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Metastatic Breast Cancer: Patterns of metastasis and novel biomarkers

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

Doctor of Medicine (MD)

By

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This thesis is dedicated to my late grandfather,
Dr. Michael O'Connor

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Abbreviations

ASCO	American society of clinical oncology
BRCA 1/2	Breast cancer susceptibility gene
CA 125	Cancer antigen 125
CA 15-3	Cancer antigen 15-3
cDNA	Complementary DNA
CEA	Carcino-embryonic antigen
C_t	Cycle threshold
CTCs	Circulating tumour cells
ctDNA	Cell free circulating DNA
DFS	Disease-free survival
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide triphosphate
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ESMO	European society for medical oncology
HER2	Human epidermal growth factor receptor
IAC	Inter-assay control
IHC	Immunohistochemistry
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
miRNA	MicroRNA
MOSAIQ	Radiation oncology software
mRNA	Messenger RNA
NAC	Neoadjuvant chemotherapy
NUIG	National University of Ireland, Galway
NFW	Nuclease free-water
NTC	No template control
OS	Overall survival
PAS	Power analysis software
pCR	Complete pathological response
PCR	Polymerase chain reaction

PDAR	Pre-developed assay reagents
PR	Progesterone receptor
PRS	Post-recurrence survival
qRT-PCR	Quantitative reverse transcriptase PCR
RNA	Ribonucleic acid
RNAseq	RNA sequencing
ROC	Receiver operator characteristic
RPM	Revolutions per minute
RQ	Relative quantification
rRNA	Ribosomal RNA
RTC	Reverse transcriptase control
SEER	Surveillance, epidemiology and end-results programme
TAM	Tumour-associated macrophages
TNBC	Triple negative breast cancer
TNM	Tumour size, Nodal status, Metastasis
UHG	University hospital Galway
µg	Microgram
µl	Microliter
UMASS	University of Massacheusetts, Boston
UV	Ultraviolet
uPA	Urokinase plasminogen activator
UTR	Untranslated region
WHO	World health organisation

Communications arising from this work

Peer Reviewed Published Manuscripts

Identification and validation of circulating microRNA distinguishing metastatic from local breast cancer

P McAnena, T Kahraman, C Curran, J Freedman, AJ Lowery, JAL Brown, MJ Kerin
BMC Cancer, December 2018

Role of micro-RNAs in breast cancer surgery
P McAnena, AJ Lowery, MJ Kerin
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doi:10.1002/bjs.10790

Breast cancer subtype discordance: Impact on post-recurrence survival and potential treatment options
P McAnena, A McGuire, A Ramli, C Curran, C Malone, R McLaughlin. K Barry, JAL Brown, MJ Kerin
BMC Cancer, February 2018, 18:203

doi.org/10.1186/s12885-018-4101-7

Circulating Nucleosomes and Nucleosome Modifications as Biomarkers in Cancer

P McAnena, J AL Brown, MJ Kerin

Cancers, January 2017, *9*(1), 5;
doi:10.3390/cancers9010005

Published Abstracts

Rate of discordance in subtype between primary and recurrence of breast cancer

P McAnena, A McGuire, A Ramli, C Curran, C Malone, R McLaughlin. K Barry, JAL Brown, MJ Kerin

Irish Journal of Medical Science (2017) 186(Suppl 2): S41

Identification and validation of circulating microRNA distinguishing metastatic from local breast cancer

P McAnena, T Kahraman, C Curran, J Freedman, AJ Lowery, JAL Brown, MJ Kerin

Mesentery Peritoneum (2018):2:AB 063. doi:10.21037/map.2018.AB063

Presentations to Learned Societies

Rate of discordance in subtype between primary and recurrence of breast cancer

25th Sylvester O'Halloran Surgical Scientific Symposium 2017, University of Limerick

Identification and validation of circulating microRNA distinguishing metastatic from local breast cancer

26th Sylvester O'Halloran Surgical Scientific Symposium 2018, University of Limerick *Plenary Session

Poster Presentations

Identification and validation of circulating microRNA distinguishing metastatic from local breast cancer

International Congress of the Association of Surgeons of Great Britain and Ireland, Liverpool 2018

Undergraduate surgical skills education: impact on attitudes to a career in surgery and surgical skills acquisition

Sir Peter Freyer Memorial Lecture & Surgical Symposium, NUI Galway 2017

Hypothesis

Metastatic breast cancer is a heterogenous disease – to optimize diagnosis and treatment we must investigate the presentation, prognosis and treatment options of the different molecular and histological subtypes and devise a more tailored diagnostic approach incorporating circulating blood based biomarkers.

Abstract

Breast cancer is a heterogeneous disease and is the leading cause of cancer-related deaths among women, with metastatic disease the principle cause of mortality. In recent years great advances have been made in stratifying breast cancer into a variety of subtypes based on morphological appearance, molecular characteristics and genomic signatures, casting light on the diverse intra-tumour and inter-tumour molecular portrait of the disease. In the era of personalized cancer management, it is imperative to further our understanding of the molecular make-up and clinical behaviour of breast cancer disease so as to tailor treatment and surveillance for recurrence appropriately.

In this work we demonstrate the disparate metastatic patterns and outcomes following metastasis of the two major histological subtypes of breast cancer. Furthermore, in a retrospective analysis of metastatic breast cancer patients we identified the initial molecular subtype of primary breast cancer may be different to the subtype of the metastatic disease. This can have significant implications for patient survival and treatment strategies.

The development of blood-based biomarkers to expedite earlier detection of breast cancer and of recurrence has been the focus of extensive international research in recent years. Mi(cro)RNAs are small, non-coding RNAs that regulate gene expression and are implicated in a variety of key processes driving both the development of breast cancer metastatic cascade. MiRNAs are stable in circulation and can be quantified using quantitative reverse transcription polymerase chain reaction (qRT-PCR). We* identified a variety of miRNAs to potentially distinguish local breast cancer from metastatic breast cancer in the commonest type of breast cancer (Luminal A). This work allowed us to identify and validate selected miRNA on an independent cohort of 74 patients. Two miRNAs (mir-331 and mir-195) showed significantly dysregulation between metastatic disease and local disease and healthy controls.

This work sheds further light on the heterogeneity of breast cancer and the diverse patterns and outcomes of metastatic disease, and highlights the potential of blood based miRNA biomarkers to contribute to the evolving management of breast cancer.

*This work was performed in conjunction with the laboratory at the University of Massachusetts, Boston, under the direction of Professor Kahraman Tanriverdi.

Chapter 1

Introduction

1.1 Breast cancer

1.1.1 Overview

Breast cancer is the most common cancer among women and is the fifth leading cause of cancer death (1). In Ireland, approximately 2,800 new cases of invasive breast cancer are diagnosed annually, accounting for 30.2% of all cancers diagnosed (2). Early detection and prompt treatment ensures the optimal outcome for patients, with survival largely dependent on the stage of breast cancer at diagnosis. Breast cancer stage is determined by size of the tumour, the nodal status and whether the tumour has metastasised to distant sites (Table 1.1). Breast cancer metastasis is the principle cause of mortality (3), with stage IV patients having significantly poorer 5-year survival compared to patients with no metastatic disease.

Stage	T	N	M	5-year survival
I	T1	N0	M0	96.1%
	T0	N1mi	M0	
	T1	N1mi	M0	
II	T0	N1	M0	89.5%
	T1	N1	M0	
	T2	N0	M0	
	T2	N1	M0	
	T3	N0	M0	
III	T0	N2	M0	66.4%
	T1	N2	M0	
	T2	N2	M0	
	T3	N1	M0	
	T3	N2	M0	
	T4	N0	M0	
	T4	N1	M0	
	T4	N2	M0	
	Any T	N3	M0	
IV	Any T	Any N	M1	28.1%

Table 1.1 Five-year survival by breast cancer stage – American Joint Committee on Cancer (AJCC), 8th Edition (4).

While the 8th edition of the AJCC staging system is based on conventional clinicopathological parameters, the committee has taken into account the increasing role of biological profiling of tumours (5). These biomarkers are incorporated into a second tier of prognostic modifiers.

Patient outcomes have been improving steadily in recent years, with 5-year survival across all stages increasing from 72.1% in the period from 1994-1999 to 81.4% from 2008-2012 in Ireland (6). Improved survival is attributable to enhanced diagnostic modalities and the introduction of

screening programmes to expedite the early diagnosis of breast cancer (7). The evolving paradigm of breast cancer treatment has also contributed to this trend, with an enhanced understanding of the molecular mechanisms underlying the heterogeneity of this disease and the introduction of targeted treatment facilitating individualised treatment regimens for patients (8, 9).

1.1.2 Breast cancer management

Surgery/surgical excision remains the cornerstone of curative treatment for breast cancer. However, the traditional surgical strategy of radical mastectomy first advocated by Halsted has been replaced by a more personalised approach, taking the stage and biology of the cancer as well as the wishes of the patient into account. Breast conservation is now established as the current standard of care (10), with the development of oncoplastic techniques, a multidisciplinary approach from a variety of specialties, and the establishment of specialized breast cancer centres all contributing to achieving the optimum clinical, oncological and cosmetic outcomes (11, 12). Minimising the risk of disease recurrence is of paramount importance in deciding which treatment strategy to use. Increasingly, genetic profiling is influencing decision making in relation to surgical strategy. High-risk individuals can now be identified at an early stage before the development of disease, meaning that risk reducing management strategies are becoming increasingly important. From a surgical perspective, this change in management is seen most obviously in the shift towards offering prophylactic mastectomy to patients with

BRCA1/2 mutations which confer a lifetime risk of 65% and 45% of developing breast cancer respectively (13).

Neoadjuvant chemotherapy (NAC) (before surgery) is a relatively recent change to the standard treatment sequence available to clinicians treating breast cancer, and where appropriate has emerged as the standard of care. NAC can downsize tumours, allowing surgeons to perform breast-conserving surgery where previously mastectomy would have been necessary. The rate of pathological complete response (pCR) following neoadjuvant therapy is excellent among certain patients and can be used as a surrogate for survival (14). NAC also allows the early recognition of patients with a poor response to chemotherapy, enabling treatment to be modified appropriately.

The introduction of targeted therapy has also been a significant advancement in breast cancer management, with endocrine therapy or Trastuzumab administered depending on the molecular subtype of the tumour.

1.1.3 Breast cancer molecular subtypes

Conventionally, breast cancer has been classified according to tumour morphology/histologic features, with invasive ductal carcinoma (IDC) the predominant histological subtype accounting for approximately 75% of all cases (15). Invasive lobular cancer (ILC) is the next most common histologic subtype making up 15% of all breast cancers. The remaining 10% include a variety of rare subtypes including mucinous, tubular, inflammatory and medullary carcinomas.

Of greater clinical significance is the classification of breast cancer into molecular subtypes. Following the initial breakthrough made by Perou et al using gene-expression profiling to identify the unique molecular portrait of breast cancer (16). While gene-expression profiling is considered the current "gold-standard" for molecular subtype characterisation, at present it is acceptable in clinical practice to perform immunohistochemical staining as a pragmatic surrogate to inform therapeutic management decisions (17).

Breast cancer is now considered to consist of at least four clinically relevant molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched and triple-negative breast cancer (TNBC) (18). The presence or absence of three established immunohistochemical biomarkers can be used as a surrogate for molecular subtype: Estrogen Receptor (ER), progesterone receptor (PR) and HER2 (human epidermal growth factor 2) receptor, with Ki67 proliferation index also used to further stratify luminal A and luminal B (19). Subtypes are can be broadly classified according to receptor status (Table 1.2), however some there is some overlap between subtypes with several changes proposed to this classification system. For example, high grade, hormone receptor positive/ HER2 negative tumours with Ki67 index greater than 14% can be classified as luminal B, while substantial PR positivity (>20%) can be an additional marker for luminal A disease (20).

Subtype	Hormone Receptor (ER/PR)	HER2 Receptor	Targeted treatment	Distribution (21)	Recurrence Pattern
Luminal A	+	–	Endocrine	74%	5-15 years
Luminal B	+	+	Endocrine + HER2 inhibitor	10%	5-15 years
HER2	–	+	HER2 inhibitor	4%	<5 years
TNBC	–	–	None	12%	<5 years

Table 1.2 Breast cancer subtypes

Each subtype exhibits distinct prognoses, rates of recurrence and responses to different treatment strategies (22). ER expression is the main indicator of potential response to endocrine therapy. Targeted drugs that inhibit the ER or oestrogen-activated pathways include the selective ER modulators (tamoxifen, raloxifene) and aromatase inhibitors (anastrazole, letrozole and exemestane) (23). Patients with HER2 overexpressing breast cancer may benefit from HER2 inhibitors such as Trastuzumab and the dual Epidermal Growth Factor Receptor (EGFR)/HER2-inhibitor Lapatinib (24). TNBC is associated with a poor prognosis and there is at present no targeted therapy to improve survival for this subtype of breast cancer (25). Response to NAC varies among the subtypes, with a high rate of pathological complete response (pCR) following NAC in patients with HER2 and TNBC subtypes (up to 60 per cent (14)) with increased long-term survival. The survival benefit is less clear in luminal disease (26).

1.2 Metastatic breast cancer

1.2.1 Overview

Despite considerable recent advances in both diagnosis and treatment, 20-30% of all breast cancer patients will develop distant metastatic disease (27). Metastatic disease involves the spread of tumour cells throughout the body and is the central clinical challenge of breast oncology as it accounts for the majority of breast cancer deaths (3). Unfortunately, most patients with stage IV disease will be diagnosed at a point where curative treatment is impossible and palliation of symptoms is the only feasible option. For high-risk patients, the goal of adjuvant chemo/hormonal/radiotherapy is to eliminate residual undetectable micrometastases to avoid disease recurrence. Targeted therapies against this elusive micrometastatic population of cells are currently suboptimal and treatment strategies to identify and treat these high-risk patients have thus far been inadequate.

To design/develop new treatment strategies for stage IV disease we must first examine the complex cellular pathogenesis of metastatic disease. The metastatic cascade paradigm is a series of intercalated steps in which cancer cells must detach from the primary tumour, intravasate into the circulatory or lymphatic system, evade detection and eradication by the immune system, extravasate at distant capillary beds and finally invade and proliferate in a distant organ(s) (28-30) (Figure 1.1).

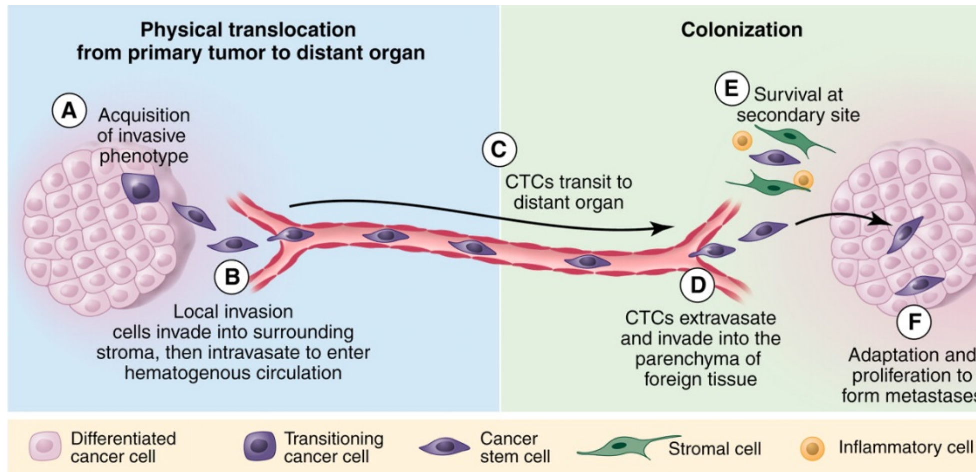


Figure 1.1 Schematic of the metastatic cascade. Image by Chaffer and Weinberg (3).

A number of theories have been suggested as to the origin of metastatic cancer cells, however none have been definitively confirmed *in vivo*. The epithelial-mesenchymal transition (EMT) posits that metastatic cells originate from epithelial cells that differentiate to tumour cells with mesenchymal features via an accumulation of genetic mutations. These dysmorphic cells lack cell-cell adhesion and are capable of distant invasion, with evidence for this key transformation being the cell-cell and cell-matrix interaction abnormalities that occur in epithelial tissues (31-33). Stem cells are potential instigators of metastasis due to their ability to proliferate and migrate during tissue morphogenesis and differentiation (34, 35). Tumour-associated macrophages (TAM) have been put forward as facilitators of the metastatic cascade by promoting tumour inflammation, angiogenesis and the eventual seeding of metastasis (36, 37).

In the era of personalised cancer therapy it is imperative to identify efficient and reliable methods of testing patients for the metastasis-promoting pathways used by their tumours and integrating this information into the monitoring of disease progression as well as designing new targeted therapies.

1.2.2 Patterns of metastatic disease in molecular subtypes

Metastatic disease location, time to metastasis and survival vary between the different subtypes of breast cancer. Across all subtypes, bone, liver, lung and brain make up the vast majority of sites of metastatic disease (38). Bone is the most common site of metastasis across all subtypes (39)(Table 1.3). HER2 positive patients are more likely to develop brain metastases compared to those with luminal A disease, and more likely to have liver metastases compared to the patients of the other three subtypes. TNBC patients are more likely to develop lung metastases compared to patients of the other three subtypes.

Subtype	Bone	Lung	Liver	Brain
Luminal A	58%	22%	16%	4%
Luminal B	47%	21%	26%	6%
HER2	35%	25%	32%	8%
TNBC	37%	32%	22%	9%

Table 1.3 Sites of breast cancer metastasis across subtypes – taken from 2017 SEER study of 17,445 patients with metastatic disease (39)

Overall survival in patients with metastatic disease also differs between subtypes. Patients with TNBC have significantly worse survival compared to the other 3 subtypes, who have comparable survival (40, 41)(Figure 1.2).

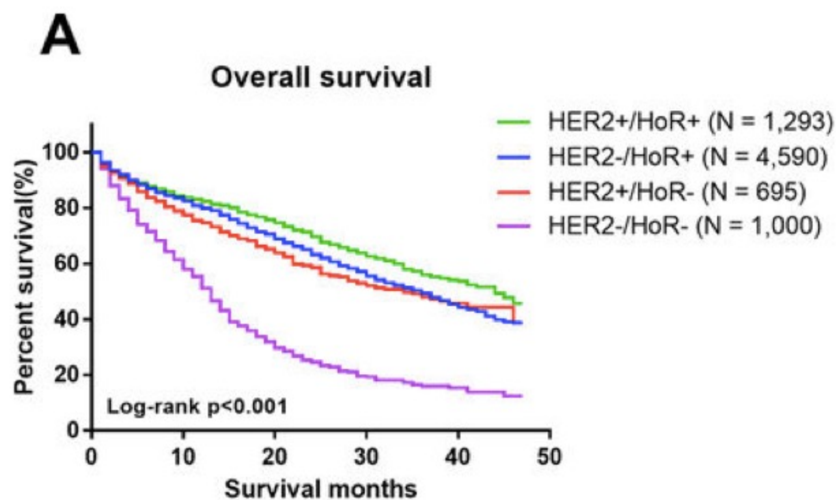


Figure 1.2 – Overall survival across subtypes – from 2017 SEER study of 7,578 patients (40)(TNBC in pink).

