Factors influencing diagnosis and outcome in HER2 receptor positive breast cancer

A thesis submitted to the National University of Ireland Galway for the degree of Doctor of Philosophy in the College of Medicine

by

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2018

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Abbreviations

ASCO	American society of clinical oncology
BAN	Bromoanisole
BCS	Breast conservative surgery
BRCA	BReast CAncer susceptibility gene
cDNA	Complementary DNA
CGA	Comprehensive geriatric assessment
CT	Cycle threshold
CTC	Circulating tumour cells
DCIS	Ductal carcinoma in situ
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
FFDM	Full field digital mammography
FISH	Fluorescent in situ hybridisation
HER2	Human epidermal growth factor receptor 2
IAC	Inter assay control
ICER	Incremental cost-effectiveness ratio
ICORG	Irish co-operative oncology research group
IHC	Immunohistochemistry
LRR	Locoregional recurrence
MRI	Magnetic resonance imaging
NACT	Neoadjuvant chemotherapy
NFW	Nuclease free water
NTC	No template control
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pCR	Pathological complete response
PDAR	Pre-developed TaqMan assay reagent
PMRT	Post- mastectomy radiotherapy
PR	Progesterone receptor
QALY	Quality adjusted life years
RNA	Ribonucleic acid
RTC	Reverse transcription control
SLNB	Sentinel lymph node biopsy
WBRT	Whole breast radiotherapy
WHO	World Health Organization
WLE	Wide local excision
µg	Microgram
µL	Microlitre

Communications originating from this work

Peer Reviewed Published Manuscripts

Metastatic breast cancer: the potential of miRNA for diagnosis and treatment monitoring.

McGuire A, Brown JAL, Kerin MJ.

Cancer Metastasis Rev. 2015 Mar;34(1):145-55. doi: 10.1007/s10555-015-9551-7.

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McGuire A, Kalinina O, Holian E, Curran C, Malone CA, McLaughlin R, Lowery AJ, Brown JAL, Kerin MJ.

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Locoregional recurrence following breast cancer surgery in the trastuzumab era: a systematic review by subtype

McGuire A, Lowery AJ, Kell MR, Kerin MJ, Sweeney KJ.

Ann Surg Oncol. 2017 Jul 28. doi: 10.1245/s10434-017-6021-1.

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Prospective assessment of microRNA as markers of response to neoadjuvant chemotherapy in breast cancer

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(Submitted to Clinical Cancer Research)

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Circulating MiRNAs: Validating Novel Breast Cancer Biomarkers For Monitoring Neoadjuvant Response To Therapy In A Prospective Clinical Cohort

McGuire A, Casey MC, Waldron R, Heneghan H, Kaliningrad O, Holian E, McDermott A, Newell J, Keane M, Miller N, Brown JAL, Kerin MJ
Sir Peter Freyer surgical symposium, September 2017 **(Plenary)**

A systematic review of locoregional recurrence by breast cancer subtype following breast cancer surgery in the trastuzumab era

McGuire A, Lowery AJ, Kell MR, Kerin MJ, Sweeney KJ
Sir Peter Freyer surgical symposium, September 2016 **(Plenary)**

Locoregional recurrence following breast cancer surgery in the trastuzumab era: a systematic review by subtype

McGuire A, Lowery AJ, Kell MR, Kerin MJ, Sweeney KJ
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Variations in survival and outcomes between her2 +ve breast cancer subtypes following the introduction of Trastuzumab

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Her-2 breast cancer treatments induced variations in the patterns of survival and metastasis in Her-2 positive breast cancers

McGuire A, Kalinina O, Holian E, Curran C, Malone CA, McLaughlin R, Lowery AJ, Brown JAL, Kerin MJ.
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Differences in metastatic patterns between luminal B and HER2 over expressing breast cancers and changes since the introduction of trastuzumab

McGuire A, Kalinina O, Holian E, Curran C, Malone CA, McLaughlin R, Lowery AJ, Brown JAL, Kerin MJ.
23rd Sylvester O'Halloran surgical symposium. March 2015

Grants and awards related to this research

National Breast Cancer Research Institute (NBCRI) Fellowship 2014-2016
Plenary Session Presentation, Sir Peter Freyer Surgical Symposium, NUI, Galway 2016
Plenary Session Presentation, Sir Peter Freyer Surgical Symposium, NUI, Galway 2017

Gold medal winner Sir Peter Freyer Surgical symposium 2017

Acknowledgements

I would like to acknowledge the contribution of the following people, who enabled me to complete this work;

Professor Michael Kerin, who provided me with the opportunity to do a PhD in breast cancer research; not only did he provide guidance throughout this thesis, but also acted as a mentor for my future career and is a model surgeon that I would hope to emulate.

Dr James Brown, who provided direction, expertise and support in the preparation of this work; his enthusiasm and advice were invaluable.

Ms Catherine Curran and Ms Emer Hennessy, who were critical in acquiring the patient data from the breast cancer database, answered all my many queries, and made all materials I needed available.

Dr Roisin Dwyer and Ms Grace Clarke, who were always accessible for discussion and advice, and whose support and encouragement are much appreciated.

Ms Emma Holian and Ms Olga Kalinina, who answered all my queries and facilitated essential components of this study.

To my parents, who have always made sure I wanted for nothing, and everything I have achieved so far in my life is thanks to them.

To my wife Rebecca, who always supported and encouraged me without question and was always there to provide advice.

I would like to thank the National Breast Cancer Research Institute (NBCRI) whose financial support enabled this project.

Finally, I would like to express my sincere gratitude to all the patients who participated in this study, and who facilitate ongoing research at the NBCRI and the department of surgery.

Andrew McGuire

Chapter 1

Introduction

Published sections

Metastatic breast cancer: the potential of miRNA for diagnosis and treatment monitoring
McGuire A, Brown JA, Kerin MJ. Cancer Metastasis Rev. 2015 Mar;34(1):145-55. doi: 10.1007/s10555-015-9551-7.

1.1 Introduction

1.1.1 Breast cancer

Breast cancer is the second most common cancer diagnosed worldwide, affecting approximately 1/8 women during their lifetime [1]. It affects 1.3 million women each year and annually accounts for 23% of all cancer cases and 14% (465,000) of all cancer deaths [2]. Furthermore, improvements in disease screening and diagnosis mean increasing numbers of the population have breast cancer detected at an earlier stage. While there has been increasing numbers of patient diagnosed with breast cancer, survival rates are similarly improving over the last decade. Currently the 5 year survival rate for patients with no metastatic or regional disease at diagnosis (Stage 1A) is 99%, this drops to 84% if there is lymph node involvement (Stage IB-IIIC) and drops significantly to 24% if there are distant metastases at time of presentation (Stage IV) (Cancer Facts & Figures 2014, American Cancer Society). Despite modern treatment a proportion of patients will have a recurrence of their disease, this can either be locoregional recurrence (LRR) or distant metastasis. Metastatic breast cancer is often incurable, with up to 5% of patients presenting with distant metastases at time of diagnosis [2]. Currently, distant metastasis (M1) occurs in 10-15% of patients within the first 3 years and approximately 30% of women with localised breast cancer without lymph node involvement at the time of diagnosis will develop distant metastases [3]. This highlights the need to not only diagnose patients at an earlier stage, but also to monitor response to treatment. The introduction of neoadjuvant chemotherapy (NACT) has allowed an insight into the response rate of breast cancer to chemotherapy. NACT is chemotherapy given prior to surgery to reduce the size the tumour and increase the chances of the patient having breast conservative surgery (BCS), as opposed to adjuvant therapy given post surgery. Assessing the response to NACT adds an extra prognostic tool, with patients that achieved a pathological complete response (pCR) having a greater disease free survival (DFS) and overall survival rates (OS) than patients with residual invasive cancer [4]. The introduction of targeted therapy such as Trastuzumab for human epidermal growth factor receptor 2 (HER2) receptor positive cancers, resulted in both a significant reduction in mortality rates and increased pCR rates when included in neoadjuvant chemotherapy regimens. However, overall only 13% of patients will

have a pCR, which means that up to 87% of breast cancers will only have a partial or no response to neoadjuvant chemotherapy.

1.1.2 Breast cancer receptors

Breast cancer is a heterogeneous disease, where the presence or absence of receptors on the surface of the breast cancer have significant impact on prognosis and treatment options.

1.1.2.1 Estrogen and Progesterone receptor

The estrogen receptor (ER) and progesterone receptor (PR) are hormone receptors that are present on up to 60-65% of all breast cancers, and have been in clinical use for over three decades [5]. Growth of hormone receptor positive breast cancers is driven by endogenous estrogen, with only 1% of tumour nuclei positivity needed to confirm positivity of the receptor [5, 6]. Hormone receptor positive breast cancers tend to occur in post-menopausal women and tend to be slower growing, with better outcomes [7]. Hormone receptor positive breast cancers have a particular metastatic pattern, and tend to spread to bone as a first site of metastasis [8].

1.1.2.2 HER2 receptor

The HER2 receptor is one of a family of four epidermal growth factors [9]. The HER2 receptor is present on the surface normal breast cells and is responsible for regulating proliferation [9]. The HER2 receptor is over-expressed in about 20-30% of all breast cancers, resulting in increased proliferation. Roughly half of all HER2 receptor positive breast cancers are also hormone receptor positive (Luminal B HER2). Although these breast cancers have worse outcomes than breast cancers that are only hormone receptor positive, greater survival and reduced recurrences are seen, when compared to the breast cancers that are only HER2 receptor positive and breast cancers that are negative for all 3 receptors. Luminal B HER2 breast cancer subtypes also tend to have similar metastatic patterns to hormone receptor positive breast cancers, with bone being the most common first sight of recurrence. The other half of the HER2 receptor positive breast cancers are negative for hormone receptors (HER2+(non-luminal)). HER2+(non-luminal) breast cancer tend to be more aggressive than Luminal cancers, and have the highest rates of locoregional

recurrence after breast conservative surgery [10]. The HER2+(non-luminal) subtype also has a distinct pattern of distant metastasis, it tends to have first site of metastasis in visceral organs such as lung or liver.

1.1.3 Breast cancer subtypes

Currently, breast cancer can be subcategorised based on the status of hormone receptors (±) ER, PR and HER2 receptor status (Table 1.1).

Table 1.1 Breast cancer subtypes.

Breast cancer subtype	**Molecular Subtypes**		
	ER	**PR**	**HER2**
Luminal A	+	+	-
Luminal B HER2	+	+	+
HER2+(non-luminal)	+	-	+
Triple negative	-	-	-

Furthermore, molecular genetic expression profiling has enabled molecular subtyping [11] [12]. Clinically, there are 4 major molecular subtypes: ~50-60% of breast cancers are Luminal A subtype (ER and/or PR +ve, HER2 –ve), 10-20% are Luminal B HER2 subtype (ER and/or PR +ve, HER2 +ve), 15-20% are HER2+(non-luminal) (ER and PR –ve, HER2 +ve) and the remaining 10-20% are Triple negative subtype (ER and PR –ve, HER2 –ve) [13]. Identifying true Luminal B breast cancer can be difficult in the absence of doing full molecular subtyping which is impractical. Some authors use high grade and high ki67 (>14%) as a surrogate for Luminal B in patients who are HER2 negative. The four molecular subtypes have differing disease progression and survival rates with the Luminal subtypes tending to have slower metastatic spread, lower recurrence rates and better outcomes than HER2+(non-luminal) or Triple negative subtypes [8, 10, 14-17]. This difference is independent of histological subtype or time of detection, with the majority of Triple negative carcinomas detected in the early stages of breast cancer. When comparing median survival from time of first distant metastasis, Luminal A & Luminal B HER2 subtypes display longer overall survival (2.2yrs and 1.6yrs). This is compared to HER2+(non-luminal) (1.3yrs), with Triple negative subtypes having the worst overall survival rate (0.7yrs) (Table 1.2).

Table 1.2 Sites of breast cancer metastasis by molecular subtypes.

Metastasis sites	Breast cancer subtype			
	Luminal A	**Luminal B HER2**	**HER2+ (non-luminal)**	**Triple Negative**
Brain	6.6%	8.2%	23.3%	18.1%
Lungs	25.1%	29.2%	32.4%	35.4%
Bone	62.1%	64.5%	47.7%	32.2%
Liver	25.1%	26%	39.9%	23.8%
References: [8, 18-21]				

The Luminal A subtype of breast cancer tends to be low grade/stage, with a good prognosis and a significantly lower relapse rate (27.8%) which occurs later than the other subtypes [8]. Luminal B HER2 has a more aggressive phenotype than Luminal A cancers, with a slightly lower overall survival rate and higher reoccurrence rate (42.9%). Similar to Luminal A breast cancers, bone is the most common primary metastatic site. Luminal B HER2 has a higher rate of metastases to other visceral organs, such as liver, than Luminal A cancers. Increasing risk factors for Luminal breast cancers identified are oestrogen exposure, nulliparity and late age of first birth [22]. Increased mutations in the TP53 gene, a gene responsible for controlling cellular response to genotoxic damage, has been linked to poor prognosis [23]. HER2+(non-luminal) subtype breast cancers clinically have a poor prognosis but since the development of anti HER2 based treatment there has been a vast improvement in survival [24]. Triple negative subtype of breast cancer tends to have a younger onset, larger mean tumour size and higher grade [25, 26] with lowest overall survival. Studies have shown HER2+(non-luminal) and Triple negative breast cancers have increased TP53 mutations [27]. Mutations in the known early onset breast cancer risk gene, Breast Cancer 1 (BRCA1), have been linked to Triple negative breast cancers with a more aggressive disease progression [28]. Current treatments mean Luminal cancers generally have better outcomes, while the HER2+(non-luminal) and Triple negative tumours are commonly more aggressive resulting in shorter overall survival rates [8, 29-31]. Due to the wide variations in outcomes seen across the breast cancer subtypes, extensive research has concentrated on development of new treatment options for breast cancer.

1.1.4 Age and breast cancer risk

Although the lifetime risk of developing breast cancer for women is approximately 1/8, this is variable, as a women's age advances so too does the risk of developing breast cancer [138]. Due to this, screening for breast cancer has been shown to have maximal benefit in reducing mortality rates for women aged 50-75years old [145-147]. Age of diagnosis has been shown to correlate with breast cancer subtype, with over half of Luminal cancers occurring in women over the age of 60 years old, while over one third of Triple negative breast cancers occur in women under 50 years old [7]. Age of patient and their menopausal status also has been shown to impact on the effectiveness of treatment regimens. Studies have shown that better disease free survival rates (DFS) are seen in post-menopausal treated with an aromatase inhibitor (AI) when compared to tamoxifen [47], while also reducing the risk of endometrial cancer [48].

1.2 Breast cancer treatments

1.2.1 Breast cancer surgery

Surgery is the primary treatment for early stage breast cancer and when used in combination with chemotherapy, radiotherapy and hormone therapy has vastly improved survival rates [32-34]. Halsted described a radical mastectomy in 1882, which involved complete removal of the breast tissue and chest wall muscles. The Patey (simple) mastectomy replaced the more radical Halsted mastectomy in 1940s and involving mastectomy along with axillary node dissection, to clear the initial draining lymph nodes of the breast. The next advancement in breast cancer surgery was with BSC introduced in the 1980s. Studies showed that for early stage breast cancer, patients that underwent BSC has equivocal survival rates to those having a mastectomy, while having reduced complication rates [35, 36]. The next major development was the introduction of sentinel lymph node biopsy (SLNB), which provides the most accurate diagnosis of lymph node involvement [37]. Sentinel lymph nodes are the first lymph node in a tumour bed that receives lymphatic drainage. The sentinel node is identified either by injection of a blue dye into the sub-areolar region, injection of a radio isotope, or a combination of both, with the first positive lymph node in the axilla excised and sent for histology. SLNB has been

proven to be beneficial and is currently recommended in early breast cancer treatments without any clinical evidence of nodal involvement [38].

1.2.2 Adjuvant treatments

Currently for patients suitable for surgical resection of their primary tumour, a combination of adjuvant treatments are available depending on the tumour stage and subtype.

1.2.3 Hormone therapy

Hormone therapy plays a large role in treatment of patients with Luminal type breast cancer. Tamoxifen prevents estrogen binding to its receptors, resulting in its inhibition. The introduction of hormone therapy has resulted in a significant improvement in survival and reduction in recurrence rates in Luminal breast cancers, with 5 years of adjuvant tamoxifen treatment reducing annual mortality by up to 31% across all age groups [39-41]. Treatment with tamoxifen can have other effects, such as prevention of bone loss in post-menopausal women and increasing the risk of endometrial cancers, hot flushes and thromboembolic events [40, 42, 43]. As tamoxifen has some paradoxical proestrogenic effects, it has been linked with an increased risk of stroke and endometrial cancer in post-menopausal women [44]. Aromatase inhibitors are a new form of hormone therapy, which work by inhibiting the synthesis of estrogen from androgens [45, 46]. Studies have shown that aromatase inhibitors have superior DFS rates when compared to tamoxifen in post-menopausal women [47]. It reduces the risk of endometrial cancer along with vaginal bleeding, cerebrovascular events, thromboembolic events and flushes [48, 49] Due to the reduction of these risk factors, aromatase inhibitors, which block the synthesis of oestrogen, are often used instead of tamoxifen in post-menopausal women. The improved outcomes using tamoxifen and aromatase inhibitors pose a question about the use of chemotherapy for Luminal cancers. In node negative Luminal breast cancer, the Oncotype Dx test, allows identification of patients that are unlikely benefit from chemotherapy [50-52].

1.2.4 Radiotherapy

Radiotherapy can be given locally to the chest wall post-surgery or regionally at the axilla. Radiotherapy is primarily used to reduce local recurrence, with analysis of

long term randomized trials have shown that radiotherapy can drastically reduce the risk of LRR in breast cancer [53]. This analysis showed that post BCS, the addition of whole breast radiotherapy (WBRT) could reduce 5 year LRR by 19% and increase 15 year survival by 5.3%. A similar impact is seen post-mastectomy, with 5 year LRR reduced by up to 17% and the 15 year overall mortality reduced by 4.4%. Recently, the ACOSOG ZOO11 trial showed that in patients with two or less positive lymph nodes undergoing BCS with WBRT, survival was equivalent in patients who had a sentinel lymph node biopsy or axillary lymph node dissection [54].

1.2.5 Chemotherapy

Chemotherapy usually involves a combination of drugs rather than individual treatments. The most common drugs used are anthracyclines (doxorubicin & epirubicin), taxanes (paclitaxel & docetaxel), fluorouracil (5-FU) and cyclophosphamide [34]. Currently there is no evidence of benefit of one regime over another, but a meta-analysis has shown a benefit of adding taxanes to an anthracycline based regime [55]. De Laurentiis demonstrated an improvement of 5% in disease-free survival and 3% in overall survival at 5 years. Patients who are HER2 receptor positive are treated with Trastuzumab (Herceptin) a monoclonal antibody in combination with chemotherapy. Trastuzumab was original used in metastatic breast cancer where it increases median survival rates from 20.3 months to 25.1 months [56]. Further to this, when used in the adjuvant setting Trastuzumab can significantly reduce the risk of mortality [24, 57].

1.2.6 Targeted therapy

Sub-categorising breast cancers, using receptor status, molecular profiling or genetic profiling has allowed clinicians to tailor treatments to each individual. Initially, this began with the development of hormone therapy for hormone receptor positive patients, but the division of breast cancer into different molecular subtypes has further stratified treatments. The introduction of genetic profiling has also had a major impact on breast cancer treatment. The discovery of the BReast CAncer susceptibility gene (BRCA) 1 & 2 genes, has allowed the identification of genetically inherited breast cancers. Although inherited cancer only represent about 5-10% of all breast cancers, a person with a mutation in these genes will have an

82% chance of having breast cancer in their life time [58]. Recently the use of Oncotype DX for Luminal breast cancers has provided insight into response and survival post chemotherapy [59, 60]. Oncotype DX is a test that evaluates 16 cancer related genes and 5 reference genes, estimating the risk of cancer reoccurring in a place other than the breast or regional lymph nodes, within 10 years of diagnosis for women with stage I or II, node negative, ER positive breast cancer treated with Tamoxifen alone. The results are used to estimate the likely reoccurrence risk in patients and are used as a diagnostic tool to determine if a patient should have chemotherapy [51]. The recurrence score was derived from reference-normalised gene-expression measurements, and ranged from 0 to 100. This has now been prospectively validated, with patients having a score between 0-10 adjuvant chemotherapy has been shown to provide no additional survival benefit [61]. There is currently an ongoing prospective clinical trial to assess if patients with a score from 11-25 derive benefit from chemotherapy, but it now appears that patients with an Oncotype score less than 25 derive no benefit from chemotherapy. One of the greatest advancements in breast cancer treatment over the last two decades has been the discovery and the development of targeted therapy for the HER2 receptor.

1.2.7 Trastuzumab

Trastuzumab is a monoclonal antibody that binds to the HER2 receptor, reducing cell signalling and resulting in immune system activation causing increased antibody-dependent cytotoxicity [62]. It was first used for metastatic breast cancer, where it was shown to prolong survival [56]. Later studies found that adjuvant treatment with Trastuzumab could reduce the risk of mortality by up to 39% [24, 57, 63]. A meta-analysis of 11,991 patients included in randomised controlled trials comparing Trastuzumab vs standard chemotherapy regimens showed significant improved DFS and OS in the Trastuzumab group [64]. The long term benefit of Trastuzumab has also been shown, as it can increase 10 year OS from 75.2% to 84% and increased 10 year DFS rates from 62.2% to 73.7% [65]. Despite both Luminal B HER2 and HER2+(non-luminal) subtypes having distinct patterns of distant metastasis, to date, no studies has investigated the impact of Trastuzumab on these metastatic patterns. In addition, to date, no study has fully investigated if the introduction of Trastuzumab has resulted in differing survival patterns in patients who were endocrine receptor positive or negative.

Development of newer monoclonal antibodies has also shown promise, by targeting different sections of the HER2 receptor. New drugs such as Lapatinib and Pertuzumab can improve response and reduce resistance [66, 67]. The introduction of targeted therapy has resulted in significant improved overall survival in breast cancer, however, these new therapies have resulted in an increased financial cost in the treatment of breast cancer.

1.3 Neoadjuvant chemotherapy

1.3.1 Role of Neoadjuvant chemotherapy

The aim of NACT is to shrink or in some cases complete eradicate the breast cancer, thus increasing the number of patient suitable for BCS. NACT also provides information on the response to chemotherapy. Patients with pCR to NACT have a better DFS and OS, with the greatest correlation seen in more aggressive tumours. Response to NACT varies greatly across the breast cancer subtypes, with the lowest rates seen in Luminal A breast cancers and the highest seen in Triple negative and HER2+(non-luminal) subtypes. Further to this, variations are seen in survival across the breast cancer subtypes that achieve a pCR. With HER+(non-luminal) subtype achieving pCR having a greater association with an increased DFS and OS when compared to the Luminal subtypes. Surprisingly, despite HER2+(non-luminal) subtype and Triple negative subtypes of breast cancers having the high pCR rates, these cancers have the poorest survival rates [68, 69]. This is due to the extremely low survival rates of the patients that do not respond to NACT.

1.3.2 Neoadjuvant Trastuzumab

Recently, NACT studies have shown that the addition of Trastuzumab to HER2 receptor positive breast cancers increases pCR rates [70-72]. A pooled analysis of NACT trials has shown that 30.9% of Luminal HER2 breast cancers and up to 50.3% of HER2+(non-luminal) patients have a pCR [4]. This analysis also showed that patients with a pCR response had a significantly improved disease free and overall survival rates compared to patients who did not have a complete response. More recently, the impact of combining a second anti HER2 agent such as Pertuzumab with Trastuzumab in the neoadjuvant setting has been shown to significantly improve pCR rates [73]. However, at five years follow up, the disease

free and overall survival between the patients that received combination compared with Trastuzumab alone showed large and overlapping confidence intervals [74].

1.4 Economics of breast cancer treatment

1.4.1 Variations in breast cancer cost

Breast cancer treatment has varied significantly over the last few decades and this has had a major impact not only on survival, but also the cost of treating breast cancer patients. The introduction of BCS, SLNB, use of neoadjuvant therapy to facilitate tumor cytoreduction and enhanced recovery programs has resulted in a large increase in the proportion of breast cancer surgeries not requiring overnight hospital stay (day case surgery). It has also resulted in reduced surgery time, resulting in an increased number of cases being performed. Overall, this has led to a reduction in the cost of treatment. There has also been multiple new factors introduced with some associated increased cost. These include breast reconstruction post mastectomy which is becoming more widely available, and this has significant psychological benefits for patients. Due to the significant lifetime risk of breast cancer for patients with the BRCA 1 and 2 mutations, there is also an increasing proportion of women undergoing prophylactic mastectomies [75]. There are multiple forms of breast reconstruction, from the use of implants and expanders, to the use of muscle flaps. Each option has increased financial impacts, from increased length of stay, higher risk of complications and the actual cost of implants. Another major impact on the cost of treatment is the use of Oncotype DX testing in node negative Luminal A breast cancers. Patients with a score from 0-18 no longer receiving adjuvant chemotherapy reducing the cost, however, each test costs $4,175. Another major impact on the cost of treatment has been the introduction of new targeted therapy, including anti HER2 receptor therapy. Adjuvant therapy with new monoclonal antibodies such as Trastuzumab and Pertuzumab has added thousands of euros to the cost of treating patients.

One of the ways of assessing the overall benefits of any new therapy, is to examine both the improved outcomes and the financial cost together. This is the objective of a cost effectiveness analysis.

1.4.2 Cost effectiveness analysis

As per World Health Organization (WHO) guidelines cost effectiveness is defined as "The estimated cost-effectiveness of a single proposed new intervention is compared either with the cost-effectiveness of a set of existing interventions reported in the literature or with a fixed price cut-off point representing the assumed social willingness to pay for an additional unit of health". The key aim of a cost effectiveness analysis is not only to assess if a new intervention has an improved patient outcome but that it is also financially viable to introduce. One of the methods of assessing effectiveness is to use quality adjusted life years (QALY). QALY is a generic measurement of disease burden including quality and quantity of life, with one QALY equating to one year of perfect health. For a cost effectiveness analysis, a price per QALY of a new intervention can be calculated and compared to current practice. A study looking at the use of QALY in patients treated with adjuvant Trastuzumab designed a utility scoring system. Treatment with adjuvant Trastuzumab was assigned a utility of 0.85, and a utility of 0.55 was assigned to breast cancer recurrence [76]. The incremental cost-effectiveness ratio (ICER), is calculated by adding average cost, making it possible to calculate the cost per QALY.

1.4.3 Cost of targeted treatments

The introduction of Trastuzumab resulted in significant improvement in survival and reduced recurrence rate in HER2 receptor positive breast cancer. However, it resulted in a substantial increase in the cost of treating patients [76]. For patients with early breast cancer, current recommendations for treatment is a regime of Trastuzumab used in combination with other chemotherapy, followed by 52 weeks of maintenance oral Trastuzumab. This treatment can add up to an extra €25,000 to the cost of treating each patient. Further to this Trastuzumab is used in the treatment of metastatic breast cancer and for recurrence of breast cancer, resulting in long term Trastuzumab therapy, which can have a significant financial burden [77]. Along with this, there is a proportion of patients treated with Trastuzumab that receive no benefit, with adjuvant studies showing up to 7.5% of patients having a recurrence in the first year alone [24]. There are also HER2 receptor positive breast cancer patients that have long term survival without Trastuzumab treatment. In prospective studies, while Trastuzumab significantly improved DFS, up to 67.1% of patients not receiving Trastuzumab were disease free at four years follow up [57].

A few studies have looked at the cost effectiveness of Trastuzumab introduction in the adjuvant setting. Trastuzumab, while increasing costs does have an ICER comparable to other widely used interventions, costing approximately $34,201-$39,982 and would be cost effective over a lifetime [76, 78, 79]. Trastuzumab treatment has also been shown to significantly reduce recurrence in HER2 receptor positive breast cancers. As a result, there is a reduction in the number of patients requiring further interventions, saving both cost and resources.

Trastuzumab used in the neo-adjuvant setting could reduce cost overall, due to the increased number of patients undergoing BCS. To date no study has assessed the cost effectiveness of neoadjuvant Trastuzumab compared to adjuvant Trastuzumab.

1.5 Prognosis in HER2 receptor positive breast cancers

1.5.1 Staging

Currently, breast cancer staging is based on the TNM staging, where T describes the tumour size, N defines the lymph node status (+/-) and M relates to any distant metastases (0/1) (Figure 1.1). Using TNM breast cancer can be also staged in groups, from stage I-V, depending on size and metastatic spread. Significantly, this staging is used to inform the choice of treatment regime. A major factor impacting survival is recurrence of breast cancer, as either distant metastasis or LRR.

