATE: ...........................................................................................................

CONSULTANT: ..............................................................................................

Keep the original of this form in the investigators file, give one copy to the participant, and send one copy to the sponsor (if there is a sponsor).
9.2 Biobank specimen form
# Chapter 9: Appendices

## Cancer Research Biobank

Surgery – Research Lab, 2nd Floor, TRF (Ext. 4202)

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<th>Culture Medium</th>
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### Tissue Specimens

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### Sample Timepoints

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<th>Peri-neoadjuvant</th>
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<th>Post-Tumour Resection</th>
<th>Review/Follow up</th>
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### Lab Use Only

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Complete this section for Cancer Genetics Research Blood only

Age at Diagnosis: ______

Family History (Please circle): Yes No

Describe Family History (Include blood relatives, maternal/paternal and cancer type)
### 9.3 Taqman Low Density Array configuration: Human miRNA Panel A

Card Name: Human miRNA Panel A

**Gene Symbols**

<p>|   | 1         | 2         | 3         | 4         | 5         | 6         | 7         | 8         | 9         | 10        | 11        | 12        | 13         | 14         | 15         | 16         | 17         | 18         | 19         | 20         | 21         | 22         | 23         | 24         |
|---|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1 | hsa-let-7a| hsa-let-7c| hsa-let-7d| hsa-let-7e| hsa-let-7f| hsa-let-7g| hsa-miR-1 | hsa-miR-9 | hsa-miR-10a| hsa-miR-10b| U6 snRNA  | U6 snRNA  | hsa-miR-15a| hsa-miR-15b| hsa-miR-16 | hsa-miR-17 | hsa-miR-18a| hsa-miR-18b| hsa-miR-19a| hsa-miR-19b| hsa-miR-20a| hsa-miR-20b| hsa-miR-21 | hsa-miR-22 |
| 2 | hsa-miR-23a| hsa-miR-23b| hsa-miR-24 | hsa-miR-25 | hsa-miR-26a| hsa-miR-27a| hsa-miR-28-3p| hsa-miR-28-28 | hsa-miR-28-29a| hsa-miR-29b| U6 snRNA  | U6 snRNA  | hsa-miR-107 | hsa-miR-122 | hsa-miR-124a| hsa-miR-125a-3p | hsa-miR-125b | hsa-miR-126 | hsa-miR-127 | hsa-miR-128a| hsa-miR-129 | mmu-miR-93 |
| 3 | hsa-miR-95 | mmu-miR-96 | hsa-miR-98 | hsa-miR-99a| hsa-miR-100| hsa-miR-101| hsa-miR-105 | hsa-miR-106a| RNU44     | hsa-miR-106b| hsa-miR-107 | hsa-miR-122 | hsa-miR-124a| hsa-miR-125a-3p | hsa-miR-125b | hsa-miR-126 | hsa-miR-127 | hsa-miR-128a| hsa-miR-129 | mmu-miR-93 |
| 4 | hsa-miR-130a| hsa-miR-130b| hsa-miR-132 | hsa-miR-133a| hsa-miR-134| hsa-miR-135a| hsa-miR-135b | hsa-miR-136 | mmu-miR-137| hsa-miR-138 | hsa-miR-139-3p| hsa-miR-140-5p | hsa-miR-140 | hsa-miR-141 | hsa-miR-142-3p| hsa-miR-142-5p| hsa-miR-143 | hsa-miR-145 | hsa-miR-146a| hsa-miR-146b-3p| hsa-miR-147b | mmu-miR-129 |
| 5 | hsa-miR-148a| hsa-miR-148b| hsa-miR-149 | hsa-miR-150 | hsa-miR-152 | hsa-miR-153 | hsa-miR-181a| hsa-miR-182 | RNU48     | hsa-miR-183 | hsa-miR-184 | hsa-miR-185 | hsa-miR-186 | hsa-miR-187 | hsa-miR-188-3p | hsa-miR-188-3p | hsa-miR-190 | hsa-miR-191 | hsa-miR-192 | hsa-miR-193a-3p | hsa-miR-193a-3p | hsa-miR-194 |
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| 195 | 196b | 197 | 198 | 199a | 199b | 200a | 200b | 200c | 203 | 204 | 205 | 208b | 210 | 214 | 215 | 216a | 216b | 217 | 218 | 219 | 221 |
|-----|------|-----|-----|-----|------|------|------|------|-----|-----|-----|------|-----|-----|-----|------|------|-----|-----|-----|-----|-----|
| hsa-miR-222 | hsa-miR-223 | hsa-miR-224 | hsa-miR-296-3p | hsa-miR-299-3p | hsa-miR-301 | hsa-miR-302b | hsa-miR-302c | hsa-miR-302d | ath-miR-159a | hsa-miR-303a | hsa-miR-303b | hsa-miR-303c | hsa-miR-303d | hsa-miR-303e | hsa-miR-303f | hsa-miR-303g | hsa-miR-303h | hsa-miR-303i | hsa-miR-303j | hsa-miR-303k |
| 7 | | | | | | | | | | | | | | | | | | | | |
| hsa-miR-335 | hsa-miR-337-3p | hsa-miR-338-3p | hsa-miR-339-3p | hsa-miR-340 | hsa-miR-341 | hsa-miR-342-3p | hsa-miR-343-3p | hsa-miR-344-3p | hsa-miR-345 | hsa-miR-346 | hsa-miR-347 | hsa-miR-348-3p | hsa-miR-349 | hsa-miR-350 | hsa-miR-351 | hsa-miR-352 | hsa-miR-353 | hsa-miR-354 | hsa-miR-355 | hsa-miR-356 | hsa-miR-357 | hsa-miR-358 |
| 8 | | | | | | | | | | | | | | | | | | | | |
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| 9 | | | | | | | | | | | | | | | | | | | | |
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| 10 | | | | | | | | | | | | | | | | | | | | |
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| 11 | | | | | | | | | | | | | | | | | | | | |
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| 12 | | | | | | | | | | | | | | | | | | | | |
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9.4 *In vivo* study project authorisation
IRISH MEDICINES BOARD ACTS 1995 AND 2006
Authorisation of Project pursuant to Part 5 of the European Union
(Protection of Animals Used for Scientific Purposes) Regulations 2012
(S.I. No. 543 of 2012) as amended

Project Authorisation Number: AE19125/P024
Case No: 7020899


Holder of the project authorisation: Dr. Róisín Dwyer of National University of Ireland Galway

subject to the provisions of the European Union (Protection of Animals Used for Scientific Purposes) Regulations 2012 (S.I. No. 543 of 2012) as amended and the terms and conditions set out in this authorisation.

The authorisation, unless amended, suspended or revoked, shall continue in force from 10 July 2015 until 09 July 2016.

Signed on behalf of the Health Products Regulatory Authority on 10 July 2015

A person authorised in that behalf by the Health Products Regulatory Authority

(NOTE: This authorisation replaces any previous authorisation in respect of this project.)
9.5 Copies of communications arising from this work