1.2.3 Subtype discordance in metastasis

Traditionally, metastatic tumours have been have been assumed to be biologically similar (i.e. the same molecular subtype) to the primary tumour. Recent studies have demonstrated that hormonal and HER2 receptor expression can change status between primary and recurrent breast cancer (42). This can impact prognosis with loss of receptor status associated with a poorer prognosis and reduced options for targeted therapy (43, 44). Conversely a gain in receptor status could potentially lead to a change in treatment options, as patients whose recurrent tumour becomes hormone

receptor positive could be candidates for hormonal therapy and similarly patients who become HER2 positive may benefit from receiving HER2 inhibitor therapy (45, 46).

Potential aetiologies of subtype discordance include:

(A) unreliability of immunohistochemical staining (47) and variability in sampling methods of the tumour tissue

(B) Intriguingly, discordance may help further explain intra-tumour and inter-tumour heterogeneity (Figure1.4) as it may demonstrate clonal genome evolution (42, 48, 49) and the clone with the more aggressive phenotype could potentially initiate the micro-metastatic process (b) (50).

(C) Biological drift is another potential cause, for example selective eradication of ER/PR positive cells by hormonal therapy could leave behind a population of ER/PR negative cells that in time could metastasize (c) (51) (Figure 3).

(D) Genuine switches in biology of the cancer appear to be a rare event based on currently available gene expression data (52, 53), however this does not exclude the potential for smaller scale genomic alterations and mutations (d) (54). Heterogeneity between patient's primary tumour and recurrence may be due to newly acquired biological characteristics that allow tumour cells to travel via the circulatory/lymphatic systems and to metastasize to new sites (55). Change in receptor status may contribute to this increased capacity

for invasion as endocrine and growth factor signalling pathways are implicated in invasion and metastasis (56, 57).

Discordance in molecular subtype may have important implications for both prognosis and therapeutic options for patients with recurrent breast cancer and may also help elicit further information on the molecular landscape of tumours that metastasize.

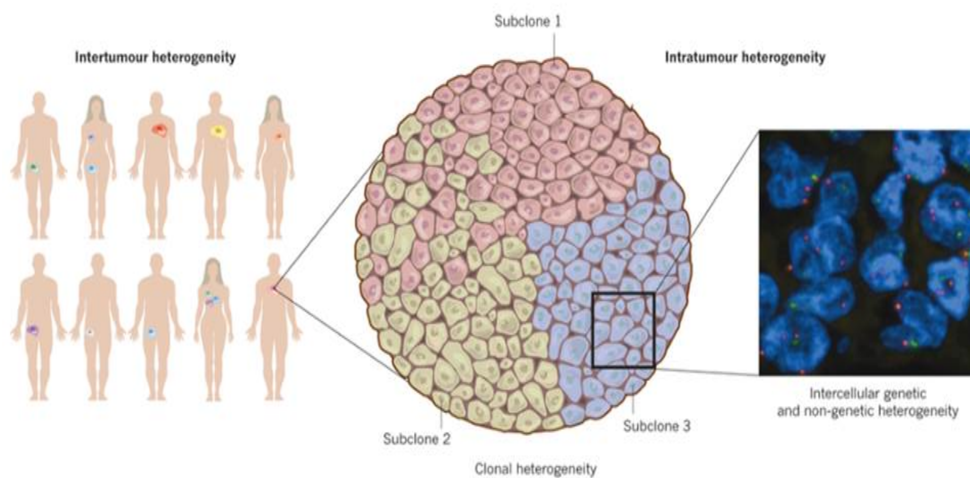


Figure 1.3 Inter and intra-tumour heterogeneity – Image by Burrell et al (58)

1.2.4 Metastatic/recurrent lobular cancer

While the vast majority (78%) of breast cancer cases constitute invasive ductal carcinoma (IDC), 10-15% of patients have invasive lobular carcinoma (ILC) (59). ILC is characterised by an older age at presentation, higher grade tumour, larger tumour size, multifocality, ER positivity, HER2 negativity, loss of e-cadherin (90%), lower cell proliferation rate and less responsiveness to chemotherapy (60-64). ILC also has a distinct profile of metastatic behaviour compared to IDC, with a tendency to develop metastases later as well as a diverse range of sites of metastasis including the gastrointestinal tract, the genitourinary tract, the peritoneum and the retroperitoneum (65, 66). In the first five years following diagnosis, ILC patients have better disease-free survival (DFS) and OS compared to patients with IDC, however this trend has been reported to reverse after five years (62, 67).

While it constitutes a small fraction of the total cases of breast cancer, ILC is still twice as common as cervical cancer and as common as multiple myeloma (68). ILC has a fundamentally different pathological profile and exhibits a distinct pattern of metastatic behaviour, however it is currently managed along the same treatment algorithms as IDC. ILC remains a distinct relevant breast cancer entity which requires consideration in making patient centred individualised decisions.

1.2.5 Breast cancer biomarkers

The WHO defines a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (69). Despite considerable investment into the development of biomarkers and advances in our understanding of the underlying molecular landscape of breast cancer, only three established biomarkers (ER, PR, HER2) are recommended for all newly diagnosed breast cancer patients. While these markers aid in the initial prognosis and therapeutic strategy of preventing distant metastatic/recurrent disease, they require invasive biopsy and cannot be used to monitor disease progression. With metastatic disease and disease recurrence the principle causes of breast cancer mortality, there is a real need for non-invasive biomarkers to identify patients with a high risk of developing metastasis, to inform clinicians of disease progression and to act as an adjunct to conventional modalities in the diagnosis of stage IV disease.

1.2.6 Biomarkers of metastasis

Biomarkers to diagnose and predict metastatic disease progression have been the focus of extensive research in recent years, with many now in clinical use and many more being analysed in clinical trials. Multigene/multiprotein tests are capable of evaluating prognosis and likelihood of disease recurrence independent of traditional prognostic factors such as grade and size and are commercially available. Oncotype DX (70, 71), Mammaprint (72) and urokinase plasminogen activator

(uPA)/PAI-1 (73, 74) have been evaluated in terms of their clinical utility in randomised prospective trials. These multi-analyte tests require invasive collection of tumour tissue and their use is limited to informing treatment decisions in early stage breast cancer.

Biomarkers accessible non-invasively that can inform clinicians of disease progression are of greater practical value in terms of identifying metastatic breast cancer. Traditional circulating markers include CA 15-3, CA 125 and CEA. While these have not been recommended for serial measurement by ASCO or ESMO (75, 76), increasing levels of these markers in breast cancer patients have been shown to precede the development of metastases, and in conjunction with prompt appropriate imaging lead to improved therapeutic options (77, 78).

A number of new circulating biomarkers of metastatic disease have been investigated in recent years such as circulating tumour cells (CTCs) (79) and cell free circulating tumour DNA (ctDNA) (80). In the era of tailored breast cancer management it is likely that clinicians will require a range of sensitive and specific tests to monitor for disease progression and achieve the optimal patient outcome. Circulating mi(cro)RNAs are an appealing adjunct to conventional diagnostic and prognostic modalities as they are stable in circulation, easily quantifiable and can reveal further information of the underlying biology of the tumour (81). The potential of miRNAs to contribute to a "liquid biopsy" has been the focus of much research in recent years.

1.3 MicroRNAs

1.3.1 Definition and synthesis

MiRNAs are small (19–22 nucleotides) non-coding RNAs that regulate gene expression. First described by Lee and colleagues in 1993 (82), research into miRNAs has grown exponentially in recent years as the role of miRNA in cancer biology is defined (83). In general, miRNA genes are transcribed in the nucleus by RNA polymerase II (Figure 1.5) (84). Primary miRNAs are then cleaved into pre-miRNA (approximately 70 nucleotides in length) by the microprocessor multiprotein complex and exported out of the nucleus by exportin 5 (85). They are cleaved into double-stranded miRNA (19–22 nucleotides) in the cytoplasm by RNase type III Dicer (86). One strand of this miRNA duplex represents mature miRNA, which forms the RNA-induced silencing complex in conjunction with other proteins (87). This complex ultimately regulates genetic expression. In mammals, this is predominantly by binding imperfectly to the 3' -untranslated region (3' -UTR) of mRNA, inhibiting translation and causing degradation of messenger RNA (mRNA) (88). Micro-RNAs play an important role in a variety of cellular processes such as apoptosis, cell-cycle control, proliferation, differentiation and response to stress (89). Over 4000 miRNAs exist in the human genome, and each has the potential to regulate a multitude of mRNAs (90). Oncogenic miRNAs (oncomirs) promote cancer progression through downregulation of tumour suppressor genes, whereas tumour suppressor miRNAs target oncogenes post-transcriptionally and impede cancer cell proliferation (Figure 1.6) (91)

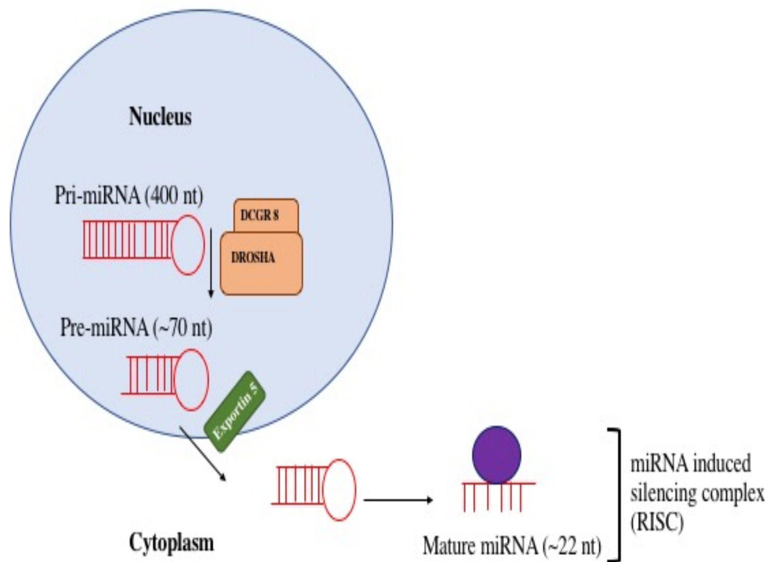


Figure 1.4 MiRNA synthesis

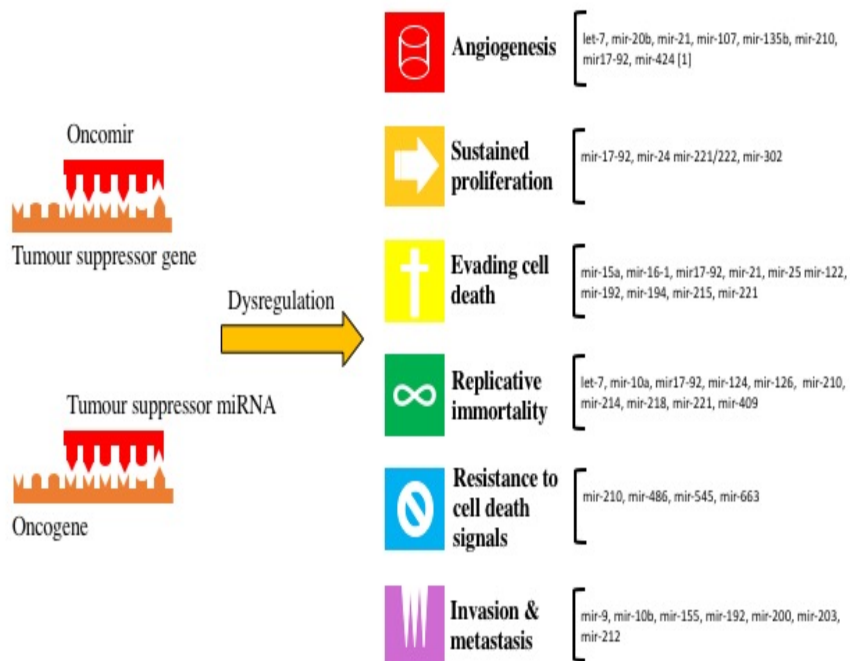


Figure 1.5 MiRNA functioning as tumour promoter/ suppressor

The discovery of miRNAs and the subsequent investigation into their role in breast cancer has uncovered another layer of molecular heterogeneity that can further stratify the disease beyond the conventional clinical subtypes. Aberrant miRNA signatures can provide information about fundamental dysregulation of key carcinogenic pathways such as epithelial-mesenchymal transition (92), revealing more about some of the unique properties and behaviours of each individual tumour.

1.3.2 Circulating miRNAs as biomarkers in breast cancer

The highly conserved nature and inherent resiliency of miRNAs make them ideal non-invasive biomarkers. They are well preserved in tissue samples following formalin fixation and embedding, and can be extracted successfully from these samples after years of preservation (93). In recent years, circulating miRNAs have been investigated in whole blood, serum and plasma as non-invasive markers of cancer (94, 95). Because of their stability, low complexity, and the relatively simple and inexpensive modern methods of detecting and profiling miRNA, such as real-time quantitative reverse transcriptase–PCR (qRT–PCR), circulating miRNAs offer an encouraging future as non-invasive markers of cancer that can help surgeons to optimize patient management individually.

Circulating miRNAs were proposed as potential non-invasive diagnostic biomarkers of breast cancer almost a decade ago (94), and are now being investigated as markers of prognosis (96, 97), of response to NAC(98, 99), of hereditary breast cancer (100), and as potential therapeutic target/agents

(101). More pertinent to metastatic breast cancer, MiRNAs can contribute to the identification of patients with early metastastatic disease - a subset of patients with otherwise undetectable metastatic disease who could potentially benefit from additional monitoring and augmented treatments. In a study examining pretreatment serum samples of 42 patients with stage II–III locally advanced breast cancer, mir-122 specifically predicted metastatic recurrence (102). Further studies investigating the ability of miRNAs to predict metastasis will allow more intensive monitoring and potentially earlier detection in high-risk patients. The majority of studies in this area have so far examined miRNAs in breast tissue, limiting their clinical value owing to the need for invasive access (96, 97, 103, 104). There is exciting potential for circulating miRNAs as liquid biopsy targets to contribute to the detection and management of patients with metastatic breast cancer.

1.4 Circulating nucleosomes and nucleosome modifications as biomarkers in breast cancer

The nucleosome is a focal point of transcription control and is fundamental to DNA structure and gene regulation (105, 106). The nucleosome is the core unit of chromatin, first described in 1974 by Kornberg (107). It consists of an octamer of the four highly conserved core histone proteins (H3, H4, H2A, H2B), joined together by a linker histone H1 with 146 base pairs of DNA wrapped nearly twice around the octamer. Nucleosomes are subject to epigenetic change - heritable, functionally relevant changes altering gene activity, and these modifications such as

acetylation and methylation alter the mechanism regulating chromatin-templated processes, ultimately influencing cell fate and pathological responses (108). For example, Acetylation acts by causing a reduction in the electrostatic interaction between negatively charged DNA and the lysine residue, leading to more "open" chromatin formation. This provides access to chromatin for process such as transcription or DNA repair. Deacetylation can promote gene repression and silencing by removing the neutralizing acetyl charge from histones, leading to chromatin condensation (109) (Figure 1.6)

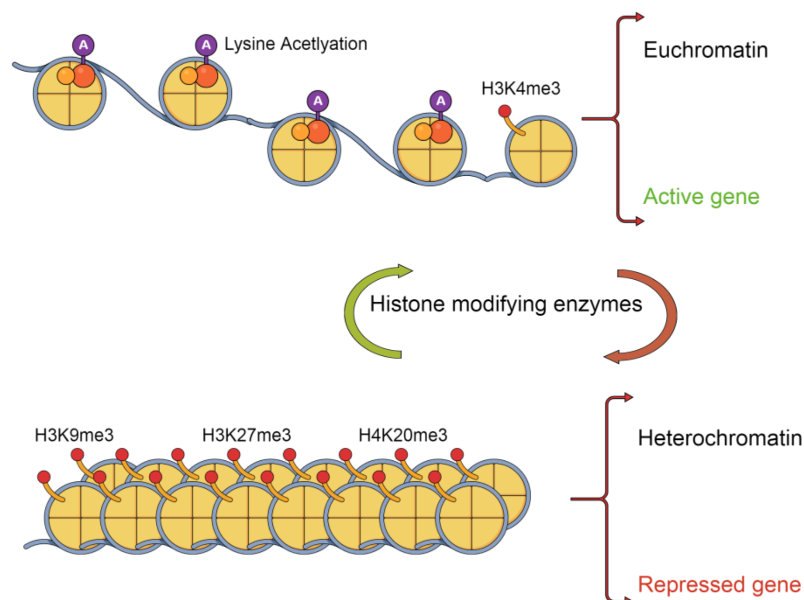


Figure 1.6 Post-translational modifications of histones regulate gene expression

The relationship between histone modifications and cancer has been the subject of much investigation in recent years, with acetylation (Ac) and methylation (Me) the most studied modifications. The loss of H4K16ac and H4K20me3 has been observed early in the tumorigenic process and occurs

across a variety of cancer cell lines, in addition to global DNA hypomethylation (110). Quantification of nucleosomes and specific PTMs show real potential as cancer biomarkers, both in tumours and importantly circulating in blood. A variety of histone modifications have been investigated as potential biomarkers in breast cancer (Figure 1.7), and show great potential in expediting the diagnosis and management of breast cancer.