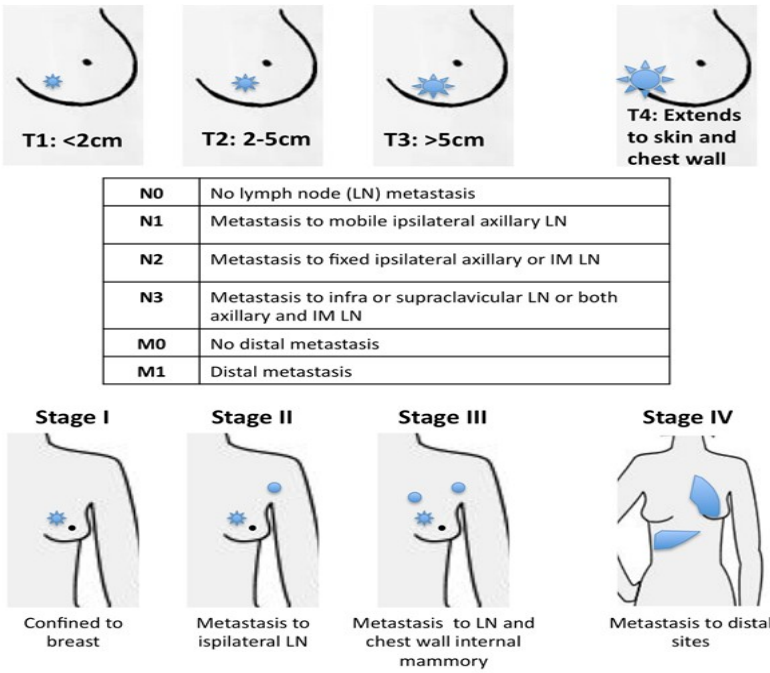


Figure 1.1 Breast cancer staging (TNM classification). T= tumour size; N= nodal status; M= metastasis.

1.5.2 Metastatic breast cancer

Breast cancer metastasises though the lymphatic system or via the circulatory system and is the overwhelming cause of mortality in patients with a malignancy, causing 90% of deaths in solid tumours [80]. Two forms of metastasis exist, firstly, LRR which is recurrence in the chest wall or the regional lymph nodes, the other is distant metastasis which in breast cancer is characterised by a distinctive spread via lungs, liver, brain and bones [81]. The rates and sites of distant metastasis can vary depending on age and stage of diagnosis [82, 83]. Approximate sites and rates at presentation are shown (Figure 1.2 A), with bone being the most common site of metastases and is often the first site of distant metastases in up to 50% of patients [84]. The second and third most common metastatic sites of breast cancer are lung and liver. Significantly, 10-15% of patients will develop brain metastases, making it the second most common source of brain metastasis [85]. Similar distribution of metastasis is seen in relapse after adjuvant treatment, with 21.9% of patients having multiple sites of metastasis at relapse (Figure 1.2 B) [18, 86].

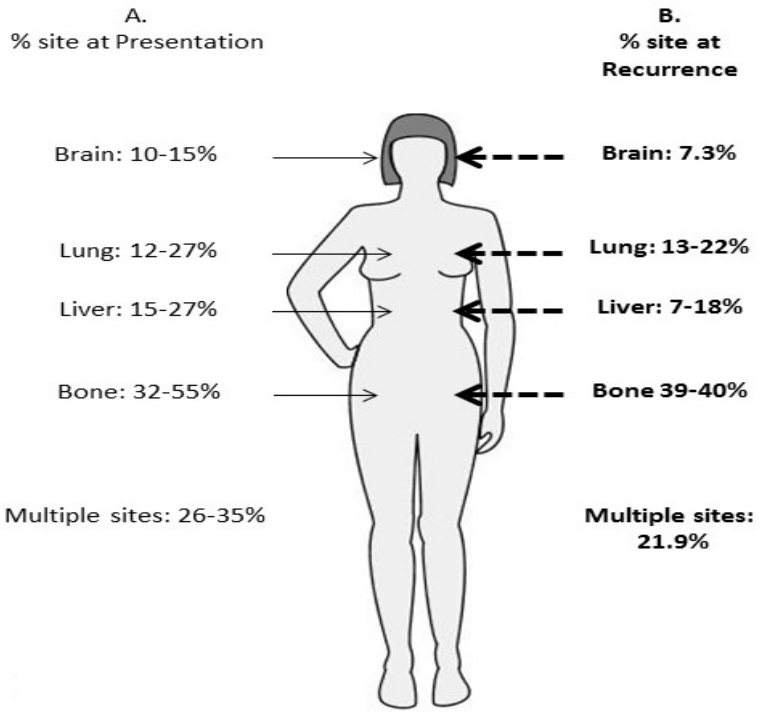


Figure 1.2 Rates of metastasis A) at presentation and B) at recurrence

Investigating metastasis by the four molecular subtypes, different patterns of metastases are seen. Bone metastases remain the most common metastatic site in Luminal A (18.7%), Luminal B HER2 (30.4%) & HER2+(non-luminal) (30.1%) breast cancers [8, 18], however Triple negative cancers primarily metastasized to the lungs (18.5%) [20]. Interestingly, Luminal cancers also tend to have a lower rate of brain metastases. While the HER2+(non-luminal) subtype retains a high rate [21], it is likely that the implementation of Trastuzumab based treatments for HER2+ receptor breast cancers in the late 00's has unduly influenced this, as Trastuzumab is not expected to cross the blood brain barrier.

1.5.3 Locoregional recurrence

One of the difficulties in assessing the impact of different variables on recurrence rates, is that recurrence occurs in very low numbers [10]. This makes it difficult to get a significant result from an analysis from a single study or centre, this is why researchers use a specific type of review called a systematic review to answer specific questions. Systematic reviews are a type of literature review that collects and critically analyses multiple research studies or papers, and is one of the highest levels of evidence. The analysis of the results of these papers can be used to answer specific questions. This type of review is important as it allows analysis of multiple papers, increasing the number of patient's data that can be analysed which reduces the risk of the results being due to chance. To perform a systematic review a specific question must be first be chosen, and an online search is then performed for all relevant papers to this question by searching for publications containing keywords related to the question. Papers are then extracted that match certain criteria, with the relevant data such as breast cancer subtype or LRR rate then extracted. This relevant data from all the studies can then be combined to answer selected questions. Using a systematic review allows new research questions to be answered. Due to the low LRR rates little research has been done to assess the LRR rates in different breast cancer subtypes, specifically the impact of targeted therapy for HER2 receptor positive breast cancers has yet to be assessed.

1.5.4 Discordance between primary and breast cancer recurrence

One of most significant developments in breast cancer treatment over the last few decades, has been the discovery that a recurrence of a breast cancer maybe a

different subtype from the primary breast cancer. This discordance in subtype can occur in over 20% of cases, and can have a significant impact on treatment [87, 88]. Studies have shown that by taking a biopsy of recurrence, it will result in a treatment change for every 1/7 patients, however, debate remains over the impact on overall survival [89]. When assessing for discordance by receptor type, significant variation is seen between the hormone receptors and the HER2 receptor. The highest rates of discordance are seen in the progesterone receptor, where discordance can occur in 31.2% of cases, while rates of up to 12.6% are seen in oestrogen receptors [88]. In the HER2 receptor low rates of only 5.5% discordance are seen, and this drops to 4.6% when assessing patients that gained HER2 status. This raises the question of what impact HER2 receptor discordance has on changing breast cancer subtype. This can impact both variation in treatment and survival, also the percentage of patients that require a biopsy to change treatment.

1.6 Monitoring response to treatment

1.6.1 Biomarkers

One of the main focuses in breast cancer research is to develop markers which will predict which patients will respond to treatment. One of the most promising new methods is the use of biomarkers, which are naturally occurring molecules, genes, or characteristics by which a particular pathological or physiological process, disease, etc. can be identified. Biomarkers can be both invasive (requiring a biopsy of tissue) or non-invasive (in circulation). An ideal biomarker, is specific, sensitive and robust and non-invasive, however, the heterogeneity of breast cancer makes finding an ideal biomarker extremely difficult [90].

Biomarkers have emerged as a possible new way of detecting, monitoring treatment and diagnosing recurrence in breast cancer. There are numerous biomarkers already used in clinical practice which have significant impact on prognosis and treatment selection. Breast tissue markers such as ER, PR and HER2 receptors provide sites for targeted therapies and their positivity impact survival and recurrence rates. More recently, the Oncotype DX gene panel analysis of breast tissue provides accurate assessment of survival in Luminal breast cancers and dictates patient selection for chemotherapy. However, these biomarkers all require an invasive and imaging

guided biopsy, so one of the major aims in breast cancer research is to identify a non-invasive biomarker identifiable in the circulation. A number of possible circulating biomarkers for breast cancer have been identified such as telomeres, free DNA and microRNA. Previous research from our institute has shown that microRNA are altered in breast cancer and may also be used to identify different breast cancer subtypes [91-93].

1.6.2 MicroRNA function

MicroRNA's (or miRNA) were originally discovered the early nineties in caenorahabditis elegans [94]. MicroRNA's are a 19-25 long class of small non-protein coding RNA that function as gene regulators by inhibiting the degradation of their target mRNAs and inhibiting translation. MiRNA have been demonstrated to be involved in cell development, differentiation, proliferation and apoptosis [94]. MiRNA's are formed from precursors called pri-miRNA which are processed in the nucleus by Drosha, an RNA III type nuclease. These pri-miRNAs are transported to the cytoplasm by exportin-5, where they are cleaved by Dicer, another RNAse III enzyme, forming an asymmetric duplex (miRNA:miRNA). This duplex is then incorporated into miRISC complex, where one strand becomes active and the other is released and degraded [94, 95] (Figure 1.3).

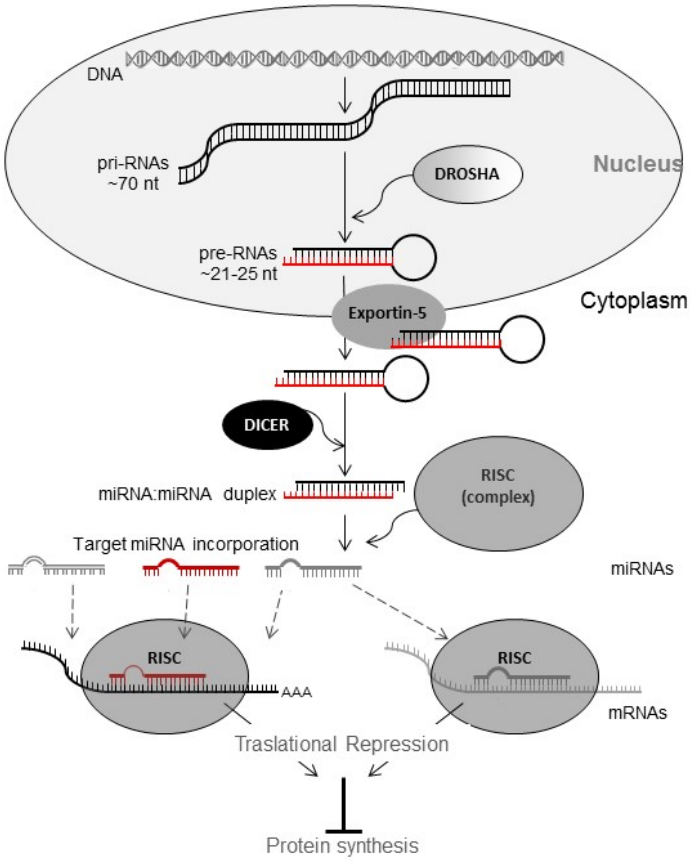


Figure 1.3 MicroRNA formation pathway.

The first disease related human microRNA was characterised in chronic lymphocytic leukaemia [96], with circulating miRNA later found in diffuse large B-cell Lymphoma [97]. Since then >2,000 microRNA's have been identified in humans, regulating approximately 30% of genes [98]. MicroRNAs can exert their action in cancers through tumour suppression and oncogenic mechanisms [94]. Fragile sites and genomic regions involved in oncogenic rearrangements in cancer are thought to partially influence cancer related miRNA [99]. MicroRNA's have been implicated in promoting cancer metastasis and may provide a diagnostic target for predicting metastasis [100]. MicroRNAs are excellent biomarker targets due to their stability in blood and ability to withstand repeat freezing and thawing cycles [101].

There are a number of limitations to using microRNA as biomarkers that have restricted their use in the clinical setting to date. MicroRNA expression can vary depending on source material. Studies analysing expression have used whole blood, serum and plasma and this may lead to differences seen across the studies [102]. The

way microRNA is extracted from samples also varies across studies, which may have resulted in discrepancies. The introduction of automated systems to extract microRNA should result in more standardized extraction process. A further limitation in the translation of results into the clinical setting, is the large variations in individual factors. Patients will often have multiple medical co-morbidities that can have a significant effect on microRNA expression levels, along with this patients can be on systemic therapy or other medication that can also impact expression levels.

1.6.3 MicroRNA in breast cancer and neoadjuvant chemotherapy

Over the last few years microRNA's have been proposed as a possible clinical biomarker for many cancers, including breast cancer [92, 103, 104]. A number of specific microRNA have been linked with the detection of breast cancer and recurrence, such as microRNA-21, microRNA-155 and microRNA-195, [91, 105-107]. Originally, microRNA identified were intracellular from breast cancer tissue specimens, but extracellular microRNA identified in the circulation have been shown to differentiate between breast cancer patients and healthy controls [92]. Not only have microRNA been shown to be a possible biomarkers for breast cancer detection, but studies have shown microRNA can be used to predict response to treatment [106]. This raises the possibility of the use of microRNA as a predictor to response to neoadjuvant chemotherapy. Studies have looked at the role of microRNA in predicting chemotherapy resistance [108, 109], but little work has been done to assess if circulating microRNA can predict which patients will respond to neoadjuvant chemotherapy.

1.6.4 Role of microRNA in cancer metastasis

The process of breast cancer metastasis follows a cascade starting with local invasion of the surrounding tissue. This is followed by spread into blood or lymphatic vessels, ending with dissemination of tumour cells to distant organs. The tumour cells then arrest within the blood vessels of the target organ and extravasate into the surrounding tissue and start proliferating [110, 111] (Figure 1.4). MicroRNAs have been linked to all stages along the metastatic cascade [112-115]. By identifying specific miRNAs involved in metastases, it is proposed that both prognostic and

therapeutic markers can be identified [116]. Significantly, it has been demonstrated that restoring expression of certain miRNAs *in vivo* can suppress metastasis [117, 118]. The existence of cancer stem cells has also been put forward as a mechanism of metastasis [119, 120].

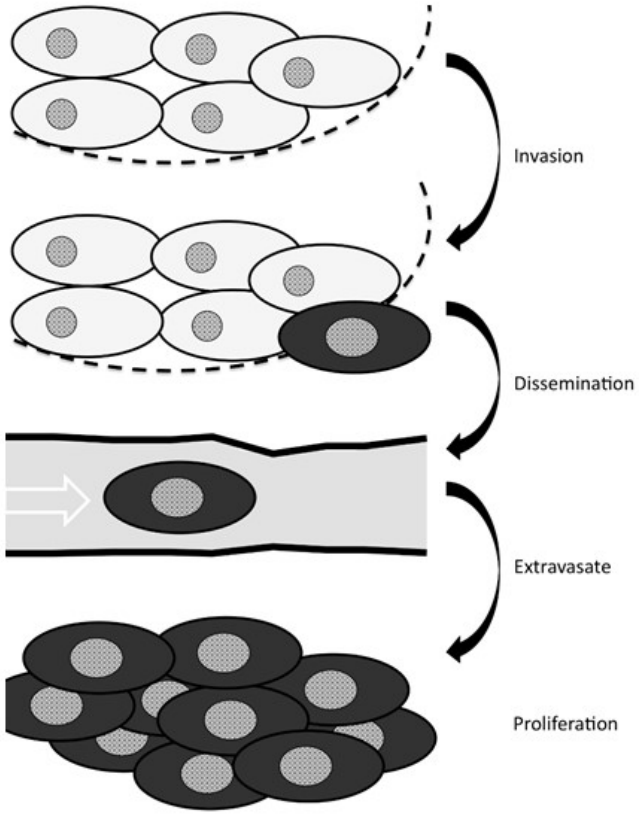


Figure 1.4 Breast cancer metastasis cascade.

1.6.5 MicroRNA as biomarkers in metastatic disease

Identifying circulating miRNA to use as biomarkers for metastatic breast cancer is currently a key priority. The first microRNA described in metastatic breast cancer was miR-10b [121]. In this study it was shown miR-10b was highly expressed in metastatic breast cancer using cell lines in both mouse and human models. Variation in microRNA-10b expression is also seen in patients with metastatic breast carcinomas. The levels of miR-10b were found to be elevated along with two other microRNAs miR-34a and miR-155 in patients with metastatic breast cancer [103]. Recently, supporting this a study demonstrated increased miR-10b and miR-373 in lymph node positive breast cancer cases [122]. Excitingly, it demonstrated a significant increase in both microRNAs in patients with positive lymph nodes when

compared to patients with no nodal involvement and healthy controls. This highlights the importance of biomarkers that can discriminate non-metastatic from metastatic breast cancer. Interestingly, miR-10b was identified as a potential biomarker for brain [123] and bone [124] metastases in breast cancer. Together, these results cast doubt on miR-10b as a metastatic specific marker, but this variation maybe due to different breast cancer subtypes and treatment.

MiR-299-5p and miR-411 were found to have significant differences in the expression in breast tumour tissue, compared to miR-299-5p and miR-411 levels in healthy controls [125]. In addition, miR-21 has been identified as a marker for breast cancer and predictor of stage [126]. Recently, eight circulating miRNA (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375 & miR-801) were found to be significantly higher in patients with circulating tumour cells (CTC) [127]. In another study, higher levels of miR-105 were found in early breast cancers that metastasised when compared to cancer that did not [128]. This study also found that over expression of miR-105 was shown to promote metastasis in-vivo. The sites of metastases are not stated, this may further account for the different microRNA identified. Significant further research is needed to confirm which microRNA can predict site-specific metastasis disease outcome or patient response to treatment [129]. Specifically, translation of basic research into clinical trials is needed to validate these findings.

1.6.6 MicroRNA and breast cancer subtypes

A previous publication from this institute, found no significant correlation between a group of target microRNA and the four intrinsic breast cancer subtypes [92]. In a number of studies microRNAs identified in the tissue of breast cancer patients have been shown to be subtype specific [93, 130, 131], however, to our knowledge no study has identified a circulating microRNA that can distinguish the different breast cancer subtypes. Although circulating microRNA have not been shown to identify the different breast cancer subtypes, a number of studies have shown correlation with hormone and HER2 receptor status. Circulating levels of microRNA-21 and microRNA-10b were found to be elevated in ER negative breast cancers [92], this result was not replicated in the study from our institute, where only microRNA-195

was shown to have significant variation between ER positive and negative breast cancers. Another study by Eichelser et al, found that increased concentration of microRNA-373 was associated with negative HER2 receptor status [132]. In evaluating the results of a microarray analysis of plasma samples from 111 breast cancer patients, another study revealed that expression levels of microRNA-130a and microRNA-146a could differentiate HER2 positive and negative breast cancers, while microRNA-107 levels were elevated in ER negative breast cancers.

1.7 Clinical trials and Translational research

1.7.1 Levels of evidence

One of the greatest challenges in breast cancer research, is translating laboratory results into clinical practice. To ensure accurate results, clinical trials must generate the highest level of evidence possible. There are currently five levels of clinical evidence (Table 1.3), these range from a meta-analysis of randomized controlled trials, which provides the highest level of evidence, to the lowest level available, expert opinion. Due to the difficulties in setting up randomized controlled trials, such as cost, length of time and recruiting patients, a large proportion of initial studies are retrospective in nature. This can result in disparity between studies, as retrospective studies are at risk of a number of biases. To ensure the accuracy of the results the highest standards of research needs to be applied in both the laboratory and the clinical setting.

Table 1.3 Levels of clinical evidence.

Strength	Level	Design	Randomization	Control
High	Level 1	**Randomized control trial (RCT)**	**Yes**	**Yes**
		Meta-analysis of RCT with homogeneous results	**No**	**NA**
	Level 2	**Prospective comparative study (therapeutic)**	**No**	**Yes**
		Meta-analysis of Level ½ studies with inconsistent results	**No**	**NA**
	Level 3	**Retrospective Cohort Study**	**No**	**Yes**
		Case-control Study	**No**	**Yes**
		Meta-analysis of Level 3 studies	**No**	**NA**
	Level 4	**Case series**	**No**	**No**
Low	Level 5	**Case report**	**No**	**No**
		Expert opinion	**No**	**No**
		Personal Observation	**No**	**No**

1.7.2 Methods of microRNA detection

Advances in research and technology, has led to newer, fast and more accurate ways of identifying potential biomarker. From the initial discovery of microRNAs, thousands of new microRNAs have been identified [133, 134]. The increasing number of microRNAs can however, add difficulty in analysing which microRNAs are dysregulated in breast cancer. One way to try identify microRNAs altered in breast cancer, is to perform an array of all known microRNAs, such as the Exiqon microarray. This involves taking a blood sample from patients with breast cancer and healthy controls and analyzing all known microRNAs to identify any significant variations. Microarrays can also provide another avenue of stratifying breast cancer subtypes, by comparing expression levels of microRNA across known breast cancer subtypes.

1.7.3 Biomarker validation

The next step after identifying a new potential biomarker is to validate these results firstly in vitro and then in vivo. It has been shown that recurrent genomic and transcriptomic characteristics in breast cancer cell lines, mirror those of breast cancer tumours [135]. This makes analysis of microRNA expression in breast cancer cell lines, an important tool in discovery of potential biomarkers. In addition to this, breast cancer cell lines can be used to predict response to therapies, and variations in microRNA expression level after treatment may predict which patients will respond to treatment [135, 136]. By analyzing expression levels before and after treatment, it may be possible to identify microRNAs that can predict which breast cancer patients will be sensitive and resistant to chemotherapy.

1.7.4 ICORG 10-11 clinical trial

Following ethical approval, a blinded, multicentre, prospective clinical trial was set up (ICORG 10-11 cohort 1 study), with 124 consecutive patients recruited. Patients with a histologically confirmed new diagnosis of breast cancer, who were counselled to undergo NACT were included. Clinicopathogical details were obtained and recorded in a perspective database, with all patient details blinded from both investigators and research staff. All patients included were aged 18 years or over, and patients were able to give written informed consent. Patients with distant metastatic disease at time of presentation were excluded. The primary aim of this study was to identify specific microRNAs ('signatures') which are associated with breast cancer intrinsic subtypes. The study also sought to identify if microRNA, detectable in the circulation, which are altered in breast cancer patients can predict response to NACT and to assess if there was a breast cancer subtype specific response to NACT.

1.8 Key Questions

Breast cancer is a heterogeneous disease, with each patient having different treatment, survival and outcomes. By exploiting these differences it may be possible to identify which patients benefit from specific treatments, leading to individualised medicine. Over the next few chapters the differences between breast cancers in the clinical setting are investigated further, along with laboratory analysis of breast

cancer cell lines and bloods from breast cancer patients to try identify novel biomarkers such as microRNAs. A number of key questions were identified prior to starting.

In the next chapter, a review was performed of the literature to assess the effects of age and subtype itself on detection and management of breast cancer.

In chapter 3, over four sections it was determined

a) Chapter 3.1: Was there a different impact on survival, recurrence rates and recurrence patterns between the two HER2 receptor positive breast cancers following the introduction of Trastuzumab?
b) Chapter 3.2: In the Trastuzumab era, do differences remain in LRR rates between the four breast cancer subtypes and is this impacted by type of surgical treatment?
c) Chapter 3.3: What are the discordance rates between primary breast cancer and breast cancer recurrence in the four breast cancer subtypes and how does this impact treatment?
d) Chapter 3.4: How does the use of neoadjuvant Trastuzumab chemotherapy impact cost effectiveness, and does this vary between the two HER2 receptor positive breast cancers?

In the fourth chapter, using the differences identified in earlier chapters, the following question were addressed.

a) Chapter 4.1: Using a microarray analysis of bloods from breast cancer patients, could a microRNA be identified that could be a biomarker for HER2 receptor positive breast cancers?
b) Chapter 4.2: Using the panel of microRNA, could differences in expression be identified between the four breast cancer subtypes using in-vitro experiments?
c) Chapter 4.3: How would treatment with Trastuzumab effect expression of microRNA in two HER2 receptor positive breast cancers?

In the final chapter, three important questions are addressed

a) Chapter 5.1: Can a panel of microRNA identify which patients will respond to NACT?
b) Chapter 5.2: Can a panel of microRNA identify the four breast cancer subtypes?
c) Chapter 5.3: How does the response to NACT, survival and microRNA expression differ between the two HER2 receptor positive breast cancers?

Chapter 2

Effects of age on the detection and management of breast cancer

Published sections

Effects of age on the detection and management of breast cancer
McGuire A, Brown JAL, Malone CA, McLaughlin R, Kerin MJ
Cancers (Basel). 2015 May 22:7(2):908-29. doi: 10.3390/cancers7020815.

2.1 Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer deaths in women worldwide, accounting for 23% of total cancer cases and 14% of all cancer related mortalities [2]. Currently, the lifetime risk of developing breast cancer for women is 1/8. However, >40% of the affected patients are currently >65 of age and remarkably, this group accounts for almost 60% of the total deaths from breast cancer [137, 138]. Interestingly, before 49 years of age the estimated risk of developing breast cancer is 1/53 however, this rises to 1/43 for 50-59 years old and rises again to 1/23 for 60-69. Significantly, for women aged >70 this risk is the highest with a 1/15 chance of developing breast cancer [138].

The number of elderly patients with breast cancer is due to rapidly increase in the near future, as more than 20% of the population are expected to be >65yrs old by 2030 [139]. Furthermore, improvements in disease screening and diagnosis mean increasing numbers of the population have breast cancer detected and at an increasingly younger age. Together, these trends are resulting in a greater number of, often elderly, patients requiring long-term treatment or management of breast cancer. The aim of this chapter is to examine the effects of modern investigations and treatment options for breast cancer across age groups and to address how this may influence changes in future research and treatment options for patients.

2.2 Population based screening and age

Currently breast cancer screening programs are running in >26 countries across the world (Table 2.1, 25 countries shown), though debate remains over the efficacy of some of these programs, what sections of the population should be screened and at what age the screening should be performed [140-144]. The introduction of early detection breast cancer screening programs has resulted in increased breast cancer detection rates for all age groups. Numerous studies investigating the benefits of screening programs have demonstrated a reduction in mortality rates, with maximal benefit seen in women aged 50-70 years [145-147].

Based on current evidence, full field digital mammography (FFDM) is the gold standard for breast cancer screening [148]. Current clinical recommendations from the U.S. Preventive Services Task Force are: biennial screening for all women aged 50-75 years old [149]. Some debate remains as to whether screening should continue past 70 years of age. However, comparison of screened versus non-screened breast cancer patients >70 years old shows a significant advantage for the screened cohort, with breast cancer diagnosed at an earlier stage, leading to a considerably reduced mortality rate [150, 151]. However, it has been suggested that the reduced mortality could be due to improved adjuvant treatment [152-154]. Currently, there is insufficient data available at present to make a formal recommendation [155, 156]. Due to the benefits observed from screening programs many countries have increased the age range of patients screened, with the United Kingdom having extended its program to cover women aged 45-73 in 2016. However, the particulars of screening remains controversial, with the best results observed using double reading and two projections [142, 143]. While mammography has an overall sensitivity of ~79%, this is reduced in younger women and women with dense breast tissue [157-159] (Table 2.2). Newer imaging techniques have emerged over the last few years such as tomosynthesis, contrast enhanced spectrum mammography and automated whole breast ultra-sound [160-162]. As yet, there is still insufficient data on these new techniques to change current practices.

Table 2.1 Countries with breast cancer screening programs.

Country	Screening introduced	Ages Screened	Interval (Yrs)	Population screened (annually)
Australia	1991	40-75+	2	1,700,000*
Canada	1988	50-69	2	196,187
China	2009	40-59	3	1,200,00
Denmark	1991	50-69	2	275,000
Finland	1987	60-64	2	N/A
France	1989	50-74	2	2,343,980
Iceland	1987	40-69	2	20,517
Israel	1997	50-74	2	220,000
Italy	2002	50-69	2	1,340,311
Japan	1977	40-75+	2	2,492,868
Korea	1999	40-75+	2	2,602,928
Luxembourg	1992	50-69	2	14,586
Netherlands	1989	50-74	2	961,786
New Zealand	1998	45-69	2	211,922
Norway	1996	50-69	2	199,818
Poland	2006	50-69	2	985,364
Portugal	1990	45-69	2	100,364
Rep of Ireland	2000	50-64	2	28,794
Saudi Arabia	2007	40-64	2	6,200
Spain	1990	45-69	2	527,000
Sweden	1986	40-74	2	1,414,000
Switzerland	1999	50-69	2	60,700
United Kingdom	1988	50-69	3	1,957,124
United States of America	1995	40-75+	1-2	416,000
Uruguay	1990	40-69	1	352,000

*50-69 year olds

Table 2.2 Breast cancer screening programs and detection rates.

DIGITAL MAMMOGRAPHY			MAGNETIC RESONANCE IMAGING	
Age Group	**Sensitivities** [157, 158, 163]	**Clinical Guidelines**	**Sensitivities** [164-166]	**Clinical Guidelines**
<40	Between 54-77%	Family history of breast cancer	71.1-77.3%	Familiar history of breast cancer
40-49	Between 77-86%			
50-59	Between 78-93%	Biennial screening (between ages of 50-75)		Biennial screening (between the ages of 50-75)
60-69	Between 78-94%			
>70	Between 81-91%			

As the sensitivity of mammography is reduced in younger women [157], it is currently recommended that high-risk patients have yearly magnetic resonance imaging (MRI) and mammograms (alternating every 6 months), which has been shown to increase detection rates [164, 166-168]. Specifically related to age, a 77% sensitivity was observed in 35-55 year old women [164]. However overall, the sensitivity of MRI ranges between 71-77.3% in breast cancer detection, however this can be increased to 94% when combined with mammography [164-166]. Based on the figures presented (Table 2.2) digital mammography is the superior method for detecting breast cancer, except in young patients where there is a high degree of variability. This is reflected in clinical practice where digital mammography is the most commonly used diagnostic tool.

Screening of high-risk patients with MRI does have drawbacks, as MRI is time consuming, expensive (compared to mammography alone) and has a lower specificity (resulting in a large number of benign biopsies) [164, 166, 169, 170].

While screening may detect cancers in high-risk age groups, patients with a family history of breast or ovarian cancer are at a higher-risk than the general population. The physical screening programs has been augmented by the recent advances in genetic analysis. Over the last decade the discovery of genetic testing for breast cancer susceptibility genes (such as BRCA1 & 2) has seen a rise in preemptive screening in many countries, particularly where a strong family history of breast cancer has been observed [171, 172] (Figure 2.1).

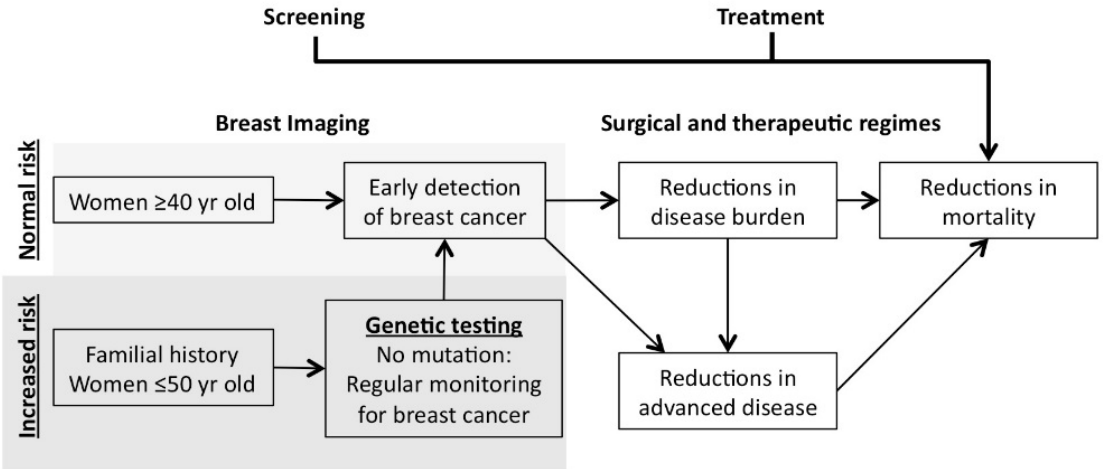


Figure 2.1 Overview of current breast cancer screening practices.

A significant amount of research conducted in the last 20-30 years has been dedicated to expanding our knowledge of the underlying molecular mechanisms and genetic risk factors influencing breast cancer susceptibility and development [28, 173]. Significantly however, very little research has focused on the effects of age on these molecular mechanisms. In this context, this research is further complicated by the additional factors such as reproductive status, menarche and menopause, which can be difficult to mimic in a research setting [141].

Moreover, many clinical trials have evaluated new diagnostic tests and treatment options for breast cancer. However, many randomized clinical trials investigating breast cancer used patients from younger age groups [174-176]. While management of younger patients has been greatly investigated, the treatment options for older patients remain largely a clinical-based decision. Often this is related to the stage of disease and the patient's general health [177-179]. Some key questions which remain

to be answered are: Do treatment options affect breast cancer patients survival based on age at diagnosis? and Is breast cancer subtype affected by age?

2.3 Breast cancer subtype risk and detection age

Molecular profiling has resulted in breast cancer being divided into 4 main subtypes, defined by differing expression levels of the estrogen receptor, progesterone receptor and HER2 receptor. The subtypes are: Luminal A (ER and/or PR+ve/HER2-ve), Luminal B HER2 (ER and/or PR+ve/HER2+ve), HER2+(non-luminal) (ER-ve and PR-ve/HER2+ve) and Triple negative (ER-ve and PR-ve/HER2-ve) [11, 180]. Luminal cancers are most common breast cancer seen (70-80%) followed by HER2 over-expressing (10-20%) and approximately 10% are Triple negative cancers [181, 182]. Currently, the incidence of each molecular subtype has been demonstrated to vary by age group (summarized in Table 2.3, [7]). Recently, molecular testing of breast cancer has further confirmed these trends [182].

Table 2.3 Breast cancer risk: by molecular subtype and age.

Breast cancer molecular subtype	**Age Group**					**Lifetime risk** (by subtype)
	<40	**40-49**	**50-59**	**60-69**	**>70**	
Luminal A	2.9%	14.2%	28.3%	**31.9%**	**22.7%**	6.79% (Luminal A & B)
Luminal B	8.1%	20.7%	32.4%	20.8%	17.9%	
HER2	5.5%	16.3%	31.6%	28.8%	17.8%	1.78%
Triple Negative	**10.8%**	**26.5%**	**35.0%**	17.5%	10.1%	1.2%

Perhaps unsurprisingly, the under 40 age group, the highest proportion per subtype was in the more aggressive triple negative breast cancer subtype (10.8 % incidence rate, at an almost ~2 fold higher risk than the next most common subtype HER2) (Table 2.3, bold text). Interestingly, this trend continues until the age of 60, however from the age of 60 onwards Luminal A has the highest incidence rate (Table 2.3, bold text). In the Luminal A breast cancer subtype, the lowest rate of diagnosis is

seen in the under 50's (Table 2.2), although this is strongly influenced by the very small numbers of breast cancers seen in these age brackets (~7% to the total cases) [1, 183]. In the 50-59 age bracket, 35% of Triple negative breast cancers occurs, while Luminal A breast cancer has the lowest rate of rate between the subtypes (28.3%) at this age, however there is an almost equal chance of developing any of the subtypes. Currently, in the 60-69 and >70's age groups the breast cancer subtypes with highest rate is in the Luminal A subtype (31.9% and 22.7%) with Triple negative now the least common subtype observed (17.5% and 10.1%). As expected, the most common form of breast cancer observed by lifetime risk is the Luminal subtype (6.79%) with triple negative the least common (1.2%) (Far right column) [184].

Notably, breast cancer survival is strongly associated with age at diagnosis (Table 2.4) [185]. Lower survival is seen in patients under 50, while patients over 70 have the lowest survival. The poor survival in the >70's group is certainly influenced by their age and their age related co-morbidities.

Table 2.4 5yr year survival rates for breast cancer by age [185].

Age Group	5 Year survival (%)
<40	84.5
40-49	89.4
50-59	90.9
60-69	90.8
>70	73

Investigating how high risk genetic mutations effect the age of onset, we find that in patients <40 years old 5.3% of breast cancer cases are due to mutations in the BRCA1 gene. In the 40-49 age bracket this falls to 2.2%, which decreases further to 1.1% for patients developing breast cancer in the 50-70 year age group [186]. Furthermore, it has been established that patients with BRCA1 mutations are more likely to develop Triple negative like breast cancers (including the triple negative molecular subtype) [187-189].

2.4 Genetics and breast cancer risk

Currently the major genes known to influence breast cancer risk are BRCA1 [190] and BRCA2 [191, 192]. These genes are tumour suppressor genes responsible for DNA damage repair [193] and mutations in these genes result in a significantly increased risk of breast cancer. It is estimated that up to 16% of all familial breast cancers are due to mutations in these genes [194] and up to 5% of all breast cancer cases [195]. BRCA1 and BRCA2 mutation carriers <70 years old face a 57% and 49% (respectively) risk of developing breast cancer [196]. Importantly, BRCA mutation carriers frequently tend to develop more aggressive breast cancer and at a younger age [197]. Screening for BRCA gene mutations in high-risk patients has become a priority and scoring systems such as the Manchester scoring system provide a means to identify which patients need increased surveillance [198]. From scoring systems like this, Genetic testing guidelines have recently been introduced for higher-risk patients (Figure 2.2).

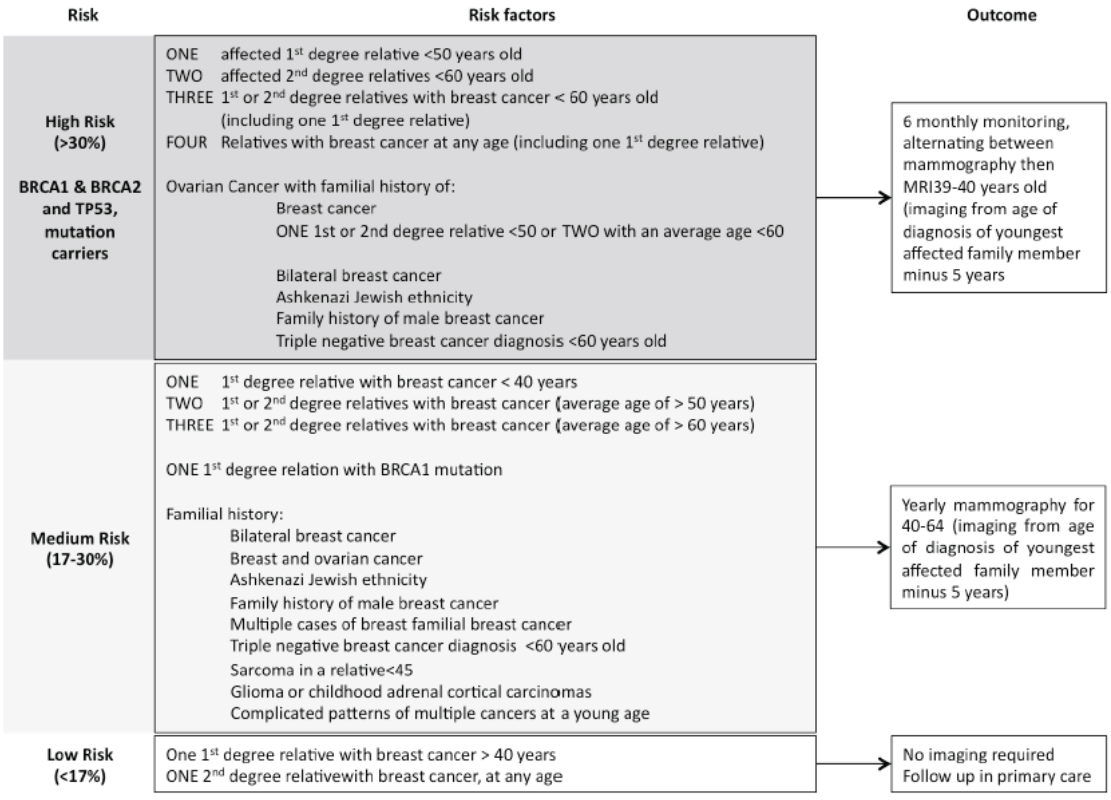


Figure 2.2 Family history influences breast cancer risk and screening.

Current recommendations for patients with detected BRCA mutations is bilateral mastectomies for carriers [199-201], with patients who decline surgery to continue

high risk screening and additionally genetic screening for first degree relatives [202]. The genetic testing of patients can have significant personal ramifications, in addition to the consequences for their families and close relatives. Due to this genetic screening is not routine worldwide, with genetic counseling recommended prior to testing [201].

2.5 Breast cancer and microRNAs

MicroRNA are 19-25 bases or nucleotides long, non-protein coding RNA involved in cell development, differentiation, proliferation and apoptosis [94, 203, 204]. Currently >2,000 distinct miRNAs have been identified in humans, where miRNAs regulate an estimated 30% of all human genes [205]. Recently age has been implicated in differential expression of miRNA [206-208]. Current research has focused on the role of miRNA and breast cancer, implicating miRNA in breast cancer initiation and progression [209-212]. Recently, a single study has investigated variations in circulating miRNA between younger and older breast cancer patients [213]. Further research into the effects of age, circulating miRNA and breast cancer may further provide insight into variations, diagnosis or treatment options for breast cancer patients with different ages of onset.

As breast cancer is a heterogeneous disease, genetic insights have complemented the research focused on sub-classifying breast cancer by molecular subtype. Molecular profiling has identified new breast cancer subtypes and has the potential to explain the difference seen in subtypes across the age groups.

2.6 Age associated treatment by molecular subtype

Luminal cancers (A & B) are the most common subtypes and tend to occur in post-menopausal patients [7, 15, 182, 214, 215] and tend to have better outcomes than the other subtypes [7, 30, 214, 216]. Furthermore, Luminal cancers have been linked to estrogen exposure, with nulliparous and women taking hormone replacement therapy displaying an increased risk [217, 218]. Due to this anti-estrogen agents (such as tamoxifen) have been developed that inhibit estrogen activity by competitively binds to estrogen receptors. This treatment has increased disease free survival and overall survival in women with hormone receptor positive cancers, with 5 years of adjuvant tamoxifen treatment reducing annual mortality by up to 31% across all age groups

[219, 220]. Interestingly, further studies have indicated that extended treatment with Tamoxifen can have other age related effects, such as prevention of bone loss in post-menopausal women [41] and increasing the risk of endometrial cancers, hot flushes and thromboembolic events [42, 43, 220, 221]. New anti-estrogen therapies (aromatase inhibitors) were developed which inhibit the synthesis of estrogen from androgens [45, 46]. Long-term studies showed that aromatase inhibitors have superior disease free survival rates than tamoxifen, in post-menopausal women [47]. Aromatase inhibitors also reduces the risk of endometrial cancer along with vaginal bleeding, cerebrovascular events, thromboembolic events and flushes [48, 49]. Currently an extended course of hormone therapy, beyond five years, is recommended due to improved survival [222-224]. Previous studies have indicated that luminal cancers have a reduced sensitivity to chemotherapy [69]. The improved outcomes using tamoxifen and aromatase inhibitors pose a question about the use of chemotherapy for luminal cancers. The results from new tests, such as the multi-gene Oncotype Dx test, provides an estimate for the risk of cancer recurrence for an estrogen positive breast cancer patient. In addition, this test also identifies patients that are either likely or unlikely to benefit from chemotherapy [51, 60, 225]. Use of this test in advanced age groups has resulted in a large proportion of elderly patients not receiving chemotherapy and improvements in quality of life.

HER2 over-expressing cancers have a higher prevalence in post-menopausal women (Table 2.3) and initially had poor outcomes [7, 226]. In patients under 40 years old, HER2 over-expressing breast cancers have been linked to a higher recurrence rate [227]. Treating patients with a monoclonal antibody that targets the HER2 receptor, like Trastuzumab, has resulted in improved survival [24, 56, 57]. Furthermore, adding Trastuzumab to neoadjuvant chemotherapy has led to significant increases in the pathological complete responses observed [71]. Development of newer monoclonal antibodies has also shown promise, Pertuzumab combined with Trastuzumab in the neoadjuvant setting significantly improves the pathological complete response rate [73].

Triple negative cancers occur at a younger age and have poorer outcomes than Luminal subtypes [7, 15, 182, 215]. Triple negative cancers have been linked to the BRCA1 gene, with studies finding 20-30% of triple negative patients having either

the BRCA1 or BRCA2 gene abnormality [189, 228, 229]. It was also found that the prevalence increases with decreasing age [228, 230]. Due to this correlation the national comprehensive cancer network recommends that all women under 60 with triple negative breast cancer be referred for genetic counselling [231]. Currently, bilateral prophylactic mastectomy has been shown to reduce the risk of breast cancer in carriers with BRCA1 and BRCA2 mutations [232].

A variation in rates of metastasis is seen across age groups, with older patients more likely to have distant metastasis [82]. However, by age (over or under 50 years old) there is little difference in the sites of metastasis except for lung metastasis which is almost twice as more common in younger patients (Figure 2.3).

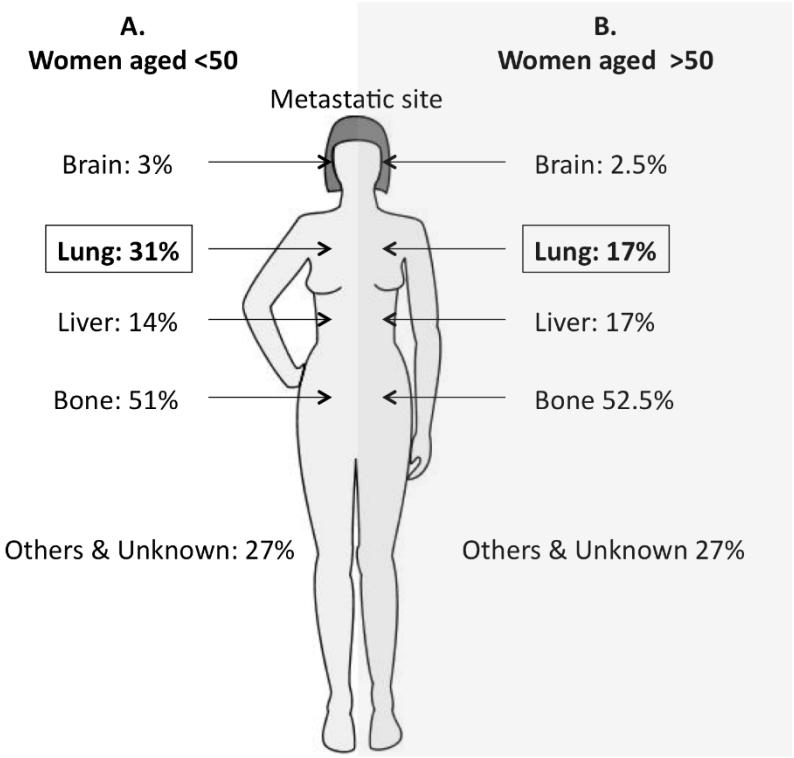


Figure 2.3 Metastatic breast cancer sites by age group. A) Site specific recurrence for women under 50. B) Site specific recurrence for women over 50.

The most common surgical intervention for breast cancer treatment is wide local excision (WLE), which in early breast cancer has similar outcomes to mastectomy while reducing surgical complications [35, 36]. While no difference in survival is

seen in premenopausal women having WLE compared to postmenopausal women, surprisingly there is a higher local recurrence rates with up to five-fold greater incidence seen in women <35 compared to women aged 45-49 [233-237]. This can make treatment decisions difficult in younger patient would prefer to have breast conserving surgery. The margin status in breast conserving surgery has been shown to be one of the most significant factors in relapse rates. Clear surgical margins have been shown to dramatically reduce recurrence rates especially in women under 40 [237, 238]. The addition of radiotherapy post WLE has resulted in recurrence rates for women <50, falling significantly from 19.4% to 11.4% [239]. This benefit was not seen in patients over 70 where radiotherapy does not improve survival [240]. It has been shown that there is an age dependent response to chemotherapy and hormone therapy, where anthracycline-based polychemotherapy reduced mortality by 38% for women <50 years old and by 20% for the 51-69 years old group [219]. A similar improvement in survival rates was seen across all groups in ER positive breast cancers treated with tamoxifen [219]. In HER2 receptor positive patients treated with Trastuzumab, no significant difference was seen in recurrence rates across different age groups [241]. Neoadjuvant chemotherapy reduces tumour size and increases the numbers of patients suitable for surgery, however no difference was seen by age for complete pathological response [242]. Neoadjuvant endocrine therapy is of benefit in post-menopausal patients with early breast cancer, improving WLE rates, disease free survival and overall survival [243, 244].

2.7 Prognosis

Prognosis of patients varies with age, as younger women tend to have more aggressive tumours (such as Triple negative) and a higher recurrence rate [245-250]. This effect is most pronounced in women <35 years old and it has been demonstrated that a younger the age of diagnosis increases the risk of mortality [251]. These effects are likely due in part to the lack of screening for younger women, meaning patients often present with larger palpable lumps and a more advanced stage [252]. Younger patients tend to have higher Ki-67 levels (an indicator of poor prognostic outcome [253]), with highest levels seen in patients <35 [254, 255]. However, contradicting this, other recent studies have shown no age related difference in mortality rates [256-258]. Current advances in screening, earlier identification of high-risk patients and improved treatment options may explain this.

In addition, recent studies have shown that women >55yrs old have a better prognosis and have a similar survival to the general population irrespective of disease status [259]. Mirroring the younger patients, women at the other end of the age spectrum (>70 years old) similarly present with more advanced tumours [179]. In younger patients chemotherapy would be used, however there is little research into which subgroups in this >70 age group this would be a suitable option for.

2.8 Age and co-morbidities

In the past many older patients were deemed unsuitable for surgery due to their age and medical co-morbidities such as diabetes mellitus, coronary heart disease, hypertension, stroke, asthma and chronic gastritis. These co-morbidities are independent risk factors for survival and are disproportionally found in older patients [178, 260, 261]. Improved surgical techniques mean a larger proportion of these patients are now able to undergo curative surgery. These medical co-morbidities may provide markers for assessing suitability for chemotherapy, in conjunction with other established factors such as a comprehensive geriatric assessment (CGA). A recent study found malnutrition and frailty to be the biggest risk factor for mortality in patients >70 years old [262]. Similar results were seen in a study using a CGA in patients >65 years old where conditions such as a low Mini Mental State Examination, Body Mass Index or high Charlson co-morbidity index scores resulted in a higher risk of chemotherapy related toxicity [263]. A CGA may provide relevant age related information indicating which patients would be suitable for chemotherapy treatment. However, limitations may include not completing a CGA prior to treatment and non-compliance with recommendations [179].

2.9 Summary

With our ever-expanding knowledge of breast cancer and age related effects, there are constant improvements in treatment guidelines and best practice. Over the last few decades, detection and survival rates have improved immensely, yet there is no consensus in the management of the very young (<35) or the increasingly elderly (>70) populations. An improved understanding of the genetics of breast cancers through molecular profiling may provide information that can be applied to the youngest and oldest patients. This has been demonstrated by identification of the high-risk BRCA genes, providing some explanation for younger patients.

Importantly, there are still no clear guidelines for the management of breast cancer patients >65 years old. In addition, scoring systems such as CGA could provide an accurate way of determining which patients should receive active or palliative treatment, however more investigation is needed to determine the feasibility and practicality of such a system.

Chapter 3

Clinical review of the impact of Trastuzumab therapy on HER2 receptor positive breast cancers

Published sections

3.2.1 Differential impact of hormone receptor status on survival and recurrence for HER2 receptor-positive breast cancers treated with Trastuzumab
McGuire A, Kalinina O, Holian E, Curran C, Malone CA, McLaughlin R, Lowery AJ, Brown JAL, Kerin MJ. Breast Cancer Res Treat. 2017 Apr 4. doi: 10.1007/s10549-017-4225-5,

3.2.2 Locoregional recurrence following breast cancer surgery in the trastuzumab era: a systematic review by subtype.
McGuire A, Lowery AJ, Kell MR, Kerin MJ, Sweeney KJ. Ann Surg Oncol. 2017 Jul 28. doi: 10.1245/s10434-017-6021-1.

3.2.3 Breast cancer subtype discordance: impact on post-recurrence survival and potential treatment options
McAnena PF**, McGuire A,** Ramli A, Curran C, Malone C, McLoughlin R, Barry K, Brown JAL, Kerin MJ. BMC Cancer. 2018; 18: 203.doi: 10.1186/s12885-018-4101-7

3.1 Introduction

Advances in molecular profiling have allowed breast cancer to be categorised into clinically relevant molecular subtypes [11, 12, 264]. In approximately 20-30% of breast cancers the HER2 receptor is over expressed [181, 182], resulting in increased cell signalling, uncontrolled cellular proliferation and poor clinical prognosis. Half of HER2 receptor positive breast cancers are also hormone receptor positive Luminal B HER2 and half are hormone receptor negative HER2+(non-luminal). Clinically it has been observed that differences in survival and outcome occur between the two HER2 receptor positive subtypes [15, 17] and they present with distinct patterns of recurrence. In the Luminal B HER2 subtype, bone is the most common distant metastasis site, which is also seen in Luminal A breast cancers [8, 30]. HER2+(non-luminal) cancers have the highest rates of locoregional recurrence (LLR) overall and tend to initially metastasize to visceral organs, such as the lung [8, 10]. However, many studies do not distinguish between the two subtypes, while others have shown no significant difference when assessing long-term survival between the two subtypes [8, 265, 266].

Trastuzumab, a targeted treatment for HER2 receptor positive breast cancer, is a monoclonal antibody that binds to the HER2 receptor and interferes with the HER2 mediated signalling cascade, preventing proliferation and eventually leading to cell death [62]. Trastuzumab was originally used to treat metastatic breast cancers and was shown to significantly improve median survival from 20.3 to 25.1 months [56]. Multiple studies have shown that Trastuzumab use in the adjuvant setting reduces recurrences and can increase survival in HER2 positive patients by up to 33-39% [24, 57, 63, 267]. However, despite increased survival and improved outcomes, 8.2% of patients will still have a breast cancer recurrence [268]. More recently, several studies have shown that when used in the neoadjuvant setting Trastuzumab significantly increases pCR rates [70-72]. While improvements in survival in HER2 receptor positive cancers have been demonstrated in multiple studies, a key question remains, does Trastuzumab treatment induce varied responses in survival and outcome between Luminal B HER2 and HER2+(non-luminal) breast cancers subtypes.

Another key question is the impact on Trastuzumab on recurrences in the two subtypes, specifically locoregional recurrence. The prevention of LRR is important, as one breast cancer death can be prevented over the next 15 years for every four local recurrences avoided [53]. Our Institute has previously performed a systematic review of LRR rates, however this was prior to the introduction of a new treatments, including Trastuzumab [10]. In this study, the highest LRR rates occurred in HER2+(non-luminal) breast cancer subtype, with significant variations seen between subtypes. To date the impact of Trastuzumab on LRR rates has yet to be compared to other subtypes in a systematic review.

Despite modern therapy, a proportion of patients will have a recurrence of their disease despite optimized treatment [8]. A possible explanation for this, is the recent discover that breast cancer recurrences can have different hormone and HER2 receptor status from the primary breast cancer, this is known as discordance [269-272]. Studies have shown discordance levels can be significant, with up to 22.7% having subtype changes between patients' primary breast cancer and the recurrence [87]. However, no study to date has examined the effect of HER2 receptor change on treatment regimens and if similar changes are seen between LRR and distant metastasis.

While the introduction of Trastuzumab has resulted in significant improvement in patients DFS and OS, it has also added a large financial burden. Cost effectiveness analysis allows for assessment of a new treatment, comparing the improvements and cost of the new treatment against current standards of care. Multiple studies have looked at the cost effectiveness of Trastuzumab in the adjuvant setting and found that Trastuzumab would be cost effective over a life time [76, 78, 79]. However, the impact of hormone receptor status on the cost effectiveness of Trastuzumab has yet to be assessed. Also the use of Trastuzumab in the neoadjuvant setting may impact cost effectiveness. The increase in pCR would result in a large proportion undergoing breast conservative surgery, conversely patients would undergo an extra course of Trastuzumab. Due to these factors, the effect of adding Trastuzumab into NACT on cost effectiveness is still unknown.

3.2 Aims

In this chapter, we look at the overall impact of Trastuzumab on HER2 receptor positive breast cancer subtypes. It is hypothesised that hormone receptor status has a major impact on survival, treatment, recurrence and cost, in HER2 receptor positive breast cancers. To assess this, four key areas were examined.

3.4.1) Examine the impact of the introduction of Trastuzumab, and compare the survival and recurrence patterns in the two HER2 receptor positive breast cancers in our patient cohort.

3.4.2) Perform a systematic review, to assess the impact of Trastuzumab introduction on LRR rates worldwide.

3.4.3) Assess the variation (discordance) in HER2 receptor status between breast cancer primaries and secondary disease.

3.4.4) Investigate the cost effectiveness of Trastuzumab therapy, specifically its use in neoadjuvant chemotherapy.

3.3 Methods

3.3.1 Patient cohorts

For the first analysis (section 3.4.1), the study group consists of all patients with HER2 receptor positive breast cancers treated at a tertiary referral unit and entered into a prospectively maintained database from 1991–2014. Only patients with a definitive HER2 receptor positive subtype were included. All clinical pathological details and treatment regimens were analysed. Hormone receptor positive patients received hormone therapy, as per treatment protocols at the time of diagnosis. Clinically, testing for HER2 receptor status began at our centre in 1999. In order to find HER2 receptor positive patients not treated with Trastuzumab we used a cohort of patients identified using retrospective testing of pathology samples. A cohort of HER2 receptor positive patients who did not receive Trastuzumab treatment was provided by retrospective testing of HER2 receptor status on patients included on a prospectively collected tissue microarray (samples from 1994-2001). Historically in our program Trastuzumab therapy was introduced as adjuvant therapy in 2006, prior to this it was available only to patients recruited on clinical trials. Patients were categorised as received adjuvant/neoadjuvant Trastuzumab (Trast +ve) or no

Trastuzumab treatment (Trast -ve). Overall survival, disease free survival and patterns of recurrence were determined.

The five year DFS & OS were determined and only patients who had completed five years of follow up were included in the analysis. Breast cancer recurrence was defined as a return of cancer after treatment and after a disease free period. Only stage I-III breast cancers were included for this section of the analysis. Recurrence was divided into LRR and distant metastasis. LRR is defined as recurrence at the same site as the primary cancer or the regional lymph nodes, while distant metastasis is recurrence at a distant site from the primary cancer.