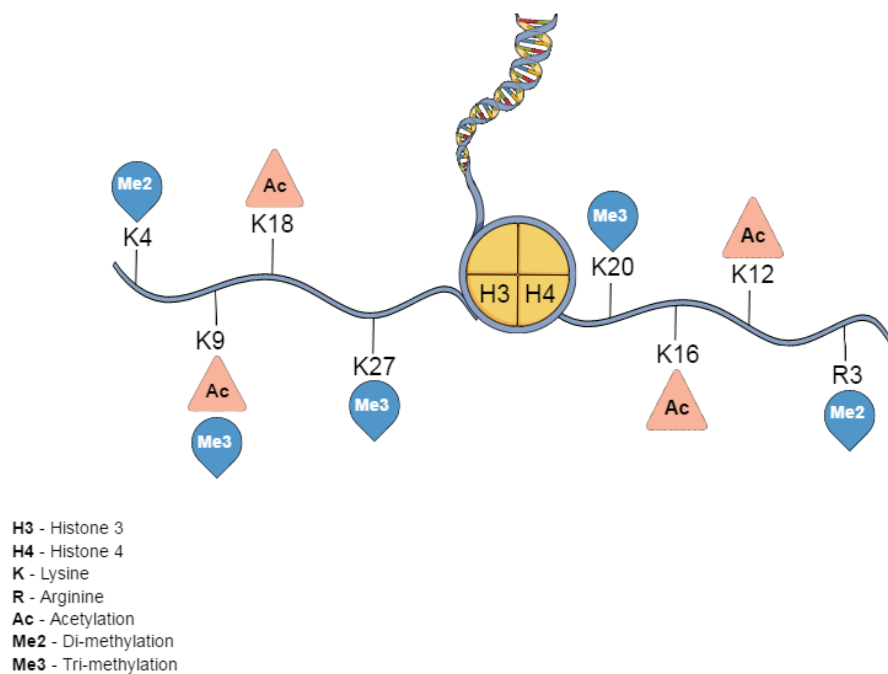


Figure 1.7 Post-translational modifications associated with breast cancer

The measurement of absolute levels of circulating histone and quantification of PTM in these circulating histones provide an exciting new avenue for the non-invasive diagnosis and monitoring of cancer progression and treatment. Further work investigating links between circulating nucleosome PTM and circulating miRNA will not only provide clinically relevant biomarkers, but reveal information related to the fundamental mechanisms underpinning cancer progression response to treatments. Clinically utilizing the real-time

(or near real-time) quantification or monitoring of circulating nucleosomes or circulating nucleosomes posttranslational modifications will provide a potentially quick, cheap and robust method for detecting cancer and monitoring the response of cancers to treatments.

1.5 Thesis aims

The molecular era of classification of breast cancer has broadened our understanding of the diverse clinical behaviour of the disease and shed light on intra-tumour and inter-tumour heterogeneity. Conventional treatment strategies are no longer appropriate and a personalized, tailored approach must now be devised for each individual patient.

Breast cancer management is rapidly evolving and continued research into the molecular portrait of the disease is required to expedite diagnosis and improve patient outcomes. With this in mind, the aims of this study were;

1. To assess long term outcomes and metastatic behaviour of invasive lobular cancer and compare metastatic ILC to metastatic IDC
2. To investigate subtype discordance between primary and metastatic breast cancer and to assess what impact this has on survival and how it may affect treatment options in specific breast cancer subtypes
3. To identify and validate novel circulating miRNAs to distinguish metastatic from local breast cancer in invasive ductal luminal A breast cancer

Chapter 2

Materials and Methods

2.1 Breast cancer database

2.1.1 Breast Unit – University Hospital Galway

The Breast Cancer Service at UHG is a multi-disciplinary service that incorporates specialist care from Surgeons, Physicians, Radiologists, Nursing staff, Research co-ordinators and Administrative staff to provide the optimal individualised treatment strategy for each patient. UHG is a high-volume tertiary care unit – in 2015 11,105 patients attended the service, of these 5,905 were new patients. There were 303 new diagnoses of breast cancer at the symptomatic breast unit and a further 217 cases referred from the BreastCheck screening service.

Newly diagnosed cases are discussed at a multi-disciplinary meeting and an appropriate treatment plan is devised to achieve the optimal individual outcome. Breast conservation is now the standard of care and is the performed where appropriate, with 69% of patients undergoing breast-conserving surgery at the unit in 2015. 70% of patients underwent sentinel-lymph node biopsy, sparing patients the deleterious effects of axillary nodal clearance.

2.1.2 Department of Surgery Biobank

The Department of Surgery biobank at Galway University Hospital contains human tissue & blood samples retrieved from patients undergoing surgery for cancer and non-cancer procedures. 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. Breast cancer-related samples have been collected by the clinical and research staff of the department since 1992. This included serum, plasma and whole blood samples retrieved from patients pre- and post-breast cancer surgery and serum, plasma and whole blood samples donated by non-cancer controls. These women were interviewed by a clinician in advance of sample collection to ensure that there was no personal history of malignancy or current inflammatory or infectious condition.

2.1.3 Patient information and pathology

Clinical and pathological data relating to all patients & controls are appropriately anonomised and recorded in the Breast Cancer Database in the Department of Surgery. Clinical data includes patient demographics, family history, menopausal status, co-morbid disease, medications. Pathological data includes oestrogen and progesterone receptor status, HER2/*neu* status which is determined by immunohistochemistry (IHC) on formalin fixed, paraffin embedded sections of clinical specimens as part of routine pathology to guide clinical decision making regarding adjuvant therapy. IHC is performed using a rabbit monoclonal antihuman ER antibody (clone

SP1, Dako, UK) and a polyclonal rabbit antihuman PR antibody (Dako, UK). The Allred scoring method(111) is used for expression scoring of ER and PR was based on proportion and intensity. In brief, the proportion score represents the estimated percentage of tumour cells staining positive as follows:

0 = 0

1 = 1%

2 = 1-10%

3 = 10-33%

4 = 33-66%

5 = >67%

Intensity of staining is defined as follows:

1 = weakly positive

2 = moderately positive

3 = strongly positive (figure 1)

The final score for ER status is derived from the equation:

$$\% \text{ Positive cells} + \text{Intensity of staining} = \text{Total score}$$

Scores of 0-1 are ER negative; scores of 2-8 are ER positive (figure 1a)

Membranous staining is scored for HER2/*neu* according to the HercepTest™ (Dako, UK) as follows:

0 = negative

1 = weak incomplete membranous staining of >10% cells (negative)

2 = weak-moderate complete membranous staining of >10% of cells (equivocal-fluorescence *in-situ* hybridisation (FISH) is used to assess amplification in these cases)

3 = strong complete membranous staining of >30% of cells (positive) (figure 1c).

Tumour size, tumour grade, nodal status and metastases status are also recorded according to the TNM classification system (112) (table 1.1, chapter 1).

ILC was identified on the basis of typical morphology, with small, relatively uniform dyscohesive single cells infiltrating stromal tissue, often in a ‘single-file’ pattern, with an absence of tubular or glandular structures. Two pathologists independently assessed the histology at a multidisciplinary meeting. Immunohistochemistry for e-cadherin is performed in occasional cases for confirmation.

2.2 Cohort selection

2.2.1 Subtype discordance cohort

Data was collected on patients who had a recurrence of breast cancer following surgery +/- chemotherapy/hormonal therapy/radiotherapy at the Galway Hospitals group between 2001 and 2014. Loco-regional recurrence after surgery was defined as the appearance of tumour in the ipsilateral chest wall or axillary, internal mammary or supraclavicular lymph nodes while distant recurrence was defined as recurrence to distant organs, confirmed by pathologists report. Only patients who had clinical pathology scoring of receptor status of both the primary and recurrent cancer were included. Exclusion criteria included presentation with bilateral tumours, biopsy results that were incomplete, and pathologist report of the recurrence as a new primary tumour. 132 patients met the inclusion criteria. PAS software was used to access pathology records with MOSAIQ software used to determine patient pathways and treatment.

Tissue samples were obtained following surgery and at recurrence, with sufficient slides taken to perform all necessary immunohistochemical and pathological analysis. Samples were reviewed by a minimum of two pathologists, with an initial assessment from at least one primary reporting pathologist and a subsequent review performed by a pathologist at a multi-disciplinary meeting.

2.2.2 Discovery cohort selection – MiRNA study

Collaboration was established with the laboratory at UMASS, Boston. This lab has extensive expertise in RNA sequencing, and kindly agreed to help us identify potentially dysregulated miRNA in metastatic breast cancer.

A discovery cohort (n=8) was seleceted that included 4 patients with metastatic luminal A breast cancer to bone and 4 patients with locally confined breast cancer (Table 2.1). Samples were age-matched and clinicopathological details were collated. Plasma was utilised as this was UMASS's preferred medium for RNAseq (113). 500µl of plasma from each sample was aliquoted, packaged in dry ice and transported by air via DHL logistics.

	Metastatic (n=4)	**Local (n=4)**
Age - mean years	61	65
Histological Subtype	Ductal (n=4)	Ductal (n=4)
Molecular Subtype	Luminal A (n=4)	Luminal A (n=4)
Stage	IV (n=4)	II (n=2) III (n=2)
Metastasis location	Bone (n=4)	-
Time Sample Taken	M1 at presentation (n=1) Metastatic at follow up (n=3)	Pre-operatively (n=4)

Table 2.1 Discovery cohort clinicopathological details

2.2.3 Validation cohort selection – MiRNA study

To validate the miRNAs identified, we interrogated our biobank and selected suitable samples. The validation cohort (n=74) was comprised of 22 patients with distant metastatic disease (17 to bone), 31 patients with locally confined breast cancer and 21 healthy controls (Table 2.2). Healthy controls were included at this stage to determine if dysregulated miRNA were breast cancer-specific or metastasis-specific. Healthy controls had no history of benign or malignant breast disease and no family history of breast cancer. RNA was extracted from whole blood for this cohort as this is the primary method of RNA extraction at our laboratory.

All breast cancer patients in the study had histologically confirmed Luminal A breast cancer; hormone receptor positive and HER2/*neu* negative. The metastatic cohort had confirmed distant metastatic disease by biopsy/imaging or both at the time blood was obtained. Blood was obtained from the locally confined breast cancer patients pre-operatively and these patients had no evidence of subsequent recurrence or metastasis at a mean follow up of 7.2 years. None of these patients received neo-adjuvant chemotherapy

	Metastatic (n=22)	**Local (n=31)**	**Healthy Controls (n=21)**
Age - mean years (SD)	60 (15)	54 (12)	52 (12)
Histological Subtype	Ductal (n=17) Lobular (n=5)	Ductal (n=31)	-
Molecular Subtype	Luminal A (n=22)	Luminal A (n=31)	-
Stage	IV (n=22)	I (n=10) II (n=17) III (n=4)	-
Tumour Grade		1 (n=6) 2 (n=17) 3 (n=8)	-
Nodal Status		N positive (n=11) N negative (n=20)	-
Metastasis location	Bone (n=17) Lung (n=3) Liver (n=2)	-	-
Time Sample Taken	M1 at presentation (n=14) Metastatic at follow up (n=8)	Pre-operatively (n=31)	-

Table 2.2 Validation cohort clinicopathological details

2.2.4 Invasive lobular breast cancer cohort

Data was collected on patients who presented with ILC to the Galway Hospitals breast cancer programme between 1985 and 2017 in a prospectively maintained database. Clinicopathological details were collected including age at diagnosis, tumour characteristics and treatment received. We then recorded all incidence of distant metastatic disease and calculated survival for the entire cohort using MOSAIQ and PAS software, with survival calculated up to March 2018.

To compare distant metastatic ILC to distant metastatic IDC, we selected a cohort of patients from the previous study investigating subtype discordance in metastatic IDC (114). From this database we included all patients who developed distant metastatic disease following curative surgery +/- adjuvant endocrine/chemotherapy (n=60) and compared this cohort to all patients from our ILC database who developed distant metastatic disease following curative surgery +/- adjuvant endocrine/chemotherapy (n=70).

2.3 MiRNA analysis

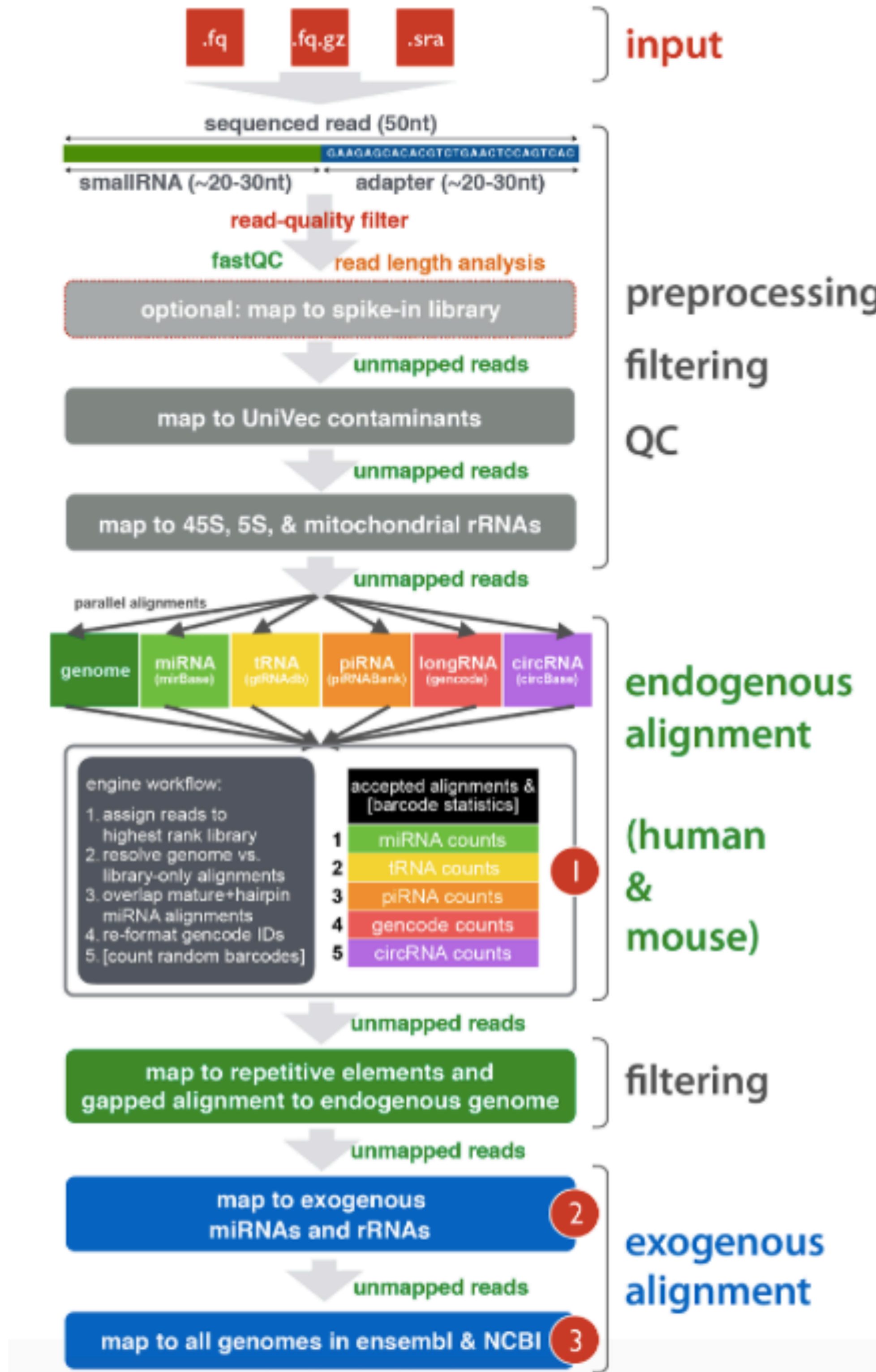
2.3.1 Sample collection and processing

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.

2.3.2 RNAseq - UMASS

Analysis of the discovery cohort (n=8) was performed in conjunction with UMASS. Following isolation of RNA from plasma, RNAseq was performed to identify miRNAs dysregulated between metastatic and locally confined luminal A breast cancer (Figure 2.1). 712 miRNAs were analysed in the array.

Figure 2.1 RNA isolation and RNAseq - UMASS



.fq
.fq.gz
.sra
input
sequenced read (50nt)
smallRNA (~20-30nt)
adapter (~20-30nt)
read-quality filter
fastQC
read length analysis
optional: map to spike-in library
unmapped reads
map to UniVec contaminants
unmapped reads
map to 45S, 5S, & mitochondrial rRNAs
preprocessing
filtering
QC
unmapped reads
parallel alignments
genome
miRNA (mirBase)
tRNA (gtRNAdb)
piRNA (piRNABank)
longRNA (gencode)
circRNA (circBase)
engine workflow:
1. assign reads to highest rank library
2. resolve genome vs. library-only alignments
3. overlap mature+hairpin miRNA alignments
4. re-format gencode IDs
5. [count random barcodes]
accepted alignments & [barcode statistics]
1 miRNA counts
2 tRNA counts
3 piRNA counts
4 gencode counts
5 circRNA counts
1
endogenous alignment
(human & mouse)
unmapped reads
map to repetitive elements and gapped alignment to endogenous genome
filtering
unmapped reads
map to exogenous miRNAs and rRNAs
2
unmapped reads
map to all genomes in ensembl & NCBI
3
exogenous alignment

2.3.2.1 RNA isolation from plasma

RNAs was isolated from plasma samples using a Qiagen miRNeasy Serum/Plasma Kit (Cat. No: 217184) for RNA isolation on QIAcube (Cat. No: 9001292).

2.3.2.2 Library preparation for RNA sequencing

Small-RNA libraries were constructed using NEXTflex Small RNA Sequencing Kit (Ion PGM & Ion Proton Compatible) Catalog #4030-02 (48 reactions) from Bioscientific.

2.3.2.3 Sequencing data analysis using Genboree-sequencing pipeline

Small-RNAseq reads were processed and quantified using the exceRpt tool available on the Genboree Workbench (http://www.genboree.org/). exceRpt incorporates several modifications to existing analysis methods used to assess cytosolic microRNAs (miRNAs) that specifically address experimental issues pertinent to exRNA profiling, such as variable contamination of ribosomal RNAs, the presence of endogenous non-miRNA small-RNAs, and the presence of exogenous small-RNA molecules derived from a variety of plant, bacteria, and viral species. Briefly, the software

processes each sample independently through a casade of read-alignment steps designed to remove likely contaminants and endogenous sequences before aligning to exogenous miRNAs:

(1) *3′ adapter clipping*. Adapter clipping is required because the majority of small-RNAs are shorter in length than the number of nucleotides sequenced. Adapter removal is performed using the FastX software (v.0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/) using the fastx_clipper tool, and the clipping requires identification of at least 7 adapter bases at the 3′ end of a read and that clipped reads must be longer than 15 nucleotides (nt).

(2) Explicit quality control and filtering is performed on the sequencing reads from each sample by removing likely contaminant sequences derived from laboratory or rRNA contamination. First, rReads are aligned, using Bowtie2 (115), to UniVec, a library of common contaminant sequences maintained by the NCBI, and reads with valid alignments counted, archived and removed from further consideration. Second, reads are aligned, again using Bowtie2, to the human ribosomal RNA (rRNA) precursor sequences (the full 45S, and 5S, and mitochondrial rRNA sequences) as these rRNAs typically constitute the source of most of the confounding contamination in any small-RNAseq experiment, especially those from extracellular preparations. Reads aligned to the rRNAs are counted, archived, and removed from further consideration in subsequent alignment steps. Bowtie2 is used in both of these steps due to its superior alignment speed, tolerance for gapped alignments, and tolerance for low-quality read sequences.

(3) At its core, exceRpt utilizes the sRNAbench software tool, which is itself based on the miRanalyzer tool (116, 117) for mapping and profiling small-RNA libraries. On input to sRNAbench, for each sample, identical clipped-read sequences are counted and collapsed to a single entry and reads containing N's are removed. Clipped, collapsed reads are mapped directly to the human genome and pre-miRNA sequences using Bowtie1 (115), allowing for only a single mismatched base in each alignment. Bowtie1 is used for this in conjunction with a 19 nt seed in order to allow local alignments at the 5′-end of a small-RNA that might exhibit 3′ non-template additions. The miRNA library used for alignment is obtained from miRBase (v21) (118). The output from this read-alignment, for each sample, is a collection of files containing the alignment outcomes (pre-miRNA and mature-miRNA IDs) for each read, the read-stack covering each pre-miRNA sequence, and the mapping of the pre- and mature-miRNA sequences with respect to the human genome. These alignments are parsed and used in conjunction with a pre-miRNA secondary-structure prediction, via RNA-fold provided in the Vienna software suite (119, 120) to enable verification through stability analysis of the various pre-miRNA hairpin sequences.

(4) Following alignment to the provided miRNA library, reads that did not align in any of the previous steps are mapped against a variety of small-RNA libraries including (with no significance to the ordering): tRNAs from gtRNAdb (120), piRNAs from RNAdb (121, 122), snoRNAs from snoRNA-LBME-db (121) and snRNAs and other RNA sequences from RFam (123). In both steps 3 and 4 reads were allowed to multi-map to different annotated

transcripts and in cases where this occurred, the read was split proportionally based on the number of equally valid alignments.

(5) Those reads that are not mapped in any of the above steps are taken forward to the final stage of the pipeline where they are aligned, again using sRNAbench, to the complete set of annotated plant and virus pre-miRNA sequences in miRBase.

Further differential expression analysis was performed using DESeq2 (version 1.6.3) on www.genboree.org (124).

2.3.3 MiRNA expression analysis – qRT-PCR

2.3.3.1 RNA extraction from whole blood

The PAXgene™ Blood RNA Kit (PreAnalytiX®) was employed to extract RNA from whole blood collected in PAXgene Blood RNAtubes. 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 micro-centrifuge 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 mico-centrifuge 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 tubecontaining the flow though was discarded. 500µl of Buffer BM4 was thenadded to the spin column and centrifuged for 2 minutes. The flow throughwas 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 micro-centrifuge 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 elute 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.

2.3.3.2 RNA analysis using nanodrop spectrophotometry

The Nanodrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE,USA) was used the quality and quantity of extracted RNA. 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. RNase free-water (or Buffer, depending on the preceding extraction protocol) was used to blank the instrument before beginning. A sample volume of 1 µl 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 nanograms of RNA per microlitre reading was used to calculate the amount of extracted RNA required for each reverse transcription reaction.

2.3.3.3 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 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 (Table 3.2) for each RNA sample was prepared in 0.2µl tubes in a cooling tray. An RT Blank was prepared for each miRNA target by using Nuclease Free water in place of RNA.

cDNA reaction mix	Volume
dDTP Mix (100mM)	0.17 µl
10x RT buffer	1.65 µl
Nuclease Free Water	4.57 µl
RNase Inhibitor (20U/uL)	0.21 µl
Multiscribe (500U/uL)	1.1 µl
Stem Loop Primer	3.1 µl
Premix	10 µl
Total miRNA	5.0 µl
Total Reaction Volume	**15µl**

Table 2.3 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 2.4.

30 mins	16 °C
30 mins	42 °C
50 mins	85 °C
∞	4 °C

Table 2.4 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.

2.3.3.4 qRT-PCR

Relative quantification polymerase chain reaction 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 (C_t) is the point at which sufficient amplified product has accumulated to produce a detectable fluorescent signal. A lower C_t value signifies more abundant template. MiRNA pre-developed assay reagents (PDAR) were obtained from Applied Biosystems (850 Lincoln Centre Drive. Foster City, CA 94404. USA). 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 2.5. 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 miR-425 were used as endogenous controls in this work (125).

ABI miRNA kit	Volume
Mastermix (Fast)	5.0 µl
NFW (Nuclease free water)	3.8 µl
miRNA PDAR	0.5 µl
cDNA	0.7 µl
Total	**10 µl**

Table 2.5 Reaction components for Polymerase Chain Reaction

The premix was centrifuged before adding 9.3µl to each well of the Fast

Optical 96-Well plate. 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 f which was to ensure consistency across all RQ-PCR runs. The IAC consisted of cDNA for miR-26b that was synthesised from a cell pellet. The standard deviation between plates was required to be <0.3.

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.

2.3.3.5 Statisical analysis of miRNA expression results

Relative expression of miRNA was calculated using the delta-delta (ΔΔ) Ct method (126). This method requires endogenous control(s) to normalise Ct values. In this study, we used miR-16 and miR-425 as endogenous controls and took the average Ct value of the sum of their Ct values to get the Average Ct EC.

The ΔCt value was calculated as follows:

ΔCt=Average Ct target-Average Ct EC

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

RQ=2- ΔΔCt

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 SPSS version 23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Macintosh, Version 23.0. Armonk, NY: IBM Corp). 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. 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. . Binary logistic regression analysis was used and receiver operating characteristic (ROC) curves generated to evaluate the ability of chosen miRNAs to distinguish between metastatic and local breast cancer patients. This was performed both individually and for combinations of miRNAs.

Chapter 3 –
Invasive Lobular Breast Cancer: Patterns of metastatic disease, survival and comparison to metastatic IDC

3.1 Introduction

While the vast majority (78%) of breast cancer cases constitute invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC) is by a distance the second most common histological subtype, accounting for 10-15% of cases (59). Epidemiological data suggests that the incidence of ILC is rising worldwide, and this has been partly attributed to the increasing use of hormone replacement therapy in post-menopausal women (59).

As previously outlined, ILC is characterised by small, round cells with scant cytoplasm that infiltrate stroma in single file (127). ILC typically does not promote a significant connective tissue response or form a discrete mass that can be diagnosed easily on palpation or by mammography (128). ILC is also characterised by an older age at presentation, lower grade tumour, larger tumour size, multifocality, ER positivity, HER2 negativity, loss of e-cadherin (90%), lower cell proliferation rate and less responsiveness to chemo therapy (60-64). ILC has a distinctly different profile of metastatic behaviour compared to IDC, with a tendency to develop metastases later as well as a diverse range of sites of metastasis including the gastrointestinal tract, the genitourinary tract, the peritoneum and the retroperitoneum (65, 66). Reports on the prognosis of ILC have produced a variety of results, with some studies demonstrating worse (129), comparable (130, 131) or better (132) outcomes compared to IDC. However, a recent analysis of a SEER database of over 260,00 cases of breast cancer (10.5% ILC) demonstrated that outcomes of stage matched cases of IDC and ILC were comparable (67).

While it constitutes a small proportion of the total cases of breast cancer, ILC is nonetheless twice as prevalent as cervical cancer and as common as multiple myeloma (68). ILC has a fundamentally different pathological profile and exhibits a distinct pattern of metastatic behaviour; however it is managed along the same treatment algorithms as IDC. ILC remains a distinct relevant breast cancer subtype which requires consideration in making patient centred individualised decisions.

3.2 Aim

The aim of this study was

(A) to analyse a large database of ILC cases over a 30+ year period to identify the clinical behaviour, metastatic patterns and prognosis of the disease.

(B) To compare the clinical course and primary tumour characteristics of metastatic ILC patients to a similar sized cohort of patients with metastatic IDC.

3.3 Methods

Case Selection

Data was collected on patients who presented with ILC the Galway Hospitals group between 1985 and 2017 in a prospectively maintained database. Clinico-pathological details were collected including age at diagnosis, tumour characteristics and treatment received. We then recorded all incidence of distant metastatic disease and calculated survival for the entire cohort using MOSAIQ and PAS software, with survival calculated up to March 2018.

To compare distant metastatic ILC to distant metastatic IDC, we selected the cohort of patients (n=60) from the previous study investigating subtype discordance in metastatic IDC (114). From this database we included all patients who developed distant metastatic disease following curative surgery +/- adjuvant endocrine/chemotherapy (n=60) and compared this cohort to all patients from our ILC database who developed distant metastatic disease following curative surgery +/- adjuvant endocrine/chemotherapy (n=70).

3.4 Results

3.4.1 ILC patient details

There were 734 recorded cases of ILC that presented to our institution from 1985-2017. Patient demographics and tumour characteristics of the study group are outlined in Table 3.1. Patients with ILC tended to have grade 2 (64.6%), node-negative (51.8%), ER and PR positive (91.5% and77.5% respectively) and HER2 negative (93.7%) tumours. 9% of patients presented with metastatic stage IV disease. The median follow-up time was 73.5 months (range 0-430 months).

Total patients	**(n=734)**
Age – mean years (SD)	61.5 (12.9)
Tumour size – mean mm (SD)	36.7 (25.5)
Grade (data on 729 patients)	**0** 14.7% **1** 5.6% **2** 64.6% **3** 15.1% Unknown - 5
T (608)	**1** 31.1% **2** 45% **3** 18.9% **4** 5% Unknown - 126
N (612)	**0** 51.8% **1** 27.6% **2** 11.9% **3** 8.7% Unknown - 122
Stage (634)	**I** 22% **II** 41.3% **III** 27.6% **IV** 9% Unknown - 95
Estrogen receptor (666)	**ER** + 91.5% **ER –** 8.5% Unknown - 68
Progesterone receptor (572)	**PR** + 77.6% **PR –** 22.4% Unknown - 162
HER2 receptor (522)	**HER2** + 6.7% **HER2 –** 93.7% Unknown - 212
Subtype (517)	**Luminal A** 89.2% **Luminal B** 5% **HER2** 1.9% **Triple-negative** 3.9% Unknown - 217
Surgery	Not available
Lymph node procedure	**Axillary Clearance** 58% **Sentinel Lymph Node Biopsy** 42%

Table 3.1 ILC patient details

3.4.2 Survival

For all ILC patients, mean OS was 92.6 months (SD 77.7). 5-year and 10-year survival were 79.1% and 61% respectively (Figure 3.1). ER negative patients (n=57) had a greater mean OS than ER positive patients (n=609), (118 vs. 88 months, p=0.027). PR negative patients (n=128) and PR positive patients (n=444) had comparable mean OS (79 vs. 80 months, p=0.87).

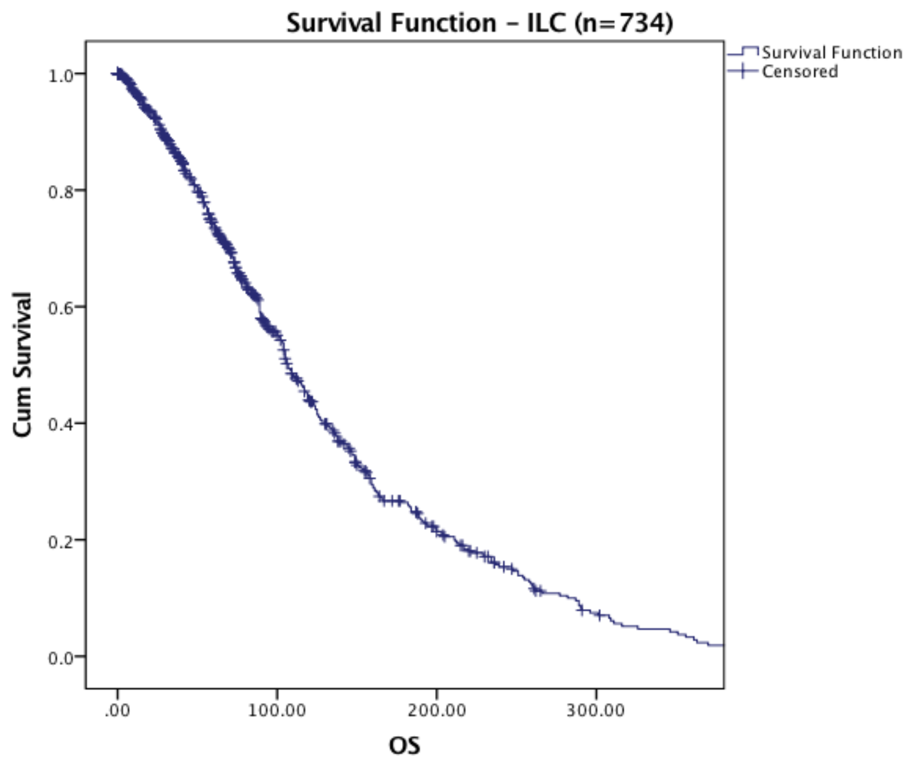


Figure 3.1 ILC overall survival

	Mean OS (months)	P-value
ER + (n=609) **ER –** (n=57)	88 118	0.027*
PR + (n=444) **PR –** (n=128)	80 79	0.87

Table 3.2 Impact of receptor status on survival

Tumour grade was an independent prognostic factor for OS. Grade 0 (n=107) and grade 1 (n=41) had comparable OS (144 vs. 146 months, p=.99). Both had significantly greater OS compared to patients with grade 2 (n=471) and grade 3 (n=110) disease ($p<0.001$ for both).

Grade	**Mean OS (m)**
0 (n=107)	143.3
1 (n=41)	145.8
2 (n=471)	78
3 (n=110)	86.4
T Stage	
1 (n=194)	127.5
2 (n=295)	87.3
3 (n=140)	81.3
4 (n=41)	72.5
N stage	
0 (n=317)	106.2
1 (n=168)	87.1
2 (n=73)	91.2
3 (n=53)	88.9

Table 3.2 ILC overall survival by Grade, T stage and Nodal stage

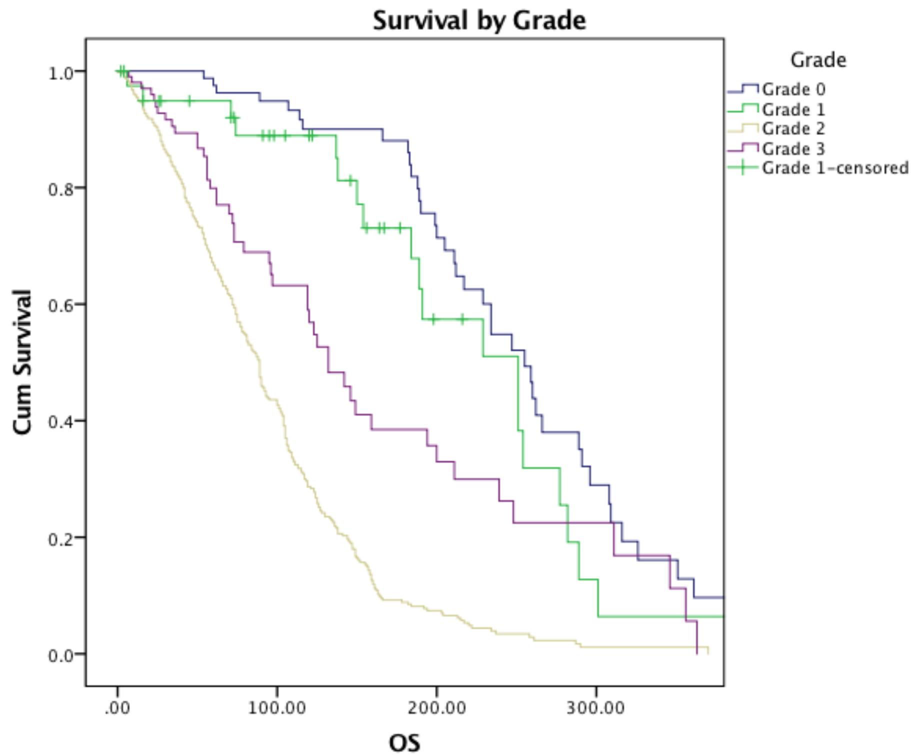


Figure 3.2 ILC overall survival by Grade

T stage and nodal status were also prognostic indicators of OS. T1 disease had a significantly greater OS compared to T2, T3 and T4 disease (Figure 5.3) (Table 5.2) ($p<0.001$). Node-negative patients (n=317) had significantly higher OS than N1 patients (n=168) (106 vs. 87 months, p=0.036). OS was comparable between N1, N2 (n=73) and N3 patients (n=53), (87 vs. 91 vs. 89 months respectively) (Figure 5.4) (Table 5.2).

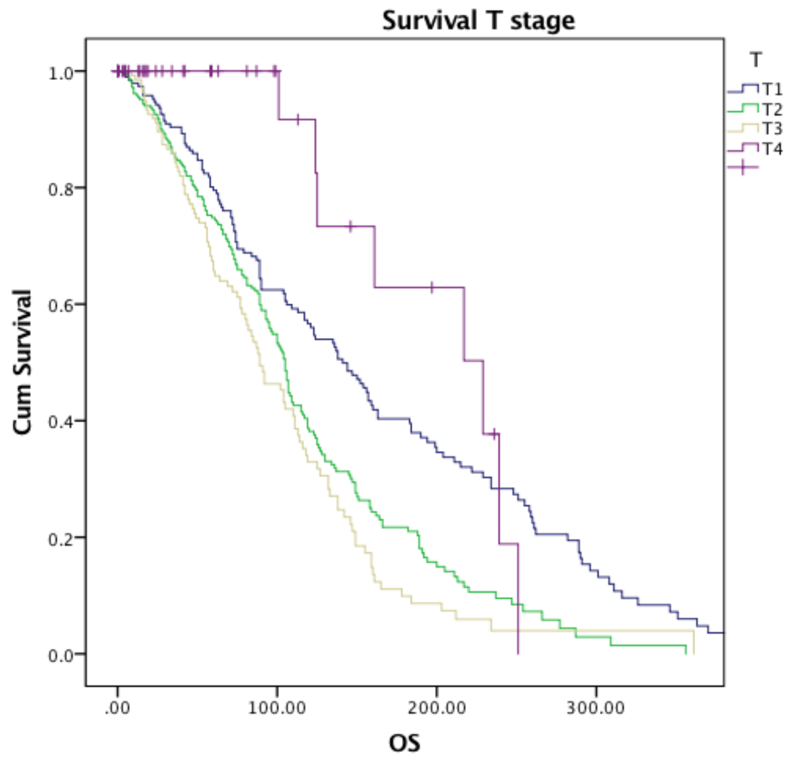


Figure 3.3 ILC overall survival by T stage

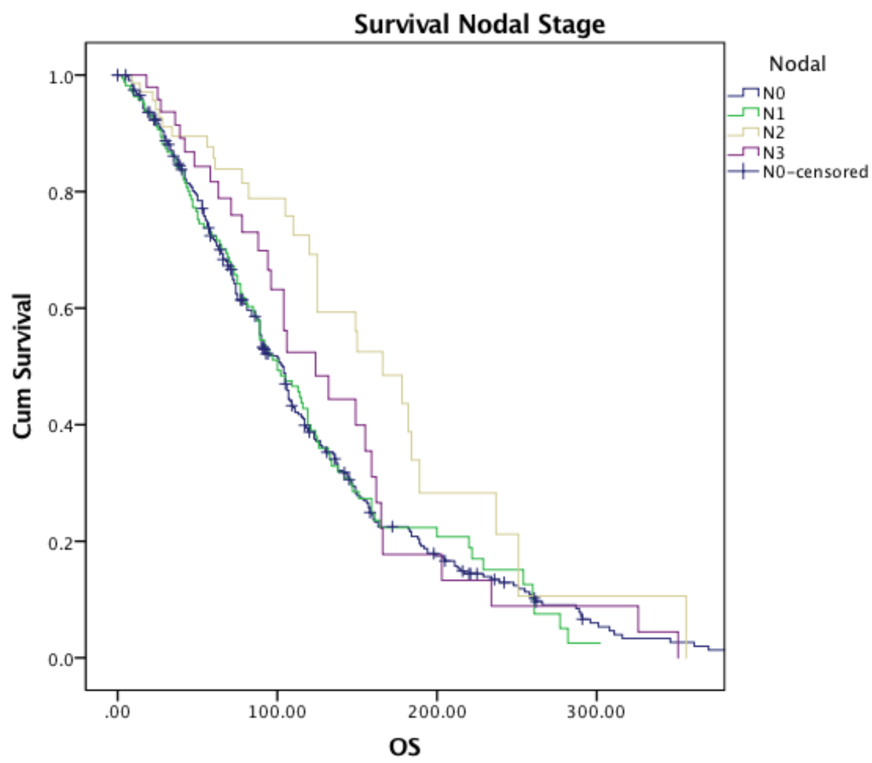


Figure 3.4 ILC overall survival by Nodal stage

3.4.3 Distant metastatic ILC

There were 101 cases (13.5%) of distant metastatic disease. Of these, pathological information was available on 77 cases. Mean time to diagnosis of metastasis was 78 months (SD 62). Bone was the most common site of metastasis (n=53), with a variety of unusual sites of metastatic disease recorded, including the omentum (n=2), the uterus (n=1), the ovary (n=1) and the bladder (n=1). Mean PRS was 21.7 months (SD 21).