For the discordance between the primary breast cancer and recurrences analysis (section 3.4.3), a similar approach was taken, all breast cancer patients with a recurrence of their cancers, were identified in a prospectively maintained patient database in a tertiary referral centre from 2001 to 2014. Patients that did not have a biopsy of the recurrence were excluded, as subtype of recurrence could not be identified. Patients were also excluded if there was metastasis at presentation or if the patient had bilateral breast cancer. The estrogen, progesterone and HER2 receptor status was recorded from the post-surgery histology report and final recurrence biopsy reports

For the cost effectiveness analysis (section 3.4.4) the database constructed for section 3.4.1 was analysed further, with the follow up updated to the start of 2016. Cost of treatment for all HER2 receptor positive patients was calculated, along with 3 year DFS. For the cost effective analysis, only cost of each individual treatment was used, and included cost of surgery, chemotherapy, radiotherapy and hormone therapy treatments. The length of stay or any extra costs such as ICU admission was not included in the data. The cost of each treatment was calculated from the cost assigned by private health insurance companies in Ireland for each treatment. Patients were excluded if the patient had metastatic disease at time of diagnosis or if Trastuzumab was given for palliative treatment only. Patients were also excluded if the information on the patients full treatment regimen was not available or if the patient received additional other anti HER2 therapy such as Lapatinib or Pertuzumab. The patients were separated into two groups, adjuvant Trastuzumab

group (no neoadjuvant Trastuzumab) and neoadjuvant Trastuzumab group (neoadjuvant +/- adjuvant Trastuzumab).

3.3.2 Subtypes definitions

Breast cancer subtypes were defined using ER, PR and HER2 receptor status (Table 3.1). Luminal A was defined as (ER and/or PR +ve, HER2 -ve), Luminal B HER2 was defined as (ER and/or PR +ve, HER2 +ve), HER2+(non-luminal) as (ER and PR –ve, HER2 +ve) and Triple negative as (ER and PR -ve, HER2 -ve) according to standard clinical pathological guidelines. The ER and PR receptor status were determined independently by clinical pathologists using immunohistochemistry as per American society of clinical oncology (ASCO) guidelines (ALLRED score >2 or more than 1% stain positive). The HER2 receptor status was identified by Herceptest, as part of the routine clinical evaluation, with a score of 3+ considered positive. Any +2 inconclusive results was confirmed using FISH (fluoresecent in situ hybridisation) testing as per ASCO guidelines, with a HER2/CEP17 ratio greater than two considered amplified.

Table 3.1 Intrinsic breast cancer subtypes

Intrinsic subtype	**Hormone receptor status**	**HER2/neu receptor status**
Luminal A	ER and/or PR positive	HER2 negative
Luminal B HER2	ER and/or PR positive	HER2 positive
HER2+(non-luminal)	ER and PR negative	HER2 positive
Triple negative	ER and PR negative	HER2 negative

3.3.3 Systematic review

An electronic based search was performed of medline using the following search algorithms: 1 “Breast cancer’’ AND (‘‘breast conserving surgery’’ OR ‘‘breast conservation’’ OR ‘‘wide local excision’’ OR ‘‘WLE’’ OR ‘‘lumpectomy’’ OR ‘‘quadrantectomy’’) AND (‘‘recurrence’’ OR ‘‘outcome’’) and 2. ‘‘Breast cancer’’ AND (‘‘mastectomy’’ OR ‘‘modified radical mastectomy’’) AND (‘‘recurrence’’

OR ‘‘outcome’’). English was chosen as a language restriction. Only publications after 2006 were included unless treatment with Trastuzumab was specifically mentioned. All abstracts and full texts were independently examined by two authors for determination of eligibility. Retrospective and prospective studies were included which reported LRR in operable breast cancer patients. Only studies with subtype specific LRR rates were included, studies failing to report rates in all subtypes were excluded. Treatment with adjuvant Trastuzumab was a further inclusion criteria, unless all included patients were treated after 2006 where Trastuzumab would have been part of standard treatment regime. Any paper that had less than fifty percent of the HER2 receptor positive patients treated with Trastuzumab was excluded. A cut off point of fifty percent was chosen as it resulted in a majority of HER2 receptor positive patients being treated with Trastuzumab. From the eligible studies information such as: authors, country of origin, year of publication, journal and surgery type were extracted. Treatment regimens where accessible such as chemotherapy, radiotherapy and hormone therapy were recorded.

Different criteria for subtype were used across the different studies, with the Ki-67 not reported in a number of studies. Due to this the four traditional subtypes based on hormone receptor and HER2 receptor status were used for this study (Luminal A, Luminal B HER2, HER2+(non-luminal) and Triple negative). The primary outcome was LRR defined as recurrence in the ipsilateral breast or chest wall or ipsilateral draining lymph nodes. To investigate the effect of breast conserving therapy (BCT) i.e. breast conserving surgery plus whole breast radiotherapy on LRR, a subgroup analysis was performed. A further subset analysis was done to compare the Luminal cancers (Luminal A and Luminal B HER2) to HER2+(non-luminal) and Triple negative breast cancers. This was done to compare results from a previous study from this institute.

3.3.4 Cost effectiveness

The quality adjusted life years adjustments for treatment and recurrence were calculated using previous published utilities [76]. Treatment with adjuvant and neoadjuvant Trastuzumab was assigned a utility of 0.85, and a utility of 0.55 was assigned to breast cancer recurrence. The QALY was calculated for all patients at

three years of follow up, with any patient not completing three years of follow up being excluded.

3.3.5 Statistics

Statistical analysis was performed using R statistical software version 3.2.3. A p-value of less than 0.05 was considered statistically significant. The Kaplan–Meier method was used to determine survival distributions. The log rank was used to determine any statistically significant differences in survival between the indicated groups. Cox regression was used for multivariate analysis, with logistic regression used to analyse categorical data. For the discordance in breast cancer recurrence, data analysis was performed using SPSS Version 21 (SPSS Inc., Chicago, IL). Data was assessed for normality using the Shaprio-Wilk test, and parametric or non-parametric tests applied as appropriate. Comparative analyses were performed between the groups. Statistical significance was accepted for $p<0.05$. For the cost effectiveness analysis, a univariate analysis was run to compare the mean cost between the two groups, with a p value of less than 0.05 once again considered significant. A multivariate linear regression was also run for cost and DFS, and once again a p value of less than 0.05 considered statistically significant.

3.3.6 Ethics, consent and permissions

This study was conducted in accordance with the granted National University of Ireland Galway and University College Hospital Galway ethical approval. Informed consent was obtained from all patients. All patients had histologically confirmed breast cancer and all relevant clinicopathological and demographic data was obtained from a prospective breast cancer database.

3.4 Results

3.4.1 The impact of the introduction of Trastuzumab on the survival, and recurrence patterns in the two HER2 receptor positive breast cancers.

The study consisted of 468 HER2 receptor positive patients eligible for this study, treated in our institute between 1991 and 2014. From these 287 (61%) were found to be Luminal B HER2, with the remaining 181 (39%) patients HER2+(non-luminal). The median age of patients was 63 and the median follow up was 49 months. The

majority of the overall cohort was recruited after Trastuzumab received approval for adjuvant treatment in 2006 (Figure 3.1).

For the purpose of this analysis patients were categorised as either Trast +ve (received adjuvant/neo-adjuvant Trastuzumab) or Trast -ve (no Trastuzumab treatment).

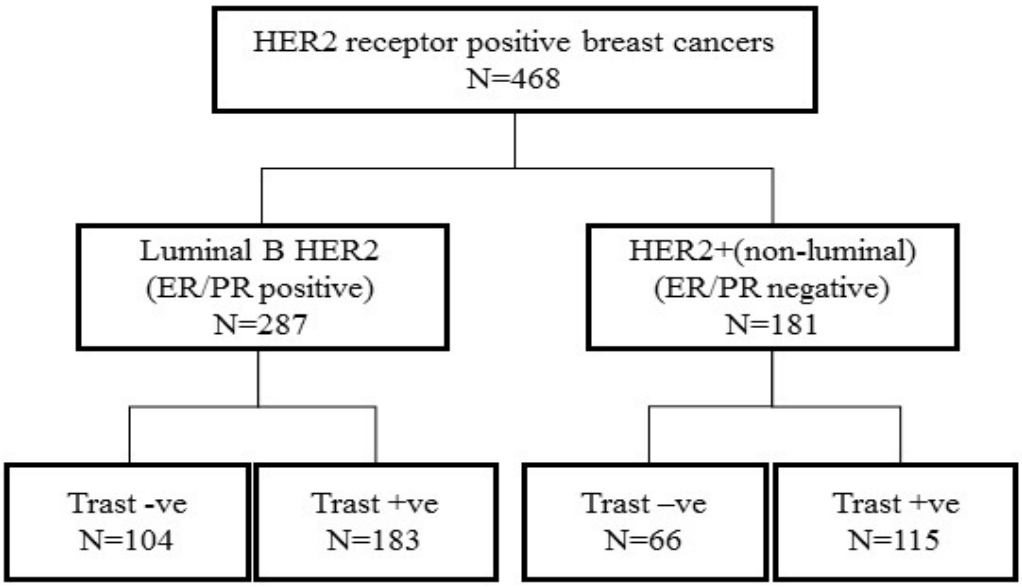


Figure 3.1 CONSORT diagram.

Overall, 299 (63.9%) patients were treated with Trastuzumab (Trast +ve). The clinicopathological details of the cohort are listed (Table 3.2), demonstrating the two subtypes are relatively matched for age, stage and treatment. The only statistical significant difference between the Luminal B HER2 and HER2+(non-luminal) was observed in the grade category, where the HER2+(non-luminal) cohort had a higher proportion of grade 3 cancers (49.8% vs 79.3% $p<0.001$). Furthermore, 263 (91.6%) of the Luminal B HER2 cancers received adjuvant hormone therapy. In the series recurrence occurred in 94 (20.1%) patients, of which 15 (3.2%) had LRR alone. 54 (11.5%) patients had distant metastasis alone and 25 (5.3%) patients had both LRR and distant metastasis. There was no significant difference in the distribution of age, stage or treatment of cancers between the two subtypes (Table 3.2).

Table 3.2: Patient demographics.

	Luminal B HER2 ($n = 287$) *N* (%)	HER2+(ER−) ($n = 181$) *N* (%)	*p* Value
Age: mean, Years±SD	64.62 ± SD	62.83 ± SD	0.160
Age category: *N* (%)	14.81	12.33	0.602
0–50	53 (18.5)	30 (16.6)	
50+	234 (81.5)	151(83.4)	
Grade: *N* (%)			**<0.001**
1,2	137 (50.2)	34 (20.7)	
3	136 (49.8)	130 (79.3)	
NA	14	17	
TNM Stage: *N* (%)			0.623
0	14 (4.9)	15 (8.2)	
1	55 (21.8)	40(26.3)	
2	103(40.9)	53 (34.9)	
3	68 (27)	43 (28.3)	
4	26 (10.3)	16 (10.5)	
NA	21	14	
Surgery: *N* (%)			0.062
Mastectomy	123 (50.8)	85(60.7)	
Wide local excision	119 (49.2)	55(39.3)	
NA	45	41	
Radiotherapy: *N* (%)			0.338
No	56 (22.1)	41 (26.3)	
Yes	197 (77.9)	115 (73.7)	
NA	34	25	
Adjuvant chemotherapy: *N* (%)			0.891
No	97 (36.2)	59 (35.5)	
Yes	171 (63.8)	107 (64.5)	
NA	19	15	
Neo-adjuvant chemotherapy: *N* (%)			0.387
No	195 (78.3)	105 (74.5)	
Yes	54 (21.7)	36 (25.5)	
NA	38	40	
Trastuzumab: *N* (%)			0.844
No	100 (35.2)	65 (36.1)	
Yes	184 (64.8)	115 (3.9)	
NA	3	1	
Neo-adjuvant Trastuzumab	38 (13.2)	32 (17.7)	0.261
pCR	10 (26.3)	13 (40.6)	0.368
Total	52 (18.1)	42 (23.2)	0.181
LRR	18 (6.3)	22 (12.2)	**0.027**
Distant	47 (16.4)	32 (17.7)	0.714

Bold values indicate significant *p* value

3.4.1.1 Trastuzumab treatment and subtype significantly affects survival

On univariate analysis, survival was similar in Luminal B HER2 compared to HER2+(non-luminal) subtypes (Figure 3.2) and Trastuzumab treatment was associated with significantly improved overall survival in both subtypes (Figure 3.3).

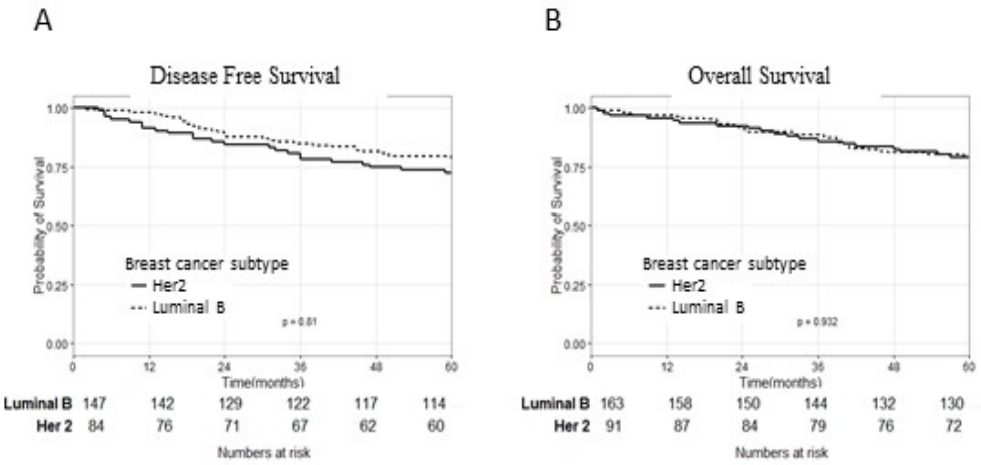


Figure 3.2 Kaplan meier curve comparing Luminal B HER2 and HER2+(non-luminal). A) DFS and B) OS between breast cancer subtypes.

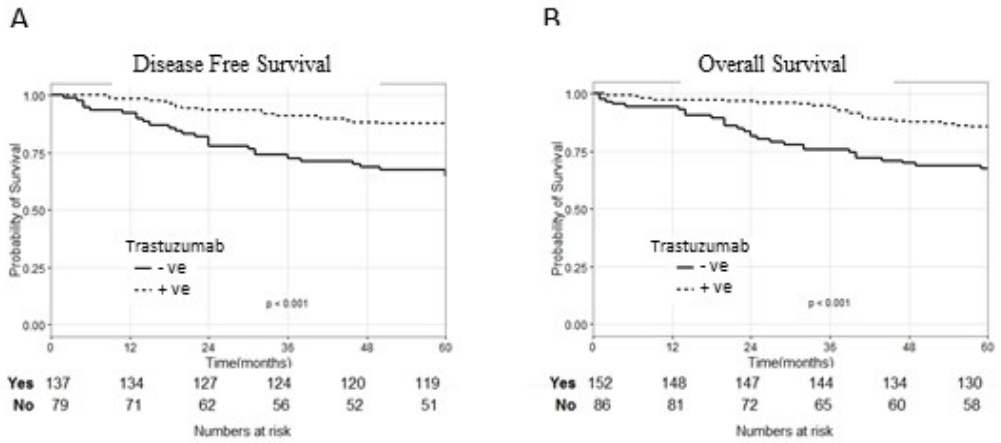


Figure 3.3 Kaplan meier curve comparing Trast +ve to Trast -ve in whole patient cohort. A) DFS. B) OS.

No difference was seen in survival between the two subtypes in either the Trast –ve or Trast +ve patients. Next to assess the impact of Trastuzumab introduction on each subtype, the 5 year DFS and OS was compared between the Trast –ve and Trast +ve groups in both subtypes. Analysing the DFS and OS by subtype, an increased survival rate is seen for Trast +ve patients in both Luminal B HER2 and HER2+(non-luminal) patients. However, a greater improvement was seen in Luminal B HER2 patients. Luminal B HER2 cancers had a statistically significant

improvement in both 5 year DFS (p<0.001) and OS (p<0.001) (Figure 3.4 A-B), while the HER2+(non-luminal) only had a significant improvement in DFS (p=0.012) but not OS (p=0.135) (Figure 3.4 C-D).

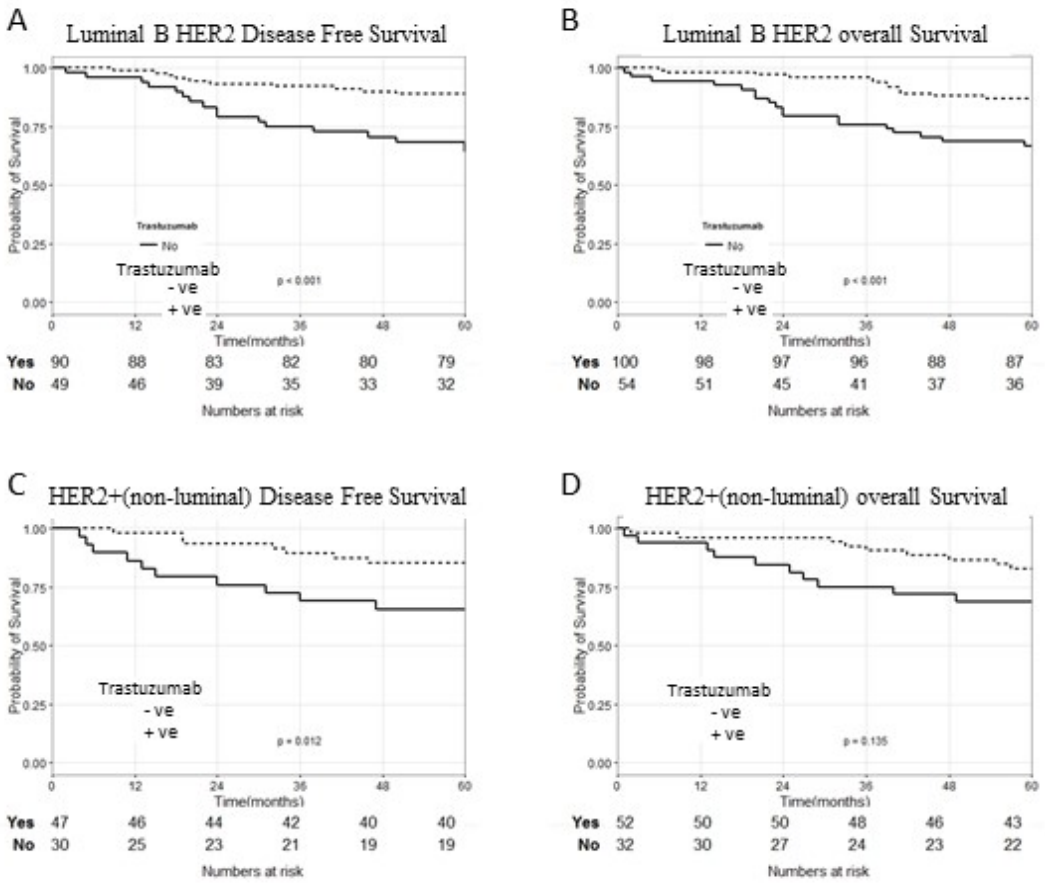


Figure 3.4 Kaplan Meier curves of individual HER2 receptor positive breast cancer subtypes. A) DFS in Luminal B HER2, B) OS in Luminal B HER2, C) DFS in HER2+(non-luminal) and D) OS in HER2+(non-luminal)

On multivariate analysis of survival, no significant increased risk was seen in the HER2+(non-luminal) subtype when compared to Luminal B HER2 cancers for either 5 year DFS (HR 1.31, 95%CI 0.42 – 4.1) or 5 year OS (HR 2.18, 95%CI 0.79 – 6.03) (Table 3.3).

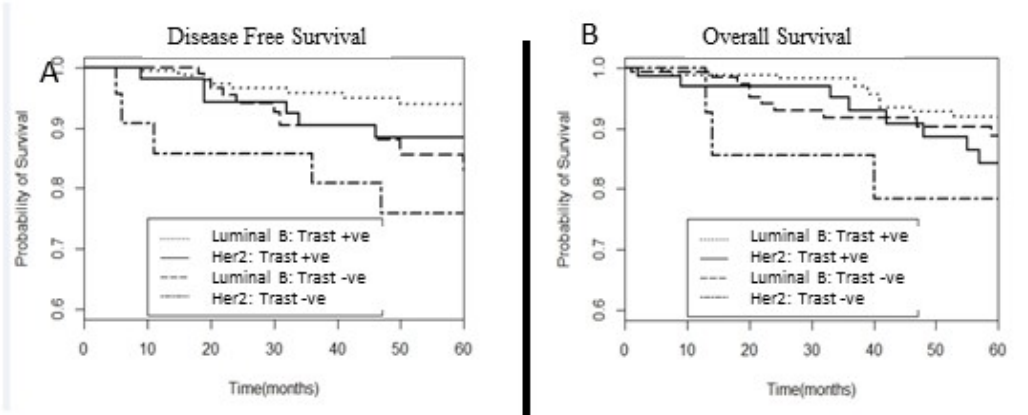
Table 3.3 Multivariate analysis of patients treated with Trastuzumab.

	DFS	**OS**
	Multivariable HR(95%CI)	Multivariable HR(95%CI)
Subtype		
Luminal B HER2	1	1
HER2(non-luminal)	1.31 (0.42 – 4.1)	2.18 (0.79 – 6.03)
Age Category		
0 to 50	1	1
50+	1.16 (0.31 – 4.29)	1.16 (0.3 – 4.46)
Grade		
1,2	1	1
3	1.16 (0.34 – 3.93)	0.56 (0.21 – 1.49)
Stage		
1	1	1
2	2.29 (0.25 – 21.01)	0.95 (0.09 -10.54)
3	12.64* (1.51 – 105.62)	7.48 (0.92 – 61.03)
4		40.68* (5.21 -317.88)
Adjuvant Chemotherapy		
No	1	1
Yes	0.42 (0.09 – 2.0)	0.40 (0.07 – 2.37)
Neoadjuvant Chemotherapy		
No	1	1
Yes	0.16* (0.04 – 0.63)	0.44 (0.12 – 1.53)

* $p<0.05$

Analysing risk factors for survival, higher grade was not associated with worse outcome but Trastuzumab given in the neo-adjuvant setting was associated with a significant improvement in 5 year DFS (HR 0.16, 95%CI 0.04 – 0.63). A multivariate cox proportional hazard model analysis of survival was performed, where the model was controlled for age, stage, grade and chemotherapy treatment

(Figure 3.5). A similar outcome is seen to the univariate analysis, with a greater improvement in survival seen in Luminal B HER2 patients treated with Trastuzumab. In Luminal B HER2 cancers, a significantly increased hazard ratio is seen in Trast –ve patients: DFS (HR 3.82, 95%CI 1.5-9.4; p=0.004) and OS (HR 2.49, 95%CI 1.1-5.6; p=0.03). However, no significant increase in hazard ratio was seen in Trast –ve HER2+(non-luminal) cancers compared to the Trast +ve group in 5 year DFS (HR 2.18, 95%CI 0.63-7.52; p=0.598) or OS (HR 1.36, 95%CI 0.39-4.72 p=0.962).



	Multivariate analysis HR (95%CI)*	P Value		Multivariate analysis HR (95%CI)*	P Value
Luminal B, Trast+ve Vs Luminal B, Trast -ve	3.82 (1.5-9.4)	0.004	Luminal B, Trast+ve Vs Luminal B, Trast–ve	2.49 (1.1-5.6)	0.03
Her2, Trast+ve vs Her2, Trast–ve	2.18 (0.63-7.52)	0.598	Her2, Trast+ve vs Her2, Trast–ve	1.36 (0.39-4.72)	0.962

*adjusted to age, group, stage ,grade, neo-adjuvant and adjuvant chemotherapy

Figure 3.5 Cox proportional analysis comparing indicated subtype and treatments. A) DFS. B) OS.

3.4.1.2 Effects of Trastuzumab treatment on recurrence rates

On univariate analysis, recurrences occurred in 52 (18.1%) of Luminal B HER2 breast cancers and 42 (23.2%) of HER2+(non-luminal) breast cancers overall. A significant reduction in recurrence rates in Trast +ve patients was observed in both Luminal B HER2 (38.3% vs 8.5%, p <0.001) and in HER2+(non-luminal) (36.7% vs 18.3%, p=0.009) (Table 3.4). Luminal B HER2 cancers displayed a significant reduction in LRR (16% vs 1.8%, p<0.001), however there was no significant

reduction in HER2+(non-luminal) breast cancers (16.7% vs 10.6%, p=0.261). Trastuzumab treatment was associated with a significant reduction in distant metastasis rates in both subtypes, with a greater reduction observed in Luminal B HER2 (36.2% vs 6.7%, p<0.001) compared to HER2+(non-luminal) (31.7% vs 12.5%, p=0.03).

Table 3.4 Recurrence Rates (Stage I-III breast cancers).

	Trastuzumab	Luminal B HER2 (n=259) N (%)	HER2+ (n=164) N (%)
Total Recurrence: n (%)	No	36/94 (38.3)	22/60 (36.7)
	Yes	14/165 (8.5)	19/104 (18.3)
		p<0.001	**p=0.009**
LRR: n (%)	No	15/94 (16)	10/60 (16.7)
	Yes	3/165 (1.8)	11/104 (10.6)
		p<0.001	p=0.261
Distant: n (%)	No	34/94(36.2)	19/60 (31.7)
	Yes	11/165(6.7)	13/104 (12.5)
		p<0.001	**p=0.003**

Analysis of the effects of Trastuzumab treatment on distant metastasis by site of recurrence and subtype was performed (Table 3.5). For Trast –ve patients, bone was the most common site of metastasis for Luminal B HER2 cancers, while lung was the most common site in HER2+(non-luminal). Following Trastuzumab treatment in the Luminal B HER2 cancers a significant reduction was seen for all distant sites of metastasis (except brain). The site with the greatest reduction in metastasis, due to Trastuzumab treatment, was in bone (22.9% vs 3.8%, p<0.001). HER2+(non-luminal) breast cancers did show decreases in all metastatic sites except for brain in Trast +ve patients, however, these reductions only approached significance in bone (p=0.075), lung (p=0.086) and liver (p=0.075).

Table 3.5 Distant Metastasis rates.

Metastasis site	**Luminal B**		p value	**HER2**		p value
	Trast -ve N=86	Trast +ve N=173		Trast -ve N=60	Trast +ve N=104	
Bone : n (%)	24 (25.5)	5 (3)	**<0.001**	6 (10)	3 (2.1)	0.075
Brain : n (%)	4 (4.3)	4 (2.4)	0.466	5 (8.3)	9(8.7)	0.999
Lung : n (%)	20 (21.3)	3 (1.8)	**<0.001**	9 (15)	7 (6.7)	0.086
Liver : n (%)	13 (13.8)	7 (4.2)	**0.005**	6 (10)	3 (2.9)	0.075

Performing a multivariate analysis of recurrence risk by treatment, metastatic site and subtype (Table 3.6), no difference was seen for LRR between Luminal B HER2 and HER2+(non-luminal) cancer in Trast –ve patients (OR 1.39, 95%CI 0.47-4.5: p=0.557). Importantly in Trast +ve patients, a significantly lower odds ratio for LRR was seen in Luminal B HER2 cancers compared to HER2+(non-luminal) (OR 0.13, 95%CI 0.02-0.59; p=0.018). Distant metastasis rates to bone was the only significant difference between the two subtypes, in the no Trastuzumab group (OR 4.63, 95%CI 1.53-17.5; p=0.012). Following Trastuzumab treatment, no difference was seen between subtypes in bone metastasis (OR 0.958, 95%CI 0.17-5.60; p=0.96) but a significantly lower risk in brain metastasis is seen in the Luminal B HER2 cancers (OR 0.19, 95%CI 0.03-0.85; p=0.041).

Table 3.6 Logistic regression analysis (site of recurrence)

	Unadjusted OR Trast –ve #		**Adjusted OR Trast –ve #***		**Unadjusted OR Trast +ve #**		**Adjusted OR Trast +ve #***	
	Luminal B vs HER2	**P value**	Luminal B vs HER2	**P value**	Luminal B vs HER2	**P value**	Luminal B vs HER2	**P value**
LRR	0.95 (0.399,2.338)	0.907	1.39 (0.471,4.50)	0.557	0.16 (0.04,0.51)	**0.001**	0.13 (0.02,0.59)	**0.018**
Distant	1.22 (0.618,2.459)	0.566	1.21 (0.508,2.95)	0.660	0.50 (0.211,1.16)	0.107	0.51 (0.17,1.44)	0.214
Bone	3.09 (1.246,8.805)	**0.022**	4.63 (1.53,17.50)	**0.012**	1.05 (0.253,5.22)	0.945	0.958 (0.17,5.60)	0.960
Brain	0.49 (0.117,1.923)	0.301	0.41 (0.09,1.76)	0.231	0.26 (0.07,0.829)	**0.029**	0.19 (0.03,0.85)	**0.041**
Lung	1.53 (0.661,3.786)	0.333	2.15 (0.78,6.688)	0.161	0.26 (0.05,0.946)	0.05	0.36 (0.06,1.57)	0.198
Liver	1.44 (0.536,4.324)	0.483	1.03 (0.33,3.39)	0.958	1.49 (0.405,7.04)	0.569	1.03 (0.22,5.64)	0.971

95%CI

* Adjusted to stage and grade

3.4.2 Systematic review of LRR rates

3.4.2.1 Included studies

In total ninety-five eligible studies were identified, that reported LRR rates by breast cancer subtype. From these studies seventy-seven were excluded, as patients in the studies were not treated with Trastuzumab, and a further eight were excluded as the LRR rates for all four subtypes could not be extracted. A further three were excluded as less than half of the HER2 receptor positive patients were treated with Trastuzumab. This resulted in 7 studies to be included in the final analysis (Figure 3.6).

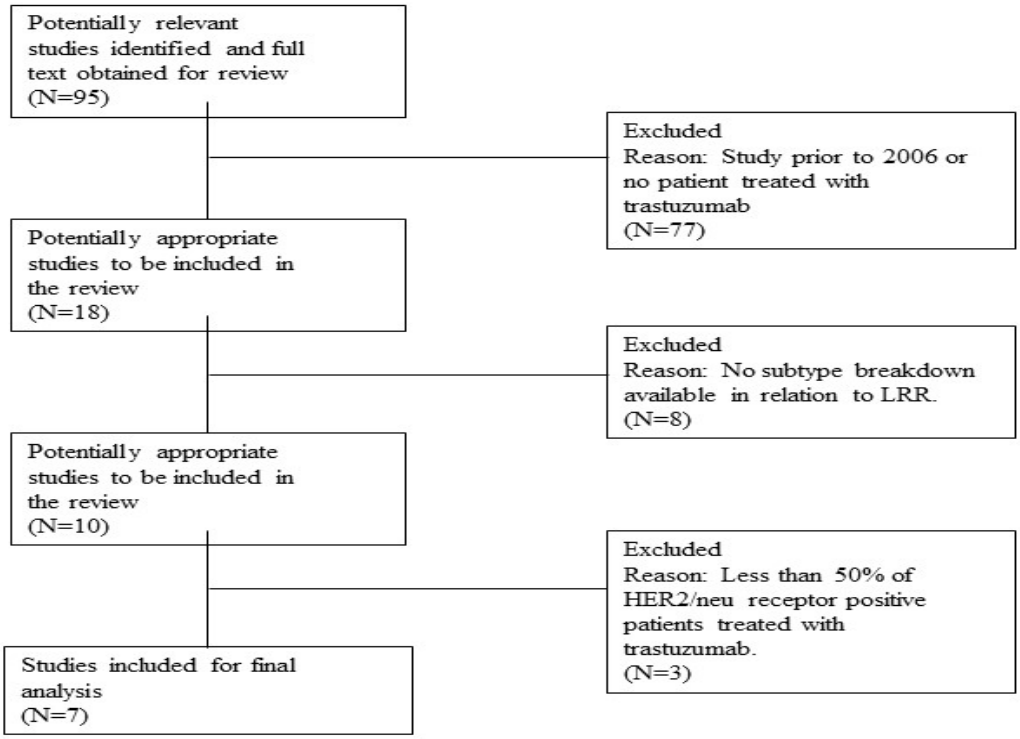


Figure 3.6 Eligible studies; quality of reporting of meta-analyses (QUOROM) statement flow diagram.

In these 7 studies data was extracted on 11,219 patients (Table 3.7) [273-279]. Radiotherapy was given to all patients who underwent BCT, radiotherapy rates after mastectomy could not be extracted across all the studies. Hormone receptor positive patients received hormone therapy as per standard guidelines. Chemotherapy was given to 82.1% of patients, with 8.6% receiving neo-adjuvant chemotherapy. The median follow up was 53 months (Range 44-84). There were 6,540 (58.3%) Luminal A patients, 1,469 (13.1%) Luminal B HER2 patients, 1,040 (9.3%) HER2+(non-

luminal) and 2,170 (19.3%) triple negative breast cancers. The total LRR rate was 3.44% across all the studies and in patients post BCT this was lower at 2.8%. The lowest rates of recurrence was seen in the Luminal A cancers 1.7%, with Luminal B HER2 having 3.3%, HER2+(non-luminal) rates 5.7% and the highest rates were seen in Triple negative 7.4%.

Table 3.7 Details of eligible studies.

Authors Ref	**Country of origin**	**No. of patients analysed**	**Luminal A**	**Luminal B**	**HER2**	**Triple Negative**	**Follow up in Months**
Mazouni et al [273]	France	791	631	50	22	88	60
Yamazaki et al [274]	Japan	217	96	43	27	51	84
Mersin et al [275]	Turkey	1,101	667	246	82	106	44
Kim et al [276]	United States	5,683	3218	636	549	1280	52
Shim et al [277]	South Korea	1,244	699	227	145	173	48
Lee et al [278]	South Korea	1,432	860	162	157	253	53
Swisher et al [279]	United States	751	369	105	58	219	75.6

3.4.2.2 Overall LRR rates

Firstly we looked at the overall data results (Figure 3.7 & 3.8). There was a significantly lower risk of LRR in patients with Luminal A subtype of breast cancer when compared to Luminal B HER2 (OR 0.54, 95%CI 0.38 to 0.76; $p<0.0004$), HER2+(non-luminal) (OR 0.32, 95%CI 0.24 to 0.45; $p<0.0001$) and Triple negative breast cancers (OR 0.25, 95%CI 0.19 to 0.32; $p<0.0001$) (Figure 3.7 A-C). When

comparing the Luminal B HER2 cancers to the HER2+(non-luminal) and Triple negative cancers a significantly lower odds ratio is seen (OR 0.61, 95%CI 0.41 to 0.89; $p = 0.0145$) and (OR 0.47, 95%CI 0.33 to 0.65; $p < 0.0001$) respectively (Figure 3.8 A-B). There was also a trend towards a significant lower risk in the HER2+(non-luminal) compared to Triple negative breast cancers (OR 0.75, 95%CI 0.55 to 1.03; $p = 0.0933$) (Figure 3.8 C)

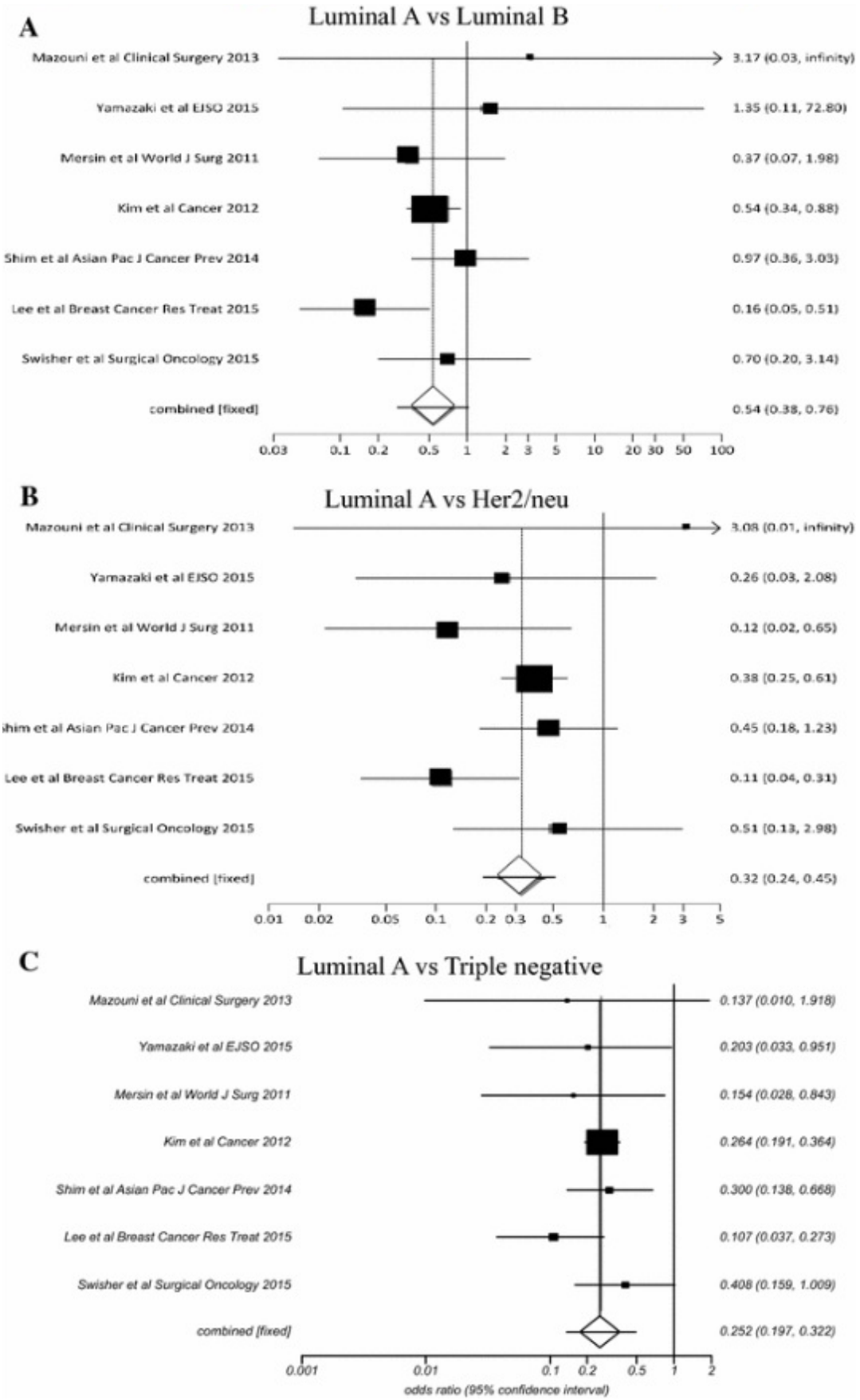


Figure 3.7 Forest plots comparing LRR rates between the Luminal A with the other breast cancer subtypes overall. A) Comparing Luminal A with Luminal B HER2, B) Comparing Luminal A with HER2+(non-luminal) and C) Comparing Luminal A to Triple negative.

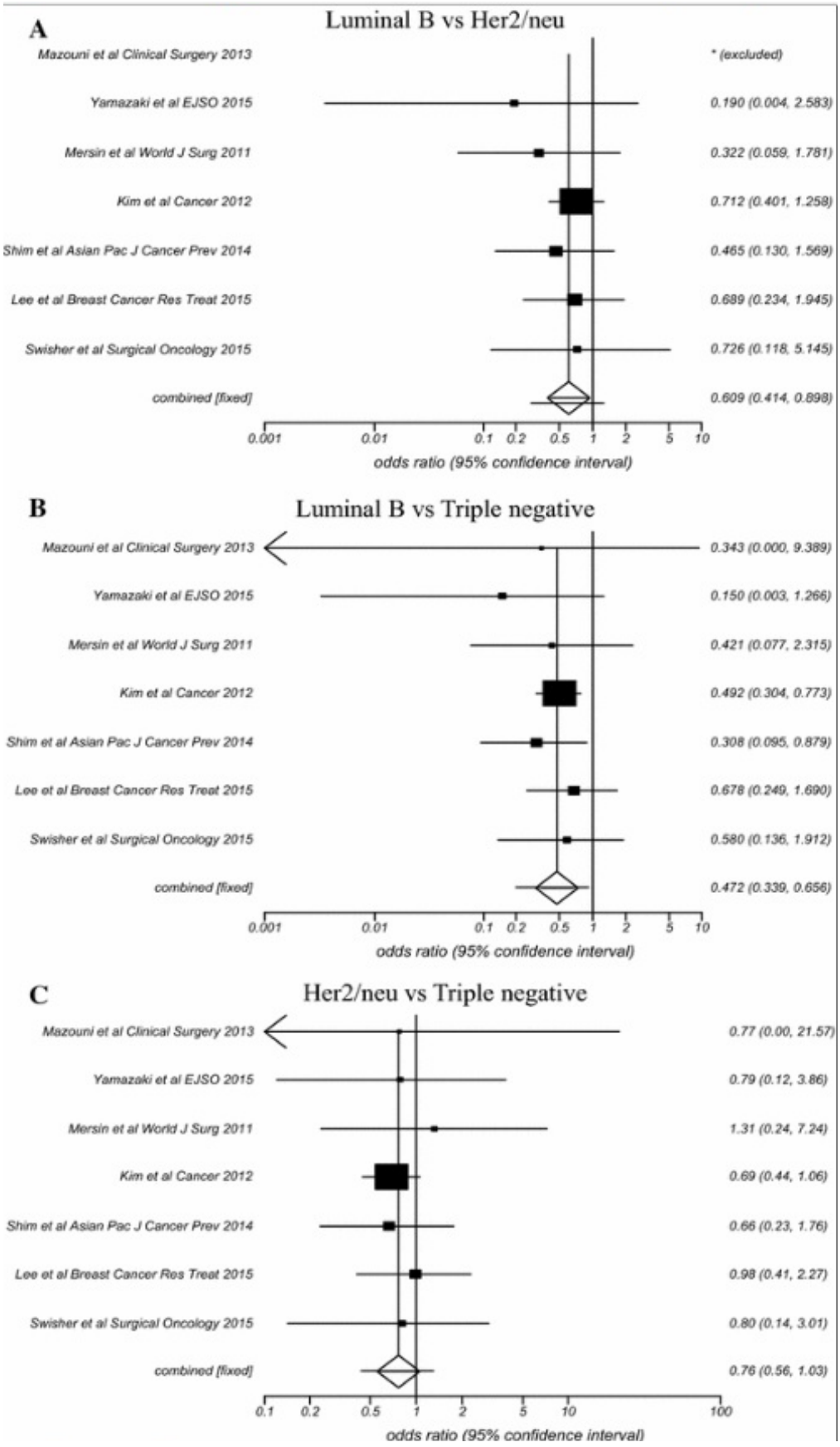


Figure 3.8 Forest plots comparing LRR rates between the non-Luminal A breast cancer subtypes overall. A) Comparing Luminal B HER2 to HER2+(non-luminal), B) Comparing Luminal B HER2 to Triple negative and C) Comparing HER2+(non-luminal) to Triple negative.

3.4.2.3 LRR rates post BCS

In total 3 studies, with 1,759 patients included (Mazouni et al (n=791), Yamazaki et al (n=217) and Swisher et al (n=751) were suitable for subgroup analysis for LRR in patients undergoing BCT (Figure 3.9). A similar trend in LRR rates are seen to the combined results with the lowest rates in Luminal A (1.3%), Luminal B HER2 rates (2.5%), HER2+(non-luminal) (5.6%) and the highest rates were in Triple negative breast cancers (6.4%). Interestingly, lower rates of LRR are seen in all four subtypes when compared to overall results. No significant difference is seen between Luminal A cancers and Luminal B HER2 cancers (OR 0.77, 95%CI0.28 to 2.09; p = 0.8123) or HER2+(non-luminal) (OR 0.37, 95%CI 0.14 to 0.97; p = 0.0794) (Figure 3.9 A-B). Only Triple negative breast cancers showed a significantly higher risk when compared to Luminal A cancers (OR 0.31 95%CI 0.16 to 0.6; p =0.0007) (Figure 3.9 C). There was also no significantly difference between Luminal B HER2 cancers and either HER2+(non-luminal) (OR 0.46, 95%CI 0.13 to 1.56; p = 0.349) or Triple negative cancers (OR 0.46, 95%CI 0.13 to 1.56; p = 0.349) (Figure 3.9 D-E). Once again no difference was seen when comparing HER2+(non-luminal) breast cancers and Triple negative (OR 0.79, 95%CI 0.31 to 1.97; p = 0.7785) (Figure 3.9 F).

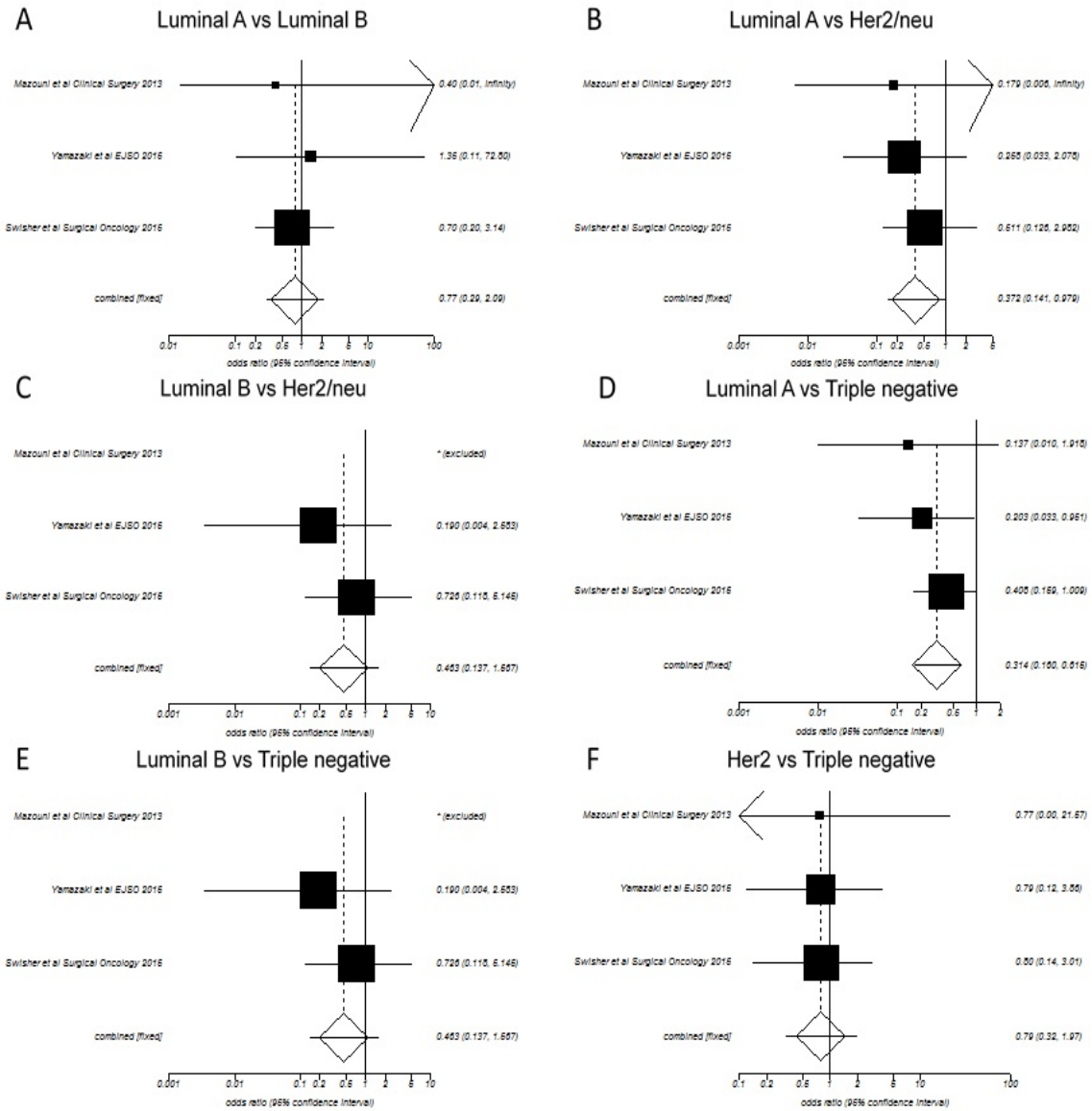


Figure 3.9 Forest plots comparing LRR rates between the different breast cancer subtypes in the breast conservative therapy cohort. A) Comparing Luminal A to Luminal B, B) comparing Luminal A to HER2+(non-luminal), C) Comparing Luminal B to HER2+(non-luminal), D) Comparing Luminal A to Triple negative, E) Comparing Luminal B to Triple negative and F) Comparing HER2+(non-luminal) to Triple negative.

3.4.2.4 Luminal vs non-luminal LRR

Next we sought to compare the luminal cancers to the non-luminal cancers (Figure 3.10). In the combined luminal group had significantly reduced LRR rates when compared to HER2+(non-luminal) (OR 0.37, 95%CI 0.27 to 0.50; p<0.0001) (Figure 3.10 A) and triple negative breast cancers (OR 0.29, 95%CI 0.23 to 0.36; p<0.0001) (Figure 3.10 B). In the BCT subgroup, no significant difference is seen between the luminal cancers and the HER2+(non-luminal) (OR 0.38, 0.15 to 0.96; p = 0.0719)

(Figure 3.10 C). However, a significant difference is seen against the triple negative (OR 0.33, 0.17 to 0.61; p = 0.0004) (Figure 3.10 D)

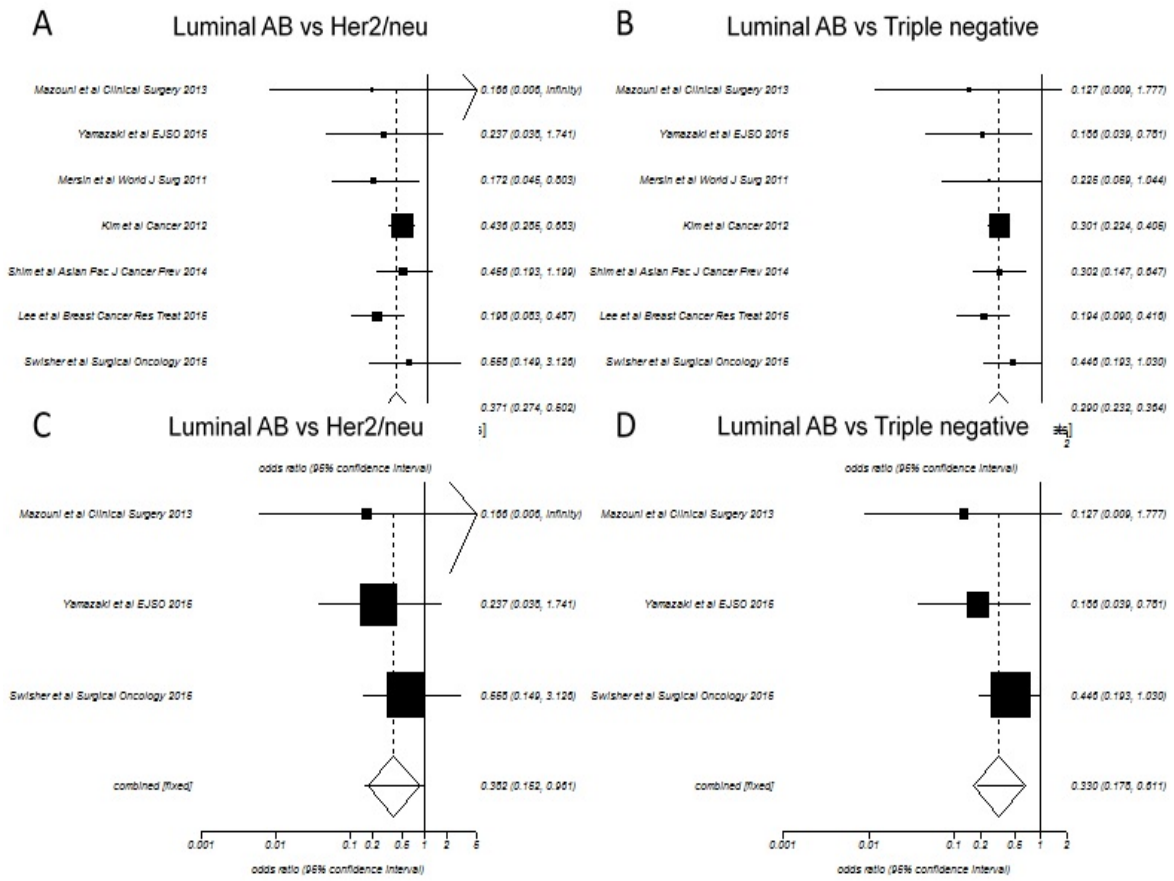


Figure 3.10 Forest plots comparing LRR rates between the different breast cancer subtypes in the luminal vs non-luminal cohort overall and in BCS cohort. A) Comparing Luminal to HER2+(non-luminal) overall, B) Comparing Luminal to Triple negative overall, C) Comparing Luminal to HER2+(non-luminal) post BCS and D) Comparing Luminal to Triple negative post BCS.

3.4.3 Discordance rates between primary and recurrence of breast cancer, and the impact on treatment in HER2 receptor positive breast cancers

In total 139 patients were included in the final analysis, with a summary of their clinicopathologicial details shown (Table 3.8).

Table 3.8 Clinical pathological details**.**

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	
Locoregional	58 (44%)
Distal	74 (56%)
Neoadjuvant Chemo Rx	
Yes	58 (44%)
No	74 (56%)
Surgery	
Mastectomy	83 (62.8%)
Wide local excision	49 (37.2%)
Primary cancer subtype	
Luminal A	67 (50.7%)
Luminal B HER2	10 (7.5%)
HER2+(non-luminal)	15 (11.3%)
Triple negative	40 (30.5%)
Recurrence subtype	
Luminal A	54 (40.9%)
Luminal B HER2	9 (6.9%)
HER2+(non-luminal)	16 (12.1 %)
Triple negative	53 (40.1 %)

The mean time to recurrence was 38.7 months (range 2-144 months), with 58 patients (44%) having LRR, and 74 (56%) had a distant recurrence. In total 25 patients (18%) primary tumours were HER2 receptor positive, of which 10 were also ER and/or PR positive. In the patients with LRR, 17 (29.3%) had discordance of

breast cancer subtype with the primary breast cancer; while in the patients with distant metastasis, 14 (18.9%) had discordance with the primary breast cancer.

3.4.3.1 Discordance rates for receptors status

Overall there were 81 (58.3%) cases of discordance in at least one receptor status, with a statistically significantly higher amount gaining status rather than losing status (67/81 vs 14/81, p>0.005) (Table 3.9).

Table 3.9 Receptor discordance.

Overall		N (% total)
Concordant		58 (41.7%)
Discordant		81 (58.3%)
	Gain	14 (17.3%)
	Loss	67 (82.7%)
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%)

There was concordance in HER2 receptor status in 128 of the patients. Only four patients (3%) in total had discordance of the HER2 receptor status, with two patients gaining and two losing HER2 status (Table 3.9). This is in contrast with the ER and PR status, where discordance is seen in 27 (20.4%) and 50 (37.8%) patients respectively. In patients with breast cancers that are both ER and PR positive, a change in receptor status in only one of the hormone receptors would not result in a change in subtype or a change in treatment, so next we compared the changes in subtype and due to this, what proportion of patients would require additional treatment.

3.4.3.2 Discordance in breast cancer subtypes

In total there was a change in subtype in 31 (23.5%) of cases, with a large variation in discordance rates between the individual breast cancers subtypes (Table 3.10).

Table 3.10 Discordance in breast cancer subtypes.

Breast cancer subtype	Discordance N (%)	Luminal A	Luminal B HER2	HER2+ (non-luminal)	Triple negative
Luminal A	20 (29.8%)	47	1	1	18
Luminal B HER2	4 (40%)	2	6	2	0
HER2+ (non-luminal)	3 (20%)	0	3	12	0
Triple negative	4 (7.5%)	4	0	0	49

Luminal subtypes showed the most discordance between the primary breast cancer and recurrence, with 20 (29.8%) of Luminal A and 4 (40%) of Luminal B HER2 changing subtype. Only 3 (20%) of the HER2+(non-luminal) subtype and 4 (7.5%) of the triple negative subtype changed subtype. The most common pattern of discordance was from Luminal A to Triple negative.

3.4.3.3 Variations in treatment

Next, we sought to assess what proportion of patients would require addition treatment due to change in receptor status. Although 31 patient had discordances in subtype, as shown above the majority of these were due to a loss of receptor status. As a result, these patients would not have a change in treatment regimens due to the primary breast cancer subtype still requiring treatment. In total, only nine patients (6.5%) had a gain in receptor status that would require an addition of treatment, with only two (1.4%) of these patients gaining HER2 status.

3.4.4 Cost effectiveness analysis of neoadjuvant Trastuzumab

In total, 225 patients were included in the analysis, with an average age of 59 years. From this, 166 patients were included in the adjuvant Trastuzumab group, while 59 patients were included in the neoadjuvant Trastuzumab group. Radiotherapy was given in 81% of patients, with hormone therapy given in 96% of hormone receptor positive breast cancers. The overall mean cost of treatment was €46,403, with the mean increasing to €58,243 in patients with a recurrence of breast cancer. For patients having neoadjuvant chemotherapy 19 (32%) had a pathological complete response (pCR), and the mean cost of treatment was €45,749 in patients having a pCR.

3.4.4.1 Cost analysis

Initially, the mean cost of patients being treated in the adjuvant Trastuzumab group was compared with patients in the neoadjuvant Trastuzumab group. The mean cost of adjuvant group was €45, 481 (95%CI 43,551 to 47,410), while the mean cost of the neoadjuvant group was €48,997 (95%CI 45,659 to 52,336) and no statistically significant difference was found between the two groups (p=0.068) (Table 3.11). Total cost was also compared by type of surgery and by breast cancer subtype (Table 3.12).

Table 3.11 Total cost by treatment group.