Patients who developed distant metastatic disease had larger tumours at presentation (38.8 vs. 31.4mm, p=0.021) and were more likely to be node positive (74% vs. 45%, p<0.001). ER and PR positivity had no association with development of distant metastatic disease (p=0.168 and p=0.194 respectively).

Distant metastasis	101 patients (13.5%) Information on 77 patients (24 cases are prior to 1995 and no data available)
Time to metastasis – mean months (SD)	78 (62)
Post-recurrence survival – months (SD)	21.7 (21.1)

Table 3.3 Distant metastatic ILC

3.4.4 Distant metastatic ILC vs. distant metastatic IDC

Of the 77 cases of distant metastatic ILC listed above, 70 received curative surgery +/- endocrine/chemotherapy prior to the development of metastasis. From the our previous study (114), there were 60 patients with IDC who developed distant metastatic disease following curative therapy (Table 3.3).

Patients in the IDC group were significantly older at diagnosis of primary breast cancer compared to patients in the ILC group (57.3 vs. 52.1 years, p=0.021). IDC primary tumours were more commonly grade III while ILC tended to be grade II (p=0.021). ILC primary tumours were more likely to be ER positive and PR positive compared to IDC (p=0.018 and p=0.003 respectively). Primary tumour size was comparable (ILC 40.6 vs. IDC 44.8mm, p=0.4) and there was no significant difference in T stage or N stage at presentation (p=0.57 and p=0.72 respectively).

Distant metastatic disease developed significantly later in ILC compared to in IDC (75.9 vs. 37.4 months, p<0.001). 5-year metastasis-free survival was 50% for the ILC cohort and 15% for the IDC cohort.

In terms of treatment strategies, surgical and axillary management were similar between the two groups. Mastectomy was performed more frequently than breast-conserving surgery in both groups (ILC 80% vs. IDC 70%, p=0.146). Axillary nodal clearance was performed more frequently than sentinel-node biopsy alone in both groups (ILC 86% vs. IDC 85%, p=0.527). Neoadjuvant chemotherapy was administered more frequently in the IDC group (48%) compared to the ILC group (14%) (p<0.001).

	ILC (n=70)	**IDC (n=60)**	**p-value**
Age at diagnosis of primary – mean years (SD)	57.3 (11.7)	52.1 (13.7)	0.021 *
Time to metastasis – mean months (SD)	75.9 (58)	37.4 (27)	<0.001 *
Size – mean mm (SD)	40.6 (24.9)	44.8 (31.6)	0.4
Grade	**0** - 3 **I** - 4 **II** - 46 **III** - 16	**0** **I** - 2 **II** - 30 **III** - 28	0.021 *
T stage	**0** - 0 **1** - 13 **2** - 32 **3** – 23 **4** - 2	**0** - 2 **1** - 13 **2** - 27 **3** – 16 **4** - 2	0.57
N stage	**0** - 18 **1** - 18 **2** - 19 **3** – 14	**0** -13 **1** - 21 **2** - 16 **3** – 10	0.72
Estrogen receptor	**ER** + 59 **ER** – 9 Unknown - 2	**ER** + 41 **ER** - 19	0.018 *
Progesterone receptor	**PR** + 45 **PR** – 17 Unknown - 8	**PR** + 33 **PR** – 27	0.003 *
Surgery	**Mastectomy** 48 **Breast-conserving** 12 Unknown - 10	**Mastectomy** 42 **Breast-conserving** 18	0.146
Axilla	**ANC** 56 **SNL** 9 Unknown - 5	**ANC** 51 **SNL** 9	0.527
Neoadjuvant chemotherapy	**Received** 10 **None** 60	**Received** 31 **None** 29	<0.001 *

Table 3.4 Distant metastatic ILC (n=70) vs. distant metastatic IDC (n=60)

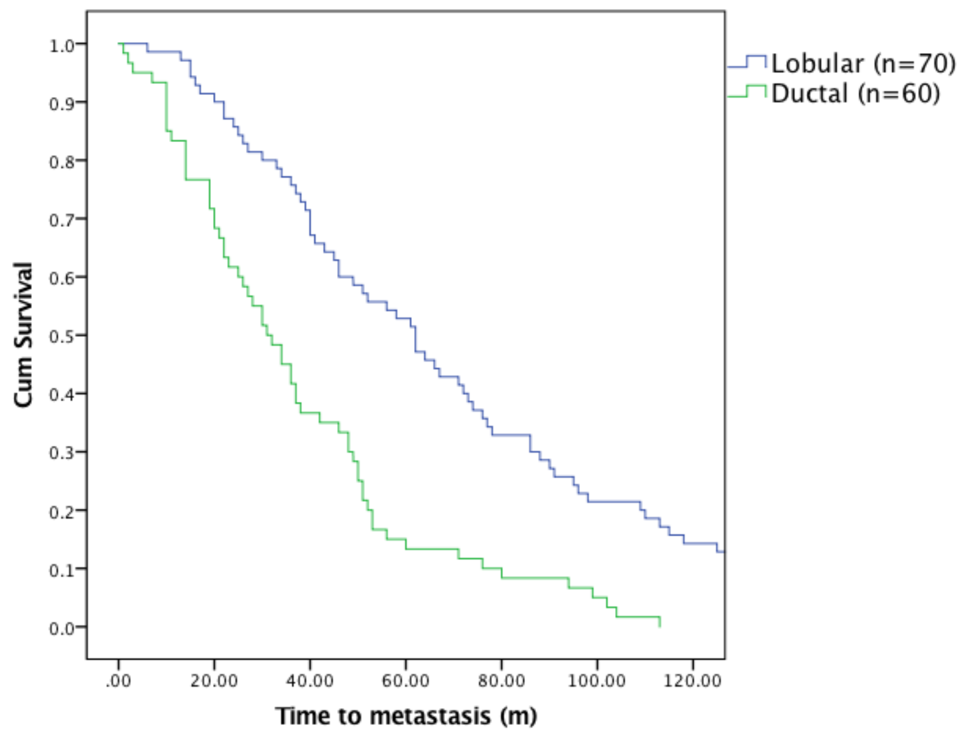


Figure 3.5 Time to distant metastasis ILC (75.9 mean months) vs. IDC (37.4 mean months)

Sites of distant metastasis differed between the two groups, with bone liver and lung the most common sites in IDC. ILC exhibited a more diverse pattern of metastasis.

	ILC (n=70)	**IDC (n=60)**
Site of metastases	**Bone –** 53 **Liver –** 21 **Lung –** 9 **Brain –** 6 **Peritoneum –** 3 **Omentum –** 2 **Ovary –** 2 **Uterus –** 1 **Stomach –** 1 **Bladder -** 1	**Bone –** 22 **Liver –** 20 **Lung** – 14 **Brain** – 2 **Adrenal** - 1

Table 3.5 Sites of metastasis ILC vs. IDC

Following development of metastatic disease, both groups had comparable PRS (ILC 21.7 vs. IDC 17.5 months, $p=0.283$). However, as metastatic disease tended to develop later in the ILC cohort, this group had a greater OS (98.7 vs. 61.9 months, $p<0.001$).

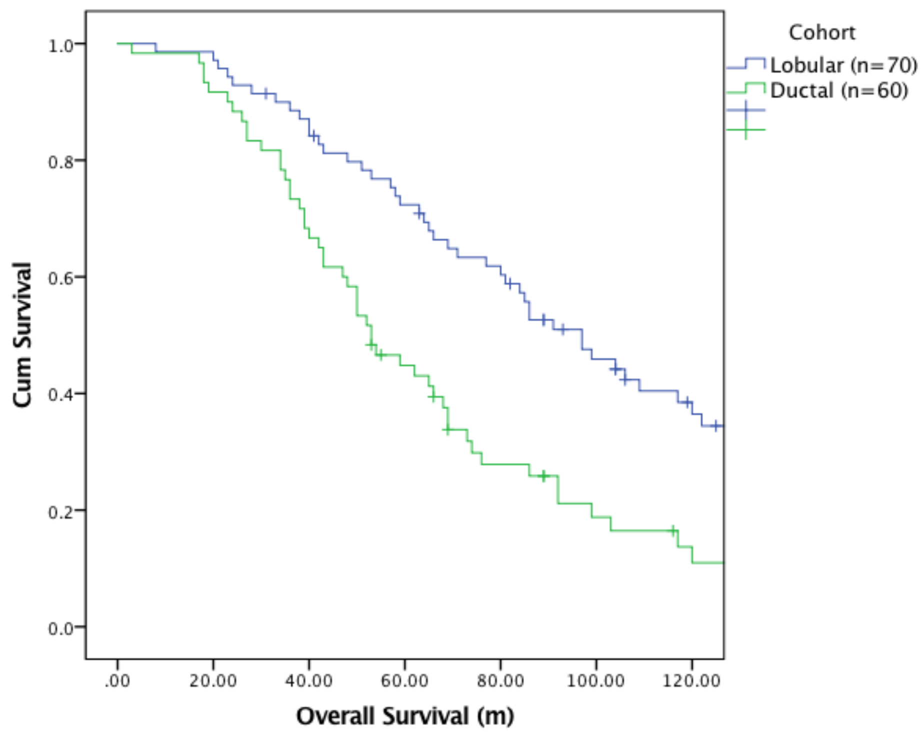


Figure 3.6 Overall survival ILC vs. IDC

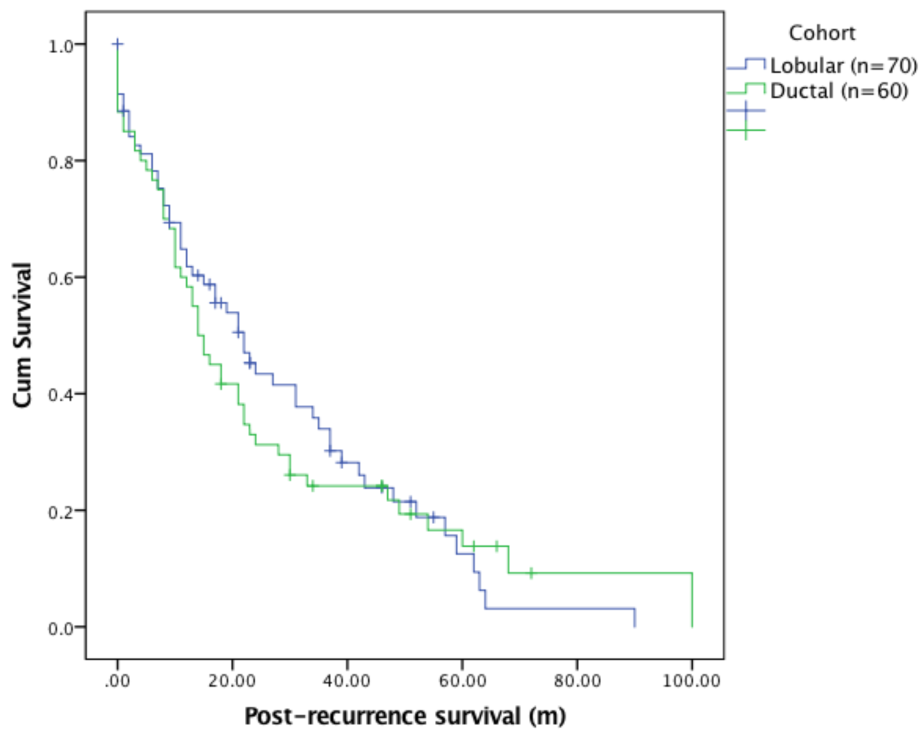


Figure 3.7 Post-recurrence survival ILC vs. IDC

3.5 Discussion

This study has demonstrated the clinical portrait of a large cohort of patients presenting with ILC over a 30+ year period at our institution, with a median follow up time of over 6 years. I have identified the rate of distant metastatic disease (13.5%) in ILC and the diverse range of sites of metastasis. Similarly to other studies, ILC at presentation is predominantly grade II, ER and PR positive and HER2 negative (62, 133-135).

I compared the clinical course of distant metastatic ILC to a similar sized cohort of patients with distant metastatic IDC, with broadly similar T and N stages. The ILC group was older at diagnosis and more commonly grade II at presentation. The ILC group also exhibited a more diverse range of metastatic sites and had a longer metastasis-free survival compared to the IDC group.

In terms of the metastatic behaviour of ILC, other studies have similarly identified a broad and diverse range of sites of metastatic disease, with ILC significantly more likely to metastasize to peritoneal and retroperitoneal organs (63, 136). The rate of metastasis to these sites may even be underestimated; a study in which experienced radiologists examined CT scans from 57 patients with metastatic ILC demonstrated that 15 patients (26%) had colon metastases while 12 patients (21%) had metastatic disease in the adnexa (66). The loss of expression of the cell adhesion molecule e-cadherin is detected in ~90% of ILC, (usually not in IDC), and may contribute to tumour growth and dissemination and explain this pattern of metastatic spread (137). Decreased cellular adhesion and increased motility

due to tumour-associated e-cadherin mutations has been observed in an experimental model (138). While loss of e-cadherin (CDH1) is the most common genetic hallmark of ILC, a recent study by the Cancer Genome Atlas Network identified mutations targeting PTEN, TBX3 and FOXA1 as distinct features of ILC (139). These mutations impact proliferation and may be related to the unique metastatic behaviour of ILC, casting further light on the distinct molecular portrait of ILC and demonstrating further fundamental differences between IDC and ILC.

This study also shows that among patients who develop distant metastatic breast cancer, those with ILC developed metastasis much later than patients with IDC (mean months - 75.9 vs. 37.4). As the vast majority of ILC patients are hormone-receptor positive, they remain at profound risk of developing metastatic disease at 10-20 years following their initial diagnosis (140). A potential cause of this phenomenon of later metastasis is the presence of disseminated tumour cells (DTCs), a surrogate for micrometastatic disease in breast cancer patients (141). A recent study of 422 patients with breast cancer found a significantly higher proportion of DTCs detected in patients with ILC compared to IDC, and further that ILC independently predicted micrometastatic disease (142).

In terms of overall survival, a number of large-scale studies have demonstrated similar survival figures for ILC and IDC (67, 143). For DFS, recent studies suggest that ILC maintains an initial advantage over IDC for the first 5 years following diagnosis, wherafter the pattern shifts to a worse outcome for ILC (61, 62). Our study suggests that among patients who go

on to develop metastatic disease, ILC patients have a longer disease-free survival compared to patients with IDC and subsequently a longer overall survival.

Currently, ILC is treated using the same management paradigm as IDC (144), however the distinct clinical course of metastatic ILC, allied to our improved understanding of ILC's molecular portrait may warrant a more tailored management regime to be developed in the future. This may include more targeted surveillance of development of metastatic disease using more specific diagnostic modalities, potentially incorporating non-invasive circulating biomarkers to contribute to the early diagnosis of metastasis. It is prudent for clinicians to consider a wider differential of sites of metastatic disease when following up patients with ILC and to investigate appropriately. Surveillance for metastatic disease may also need to be planned for increased periods, as we have demonstrated that metastatic disease may develop as much as 20 years after the diagnosis of primary ILC.

Our study by virtue of the retrospective design is inherently limited. Pathological information could not be collated comprehensively for all patients due to the long time-frame of the study, meaning that a number of records could not be retrieved. All ILC cases were pooled as one disease entity whereas in reality a variety of subtypes of ILC exist that stratify the disease further, each with different prognoses (139). This more modern approach will allow better patient and lobular subtype evaluations to be performed in the future but unfortunately was not feasible for this cohort. The exact treatment received by every patient could not be collected, and

indeed a variety of alterations to standard practice of breast cancer treatment would have been implemented over the timeframe of the study, such as the introduction of Trastuzumab and anti-HER2 targeted therapy for HER2 positive breast cancer. In terms of matching the two metastatic cohorts, they were not ideally matched to clinic-pathological parameters and were from slightly different time periods, potentially impacting the treatment received and the follow-up regime.

This study holds several strengths. Patient and tumour details were collated in the same prospectively maintained database. As this is a single-centre study, patients underwent the same treatment protocols and went through the same multi-disciplinary meeting management strategy. Pathological review was consistent over the time frame of the study with many of the same pathologists reviewing tumour samples. The long median follow up time of over 6 years gives a broad perspective of the long term outcome of ILC. Survival and metastatic recurrence data were systematically updated up to March 2018.

In conclusion, this study has examined long term outcomes of 734 cases of ILC treated at a tertiary referral centre over a 30+ year period. We have also compared the clinical course of metastatic ILC to metastatic IDC, demonstrating a different profile of sites of metastatic disease and a longer DFS for ILC. Further prospective studies examining the clinical course of ILC are required to determine the exact metastatic behaviour of the disease, taking into account what treatment is received and how patients are followed up. Further studies are also required to unravel more of the unique

molecular portrait of ILC will enable us to treat and monitor the disease more effectively in the future.