Treatment Group	No. of patients	Mean cost (€)	95% CI
Adjuvant	166	45,480	43,550 - 47,410
Neoadjuvant	59	48,996	45,658 - 52,334
Combined	225	46,402	44,732 - 48,072

Table 3.12 Total cost by surgery/subtype/treatment regime.

	No. of patients	Mean cost (€)	95% CI	P value
Surgery type				
WLE	118	45,676	43,898 - 47,454	0.286
Mastectomy	103	47,492	44,518 - 50,465	
Tumour subtype				
Luminal B HER2	138	47,298	45,318 - 49,279	0.183
HER2+(non-luminal)	87	44,980	41,987 - 47,974	
Adjuvant group				
Luminal B HER2	105	45,900	43,888 - 47,912	0.574
HER2+(non-luminal)	61	44,757	40,725 - 48,788	
Neoadjuvant group				
Luminal B HER2	33	51,747	46,553 - 56,940	0.062
HER2+(non-luminal)	26	45,505	41,818 - 49,193	

No significant difference was seen in cost between patients undergoing breast conservative surgery (BCS) €45,676.4 (95%CI 43,898 to 47,454), when compared to patients who had a mastectomy €47,492.14 (95%CI 44,518 to 50,465) (p=0.286). Again, no significant difference in cost are seen between the Luminal B HER2 patients €47,298.82 (95%CI 45,318 to 49,279), and the HER2+(non-luminal) subtype €44,980 (95%CI 41,987 to 47,974) (p=0.183). Comparing the cost by breast cancer subtype and Trastuzumab treatment group, in the adjuvant group no significant difference was seen between the Luminal B HER2 €45,900 (95%CI 43,888 to 47,912) and the HER2+(non-luminal) €44,757 (95%CI 40,725 to 48,788) breast cancer subtype (p=0.574). In the neoadjuvant group, a higher cost was seen in the Luminal B HER2 €51,747 (95%CI 46,553 to 56,940) compared to the HER2+(non-luminal) €45,505 (95%CI 41,818 to 49,193) breast cancer subtype, with the p value approaching significance (p=0.062).

Next a multifactorial linear regression factoring for age, stage, grade and type of treatment was run, analyzing factors impacting total cost (Table 3.13).

Table 3.13 Multifactorial linear regression for total cost.

Variable	Coef.	95% CI	P value
Neoadjuvant group	3,921	33.3 to 7,810.3	0.048
Stage 2	2,463	-450.1 to 5376.8	0.097
Stage 3	9,708	5107.8 to 14,309.9	0.001
Age <50	1,603	489.6 to 2,717.6	0.005
Age >50	-13.57	-22.9 to -4.2	0.005
Grade 2	9,741	-6,252.7 to 25,735.4	0.231
Grade 3	9,401	-6,497.2 to 25,301.1	0.245

Patients treated by neoadjuvant Trastuzumab were found to have significantly higher cost of treatment compared to the adjuvant group (Coef €3,921.84, 95% CI 33.34 to 7,810.34; p=0.048). Other significant factors increasing cost included later stage (Coef €9,708.85, 95%CI 5,107.8 to 14,309.91; p >0.001) and age less than 50 years (Coef €1,603.64 95%CI 489.60 to 2,717.68; p=0.005), while age over 50 years was found to significantly reduce total cost (Coef €-13.58, 95%CI -22.95 to -4.20; p=0.005).

3.4.4.2 Survival analysis

The 3 year DFS for both patients in the adjuvant and neoadjuvant Trastuzumab groups was analysed next. For patients in the adjuvant Trastuzumab group, there was 97% DFS, compared with a 92% DFS for patients in the neoadjuvant Trastuzumab group. Next a multifactorial linear regression analysis for disease free survival was run (Table 3.14).

Table 3.14 Multifactorial linear regression for total disease free survival.

Variable	Coef.	95% CI	P value
Neoadjuvant group	-0.928	-8.897 to 7.040	0.819
Stage 2	-0.035	-7.682 to 7.612	0.993
Stage 3	-10.047	-18.147 to -1.948	0.016
Age <50	0.355	-1.664 to 2.376	0.729
Age >50	-0.005	-0.022 to 0.011	0.507
Grade 2	26.287	7.113 to 45.46	0.007
Grade 3	24.857	6.154 to 43.560	0.009

The neoadjuvant Trastuzumab group had no significant difference in DFS when compared to the adjuvant Trastuzumab group (Coef -0.93, 95%CI -8.90 to 7.04; p=0.819). Later stage was the only variable shown to have a significant impact on reducing DFS (Coef -10.05, 95%CI -18.15 to -1.95; p=0.016).

3.4.4.3 Cost effectiveness

Finally, the cost effectiveness analysis was done comparing adjuvant Trastuzumab treatment with neoadjuvant Trastuzumab (Table 3.15).

Table 3.15 QALY and Cost/QALY.

Treatment group	No.	QALYs (Range)	Average Cost (€)	Cost/QALY
Adjuvant Trastuzumab	136	2.53 (0.24 – 2.55)	45,481	17,977
Neoadjuvant Trastuzumab	41	2.54 (2.31 – 2.55)	48,997	19,290

The three year QALYs for adjuvant Trastuzumab was calculated as 2.53 (range 0.24 – 2.55), while for neoadjuvant Trastuzumab it was found to be 2.54 (range 2.31 – 2.55). The cost per QALY was calculated for each group, with the adjuvant Trastuzumab group costing €17,977/QALY and the neoadjuvant Trastuzumab group costing €19,290/QALY.

3.5 Discussion

3.5.1 Impact of hormone receptor status on response to Trastuzumab

The impact of Trastuzumab treatment has been well established, greatly improving survival and significantly reducing recurrence in patients with HER2 receptor positive breast cancers [57, 268, 280]. It has also been shown to improve pathological complete response rates, and in our study pCR was associated with an improved DFS. A pooled analysis of 11,955 patients in neoadjuvant chemotherapy trials, found that overall patients that achieved pCR had a higher level DFS than those with residual cancer [4]. However, few studies have investigated the differential effects of Trastuzumab treatment on Luminal B HER2 and HER2+(non-luminal) breast cancers.

Few previous studies have compared survival or recurrence rates between the two classes of HER2 receptor positive breast cancers since the introduction of Trastuzumab, Romond et al found at 4 years of follow up that hormone receptor status minimally influenced the response to Trastuzumab, although hormone receptor status was reported as a significant predictor of DFS [57]. A previous systematic review from our centre has shown LRR rates vary between breast cancer subtypes, with the HER2+(non-luminal) and Triple negative subtypes reported to have the highest LRR rates [10]. This study found the average LRR rate for breast cancer is 7.9%, however, the rates in HER2+(non-luminal) subtypes post breast conserving surgery, were as high as 15.7%. Our findings support previous studies where Trastuzumab treatment led to a reduction in LRR only in Luminal B HER2 cancers [276]. We observed a statically significant reduction in LRR rates in Luminal B HER2 patients, while no significant difference was seen in the HER2+(non-luminal) subtype.

Importantly to our knowledge we are the first to demonstrate difference in recurrence rates and sites since the introduction of Trastuzumab treatment for HER2 receptor positive breast cancers patients. The Kennecke et al study of 3,726 patients reported that rates of distant metastasis vary by subtype [8], although this was in the pre Trastuzumab era. Previous studies demonstrated that bone was the most common recurrence site in Luminal B HER2 breast cancers and lung was most common for HER2+(non-luminal) [8, 265]. We show Trastuzumab treatment resulted in a

reduction in metastasis to all sites, except the brain which might be explained by the fact that Trastuzumab does not cross the blood/brain barrier [281, 282]. Surprisingly, Luminal B HER2 cancers treated with Trastuzumab showed the greatest reduction in distant metastasis to bone. In the Trastuzumab era no significant reduction in brain metastasis rates overall were seen, it was found that Luminal B HER2 cancers had a significantly reduced odds ratio of brain metastasis when compared to HER+(non-luminal) cancers. This correlates with previous studies which show a higher rate of brain metastasis in hormone receptor negative tumours [282].

The variation in recurrence rates found in our study may be due to intrinsic differences between the subtypes. Luminal B HER2 cancers can metastasise 10-15 years after treatment, while the majority of HER2+ve(non-luminal) cancers metastasise in <5 years [8]. However, the proportion of patients with Luminal B HER2 breast cancer subtype who develop metastasis more than 5 years after treatment is low [8]. Another potential reason for variation between subtypes could be that HER2 receptor over expression reduces the response to hormone therapy. Studies have observed increased hormone resistance rates in Luminal B HER2 cancers compared to Luminal cancers [283, 284]. Cross talk between HER2 receptors and hormone receptors results in activation of the hormone receptor, even in the presence of endocrine therapy [285]. Clinical trials have shown that the addition of Trastuzumab to endocrine therapy improves survival in metastatic breast cancer [286]. In our study the introduction of Trastuzumab resulted in significantly reduced incidence of distant metastasis, especially bone metastasis rates. By reducing the activity of HER2 receptors, Trastuzumab may restore the response to hormone therapy in Luminal B HER2 cancers [287]. In the neoadjuvant setting, this may provide an explanation as to why lower levels of pCR are seen in Luminal B HER2 cancers compared to HER2+(non-luminal) cancers [4].

Another potential difference between subtypes could be the increased number of grade 3 cancers seen in the HER2+(non-luminal) group. It has been shown that both HER2+(non-luminal) and Triple negative breast cancers have worse outcomes and present with higher-grade cancer than Luminal cancers [15]. Although, in a pooled analysis of neoadjuvant Trastuzumab trials (where it is always given in conjunction with chemotherapy) grade 3 cancers had a higher pCR rate than grade 1 and 2

cancers [4]. Importantly, our study revealed that on multivariate analysis of patients treated with Trastuzumab, grade 3 cancers did not have a higher DFS or OS risk when compared to grade 1 and 2 breast cancers.

In patients receiving neoadjuvant Trastuzumab chemotherapy, around 40% of patients will have a complete pathological response [70, 288], confirming that a large proportion of patients only have a partial or no response to Trastuzumab treatment. This resulted in the development of new anti-HER2 receptor treatments targeting different pathways such as Pertuzumab, which has been shown to increase response [73]. Our study highlights the improved prognosis associated with anti HER2 receptor therapy, it also demonstrates that a large proportion of patients survived despite not being treated with Trastuzumab. We believe this clearly indicates that Trastuzumab treatment is not required for all HER2 receptor positive breast cancers and demonstrates the clear need to develop a molecular or genetic scoring system to identify which patients will benefit from Trastuzumab treatment and those that will not.

This study also highlights how a targeted therapy has altered responses in related breast cancer subtypes, emphasising that molecular differences defining efficacy remain to be defined. This work highlights the need to fully understand the subtype specific effects and mechanisms of action of Trastuzumab therapy, which will allow truly individualised breast cancer management regimes to be implemented.

3.5.2 Variations in LRR between breast cancer subtypes

Previous work by this institute has shown significant variations in LRR rates across breast cancer subtypes [10]. This review was done prior to the routine availability of Trastuzumab, with less than six percent of HER2 receptor positive patients in the study receiving Trastuzumab. The introduction of Trastuzumab treatment has been shown to significantly improve survival and outcomes in HER2 receptor positive breast cancers. However, the impact of Trastuzumab introduction on LRR across the different breast cancer subtypes has yet to be explored. In this systematic review of 11,219 patients, the impact of the introduction of the targeted therapy Trastuzumab on LRR is demonstrated.

When comparing results with the previous systematic review by this institute, a reduction in LRR is seen across all subtypes [10]. The overall LRR rates decrease from 7.9% to 3.44%, with LRR rates post BCT reducing from 7.12% to 2.8%. The reduction in LRR across the two studies highlights the effect of multiple changes in breast cancer management over the last two decades. An early study reporting LRR rates from 1986-1992 reported overall rates as high as 18.5% and rates post BCS at 16% [30]. While an overview of all randomised radiotherapy trials beginning prior to 1995, found that the five year risk LRR post BCT was 7% [53]. Improvements in imaging, earlier diagnosis, surgical planning and adjuvant therapy has resulted in significant improvements in survival and outcomes for breast cancer patients. This reduction in LRR is seen across all the breast cancer subtypes, with the greatest reduction seen in HER2+(non-luminal) breast cancer post BCT with rates reducing from 15.7% to 5.6%. The highest rates of LRR are seen in Triple negative breast cancers both in the overall analysis and post BCT. Post BCT there is no longer any significant difference seen between luminal B HER2 and HER2+(non-luminal) subtypes, though a significant difference is seen in overall rates of local recurrence with a reduction in the Trastuzumab era.

In our study, Luminal A breast cancers had the lowest rate of LRR both overall and post BCT when compared to the other three subtypes. The introduction of endocrine therapy has resulted in a significant reduction in recurrence rates in Luminal breast cancers [39, 40]. Surprisingly, in the overall results despite the introduction of Trastuzumab, there was still a significant difference in LRR between Luminal A and Luminal B HER2 breast cancers. This could be explained by the large proportion of Luminal B HER2 patients that have only a partial or no response to Trastuzumab therapy. Neoadjuvant Trastuzumab trials found that only 30.9% of Luminal B HER2 breast cancer had a complete pathological response [4].

Multiple studies have examined the effect of Trastuzumab on LRR rates in HER2 receptor positive breast cancers [57, 70, 267, 289, 290]. Reduced LRR are seen across all these studies with the rate of reduction varying from 1-7%. One study reported a 40% relative reduction in the number of LRR cases but this only resulted in a 1.8% reduction in LRR overall [57]. Only Keiss et al showed a significant reduction in LRR rates after the introduction of Trastuzumab. The largest study with

over 3,000 patients treated with one year of adjuvant Trastuzumab, showed little improvement in LRR, with rates reducing only from 4% to 3% [267]. However, in a study that compared reduction in LRR in both of the HER2 receptor positive subtypes, a difference was seen between them [276]. In Luminal B HER2 cancers, LRR rates dropped from 6% to 3% following the introduction of Trastuzumab, while HER2+(non-luminal) breast cancers demonstrated the same rate of LRR (6%) before and after treatment. This matches the result from this systematic review, where a significantly lower risk of LRR is seen in Luminal B HER2 breast cancers when compared with HER2+(non-luminal).

Triple negative breast cancers had the highest rates of LRR in both the combined results and the BCT subgroup overall. Triple negative breast cancers are known to be aggressive and to have the lowest survival rates of the breast cancer subtypes. This is highlighted in neoadjuvant chemotherapy studies, where it was found that despite Triple negative breast cancers having the highest pathological complete response rate, these cancers have the poorest survival rates [68, 69]. This is due to the aggressive nature of these cancers, whereby the patients who do not respond to treatment have poorer outcomes. Triple negative breast cancers have also been linked to genetically inherited cancers such as BRCA 1 and 2, which are known to be more aggressive and tend to occur at an earlier age [189, 291].

Our analysis of Luminal vs non-luminal cancers has highlighted that there are significantly higher LRR rates in non-luminal breast cancers, indicating a possible need for more aggressive local therapy. Multiple randomized controlled trials have shown equivalent survival in stage 1 & 2 breast cancer patients undergoing BCT as with mastectomy [35, 292]. Further to this, for stage 1 & 2 breast cancers undergoing BCT, wider margins are not indicated based on tumour subtype under current recommendations [293]. This is based on a number of studies, which showed lower LRR rates in patients undergoing BCT compared with mastectomy in Triple negative breast cancers [294, 295]. Comparing the results of our subset analysis of patients who had BCT with the overall combined results, lower LRR rates are seen in the BCT subset in all subtypes. This further highlights the safety of current guidelines for selection of patients suitable for BCT.

Although the benefit of BCT in reducing LRR has been shown, the use of post-mastectomy radiotherapy (PMRT) remains a topic of debate. Significant improvement in LRR for high risk patients having PMRT are seen in long-term survival studies [296, 297]. However, the use of PMRT in intermediate risk patients is still unclear, with studies showing varying benefit [298, 299]. Current guidelines for patients with 1-3 positive lymph nodes, recommend patients undergo PMRT [299]. In our study, the significant variation in LRR between luminal and non-luminal subtypes may provide a way to stratify intermediate risk patients that would benefit from PMRT. One study looked at LRR rates before and after the introduction of PMRT in the four subtypes [300]. In this study, while the greatest reduction in LRR was seen in Luminal cancers, there was still over 50% reduction in LRR in HER2+(non-luminal) and Triple negative breast cancers. A retrospective study comparing LRR free survival between patients with Triple negative breast cancers, found that the highest rates of LRR were in post-mastectomy patients without adjuvant radiotherapy [294]. This highlights the need for a prospective study to analyze PMRT in Triple negative breast cancers to assess what cohorts may derive benefit.

There are a few limitations in this review that need to be acknowledged. The breast cancer subtypes were not standardized across the series and were assigned based on hormone receptor and HER2 receptor status. The use of Ki-67 to further stratify the Luminal cancer subtypes, may provide further information on differences in LRR between the breast cancer subtypes. A potential bias is the variation in follow-up between the studies which varied from 44-84 months, with LRR in Luminal breast cancers tending to reoccur at a later stage. Important risk factors such as patient age, tumour stage and tumour grade could not be independently assessed and further analysis accounting for these risk factors may provide a more accurate results. Further to this, radiotherapy post BCT is reported as whole breast radiotherapy. Ideally radiotherapy rates would be separated into local and regional radiotherapy. Finally, LRR after different surgery types was not reported in four of the studies, and only one study Mersin et al reported LRR rates after mastectomy. Due to this, direct analysis of LRR rates post BCT cannot be compared with post mastectomy rates.

3.5.3 Discordance in breast cancer recurrence

This study found a large discordance rates in receptor status between primary breast cancer and recurrence, with gain of status far more common than loss (82.7% vs 17.3%), with HER2 loss of positivity only occurring in 4.6% of patients matching multiple other studies [87, 88, 269]. Similar discordance rates of 20.4%, 37.8% and 3% for ER, PR and HER2 receptors found in this study, match rates seen in both a recent meta-analysis and a prospective study analysing discordance rates by receptor status [269] [88].

Although high levels of discordance can be seen in receptor status, this may not result in a change of breast cancer subtype or imply necessity to change treatment. In patients positive for both ER and PR status, a loss in only one of these receptor status will mean the patient will remain a Luminal cancer, though of course loss of particularly Progesterone receptor positivity will reduce the likelihood of response to endocrine therapy. On assessing the impact on subtypes, discordance occurred in 23.5% of cases in our study, which matches previously reported rates [87, 301]. To date, there has yet to be a prospective study to investigate subtype discordance, with current prospective studies only reporting variations in receptor status only.

Investigating the discordance across the different subtypes, the highest rates were seen in Luminal B HER2 subtype and the lowest rates were seen in Triple negative breast cancers. The most common change in subtype between the primary and recurrence was from Luminal A to Triple negative subtypes, although rates are too low to test for significance. Overall, only two patients had a recurrence that changed to a HER2 receptor positive subtype, with no Triple negative breast cancers becoming HER2 receptor positive. A similar trend was seen by Shiino et al, with only 4% of patients changing from a HER2 negative to a HER2 positive subtype and only 2 patients (1%) of triple negative cancers becoming HER2 positive on recurrence. This leads to the question, what impact discordance has on treatment regimens?

Our study found that change in receptor status would only result in treatment change in 9 (6.5%) of patients, with the addition of targeted HER2 therapy being appropriate in only 1.4% of all patients biopsied. This means fifteen patients having a biopsy of a

recurrence for every one patient having an additional treatment and fifty-five patients requiring a biopsy to diagnosis one patient gaining HER2 positivity. A pooled analysis of two prospective studies reported a 14% change in treatment compared with a pre-biopsy treatment plan [88]. However, on further analysis half of the changes in regimen was due to loss of receptor status, new primary diagnosis or benign disease on biopsy. In total only 6.9% of patients had a treatment added due to gain in receptor status and further to this, only one in twenty six patients biopsied had anti HER2 therapy added due to a gain in HER2 receptor status. This raises the question, should all recurrences be biopsied?

Many studies have shown the benefits of taking a biopsy of a recurrence, firstly it can differentiate a recurrence from a new primary breast cancer, change treatment and can provide further information on prognosis [87, 88, 301]. As part of the current ASCO guidelines, it is recommended that a recurrence be biopsied when feasible [302]. However, this recommendation is deemed moderate, as debate remains over the long-term survival benefit of changing therapy based on biopsy. In addition to this, it may not always be possible to biopsy a recurrence due to technical difficulty and studies have reported a complication rate of 2% for image guided percutaneous biopsies [303]. This, along with the low numbers of additional treatment due gain of status seen in our study, highlights the need for non-invasive biomarkers to diagnose discordance.

A number of reasons for discordance between primary breast cancer and recurrence have been researched. One potential reason is the handing, processing and interpretation of the immunohistochemistry results, which can all have an impact on the final immunohistochemistry results [304]. A more commonly excepted position, is that there may be an element of heterogeneity within the primary tumour itself, with clones of resistant cells or multiple different subtypes in a single tumour [305-307]. This could allow the small proportion of cells in a tumour that are resistant to treatment to propagate during treatment. This may explain the higher rate of recurrence with loss of status, with clones that have negative receptor status not being eradicated with targeted hormone and HER2 therapy.

3.5.4 Cost effectiveness of neoadjuvant Trastuzumab

In this section, the economic impact of treating HER2 receptor breast cancer with Trastuzumab was examined, specifically assessing if there was any variation in cost effectiveness between patients who received adjuvant Trastuzumab compared to neoadjuvant Trastuzumab. As shown in section 2.4.1, a variation in survival and outcomes is seen between the two HER2 receptor positive breast cancers subtypes. Due to this, the costs of treatment of Luminal B HER2 and HER2+(non-luminal) breast cancer subtypes were compared, as well as assessing if there was any variation between the costs of therapy in the adjuvant Trastuzumab group and the neoadjuvant Trastuzumab group.

Studies have shown that the addition of adjuvant Trastuzumab to the treatment of HER2+ breast cancers can significantly improve survival and reduce recurrence [267, 308]. Further to this, it has been shown that it can increase life expectancy by 1.54 QALYs [309]. This was also shown in section 3.4.1, where a significant improvement in both DFS and OS was seen after the introduction of Trastuzumab. The addition of neoadjuvant Trastuzumab to one year of adjuvant Trastuzumab chemotherapy has been shown to improve DFS, one study comparing neoadjuvant combined with adjuvant Trastuzumab to adjuvant Trastuzumab alone found it could significantly improve DFS [70]. However, pooled analysis of prospective neoadjuvant chemotherapy trials have shown survival is dependent on response to treatment, with patients having a pCR having an improved DFS [4]. In our study after 3 years follow up, no significant difference was seen in survival between the adjuvant Trastuzumab group and the neoadjuvant Trastuzumab group on multivariate analysis. To assess for a variation in cost effectiveness between the two groups, the cost of treatment for the adjuvant Trastuzumab group and neoadjuvant Trastuzumab group was also analysed.

Numerous studies have assessed the cost effectiveness of adjuvant Trastuzumab treatment in HER2 receptor positive breast cancer, with the majority of these studies suggesting it would be cost effective, running under $50,000/QALY [76, 78, 310]. In our analysis a favourable cost effectiveness of €17,997/QALY is seen, once again showing the benefit of adjuvant Trastuzumab.

There are a number of issues with using cost/QALY for analysing cost effectiveness of Trastuzumab. A systematic review of cost effectiveness found large variation in cost per QALY across studies, ranging from $5020/QALY to $134,610/QALY and almost 85% of studies used a predictive Markov model to obtain results [310]. The large variation in reported cost per QALY in the literature may be due to publication bias, where studies might only publish positive or negative results but not intermediate results [311].

The addition of Trastuzumab to neoadjuvant chemotherapy regimens for HER2 receptor positive breast cancer has been shown to significantly improve pathological complete response rates [71, 288]. It also increases the proportion of patients undergoing breast conserving surgery and may improve DFS rates, which may improve cost effectiveness. To date, no study has assessed the impact of adding neoadjuvant Trastuzumab to cost effectiveness in treating HER2 receptor positive breast cancers. In our study when comparing direct cost of treatment, no significant difference was seen between the adjuvant Trastuzumab group and the neoadjuvant Trastuzumab group (€45, 481 versus €48,997, p=0.068). On multivariate analysis though, neoadjuvant Trastuzumab was found to be a significant factor for increasing cost of treatment. Finally on assessing cost effectiveness, the neoadjuvant Trastuzumab group had a favourable result of €19,290/QALY, with minimal difference seen compared to the adjuvant Trastuzumab group.

As the final part of the study, the cost of treatment between the two HER2 receptor positive subtypes was compared. As shown in section 3.4.1 – 3.4.3 above, significant differences in survival, recurrence rates, and recurrence patterns are seen between Luminal B HER2 and HER+(non-luminal) breast cancers. On assessing cost of treatment, no significant difference was seen overall and on subgroup analysis of the adjuvant Trastuzumab group. On evaluating the neoadjuvant Trastuzumab group, while not significant, there is a trend towards reduced cost in the HER2+(non-luminal) breast cancer subtype. This may be due to the increased pathological complete response rate seen in HER2+(non-luminal) subtypes [4], resulting in reduced surgical intervention and reduced recurrence rates.

There are a number of limitations with this study that need to be acknowledged. Firstly, due to the retrospective nature of the study, length of stay and in hospital treatment were not available and excluded from the over cost. Also the adjuvant Trastuzumab and neoadjuvant Trastuzumab groups were not matched for age and stage. A large proportion of the neoadjuvant group had late stage or node positive disease, increasing the need for mastectomy and axillary node clearance, along with the risk of disease recurrence. This would have contributed to the increased cost seen in the neoadjuvant Trastuzumab group, as later stage was found to be a significant factor for increased cost on multivariate analysis. Finally, the DFS rate was calculated after three years of follow up, a long-term follow up may provide a more accurate assessment of cost effectiveness in the neoadjuvant Trastuzumab group.

3.6 Overall discussion and key questions

In this chapter the survival, outcomes and response to treatment are compared between the two HER2 receptor positive breast cancers.

In section 3.4.1, it was shown that the Luminal B HER2 and HER2+(non-luminal) breast cancer subtypes responded differently to the introduction of Trastuzumab. Overall this study addressed its key aim, of identifying that hormone receptor status has a profound influence on survival and outcome in HER2 receptor positive breast cancers.

In section 3.4.2, a systematic review compared the LRR rates in breast cancer across the breast cancer subtypes, and once again a significant difference was found between the two HER2 receptor positive subtypes. Overall, this systematic review has demonstrated vast improvements in reducing LRR across all breast cancer subtypes over the last few decades.

In section 3.4.3, it was shown that there can be discordance in subtype between a patient's primary breast cancer and the recurrence. Loss of receptor status, is far more common than gain of receptor status, this resulted in only a small proportion of patients having new treatments added. Due to the risks involved with taking a biopsy, further investigation and long-term follow up is needed to assess if all

patients benefit from biopsy of breast cancer recurrence, and highlights the need for a biomarker to accurately predict discordance.

Finally in section 3.4.4, the cost effectiveness of neoadjuvant Trastuzumab treatment was compared to adjuvant Trastuzumab therapy. No significant difference was seen in DFS between the two groups on multivariate analysis. On comparing cost effectiveness, little difference is seen between the two groups and both were found to be within the excepted cost per QALY. On comparing the two HER2 receptor positive subtypes, despite difference in survival and recurrence rates, no significant difference in cost was found between the two subtypes. Overall, this study has shown that treatment with neoadjuvant Trastuzumab is cost effective, however a long-term prospective study may provide further evidence.

This leads to a number of key questions to be addressed in future work. With only about a third of patients having a complete response to neoadjuvant chemotherapy, would it be possible to identify a biomarker in a patient's blood sample prior to treatment that could predict which patient would respond? With the large variations seen between the breast cancer subtypes, would it be possible to identify a biomarker that could identify the four breast cancer subtypes at diagnosis? Further to this, with such a significant impact seen in treating HER2 receptor positive breast cancers with Trastuzumab, would it be possible to discover a biomarker that could not only identify HER2 receptor positive breast cancer but also predict response to Trastuzumab?

Over the next two chapters, we sought to address these key questions by running both in-vitro experiments in the laboratory and a prospective clinical trial.

Chapter 4

HER2 positive breast cancer biomarker discovery and validation

4.1 Introduction

Survival from breast cancer has been improving over the last few decades, due to improvements in diagnosis and treatments [312]. However, breast cancer is still responsible for 690 deaths each year (17% of all cancer deaths in women) in Ireland alone (National cancer registry Ireland, March 2016). There is also a diverse response to treatment seen in breast cancer patients, but the introduction of molecular profiling has provided great insights into the differences seen. Dividing breast cancer into different subtypes, has allowed a more targeted approach to treatment [313]. A current major aim in breast cancer research is to identify biomarkers that can aid diagnosis, predict response to treatment or predict recurrence. Biomarkers are a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified or monitored.

Numerous new potential biomarkers for breast cancer have been identified such as telomere length or circulating free DNA [314, 315], but a biomarker with the greatest potential is microRNAs. Previous research from our own group, has shown that microRNAs are altered in breast cancer and may be able to differentiate different breast cancer subtypes [92, 93, 316, 317].

One of the greatest difficulties with using microRNA as biomarkers, is the number of microRNA identified, with over 6700 microRNAs identified to date and the number is continuously increasing [318]. Researchers use microarrays, to profile all known microRNAs expression [319]. This involves profiling multiple patient and control samples for all known microRNAs. The results are analysed for variations in microRNA expression in breast cancer patients. This also gives us the opportunity to analyse for variations within breast cancer subtypes, discovering markers that may predict different responses to treatment or survival.

To confirm variations in microRNA expression identified on a microarray, the changes in expression levels need to be validated. One way involves, analysing the microRNA expression levels using quantitative polymerase chain reaction (PCR) in breast cancer cell lines. Apart from validating microRNA expression, the in vitro

model also allows the investigation of the impact of chemotherapy on growth and expression levels of microRNA levels [320, 321].

4.2 Aims

The aims of this chapter were firstly to identify a new microRNA biomarker that can detect breast cancer patients from health controls using a microarray, and to assess if this microarray data could also identify a biomarker for HER2 positive breast cancers (Section 4.4.1). Next, we sought to validate if previous published possible biomarkers (ICORG targets: let-7a, miR-10b, miR-21, miR-145, miR-155 & miR-195) could separate breast cancer cell lines from a control normal breast tissue cell line, and assess if these microRNA could also separate breast cancer subtypes, specifically if these microRNA could identify HER receptor positive versus negative subtypes (Section 4.4.2). The final aim was to analyze the impact of Trastuzumab therapy on growth and microRNA expression on hormone receptor positive and negative, HER2 receptor positive breast cancers (Section 4.4.3).

4.3 Methods

4.3.1 Cohort selection

Whole blood samples were consecutively collected from patients at diagnosis, in a prospective study. Clinicopathological data, such as menopausal status, stage, grade, tumour size and subtype were collected. Healthy controls were selected from an age matched population. Ethical approval was obtained, along with consent from participating patients and controls.

4.3.2 MicroRNA array

For the microarray analysis, 38 whole blood samples (controls and breast cancer) in PAXgene tubes were sent to Exiqon headquarters in Vedbeak, Denmark. The microRNA profiling was performed on the Exiqon miRCURY™ LNA array identified >5000 microRNAs targets that are differentially expressed in the different samples. Reversal of these pass the Benjamini-Hochberg correction for multiple testing.

4.3.3 Statistical analysis of microarray

Analysis was performed on control versus cancer, and along with subgroup analysis of each breast cancer subtype against each other. Analysis was performed by Exiqon and Dr Phil O'Brien. For comparative analysis, both heat maps and correlation circle plots were used to identify potential biomarkers. In the heat maps, variations in colour from red to green indicate the variation in microRNA expression across variables. In the correlation plots, the inner eclipse indicates 50% of the explained variance. The outer eclipse indicates 100% explained variance, so the nearer the variable to the outer eclipse the greater the likelihood of explained variance between the two groups.

4.3.4 Subtypes definitions

Breast cancer subtypes were defined using ER, PR and HER2 receptor status. Luminal B HER2 was defined as (ER and/or PR +ve, HER2 +ve) and HER2+(non-luminal) as (ER and PR –ve, HER2 +ve) according to standard clinical pathological guidelines. The ER and PR receptor status were determined independently by clinical pathologists using immunohistochemistry as per ASCO guidelines (ALLRED score >2 or more than 1% stain positive). The HER2 receptor status was identified by Herceptest, as part of the routine clinical evaluation, with a score of 3+ considered positive. Any +2 inconclusive results were confirmed using a FISH testing as per ASCO guidelines, with a HER2/CEP17 ratio greater than two considered amplified.

4.3.5 In-vitro breast cancer cell lines

Four cell lines [MCF10A – normal breast tissue, T47D - Luminal A subtype, BT474 – Luminal B HER2, and SKBR3 – HER2+(non-luminal)] were recovered from liquid nitrogen. The two HER2 positive cell lines (BT474 and SKBR3) are known to have the highest amplification of HER2 on FISH and have been shown to be sensitive to Trastuzumab therapy [135, 322].

4.3.6 Cell line growth and maintenance

To recover cells, separate media was made for each cell.

MCF10A: DMEM/Hanms F-12 medium + 5% Horse serum, 20ng/ml Epidermal Growth Factor + 100ng/ml Cholera Toxin + 0.01mg/mL insulin + 500ng/ml

Hydrocortisone + Pen/Strep (100IU/mL Penicillin G/100mg/mL Streptomycin sulfate)

T47D: RPMI 1640 w/L-glutamine + 10% Fœtal bovine serum (FBS) + Pen/Strep

BT474: RPMI 1640 w/L-glutamine + 10% FBS + Pen/Strep

SKBR3: McCoys 5A w/L-glutamine + 10% FBS and Pen/Step

Following this 10mls of each media was added to a T25 incubation flask and heated in an incubator. The cell pellets were then defrosted rapidly in a water bath and added to the flasks. The flasks were then placed in an incubator at 37 degrees Celsius, with 5% CO2 and humidified overnight to allow cells to adhere to the flask. The following day, the media was poured off and the cells were washed using 5mls of PBS, 10mls of fresh media was add to the flask the cells were placed back in an incubator to grow. The washing and feeding steps were repeated until the cells became 70-90% confluent. The cells were then washed with PBS and 2mls of trypsin was added to the flask to lift the cells off the base of the flask, the cells were then split and replanted in a T75 flask filled with 15mls of fresh media and placed back into the incubator to allow to grow. The cells were washed and feed until cells reached a confluence of around one million cells.

4.3.7 Ribonucleic acid extraction

Once cells had reached a confluence of approximately one million cells, the cells were trypsinized and then growth media was added to deactivate the trypsin. Following this the cells were spun down using a centrifuge at 1500rpm for 5 minutes, the media was then removed leaving only the pellet, the pellet was washed again with PBS and the spin repeated. The PBS was removed leaving only the pellet which was then immediately frozen at -80 degrees Celsius.

To extract the mRNA, an automated system (Roche magNA pure compact) was used. Cell pellets were defrosted on ice, then in an extraction hood 100µls of PBS and lysis buffer were added to each pellet and mixed well. This 200µls mixture was added to the magNA pure, along with 20µls of DNase. The extraction of mRNA from cells protocol was run, with mRNA extracted in a 50µL volume of buffer. The mRNA was labelled and frozen at -80 degrees Celsius, until ready for use.

4.3.8 Complementary DNA synthesis for gene expression

The dTNP mix/Nanomer random primer/Superscript III/5X RT buffer/0.1M DTT/RNaseOut (200U/ml) were defrosted on ice, along with each sample. Using NanoDrop 1000 spectrophotometer, the mRNA concentration and purity for each sample was assessed. A 1μL aliquot of RNA was pipetted onto the apparatus pedestal, with a RNA absorbance ration at 260 and 280 nm between 1.8 to 2.2 deemed indicative of pure RNA. The concentration of each sample for 1μg of RNA was calculated using the equation: 1μg= 1/RNA conc (μg/μl) x1000. Nuclease free water (NFW) was added to each sample to make up a final volume of 11.67μL plus a control and added to 200μL tubes and mixed by pipetting. Premix A was made up using 0.33μL/sample of Namomer random primer and 1μL/sample of dNTP (Table 3.1), then 1.3μL of premix A was added to all samples and the control, mixed and any secondary structures that may have formed in the RNA were denatured by running sscript denature program on the GeneAmp PCR system 9700. During this denature program, premix B is made up using 4μL/sample 5X RT buffer and 1μL/sample of DTT/RNaseOut/SuperScript III. Once the denature run is complete 7μL of premix B was added to each sample and the control and mixed by pipetting. The samples were then added back to the GeneAmp PCR system 9700 and run on the sscript program. Once completed, the samples are spun to bring down condensation, transferred to a 200μL tube with 30μL of nuclease free water (NFW) and frozen at -20 degrees Celsius for storage.

Table 4.1 cDNA synthesis reagents and volumes.

Reagent	Volume
Premix A	
Nanomer Random Primers (3 μg/μL)	0.33μL/sample
dNTP mix (10mM)	1μL/sample
Premix B	
5X RT Buffer	4μL/sample
DTT (0.1 M)	1μL/sample
RNaseOut (200U/μL)	1μL/sample
SuperScript III (200U/μL)	1μL/sample

4.3.9 Complementary DNA synthesis for microRNA

The dTNP mix/RT buffer/stem loop primer (for each target)/multiscribe/Rnase inhibitor, along with each sample were taken out of the freezer and allowed to defrost on ice. Using a NanoDrop 1000 spectrophotometer, the mRNA concentration and purity for each sample was assessed. A 1μL aliquot of RNA was pipetted onto the apparatus pedestal, with a RNA absorbance ration at 260 and 280 nm between 1.8 to 2.2 deemed indicative of pure RNA. Using the miRNA concentration, the total/miRNA needed for each 5μL sample was calculated. The volume of each reagent needed to make 10μL premix per target was also calculated (Table 4.2), and made up for each target microRNA, each premix is then votexed to mix and spun down. Next 10μL of premix is added to each sample. Following this, the calculated 5μL mix (total/miRNA+NFW) was added to each sample. To out rule contamination of any of the premix components a RTC was also made for each target, containing 10μL of target premix and 5μL of NFW. The samples were then spun again in a mini centrifuge and loaded into an Applied Biosystems GeneAmp PCR system 9700 and the cDNA generation program was run, samples are heated to 16 ^{o}C for 30 minutes, 42 ^{o} C for 30 minutes, 85 ^{o} C for 5 minutes and then cooled to 4 degrees. Following completion of cDNA generation, samples were then

transferred into sealed tube and diluted with 30μL of NFW to get a final total of 45μL of cDNA to measure. The cDNA is then stored at -20 C.

Table 4.2 cDNA reaction mix.

Reagent	Volume
NFW	4.57μL/sample
Stem Loop Primer	3.1μL/sample
10X RT Buffer	1.65μL/sample
Multiscribe (50U/μL)	1.1μL/sample
RNase Inhibitor (20U/μL)	0.21μL/sample
dNTP Mix (100mM)	0.17μL/sample

4.3.10 Real time quantitative polymerase chain reaction

To show there was no variation between PCR plates, an interassay control (IAC) was added to every PCR plate. MicroRNA 26b was chosen as the IAC, with cDNA from a control sample made up as above along with an RTC. For each plate of PCR, 1μL of cDNA of the IAC was run in triplicate. Consecutive PCR plates could then be linked, by having the average CT of the IAC within 0.3 standard deviations on each plate.

The cDNA for each sample, along with each target microRNA PDAR was slowly defrosted on ice. Premix for each sample and the reverse transcription control (RTC) for each target was made up using fast mastermix, NFW and pre-developed TaqMan assay reagent (PDAR) for each target microRNA (Table 3.3). Following this each premix was votexed, down and using an electronic pipette, premix was added to the PCR plate. Next 1μL of cDNA for each sample was added in triplicate to the PCR plate. To out rule contamination of the PCR plate, a no template control (NTC) was run on each plate, which contains premix for each target and 1μL of NFW in triplicate. After cDNA for each sample, RTC, IAC and NFW for the NTC were

added for each sample, the plate was then sealed using an optical adhesive cover. Next the plate was spun down using a centrifuge for one minute at 2000RPM.

Amplification of the cDNA was done using an Applied Biosystems 7900HT fast-time PCR system. Using Applied Biosystems SDS 2.4 software, the samples and target microRNA were assigned as per PCR plate set up and saved on the system. Next the PCR plate was added to the machine and PCR was amplified by the plate being heated up to 95 ^{o}C for 20 seconds and them cooled to 60 ^{o} C for 20 seconds and this process was repeated for 40 cycles. Following completion of the run, the cycle threshold for detection of each target microRNA was analyzed using Applied Biosystems RQ manager software. The results were then saved and then converted to Microsoft excel for further analysis. The average CT value of the three replicates of each sample was calculated, along with the standard deviation between the 3 replicates. Any sample outside of 0.3 standard deviations of each other were excluded and the sample were repeated. Samples were also excluded if either the RTC or NTC for the target microRNA were contaminated, new cDNA was made for these samples and PCR was repeated.

Table 4.3 PCR premix.

Reagent	Volume
Fast Mastermix	5.0μL/sample
NFW	3.8μL/sample
microRNA PDAR	0.5μL/sample

4.3.11 Confirming HER2 gene expression in breast cancer cell lines

To confirm variation in expression of the HER2 gene, a Luminal A breast cancer cell (T47D), a Luminal B HER2 breast cancer cell line (BT474) were grown, along with a normal breast tissue cell line (MCF10A) and a HER2+(non-luminal) breast cancer line (SKBR3) as controls. HER2 receptor status was confirmed in all four cell lines by analyzing HER2 gene expression (Figure 4.1). A significantly lower HER2 gene expression was seen in the MCF10A cells compared to the T47D ($p=0.03$), BT474

(p=0.004) and SKBR3 (p=0.004) breast cancer cell lines. A significantly lower expression of the HER2 gene was also seen between the T47D breast cancer cell lines and both the BT474 (p=0.029) and the SKBR3 (p>0.001) breast cancer cell lines. No significant difference in HER2 gene expression was seen between the BT474 and SKBR3 breast cancer cell lines (p=0.529). This confirmed that the both the BT474 and SKBR3 breast cancer cell lines over-expressed the HER2 receptor.

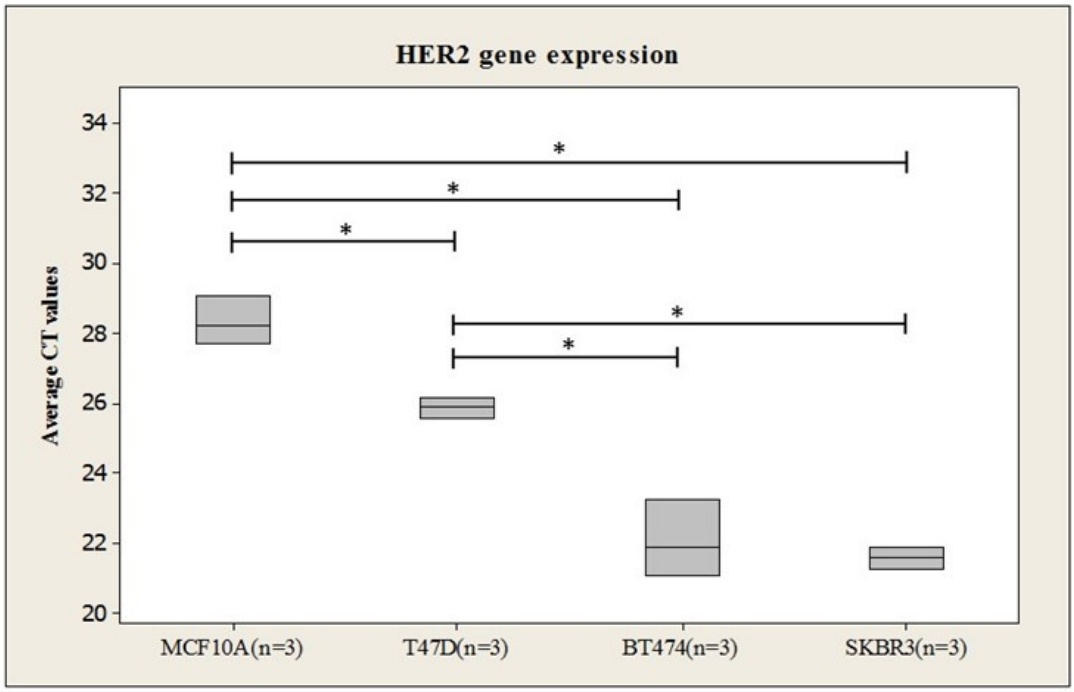


Figure 4.1 HER2 gene expression by breast cell line.

4.3.12 Growth Inhibition 50% of Trastuzumab

Firstly, BT474 were seeded at a concentration of 50,000 cells per well in a six well plate. To do this, a T75 flask of cells were trypsinized and added to media to deactivate the trypsin. The concentration of cells/ml was then calculated using a Chemometic nucleocounter, 100µL of solution A and solution B were added to 100 uL of cells and mixed. Using a nucleocassette a sample was taken from this mixture and the concentration per ml of cells was calculated. A sample of cells were diluted with media to 500,000 cell/ml. Next 100µLs from this concentration was added to 900mls of media in each well of a six well plate to give a concentration of 50,000 cells per well. The cells were then placed in an incubator overnight to allow the cells to adhere. The following day, the old media was removed, cells washed with 1ml of

PBS and fresh media added. Trastuzumab was then added at concentrations shown below.

Well 1: Trastuzumab 500 μg/ml
Well 2: Trastuzumab 100 μg/ml
Well 3: Trastuzumab 50 μg/ml
Well 4: Trastuzumab 10 μg/ml
Well 5: Trastuzumab 5 μg/ml
Well 6: Control

The six well plates were then placed back into the incubator for 72 hours. After 72 hours the cells were taken out, the old media removed, washed with 1ml of PBS and the cells were lifted off the plate using 1ml of trypsin in each well. Using a nucleocounter the amount of cells in each well was calculated.

To confirm the GI50% concentration, cells for all cell lines were seeded in two T25 flasks (control and test) in 10mls of media at a concentration of 400,000 cells per flask. After being left in an incubator overnight to adhere, the old media was removed, cells washed with 5mls of PBS and media with Trastuzumab at a concentration of 100micrograms per ml added to the test flask and regular media for the control flask for 72hrs. At 72hrs, the old media was removed, cells washed with PBS again and trypsinised with 2mls of trypsin. The cells were then added to media to deactivate the typsin and a cell count performed, the remaining cells were spun down using a centrifuge at 1500rpm for 5mins, media was discarded and then the cell pellet was frozen at -80 degrees Celsius to test the microRNA expression post treatment.

4.3.13 Statistical analysis

Minitab version 16 statistical software was used for boxplot generation and all statistical analysis. MicroRNA expression between cell lines was compared using 2-sample t-test. A p value of 0.05 considered significant.

4.4 Results

4.4.1 MicroRNA microarray

In total 33 consecutively collected whole blood samples were sent for analysis (7 controls and 26 breast cancer) (Table 4.4).

Table 4.4 Clinicopathological data for patients blood used in microRNA array.

Cases	Age (yrs)	T size (mm)	Nodal Status	Grade	UICC Stage	ER	PR	HER2/neu	Controls*	Age (yrs)
1	52	70	ypN3a	2	3C	+	+	−	1	70
2	37	67	pN2a	3	4	+	+	−	2	68
3	46	22	pN0	2	2A	+	+	−	3	54
4	81	15	pN0	2	1A	+	+	−	4	Unknown
5	56	23	pN0	2	2A	+	+	−	5	70
6	68	20	pN0	3	1A	+	+	−	6	57
7	54	9	pN0	2	1A	+	+	−	7	62
8	58	19	pN0	1	1A	+	+	−		
9	51	33	pN1a	2	2B	+	+	+		
10	82	18	pN0	3	1A	+	+	+		
11	78	36	pN2a	3	3A	+	+	+		
12	76	19	pN0	3	1A	+	+	+		
13	62	13	pN0	2	1A	+	+	+		
14	69	0		0	0	−	−	+		
15	58	0		0	0	−	−	+		
16	70	0	ypN0	3	Unknown	−	−	+		
17	62	1	pN0	0	1A	−	−	+		
18	41	20	pN0	3	1A	−	−	+		
19	54	0	ypN3a	2	3C	−	−	+		
20	71	7	pN1a	3	2A	−	−	−		
21	53	36	pN1a	3	2B	−	−	−		
22	56	6		2	1A	−	−	−		
23	82	13		3	1A	−	−	−		
24	78	26	pN0	2	2A	−	−	−		
25	60	7		3	1A	−	−	−		
26	54	0	pN1mi	2	Unknown	−	−	−		

4.4.1.1 Breast cancer versus control, microRNA expression

Overall there was poor distinction in the microRNA expression between breast cancer samples and controls, with no significant target microRNA identified. The top ten ranking microRNAs, showing variation between breast cancer patients and controls are shown (Figure 4.2 & 4.3). Overlay of clinicopathological information showed no distinct clustering of samples based on nodal status, tumour size, grade, or stage.

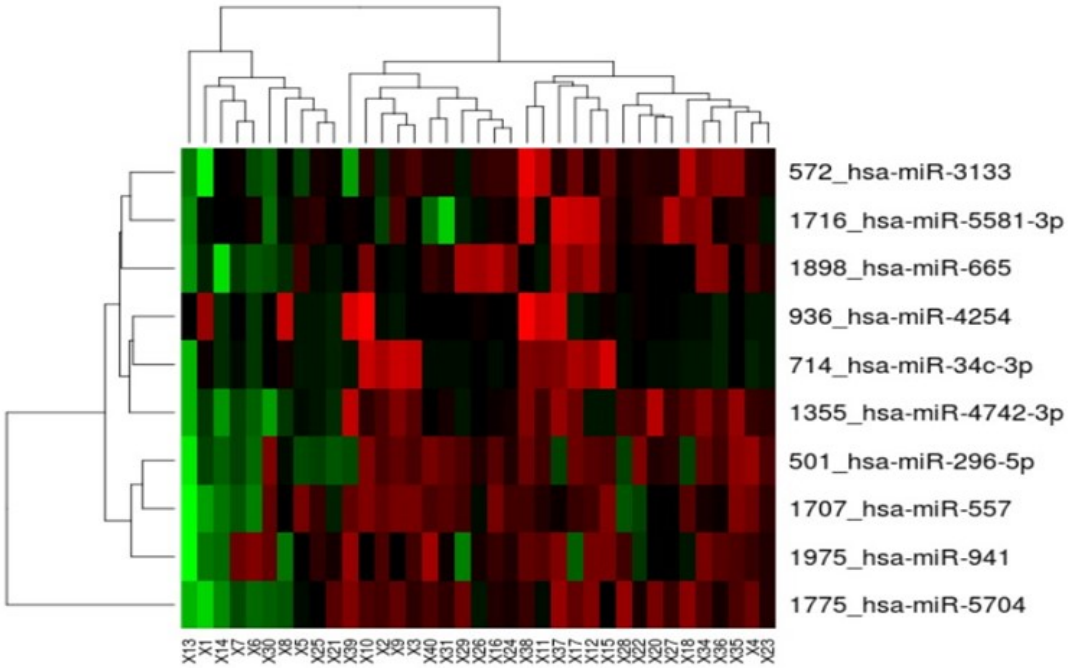


Figure 4.2 Heat map top ranking microRNA expression in breast cancer patients versus health controls.

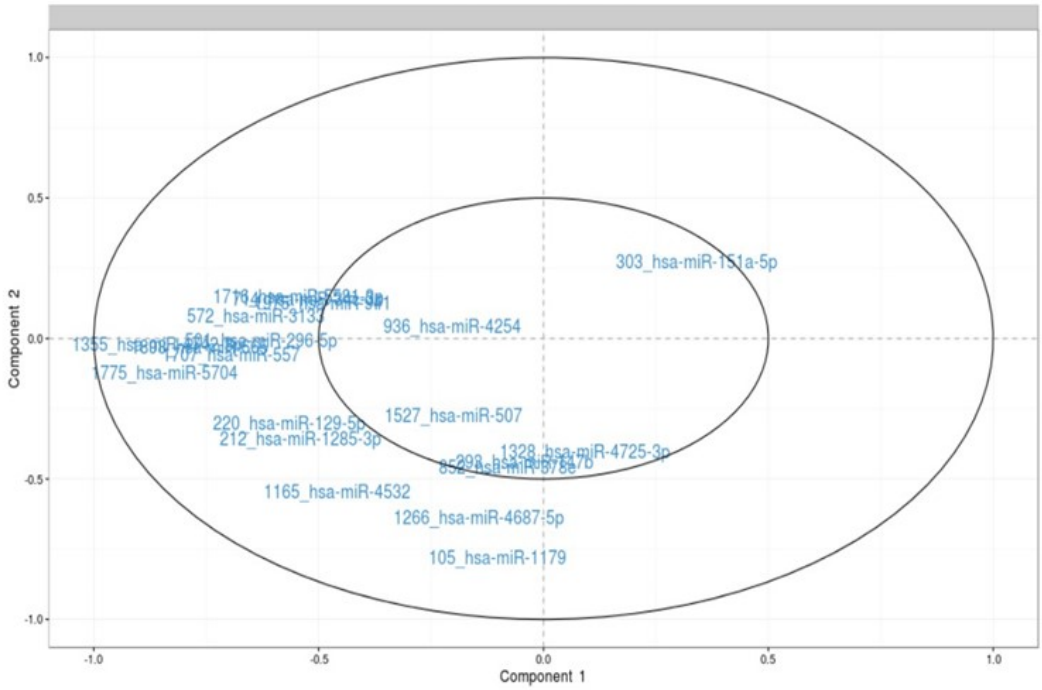


Figure 4.3 Correlation circle plot of microRNA expression in breast cancer patients versus health controls.

4.4.1.2 Subtype specific analysis

Next a subgroup analysis was performed, comparing the microRNA correlation between the different breast cancer subtypes. Due to the low number of HER2+ve (non-luminal) breast cancer subtype samples (n=3), variation between Luminal A and Luminal B HER2 breast cancer subtypes was used to identify a microRNA which could possibly separate HER2 receptor positive and negative subtypes. On comparison between Luminal A and Luminal B HER2, microRNA-4308 showed the highest variation on correlation circle plot and heat mapping, being up regulated in Luminal A subtype and down regulated in Luminal B HER2 subtype (Figure 4.4 & 4.5). MicroRNA-3188 showed the next highest variation, being up regulated in Luminal B HER2 and down regulated in Luminal A. For this reason, microRNA-4308 and microRNA-3188 were chosen for validation using in vitro breast cancer cell lines. The microRNA-4308 is located on chromosome 14q22.2, and has not been previously linked to any cancers. The microRNA-3188 is located on chromosome 19p13.11 and has been previously reported to have a higher expression in hepatocellular carcinoma [323].

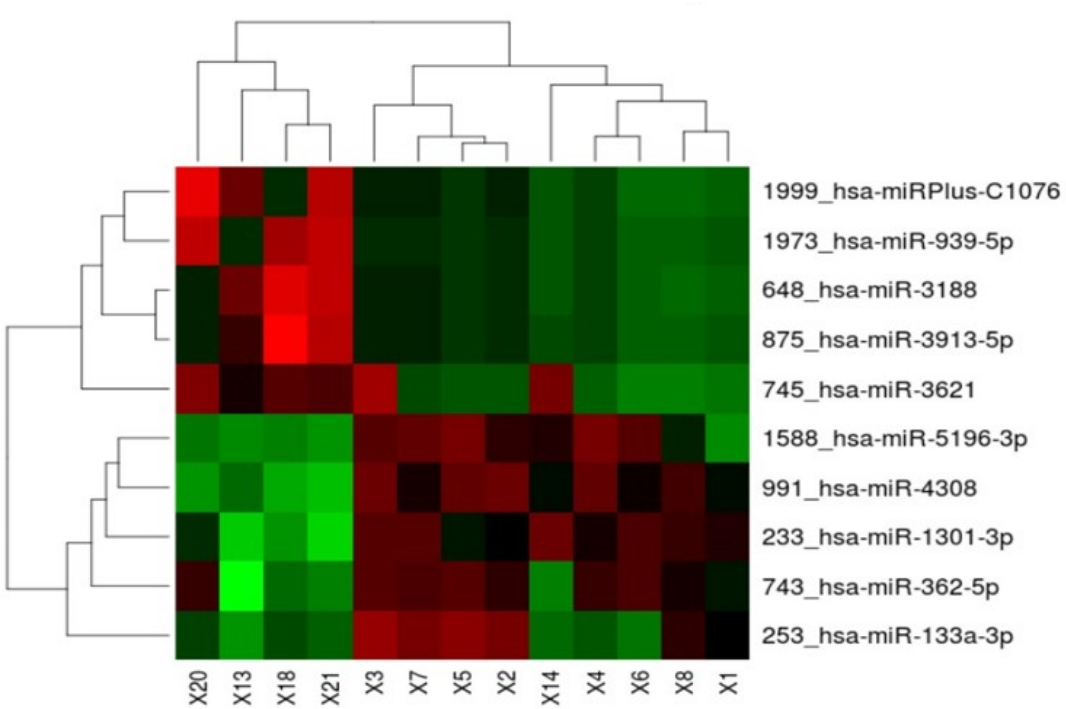


Figure 4.4 Heat map top ranking microRNA expression in Luminal A versus Luminal B HER2 breast cancer patients.