Chapter 4

Breast cancer subtype discordance in metastatic disease: impact on post-recurrence survival and potential treatment options

4.1 Introduction

Risk of recurrence and outcome in breast cancer have conventionally been stratified according to the tumour size, grade, nodal status and especially tumour subtype (145). Breast cancer is a heterogeneous disease with 3 established immunohistochemical biomarkers: Estrogen Receptor (ER), progesterone receptor (PR) and HER2 (human epidermal growth factor 2) receptor. The presence or absence of these receptors defines the four distinct molecular subtypes of breast cancer- luminal A (ER/PR positive, HER2 negative), luminal B (ER and/or PR positive, HER2 positive), HER2 over-expressing (HER2 positive alone) and triple negative (negative for all 3 receptors) (146). Each subtype exhibits distinct prognoses, rates of recurrence and different treatment strategies (22). Following treatment, breast cancer recurrence can be classed as either loco-regional (LRR; confined to the ipsilateral breast/lymph nodes) or distant. Recurrence rates are influenced by the original breast cancer subtype, the specific therapy received and the response to the therapy (147). Traditionally, recurrent tumours have been have been assumed to be biologically similar (the same subtype) to the primary tumour. Recent studies have demonstrated that hormonal and HER2 receptor status can change status between primary and recurrent breast cancer (42). This can impact prognosis with loss of receptor status associated with a poorer prognosis (43, 44). A change in receptor status could potentially lead to a change in treatment options, as patients whose recurrent tumour becomes hormone positive could be candidates for hormonal therapy and similarly patients who become HER2 positive may benefit from receiving Trastuzumab (45, 46).

4.2 Aim

The aim of our study was to identify subtype change in recurrent breast cancer at our institution, to assess the impact of discordance on patient outcomes, and to identify any potential changes in treatment due to a subtype change and if in reality patients who changed subtype experienced a change in treatment strategy.

4.3 Methods

Data was collected on patients who had a recurrence of breast cancer following surgery +/- chemotherapy/hormonal therapy/radiotherapy at the Galway Hospitals group between 2001 and 2014. Only patients who had clinical pathology scoring of receptor status of both the primary and recurrent cancer were included. Exclusion criteria included presentation with bilateral tumours, biopsy results that were incomplete, and pathologist report of the recurrence as a new primary tumour. PAS software was used to access pathology records with MOSAIQ software used to determine patient pathways and treatment. Overall survival and post-recurrence survival were estimated using the Kaplan-Meier product limit method. The log rank was used to determine any statistically significant differences in survival between the indicated groups. Comparative analyses were performed between groups using Chi-squared and T-tests.

4.4 Results

4.4.1 Patient and primary tumour characteristics

132 patients met the inclusion criteria. Mean age at diagnosis was 53.3 years. 58 patients (44%) had a loco-regional recurrence while 74 (56%) had a distant recurrence (Table 4.1). The majority of patients in our cohort were stage 2 or stage 3 (41.6% and 29.5% respectively), grade 2 or 3 (40.1% and 52.3% respectively (Table 4.2). Bone was the most common distant recurrence (n=27), followed by liver (n=22) and lung (n=16) (Table 3.3). 49 patients (37.2%) had breast-conserving surgery while 83 (62.8%) underwent mastectomy. 58 patients (44%) received neo-adjuvant chemotherapy prior to their primary surgery, with a mean time of 181 days (SD ±89.7) between diagnosis and surgery in this group. Mean time from diagnosis of primary disease to diagnosis of recurrence was 38.7 months (Table 4.1). Mean overall survival (OS) was 60.1 months (SD ±38.2 months) while mean post-recurrence survival (PRS) was 20.8 months (SD ±21.1 months).

Patient Details	Total (n=132)
Age at diagnosis: mean years (SD ±)	53.3 (SD ±13.6)
Time to recurrence: mean months (SD ±)	38.7 (SD ±27.7)
Recurrence location	
Loco-regional	58 (44%)
Distal	74 (56%)
Neoadjuvant Chemo Rx	
Received	58 (44%)
Did not receive	74 (56%)
Surgery	
Mastectomy	83 (62.8%)
Wide local excision	49 (37.2%)
Survival : Months	
Overall: mean (SD ±)	60 (38.3)
Post-recurrence survival: mean (SD ±)	20.7 (21.1)

Table 4.1 Patient details

Tumor details	n	(%)
Stage		
I	15	11.3 %
II	55	41.6 %
III A/B	39	29.5 %
III C	23	17.4 %
Grade		
1	10	7.6 %
2	53	40.1%
3	69	52.3 %
T		
1	37	28 %
2	58	43 %
3	34	25.7 %
4	3	2.3 %
N		
0	34	25.8 %
1	46	34.8 %
2	29	21 %
3	23	14.4 %

Table 4.2 Primary tumour details

Distant recurrences (n=74)	**N (%)**	**Proportion that changed subtype**
Bone	27 (36%)	4 (14%)
Liver	22 (30%)	5 (23%)
Lung	16 (22%)	4 (25%)
Lymph node distant	6 (8%)	1 (17%)
Brain	2 (3%)	0
Adrenal	1 (1.5%)	0

Table 4.3 Distant recurrence sites

4.4.2 Receptor discordance & survival

Rates of single receptor discordance for ER, PR and HER2 receptors were 20.4% (n=27), 37.8% (n=50), and 3% (n=4) respectively (Table 4.4).Overall survival (OS) was comparable between the ER discordant group (n=27) and the ER concordant group (n=105), (60.2 vs. 59.3 months), while post-recurrence survival (PRS) was shorter in the discordant group, but this was

not statistically significant (21.6 vs. 17.4 months, p=0.36). There was no statistically significant difference in OS or PRS between the PR discordant (n=50) and concordant (n=82) groups (OS 67.1 vs. 55.7 months, p=0.096, PRS 23.3 vs. 19.1 months, p=0.096). There was a statistically significant loss compared to gain of both ER and PR receptor status (ER loss n=21 (15.9%) vs. gain n=6 (4.5%), p=0.04; PR n=44 (33.2%) vs. n=6 (4.5%), p=0.01).

ER	
Concordant	105 (79.6%)
Discordant	27 (20.4%)
Gain	6 (4.5%)
Loss	21 (15.9%)
PR	
Concordant	82 (62.1%)
Discordant	50 (37.8%)
Gain	6 (4.5%)
Loss	44 (33.2%)
HER2	
Concordant	128 (97%)
Discordant	4 (3%)
Gain	2 (1.5%)
Loss	2 (1.5%)
Subtype	N (%)
Concordant	101 (76.5%)
Discordant	31 (23.5%)

Table 4.4 Receptor discordance

4.4.3 Subtype discordance & survival

31 patients (23.5%) changed subtype on recurrence, 17 were loco-regional recurrences and 14 were distant. The majority of subtype changes were from luminal A to triple-negative (n=18) (Figure 4.1).

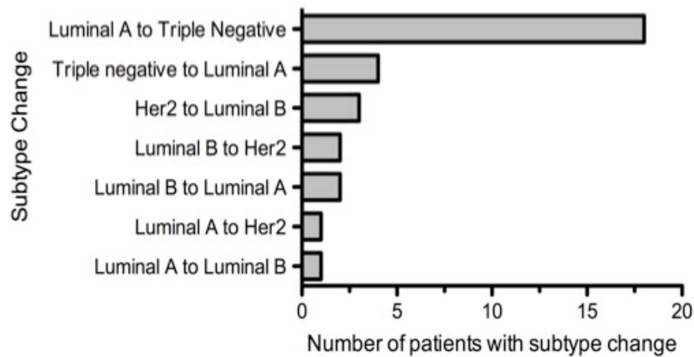


Figure 4.1 Subtype changes

The group who changed subtype (n=31) had a longer mean time to recurrence compared to the concordant group (n=101) (44.9 vs. 36.9 months, p=0.16) (Table 4.5).

Patient Details	**Total** **(n=132)**	**Change subtype** **(n=31) 23.5%**	**Gain of Receptor** **(n=9) 6.8%**
Survival : Months	**N (%)**	**N (%)**	**N (%)**
Overall: mean (SD ±)	60 (38.3)	64.9 (40.3)	76.9 (56.3)
Post-recurrence survival: mean (SD ±)	20.7 (21.1)	18.5 (22.8)	30.6 (30.3)

Table 4.5 Impact of subtype change on survival

Recurrence location, type of surgery received and neo-adjuvant therapy were not associated with subtype change (p=0.3, p=0.83, p=0.674 respectively) (Table S3.1). A change from luminal A to triple negative (n=18) subtype resulted in poorer 10 year OS versus the concordant luminal

A group (n=46) which approached statistical significance (46.8 vs. 67 months, p=0.064) (Figure 4.2) and there was a statistically significant shorter 5 year PRS between the two groups, (8.6 vs. 22.5 months, p<0.05) (Figure 4.3).

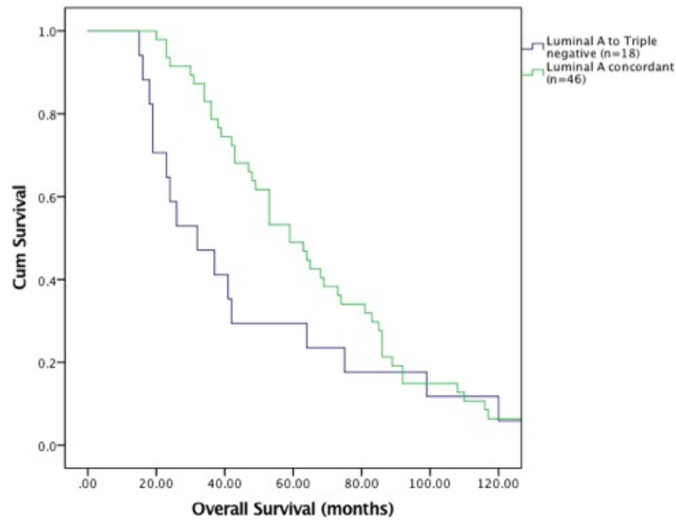


Figure 4.2 Overall survival (10 years) – Luminal A – TN (n=18, blue line) vs. Luminal A concordant (n=46, green line)

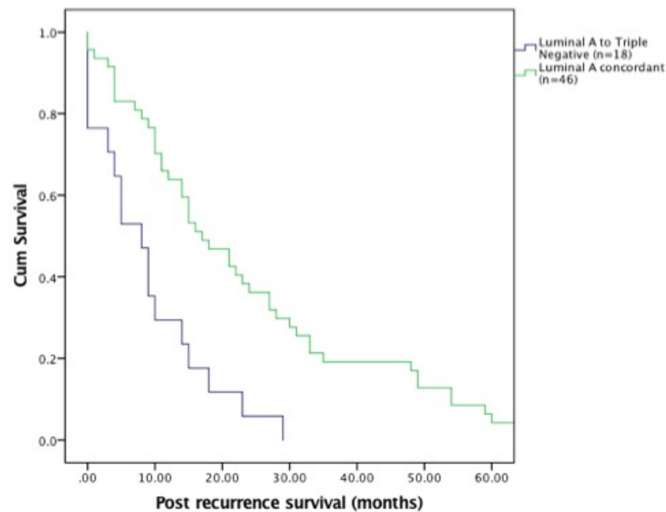


Figure 4.3 Post recurrence survival (5 years) – Luminal A – TN (n=18, blue line) vs. Luminal A concordant (n=46, green line)

Comparing patients who changed from triple-negative to luminal A (n=4) to the concordant triple negative group (n=35), there was no significant difference in 10 year OS (35 vs. 49 months, p=0.378) or 5 year PRS (13.5 months vs. 14.2 months, p=0.919).

4.4.4 Potential changes to treatment

In terms of changes in subtype that could potentially lead to a change in treatment, nine patients (6.8%) gained receptor status on recurrence. Seven went from HR negative to positive, with 6 patients going from ER negative to positive (ALLRED score 0 in the primary to >2 in the recurrence). One went from PR negative to positive. Of these seven patients, five had a loco-regional recurrence and two had distant recurrences (one liver, one lung). None of these patients received additional endocrine therapy following the biopsy results of the recurrence. All nine patients are deceased with a mean OS of 52 months and a mean PRS of 21 months.

Two patients gained HER2 receptor status, both going from HER2 score of 0 on Herceptest of the primary to 1 in the recurrence, with both subsequently testing positive on FISH. One patient had a distant recurrence in bone, and was enrolled in the TRIO 022 trial (148), subsequently receiving Letrozole, Denosumab and a CDK inhibitor without receiving Trastuzumab. This patient is alive with an OS of 145 months and a PRS of 33 months. The other patient had a loco-regional recurrence and subsequently received one year of Trastuzumab. This patient is alive with an OS of 179 months and a PRS of 96 months.

In summary, only one patient in our study of nine who gained receptor status ultimately received additional targeted therapy.

3.5 Discussion

In our single-centre analysis the rate of subtype change of was 23.5%, supporting previously published figures (149, 150). In terms of the specific changes in subtype, the most frequent change was from luminal A to triple negative, and this group had a significantly poorer 5 year PRS. Despite initially diverging OS, ultimately both groups have similar 10 year OS. Other studies have demonstrated a similar reduced survival in patients who change form HR positive to negative on recurrence (150-154).

Single receptor discordance was 20.4%, 37.8%, and 3% for ER, PR and HER2 receptor respectively, similar to that reported in a recent meta-analysis examining 48 papers, which reported pooled discordance rates of 20%, 33% and 8% for ER, PR and HER2 receptor (42). HER2 receptor exhibits the lowest rate of discordance between primary and recurrence (155). Loss of single receptor status was more common than gain for ER ($p=0.04$) and PR ($p=0.01$), in line with published data (156).

There are a number of possible aetiologies for receptor discordance. Firstly, variability exists in the reproducibility and accuracy of immunohistochemical staining (47). There is also variability in sampling methods, for example fine needle aspiration or core biopsy versus surgical extraction in the primary tumour and in sampling of the recurrence that can

contribute to the discrepancy. With the advent of next generation sequencing technology, it has become apparent that breast cancer demonstrates both intra-tumour and inter-tumour heterogeneity to a greater extent than previously understood. The discordance in receptor status may demonstrate clonal genome evolution (42, 48, 49) and the clone with the more aggressive phenotype could potentially initiate the micro-metastatic process (50). Biological drift is another potential cause, for example selective eradication of ER/PR positive cells by hormonal therapy could leave behind a population of ER/PR negative cells that in time could metastasize (51) (Figure 4.3). Genuine switches in biology of the cancer appear to be a rare event based on currently available gene expression data (52, 53), however this does not exclude the potential for smaller scale genomic alterations and mutations (54). Heterogeneity between patient's primary and recurrence may be due to newly acquired biological characteristics that allow tumour cells to travel via the circulatory/lymphatic systems and to metastasize to new sites (55). Change in receptor status may contribute to this increased capacity for invasion as endocrine and growth factor signalling pathways are implicated in invasion and metastasis (56, 57)

In terms of potential alterations to treatment and survival benefits of performing a recurrence biopsy, there is conflicting data with much of the literature being retrospective and examining small populations with variability in assay used, site of metastasis and definition of recurrence (43, 152, 157, 158). Two prospective studies aimed to address these limitations - the BRITS study (159) in the United Kingdom which was carried out at 20

secondary care sites, and the DESTINY study (46) conducted at a single centre in Toronto, Canada. Both were conducted using similar eligibility and exclusion criteria. A pooled analysis of the two studies examined the proportion of patients who underwent a change in management based on the results of the recurrence biopsy (160). 289 patients underwent biopsy of recurrence, consisting of 48% loco-regional recurrences and 52% distal metastases. 14.2% of patients had a change in management based on their results. However, on further analysis, half of the changes in treatment regime were due to loss of receptor status, new primary diagnosis or benign disease on biopsy. In total only 7.1% of patients had a treatment added due to gain in receptor status.

In terms of the effect that changing management had on patient outcomes, the results were unclear and only the DESTINY trial looked at overall survival. There was no significant association between overall survival and discordance (median OS 27.6 vs. 30.2 months in the concordant and discordant groups respectively). Other retrospective studies have identified a change in management plan in 12-20% of patients where there was a gain in receptor status (149, 158, 161).

Current guidelines by the American Society of Clinical Oncology (ASCO) (162) advise offering biopsy where feasible to patients with recurrence for receptor status. Treatment should be guided preferentially by the ER/PR/HER2 status of the recurrence if justified by the clinical scenario and conforming to the patient's wishes. The panel's recommendations are

deemed to be “moderate” due to the paucity of clinical evidence demonstrating that altering therapy based on receptor change has significant health outcomes. A number of barriers exist to routine biopsy of tumour recurrence – it may not be technically feasible or safe to perform, there is a 2% risk of major complications (163), and the patient or physician may decide against it.

Limitations of our study include the relatively small sample size. The retrospective nature of the study made it difficult to accurately collate data on patient’s precise treatment regimes. Furthermore, as discussed above technical misclassification is a significant contributor to receptor discordance. Gain in receptor status may be attributable to this misclassification as opposed to a genuine change in tumour biology (163). It may be beneficial to carry out an independent re-review of the pathology slides from this study to identify what proportion of subtype change was due to this misclassification.

In summary, our study demonstrates the discordance of receptor and subtype between primary and recurrent breast cancer at our institution and sheds further light on the topic of potential intra-tumour heterogeneity. It highlights the importance of performing a biopsy of recurrent breast cancer, due to the implications that change in subtype has on survival. Further research is required to investigate the aetiology and biology of subtype discordance and the optimal strategy for treatment change based on this discordance. Our results highlight the need for a prospective, multi-centre

trial collecting data on patients who experience recurrence (including routine biopsies of recurrence) to establish if all recurrent patients should be biopsied, or only a subset of patients most likely to benefit from additional treatment options.

Chapter 5

Identification and validation of circulating miRNAs to distinguish metastatic from local luminal A breast cancer

5.1 Introduction

Breast cancer is the most common cancer among women and the fifth leading cause of cancer death, with metastasis the principle cause of mortality (1). Despite considerable recent advances in both diagnosis and treatment, 20-30% of breast cancer patients will develop distant metastatic disease (27). The risk of developing metastatic disease is determined by the initial stage as well tumour subtype. Breast cancer consists of at least four clinically relevant molecular subtypes: luminal A, luminal B, HER2-enriched and triple-negative (18). Luminal A is the most common subtype comprising up to 60% of all breast cancers (164). Bone is the most frequent site of metastasis among all subtypes, with a 2017 SEER study of over 240,000 breast cancer patients finding that 3.1% of those with luminal A breast cancer developed bone metastasis (39). Luminal A patients remain at considerable risk of metastasis after 5 years in contrast to triple-negative patients, who tend to develop metastases in the first 3 years following diagnosis (38). The search for non-invasive biomarkers capable of augmenting conventional diagnostic and prognostic modalities in metastatic breast cancer is a priority in the era of individualised treatment regimens.

Mi(cro)RNAs are small, non-coding RNAs that regulate gene expression by targeting messenger RNA, resulting in either translational repression or RNA degradation (88). Over 4,000 miRNAs have been described and it is estimated that they regulate up to 30% of all human genes (165). MiRNAs can operate as tumour-suppressors or as tumour-promoters and their dysregulation is intricately linked to cellular processes involved in the metastatic cascade such as sustained proliferation, angiogenesis and

epithelial-mesenchymal transition (EMT) (166, 167). Circulating miRNAs show great promise in contributing to the diagnosis, prognosis, evaluation of response to therapy and treatment of breast cancer (168). MiRNAs are stable in circulation and can be quantified relatively simply and inexpensively by real-time quantitative reverse transcriptase PCR (qRT-PCR) (94, 95).

5.2 Aim

The aim of our study was to identify and validate circulating miRNAs capable of distinguishing metastatic breast cancer from locally confined breast cancer. This was a collaborative project in conjunction with UMASS Boston in which we utilised their expertise in deep sequencing (RNAseq) to initially identify these miRNAs (113). Validation of candidate miRNAs was performed on an independent cohort of patients with metastatic disease, patients with local disease and healthy controls. In an effort to expand on previous work from our lab (94), a secondary aim of our study was to investigate expression of mir-195 in our validation cohort to identify if this miRNA had any association with metastasis or if it could contribute to the identification of metastatic disease in combination with other miRNAs.

5.3 Materials and Methods

Patient selection

Patients were selected from the Lambe Institute UHG biobank. Samples were prospectively collected from 2008-2015. The discovery cohort (n=8) included 4 patients with metastatic disease to bone and 4 patients with locally confined breast cancer.

The validation cohort (n=74) was comprised of 22 patients with distant metastatic disease (17 to bone), 31 patients with locally confined breast cancer and 21 healthy control*s*.

MiRNA analysis

Refer to chapter 2 – methods and materials.

5.4 Results

5.4.1 Discovery cohort RNAseq – UMASS

712 miRNAs were analysed in the discovery cohort array. 16 miRNAs were found to be significantly differentially expressed between the metastatic and local groups (p<0.005) (Table 5.1). We selected 5 miRNAs from these to validate. 3 miRNAs (mir-181a, mir-329 and mir-331) were selected based on evidence from the literature of their involvement in metastatic processes (Table 5.2). 2 miRNAs (mir-6734 and mir-4433) were chosen speculatively as they had no confirmed targets or functions in the literature and may have proven to be novel miRNAs warranting further investigation.

Rank	miRNA	baseMean	log2FoldChange	lfcSE	stat	P value
1	hsa-miR-487a-5p	10.204322	-6.5949809	2.08622397	-3.1612046	0.00157118
2	hsa-miR-376c-3p	9.66930256	-6.2249402	2.16041009	-2.8813697	0.00395951
3	**hsa-miR-181a-2-3p**	10.0812243	6.10481296	2.17498667	2.80682775	0.0050032
4	hsa-miR-6721-5p	11.0766671	-5.5985336	2.00252808	-2.7957329	0.00517822
5	**hsa-miR-329-3p**	40.6587497	-4.2606132	1.5758723	-2.7036539	0.00685817
6	hsa-miR-665	4.33646293	-5.5104353	2.17214494	-2.5368635	0.01118505
7	**hsa-miR-331-3p**	9.380562	-4.2942354	1.79092278	-2.3977781	0.01649485
8	**hsa-miR-4433a-5p**	15.8475709	3.90544954	1.65092455	2.36561359	0.01800022
9	hsa-	3.59270664	-4.7490645	2.08789239	-2.2745734	0.02293153

	miR-2277-3p					
10	**hsa-miR-6734-5p**	25.3078788	3.92134038	1.73154349	2.26465024	0.02353415
11	hsa-miR-4446-5p	2.07468503	-4.7899413	2.19677719	-2.1804402	0.02922485
12	hsa-miR-636	6.03478118	4.19953728	1.97006819	2.13167102	0.0330339
13	hsa-miR-1273h-3p	24.0903406	-3.6161293	1.70742406	-2.1178859	0.03418473
14	hsa-miR-4701-5p	1.70465123	-4.6347346	2.19807189	-2.1085455	0.03498383
15	hsa-miR-212-3p	2.0182045	4.61548377	2.2188873	2.08008932	0.03751734
16	hsa-miR-323b-3p	1.14207066	-4.3254517	2.16070262	-2.0018728	0.04529842

Table 5.1 RNAseq data - MiRNAs differentially expressed between local and metastatic disease in discovery cohort (*Selected miRNAs in **bold)**

miRNA	**Metastatic process**	**Expression Pattern**	**Cancer (Reference)**
Mir-181a	EMT Migration and Invasion	↑ ↑	Breast (169), Colorectal (170) Breast (171)
Mir-329	Proliferation and migration Apoptosis	↓ ↓	Neuroblastoma (172), Gastric (173) Lung (174)
Mir-331	Proliferation and EMT	↑	Liver (175)

Table 5.2 Candidate MiRNAs implicated in the metastatic cascade

5.4.2 Validation cohort qRT-PCR – NUIG

5.4.2.1 Biomarkers of metastatic and local disease

Expression of mir-331 was significantly higher in the metastatic group (n=22) compared to both the local group (n=31) and the healthy control group (n=21), ($p<0.001$ and $p<0.001$, ANOVA and post-hoc Tukey analysis), corresponding to an average fold-change of 2.58 and 2.94 respectively *(*Figure 5.1*)*. There was no significant difference in mir-331 expression between the local group and the control group ($p=0.825$).

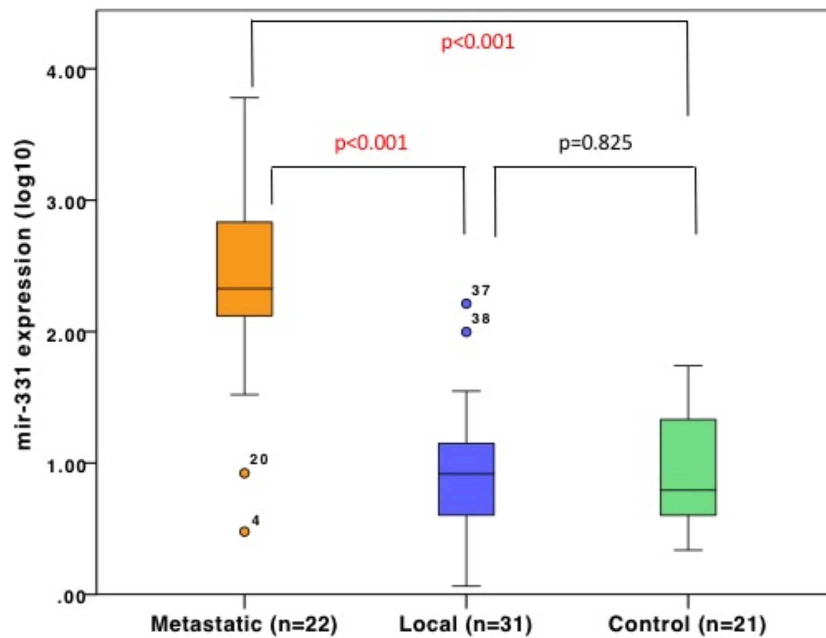


Figure 5.1 Mir-331 expression

Expression of mir-181a was significantly higher in the healthy control group in comparison to the metastatic group and the local group (p=0.001 and p=0.02, average fold-change of 1.4 and 1.19 respectively) *(*Figure 5.2*)*. Expression of mir-181a was lower in the metastatic group compared to the local group, approaching significance (p=0.059). Pooling the metastatic and local groups together (n=53), this group with breast cancer had significantly lower expression of mir-181a compared to the healthy controls (p<0.001).

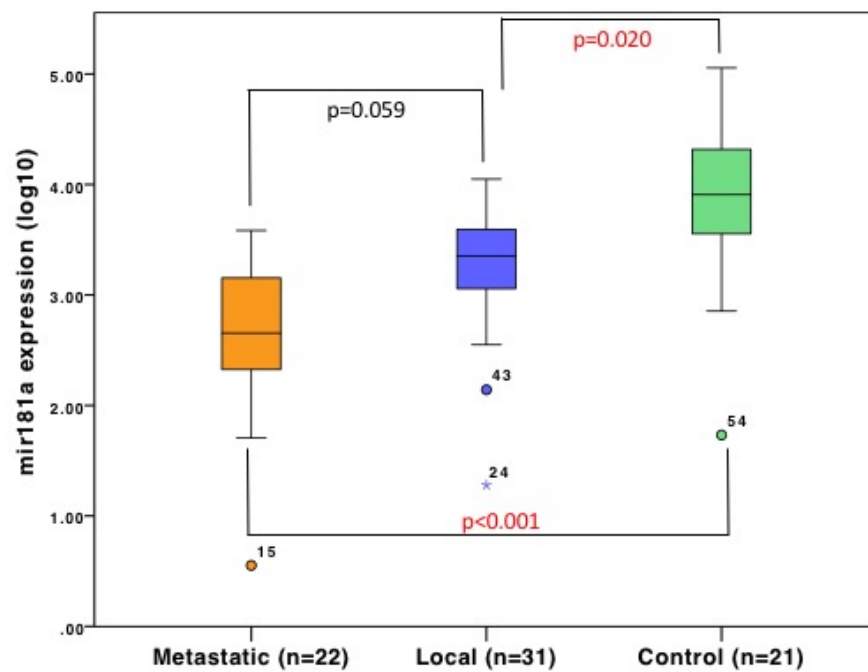


Figure 5.2 Mir-181a expression

Expression of mir-195 was significantly lower in the metastatic group compared to both the local and the healthy control groups (p<0.001 and p=0.043, average fold-change of 0.6 and 0.73 respectively) (Figure5.3). There was no significant difference in mir-195 expression between the local and healthy control groups (p=0.087).

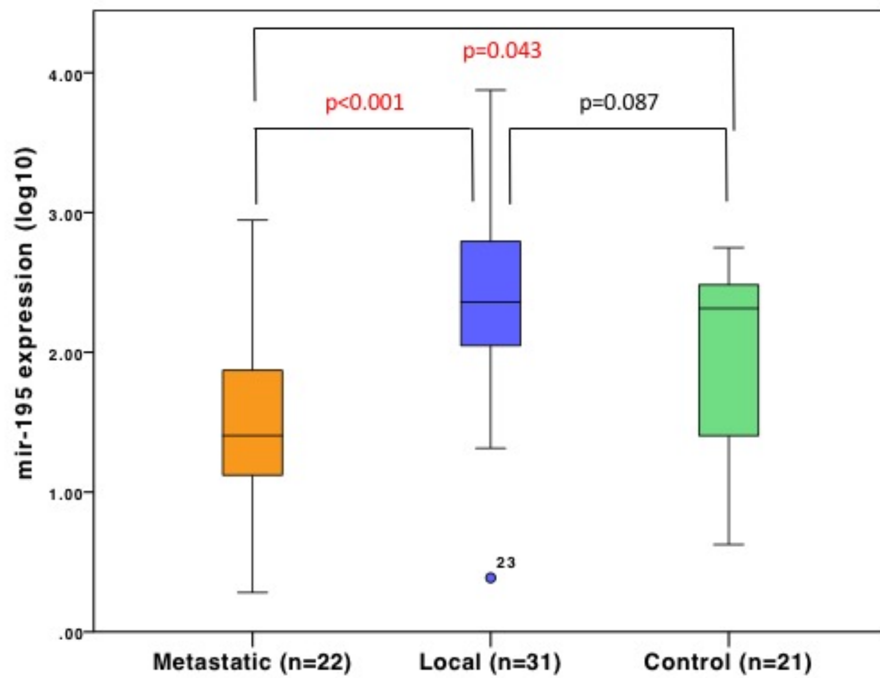


Figure 5.3 Mir-195 expression

Mir-329 could not be detected reliably across all 3 groups, with a persistent C_T value >35 in over 50% of samples. Mir-4433 and mir-6734 demonstrated no difference in expression between the local and metastatic groups (Figure 5.4).

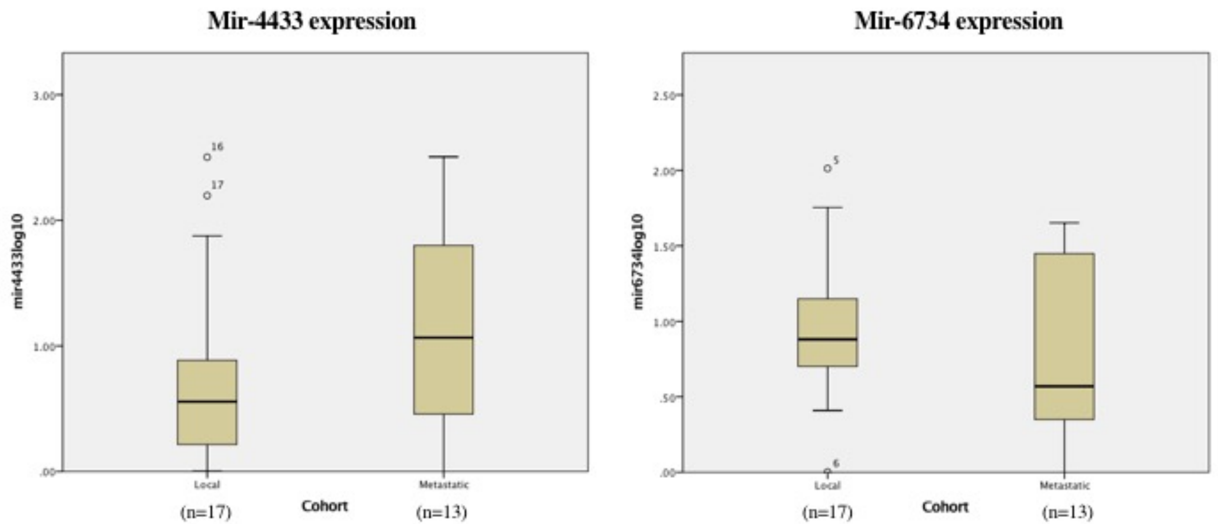


Figure 5.4 Mir-4433 and mir-6734 expression

5.4.2.2 MiRNAs – Relationship with Clinicopathological Parameters

Investigating the clinicopathological details of the local group (n=31), those with lymph node positive disease (n=11) had higher expression of mir-331 compared with those with lymph node negative disease (n=20), approaching significance (p=0.099) (Figure 5.5). There was a trend towards higher expression of mir-331 in higher grade tumours (Figure 5.6), but this did not reach statistical significance (p=0.274). Mir-331 expression had no significant association with lymphovascular invasion or tumour size in the local group.

Mir-181 expression showed no association with lymph node status, tumour grade, lymphovascular invasion or tumour size.

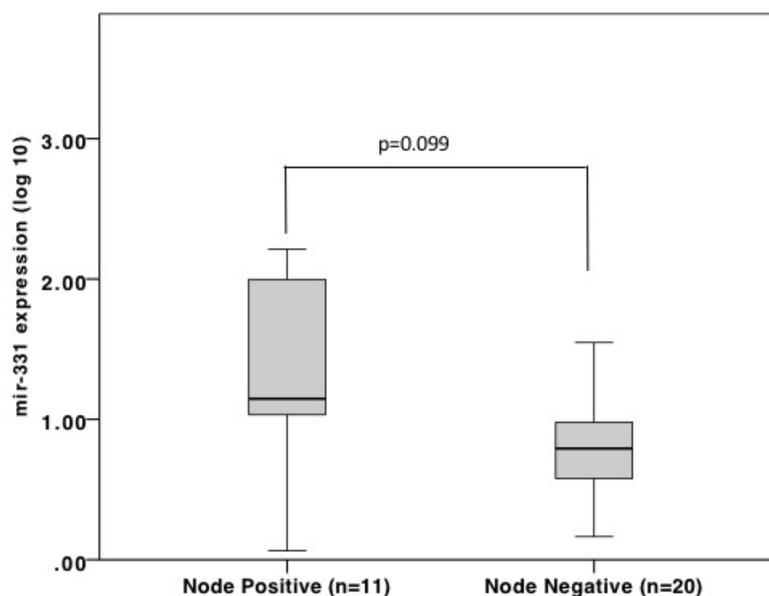


Figure 5.5 Mir-331 expression – comparing node positive vs. node negative patients in the local breast cancer group

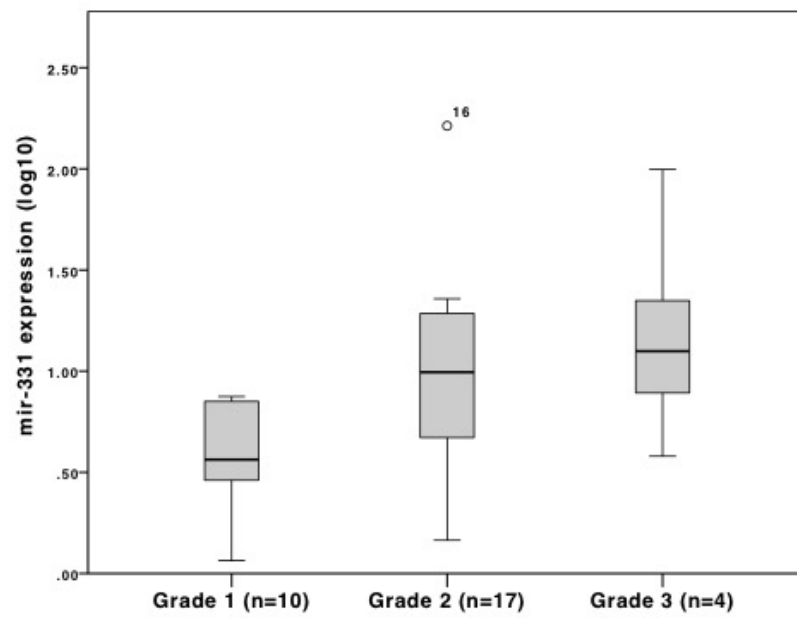


Figure 5.6 Mir-331 expression – association with tumour grade in the local breast cancer group

5.4.2.3 MiRNA as biomarkers of metastatic luminal A breast cancer

A logistic regression analysis was performed to ascertain the potential of circulating miRNAs in distinguishing metastatic from local disease. Analysing each individual miRNA and combination of miRNAs, we compared the area under the curve (AUC) produced from receiver operator characteristic (ROC) curve generation using binary logistic regression. The highest AUC of 0.902 was achieved combining mir-331 and mir-195, providing a sensitivity of 95% and a specificity of 76% (Figure 5.7). The logistic regression model was significant (x^2 (4) =28.98, $p<0.001$). Mir-181a did not contribute to the biomarker profile.

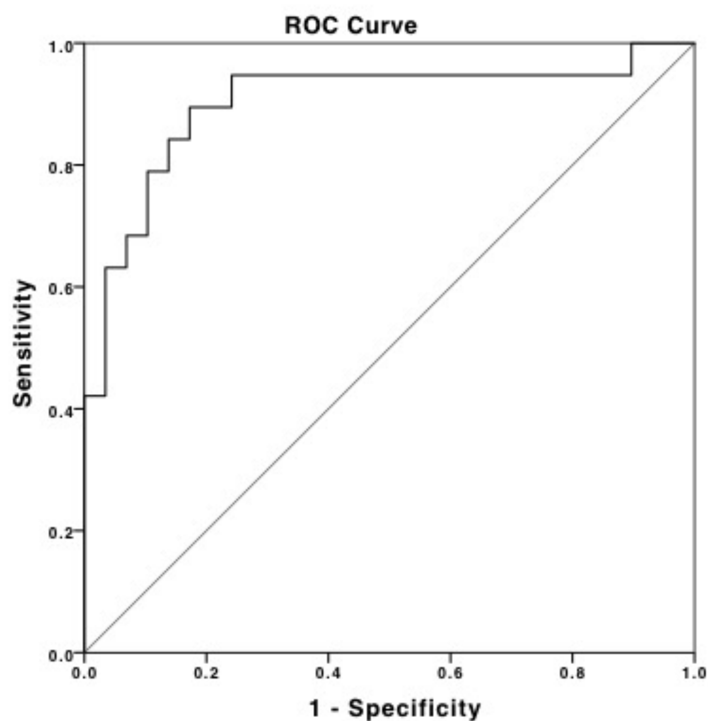


Figure 5.7 Mir-331 and mir-195 in combination to distinguish metastatic from local breast cancer (AUC=0.902)

5.5 Discussion

Circulating miRNAs are an appealing adjunct to conventional diagnostic and prognostic modalities as they are stable in circulation, easily quantifiable and can reveal further information of the underlying biology of the tumour (81). The potential of miRNAs to contribute to a "liquid biopsy" has been the focus of much research in recent years.

In this study we have identified 2 novel miRNAs (mir-331 and mir-195) dysregulated in the circulation of patients with distant metastatic Luminal A breast cancer compared to patients with locally confined Luminal A breast cancer. In addition, we have shown that levels of these miRNA are comparable in age-matched healthy controls to the patients with local breast cancer.

Mir-331 was identified as dysregulated in metastatic patients in our discovery cohort using deep-RNA sequencing. Mir-331 was subsequently validated as a marker of metastatic disease on a large independent cohort, with higher expression in the metastatic group. Expanding on previous work from our laboratory (94), mir-195 was also examined in the validation cohort and similarly distinguished metastatic from local breast cancer, with lower expression in the metastatic group.

Confirmed target of Mir-331 include HER2, HOTAIR, E2F1 and DOHH (176), with established links to metastatic processes such as cell proliferation, evasion of apoptosis, angiogenesis and EMT. A recent study investigating mir-331 in hepatocellular carcinoma demonstrated that high expression of mir-331 in HCC was associated with poor clinicopathological

details and worse survival (175). In-vitro, the study showed that mir-331 targets PHLPP, resulting in stimulation of protein kinase B (AKT) and subsequent EMT promoting proliferation and metastasis. Finally, the group inhibited mir-331 in xenograft mice using an anti-mir-331 vector resulting in marked inhibition of proliferation and metastasis, further supporting the putative role of mir-331 as a tumour-promoting miRNA.

Mir-195 has previously been implicated as a diagnostic biomarker of breast cancer (94, 177), and has more recently been investigated as a tumour suppressor. Mir-195 has been shown to target Bcl-2 and induce apoptosis, thereby suppressing tumour growth (178). Other studies have shown that mir-195 regulates biological processes such as cell proliferation and cell cycle by targeting CDK4, CDK6, cyclin D1 and others (179-181). More pertinently, mir-195 has recently been shown to target FASN, HMGCR, ACACA and CYP27B1 in hormone-receptor positive breast cancer cell lines, acting as a suppressor of cell proliferation, EMT, invasion and metastasis (182).

A variety of challenges must be overcome before circulating miRNA can be implemented into clinical practice. Individual factors, such as age, ethnicity and lifestyle factors (for example smoking, alcohol and diet), can influence miRNA expression (183-185). This represents a background heterogeneity that limits the significance drawn from small samples. Even with large-scale studies there exists no consensus on the ideal tissue type from which to extract miRNA. Whole blood is not currently considered an ideal biological fluid as constituent (non-malignant) cells contribute to miRNAs in the

circulation (186). Neither plasma nor serum has yet been delineated as ideal either (186, 187). Different methods of sample preparation, anticoagulation, centrifugation and storage have contributed to interstudy variability (188-190). Standardized procedures would be an excellent starting point from which to draw conclusions on the valuable resource of biological samples.

Debate continues as to the optimal detection platform for detecting circulating miRNAs. The current approach to quantifying miRNA is by use of qRT–PCR. Although user-friendly, with as little as 100 ng of RNA required as input, it has a low-to-medium throughput and can detect annotated miRNA only (186, 191). Hybridization-based miRNA microarrays analyse up to 1000 miRNAs per assay and are cheaper than qRT–PCR, but have a comparatively lower dynamic range and specificity. Quantification by miRNA sequencing (miRNAseq) is a rapidly developing approach and is particularly useful for miRNA families that differ by a single nucleotide. It is capable of detecting novel miRNAs, giving it a dual function of discovery and quantification for optimal large-volume testing (192). However, miRNAseq is expensive, requires extensive specialist input, and widespread use is limited at present.

Normalization of miRNA presents a problem owing to the lack of a universally accepted reference miRNA. Examples of RNAs that have been used to normalize results include mir-16, mir-26a, and other non-coding RNA such as RNU6 and RNU48. Mir-16, probably the most used 'house-keeping' miRNA, is affected by haemolysis so may not be ideal for use as a reference (186). The diverse criteria for normalization and cut-off values for

statistical methods are impediments to reproducibility and clinical application.

Future areas of research include the use of miRNAs as therapeutic adjuncts. This may be possible through the restoration of tumour suppressor miRNAs or the inhibition of oncomirs. The precise delivery of miRNAs remains a challenge. Delivery mechanisms proposed include via lipid-based vehicles (193) or potentially as exosome-encapsulated miRNAs delivered via mesenchymal stem cells (194). Whether it is possible for miRNAs to be used to manipulate genes directly linked to carcinogenesis remains to be seen.

The results of this study are encouraging and further substantiate the potential of circulating miRNAs to contribute to breast cancer management. However, we must acknowledge a number of limitations of the study. The sample size is small and a larger, blinded prospective study is required to draw more concrete conclusions about the utility of these miRNAs as biomarkers. Our study was limited to ER positive, Luminal A breast cancer patients and it is unclear if the dyseregulated miRNAs are subtype specific or if the same pattern of expression would persist across all breast cancer subtypes. Blood samples were taken in the metastatic cohort at the time the patient had confirmed distant metastatic disease, so that while our results suggest that these miRNAs reflect the presence of metastasis, prospective collection of blood samples from patients with locally confined disease need to be conducted to determine if dysregulated miRNA expression preceded metastatic disease.

5.6 Conclusion

This study has identified and validated two miRNAs with differential expression in the circulation of patients with distant metastatic Luminal A breast cancer compared to patient with locally confined Luminal A breast cancer. Our results suggest that patients with metastatic disease have a higher expression of mir-331 and a lower expression of mir-195 in their circulation. In combination, these markers distinguish metastatic from local breast cancer with a high sensitivity and specificity. While mir-195 has previously been investigated as a suppressor of metastatic disease in breast cancer (182), to our knowledge this is the first study to identify mir-331 as a potential promoter of breast cancer metastasis. Further research is required to elucidate the precise mechanism of mir-331 in breast cancer, and also to establish if dysregulation of this miRNA profile precedes the development of metastatic disease and can contribute to the evolving paradigm of breast cancer management.

Chapter 6

Final discussion and future directions

The field of circulating miRNAs as biomarkers in breast cancer is evolving rapidly and offers great potential to contribute to earlier diagnosis and tailored therapy, ultimately improving patient outcomes. Over 4,000 miRNAs are currently described and the precise functions and targets of many of these are yet to be elucidated, making identifying potentially clinically relevant miRNAs a daunting task. While many previous studies have used microarrays to discover potentially relevant miRNAs, we have utilized RNAseq (195, 196) technology in collaboration with the laboratory at UMASS, Boston to identify miRNAs dysregulated between metastatic and local breast cancer. The advantages of RNAseq mean it is rapidly replacing gene expression microarrays in many laboratories. RNAseq enables investigators to look at coding and non-coding RNA, at splicing and allele specific expression. Probes and primers are not used, limiting the bias suffered compared to microarrays. Digital data is provided in the form of aligned read-counts, which improves the sensitivity for rare transcripts and increases the dynamic range, and finally re-analysis of the dataset produced is possible when new information about the transcriptome becomes available.

Individual factors such as age, ethnicity, and lifestyle factors (e.g. smoking, alcohol, and diet) can impact miRNA expression (183-185). This represents a background heterogeneity that limits the significance drawn from small sample sizes. Even with large scale studies there exists no consensus on the ideal tissue type from which to extract miRNA. Whole blood is not currently considered an ideal biological fluid as constituent (non-malignant) cells contribute to miRNAs in circulation (186). Neither plasma nor serum

has been delineated as the ideal as yet either (186, 187). Different methods of sample preparation, anticoagulation, centrifugation and storage have contributed to inter-study variability (188-190). Standardised procedures would be an excellent starting point from which to draw conclusions on the valuable resource of biological samples.

Following the identification of dysregulated miRNAs, we sought to validate these results on an independent cohort. Bloods were collected and RNA was extracted from patients with metastatic disease, with local breast cancer and healthy controls with no history of breast disease. Two miRNAs showed promising results. Mir-331 was significantly over-expressed in the circulation of patients with metastatic disease compared to both patients with local breast cancer and healthy controls, while mir-195 was significantly under-expressed. While mir-195 has previously been described as a tumour-supressor in breast cancer (94, 177), to my knowledge this is the first report suggesting a potential tumour-promoting role of mir-331 in breast cancer.

A recent study has demonstrated a similar trend of over-expression of mir-331 in hepatocellular cancer, further demonstrating its tumour-promoting role in-vitro and most pertinently halting the progression of metastasis in a murine model using an anti-mir-331 vector (175).

To elucidate the function of mir-331 in breast cancer, future studies will need to examine the impact of dysregulation on breast-cancer cell lines in-vitro, and what if any aspect of the metastatic cascade is promoted by

overexpression of this miRNA. Knockdown of mir-331 and/or its targets may offer a potential therapeutic target for inhibition of metastasis. It remains to be seen if these results are reproducible across different breast cancer subtypes, as all patients studied had luminal A breast cancer.

It is becoming apparent that no single circulating miRNA will contribute sufficiently to be utilized in clinical practice and the majority of studies now examine miRNAs in combination. A 2016 study investigated a panel of five miRNAs in 1280 patients with breast cancer, and was able to diagnose malignancy with a sensitivity of 97.3 per cent, specificity of 82.9 per cent and accuracy of 89.7 per cent, figures superior to those of conventional mammography (197). In our study, the combination of expression values of mir-195 and mir-331 produced an AUC of 0.901 in distinguishing metastatic from local breast cancer. Should the results of our study be definitively validated it is possible that mir-331 and mir-195 may contribute to a battery of circulating miRNA and/or other circulating markers to monitor for the development of metastatic breast cancer. While this result is encouraging, future prospective studies are necessary to determine if this is reproducible across a larger sample of patients, and indeed if dysregulation of these circulating miRNA precede the clinical diagnosis of metastasis. The issues pertaining to the validity of miRNAs raised earlier (patient factors, medium of choice, detection platforms, normalization of data) must also be definitively addressed before circulating miRNAs can be considered as potential adjuncts to the management of breast cancer.

The two retrospective studies of metastatic breast cancer have raised important issues going forward as to the management and surveillance of

metastatic breast cancer. A significant rate of discordance (23.5%) of molecular subtype between primary and metastatic breast cancer was demonstrated, with profound implications for both treatment strategies and post-recurrence survival. Increasingly, patients with metastatic breast cancer undergo repeat biopsy of the recurrent lesion where feasible to determine the receptor status, as per ASCO guidelines (162). Treatment should be guided preferentially by the ER/PR/HER2 status of the recurrence if justified by the clinical scenario and conforming to the patient's wishes. Two changes in treatment strategy based on subtype change were identified, however there is a need for prospective studies to determine what impact, if any, that altering patient's treatment regiments will have on long-term outcomes. Due to the relatively small number of qualifying cases, it is likely that these trials will require pooled data from multiple institutions, similar in design to the recent DESTINY and BRITS studies (159, 160). The findings herein also raise questions pertaining to the degree of intra-tumour heterogeneity and the impact of targeted therapy on subtype change which should be addressed in future studies.

The analysis of outcomes of ILC at our institution over 30 years, long-term survival following diagnosis of ILC was demonstrated with a long median follow-up time of over 6 years. ILC metastasized to a wider spectrum of distant organs compared to the classical metastatic profile of IDC. On comparison to a cohort of patients with distant metastatic IDC with similar age, T and N stages, disease-free survival was almost twice as long in patients with ILC. While currently both histological subtypes are managed using broadly similar strategies (198), these distinctly different metastatic

profiles allied to emerging evidence of fundamental differences in the genomic profile of the two subtypes (139) suggests that treatment and surveillance strategies may need to be tailored appropriately according to histological subtype. Further prospective studies collating comprehensive tumour characteristics and treatment received are required to determine the distinct characteristics of each subtype and to inform future clinical trials.

Chapter 7

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Chapter 8

Appendices

8.1 Biobank ethical approval and patient consent form

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Ospidéal Réigiúin Pháirc na Muirlinne
Merlin Park Regional Hospital,
Galway, Ireland.

Tel: (091) 757 631

Research Ethics Committee
Unit 4
Merlin Park Hospital
Galway.

27th January, 2006.

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 overcome.

Yours sincerely,

Dr. S. T O'Keeffe
Chairman Research Ethics Committee

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 CONSENTER, 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

………………………………………………………

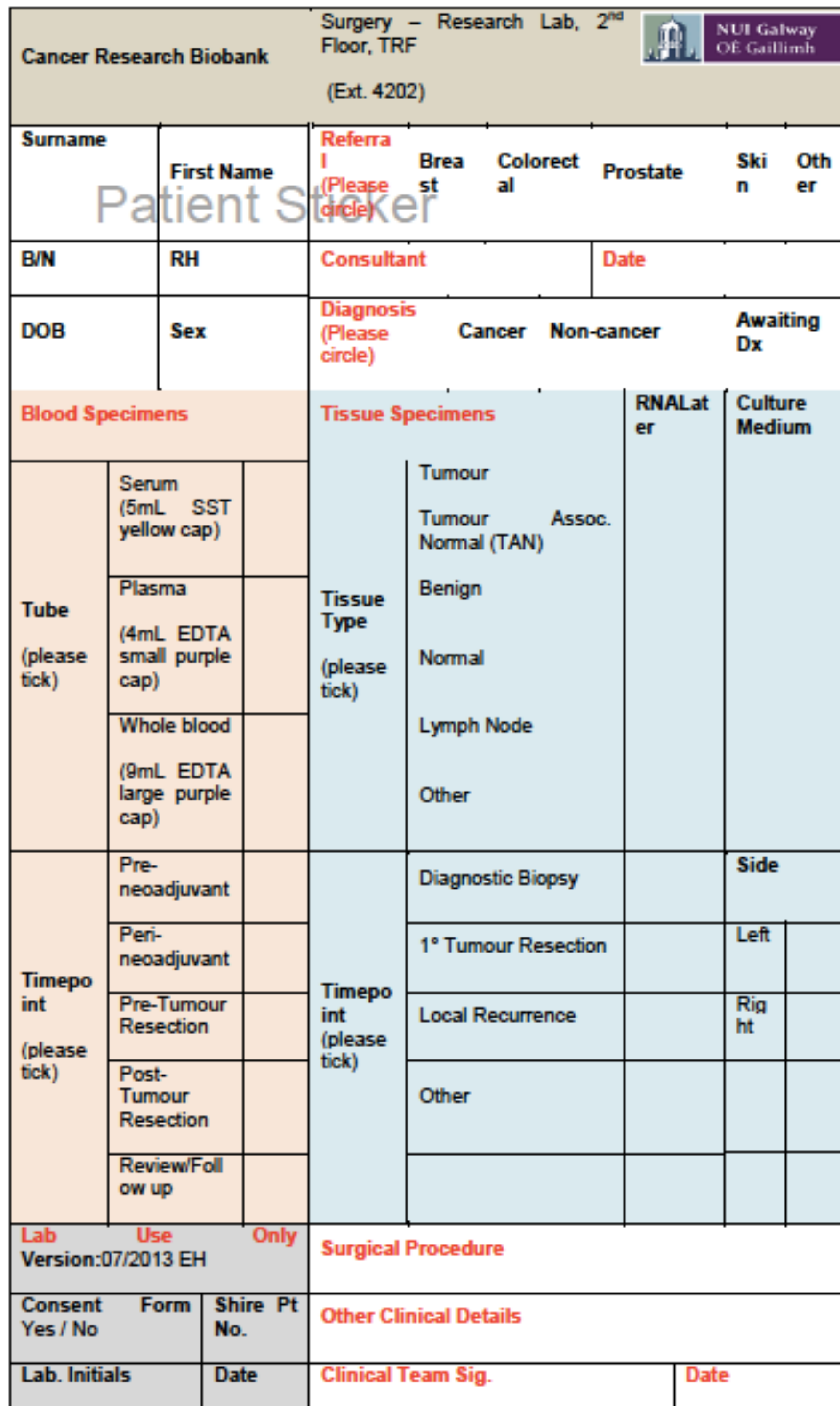
INVESTIGATOR:

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).



Cancer Research Biobank	Surgery – Research Lab, 2nd Floor, TRF (Ext. 4202)	NUI Galway OÉ Gaillimh

Surname	First Name	Referral (Please circle)	Breast	Colorectal	Prostate	Skin	Other
Patient Sticker							
B/N	RH	Consultant			Date		
DOB	Sex	Diagnosis (Please circle)	Cancer	Non-cancer		Awaiting Dx	

Blood Specimens			Tissue Specimens		RNALater	Culture Medium	
Tube (please tick)	Serum (5mL SST yellow cap)		Tissue Type (please tick)	Tumour			
				Tumour Assoc. Normal (TAN)			
	Plasma (4mL EDTA small purple cap)			Benign			
				Normal			
	Whole blood (9mL EDTA large purple cap)			Lymph Node			
				Other			
Timepoint (please tick)	Pre-neoadjuvant		Timepoint (please tick)	Diagnostic Biopsy		Side	
	Peri-neoadjuvant			1° Tumour Resection		Left	
	Pre-Tumour Resection			Local Recurrence		Right	
	Post-Tumour Resection			Other			
	Review/Follow up						

Lab Use Only Version:07/2013 EH		Surgical Procedure	
Consent Form Yes / No	Shire Pt No.	Other Clinical Details	
Lab. Initials	Date	Clinical Team Sig.	Date

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)