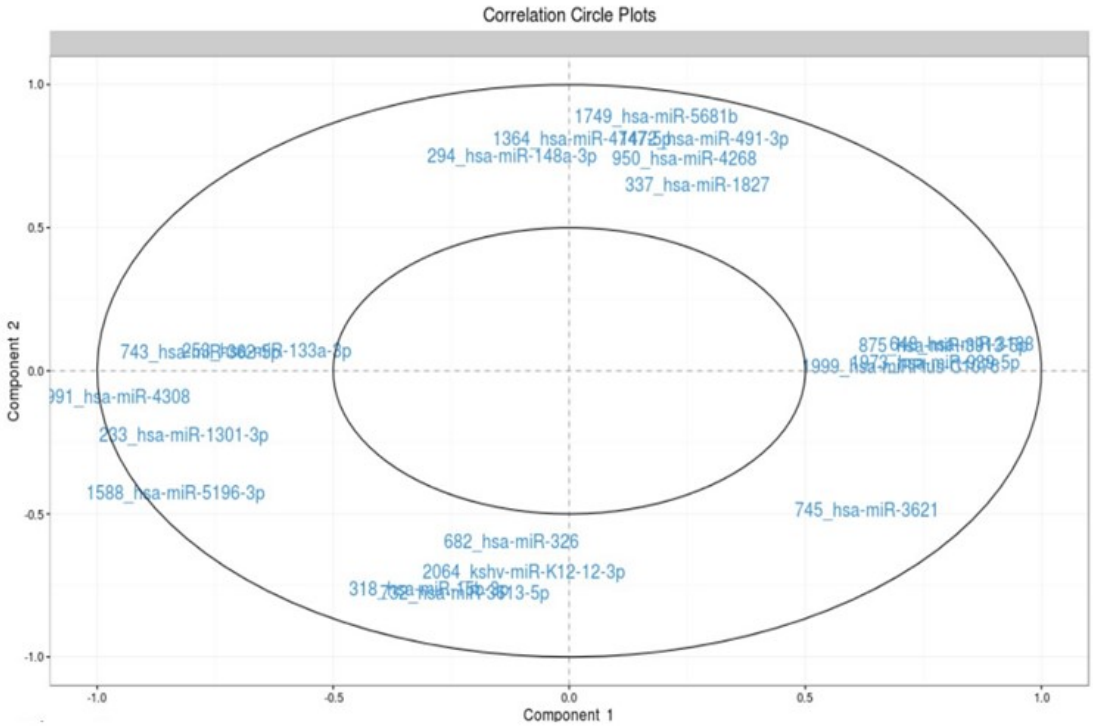


Figure 4.5 Correlation circle plot of microRNA expression in Luminal A versus Luminal B HER2 breast cancer patients.

4.4.1.3 MicroRNA-3188 expression in breast cancer cell lines

Using cDNA and PCR amplification, the expression levels of microRNA-3188 were tested in all four cell lines (Figure 4.6).

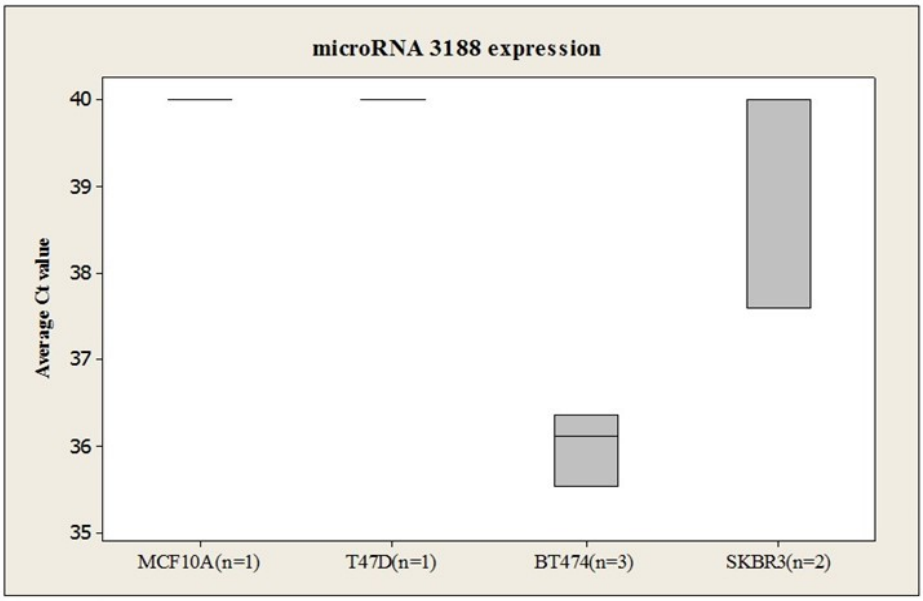


Figure 4.6 MicroRNA-3188 expression by breast cancer cell line.

For the microRNA-3188 no levels were detected in either the MCF10A or the T47D cell lines. MicroRNA-3188 was also expressed very late in BT474 and SKBR3 breast cancer cells with no significant difference seen between them (p=0.061). Overall this made microRNA-3188 a poor candidate as a biomarker for HER2 positive breast cancer and no further analysis was performed in patient samples.

4.4.1.4 MicroRNA-4308 expression in breast cancer cell lines

The expression levels of microRNA-4308 was also analysed in all four breast cancer cell lines (Figure 4.7).

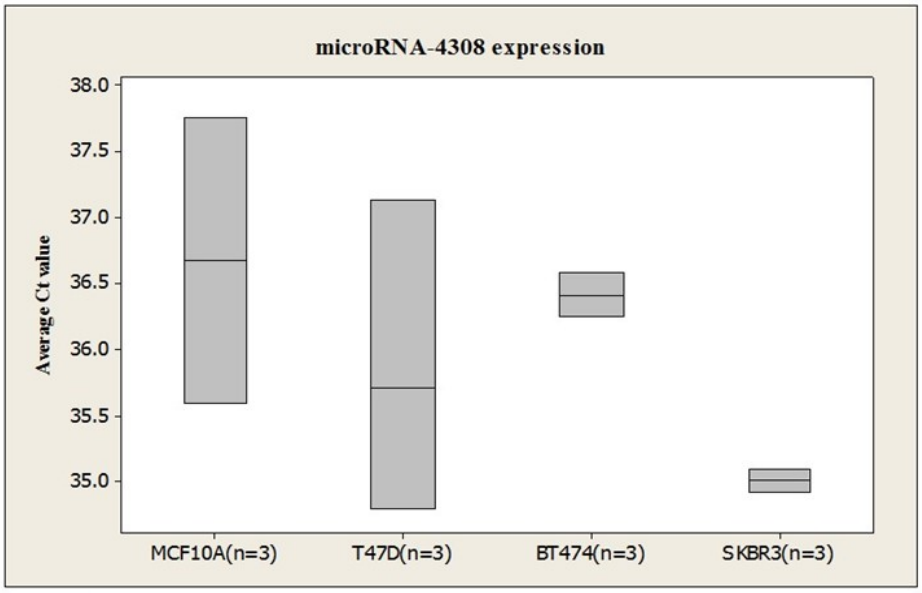


Figure 4.7 MicroRNA-4308 expression by breast cell line.

No significant difference in HER2 were seen between the MCF10A cell line and the T47D (p=0.644), BT474 (p=0.849) or the SKBR3 (p=0.366) breast cancer cell lines. Similarly, no significant difference in microRNA-4308 expression was seen between T47D cell lines and BT474 (p=0.527) or SKBR3 (p=0.333) breast cancer cell lines, and between BT474 and SKBR3 (p=0.084) breast cancer cell lines. This also made microRNA-4308 a poor candidate as a biomarker for HER2 expression and no further analysis was performed on patient samples.

4.4.2 ICORG microRNA panel expression across breast cancer cell lines

To assess the expression of the ICORG microRNA targets (Let7a, microRNA-10b, microRNA-21, microRNA-145, microRNA-155 and microRNA-195), in a control normal tissue breast cell line MCF 10A, along with breast cancer cell lines T47D (luminal A), BT474 (luminal B HER2) and SKBR3 (HER2+(non-luminal)) were grown. Two sets of analysis were performed for each microRNA, a) to compare the microRNA expression levels between the MCF10A cell line "control" and three breast cancer cell lines combined "cancer", b) to compare microRNA expression across all four cell lines.

Two control microRNA (microRNA-16 and microRNA-425) were also analysed and the target microRNA expression was expressed in logarithmic scale relative to the average of the control microRNA. The microRNA-10b levels were not detected in

either MCF10A cell lines or in any of the three breast cancer cell lines, so it was not possible to run analysis on this microRNA.

4.4.2.1 MicroRNA Let 7a expression

The expression of Let 7a was compared between control and the cancer cell lines (Figure 4.8). A significantly higher expression of Let 7a was seen in the control cell lines compared to the combined cancer cell lines (p=0.001).

Next the Let 7a expression levels were assessed across all individual cell lines (Figure 4.9). A significant difference was seen between MCF10A expression and T47D (p=0.004), BT474 (p=0.013) and SKBR3 (p=0.018) breast cancer cell lines. A significantly lower expression was seen between the T47D and SKBR3 cell lines (p=0.03), however there was no significant difference found between T47D and BT474 cell lines (p=0.809). No difference in expression was seen between the BT474 and the SKBR3 cell lines (p=0.126)

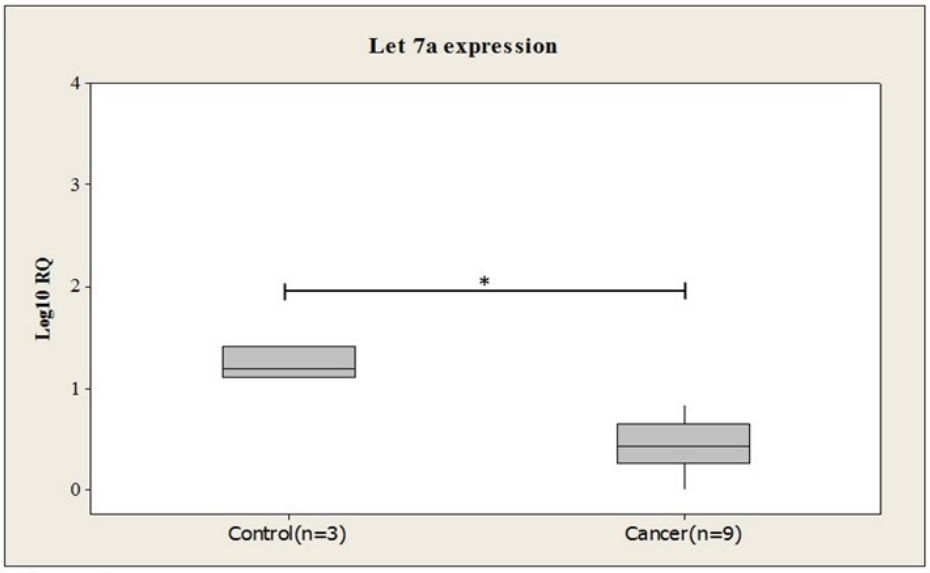


Figure 4.8 Let 7a expression in cancer versus control cell lines.

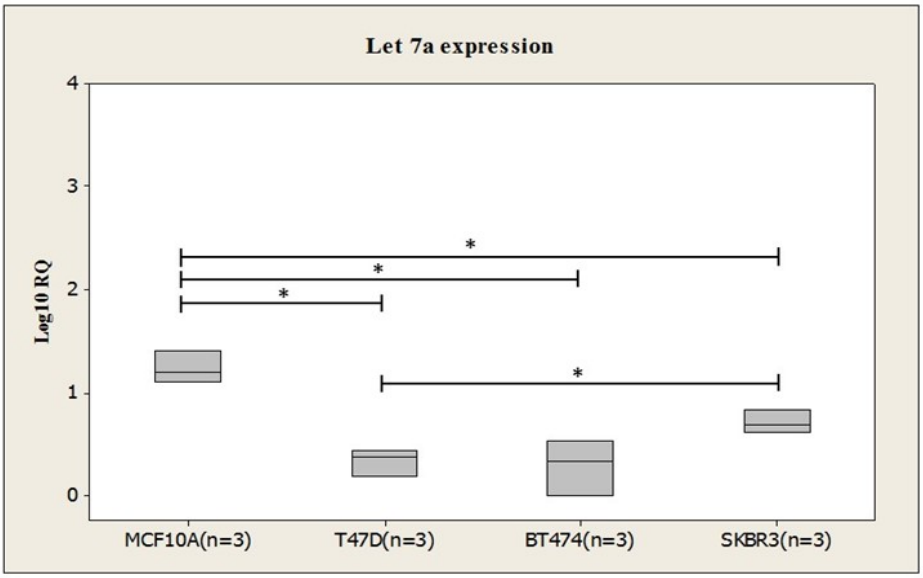


Figure 4.9 Let 7a expression by cell line.

4.4.2.2 MicroRNA-21 expression

Comparing microRNA expression between control and cancers, a significantly higher expression was seen in the control cell line (p=0.002) (Figure 4.10). This trend was seen across all the breast cancer cell lines, with higher expression of microRNA-21in seen in the MCF10A cell line compared to T47D (p=0.009), BT474 (p=0.01) and SKBR3 (p=0.006) breast cancer cell lines (Figure 4.11). A significantly lower expression is was seen in the T47D and BT474 (p=0.012) breast cancer cell lines but no difference was found between T47D and SKBR3 (p=0.124) cell lines. A significantly higher expression of microRNA-21 was seen between BT474 and SKBR3 (p=0.009) cell lines.

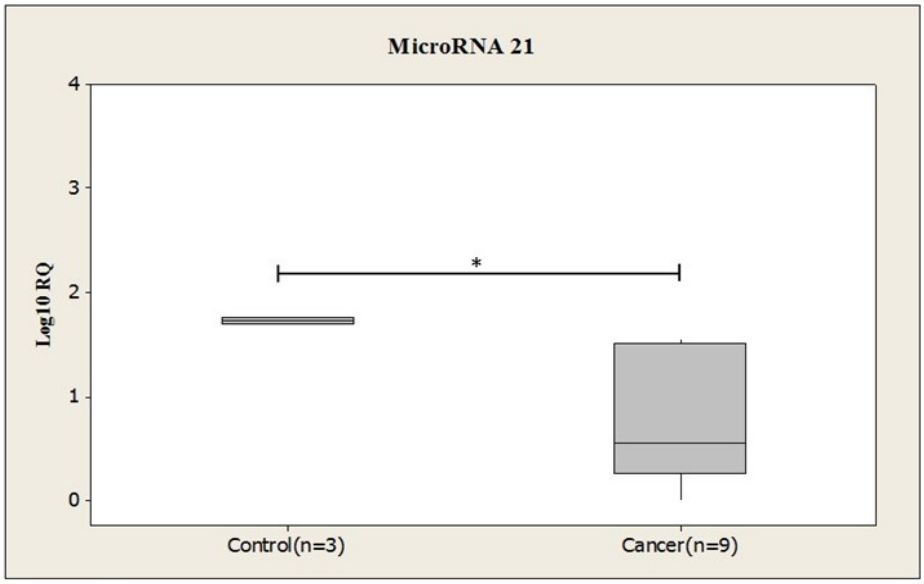


Figure 4.10 MicroRNA-21 expression in cancer versus control cell lines.

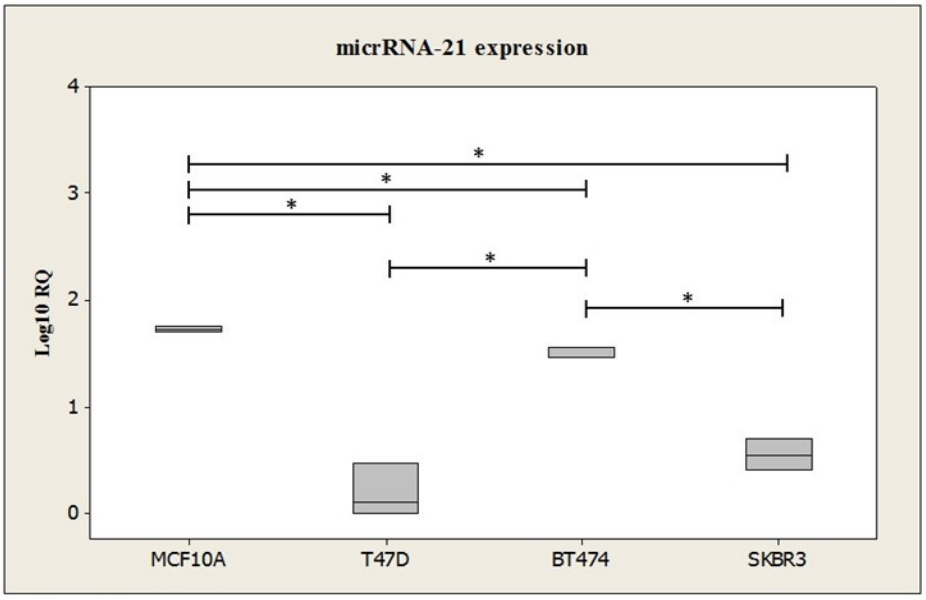


Figure 4.11 MicroRNA-21 expression by cell line.

4.4.2.3 MicroRNA-145 expression

Comparing cancers with controls, a significantly higher expression of microRNA-145 was seen in the control group (Figure 4.12). On analysis comparing the MCF10A cell lines with the individual breast cancer cell lines, no significant difference was found with the T47D (p=0.339) or the BT474 (p=0.309) cell lines, while microRNA-145 was only detected in one of three replicates of the SKBR3 cell line so no analysis could be performed (Figure 4.13). No difference in microRNA-145 expression was seen between the T47D and the BT474 breast cancer cell lines (p=0.797).

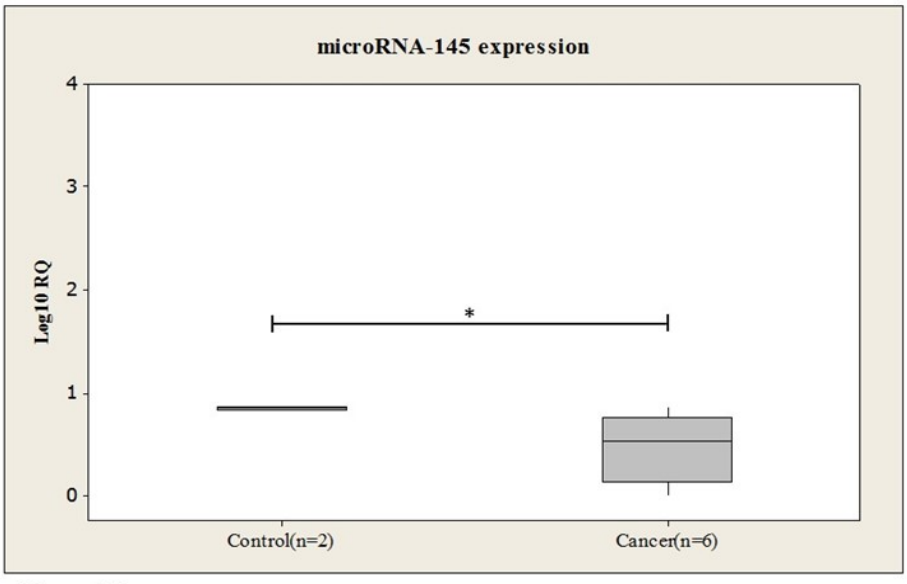


Figure 4.12 MicroRNA-145 expression in cancer versus control cell.

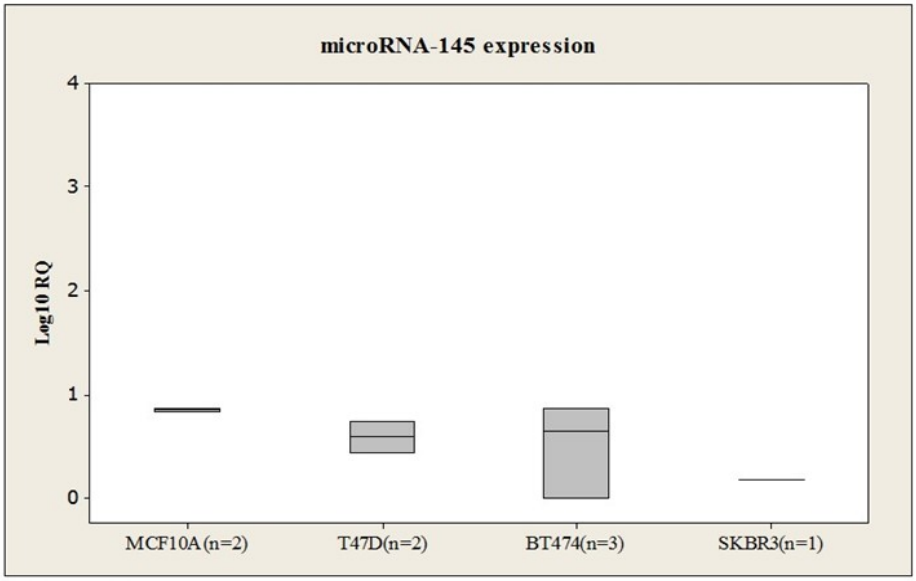


Figure 4.13 MicroRNA-145 expression by cell line.

4.4.2.4 MicroRNA-155 expression

No significant difference in expression of microRNA-155 was seen between cancers and controls (Figure 4.14). This was also seen across the individual cell line analysis, with no difference seen between the MCF10A normal breast tissue cell line and T47D (p=0.775), the BT474 (p=0.181) or the SKBR3 (p=0.358) breast cancer cell lines (Figure 4.15). No difference in microRNA-155 expression was found between any of the breast cancer cell lines, comparing the T47D with BT474 (p=0.157), the T47D with SKBR3 (p=0.105) or the BT474 with SKBR3 (p=0.066).

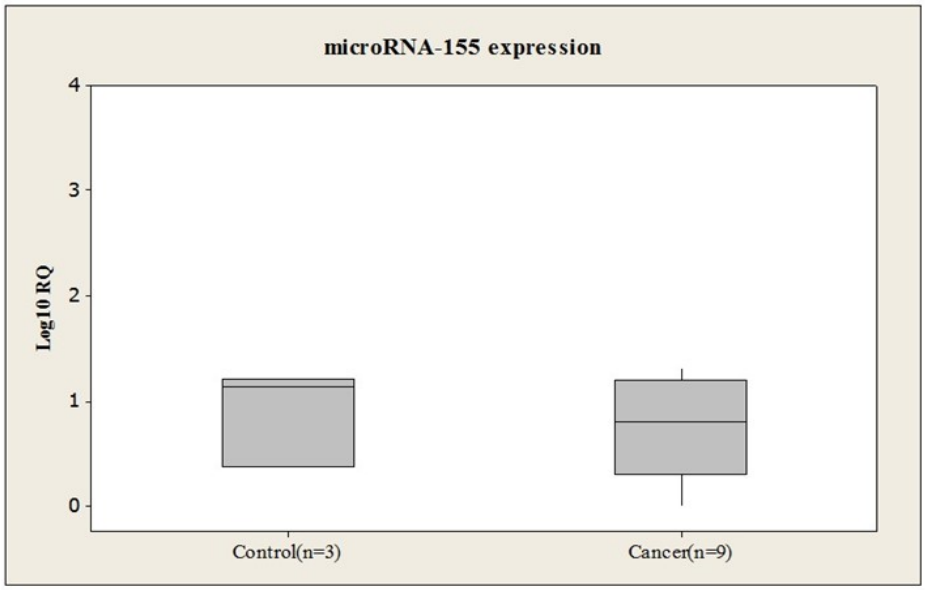


Figure 4.14 MicroRNA-155 expression in cancer versus control cell lines.

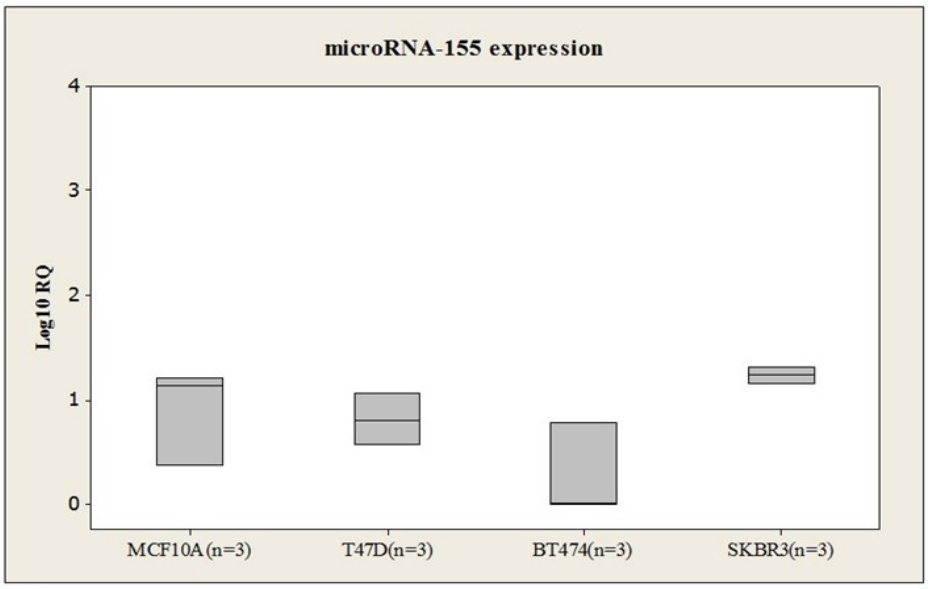


Figure 4.15 MicroRNA-155 expression by cell line.

4.4.2.5 MicroRNA-195 expression

Finally the expression of microRNA-195 was compared with the control and cancer groups, with no significant difference seen in expression levels (p=0.151) (Figure 4.16). There was also no significant difference seen in microRNA-195 expression between MCF10A cell line and the T47D (p=0.052), the BT474 (p=0.774) or the SKBR3 (p=0.726) breast cancer cell lines (Figure 4.17). No difference in expression

was found between the T47D and the BT474 (p=0.097) or the SKBR3 (p=0.082) breast cancer cell lines, or between the BT474 and the SKBR3 (p=0.924) cell lines.

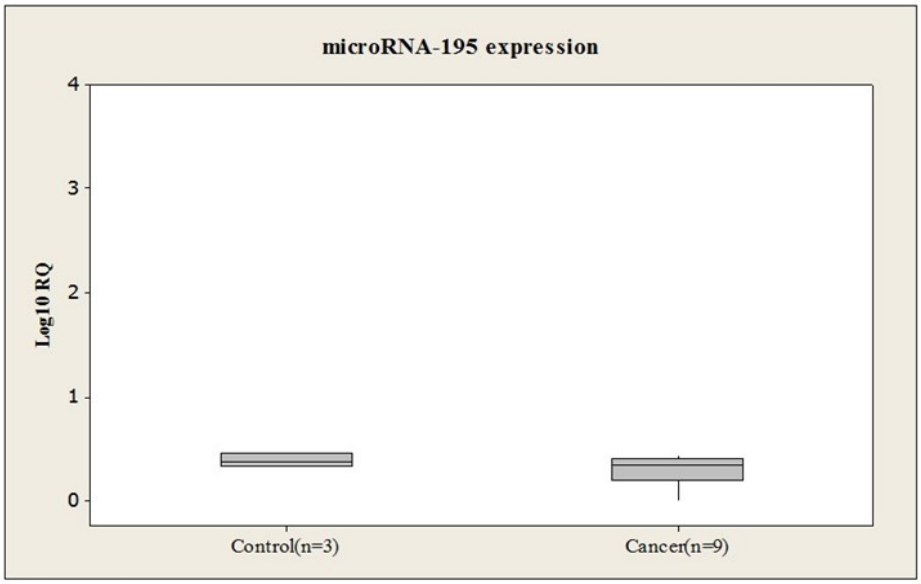


Figure 4.16 MicroRNA-195 expression in cancer versus control cell.

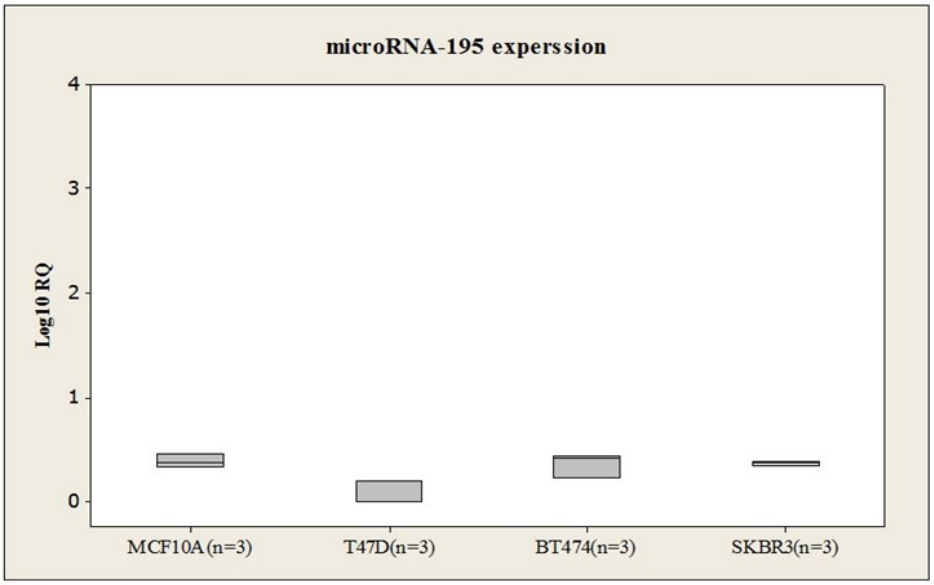


Figure 4.17 MicroRNA-195 expression by cell line.

4.4.3 Impact of Trastuzumab on microRNA expression levels in HER2 receptor positive cell lines

The impact of Trastuzumab on the expression of ICORG panels of microRNA targets was assessed in both hormone positive (BT474) and hormone negative (SKBR3) HER2 receptor positive cell lines. The effect of Trastuzumab on

microRNA was also assessed in a control HER2 receptor negative breast cancer cell line T47D.

4.4.3.1 Determining optimum Trastuzumab concentration

Firstly the optimal Trastuzumab dose to inhibit growth in HER2 receptor positive breast cancer cell lines was calculated. Increasing concentrations of Trastuzumab were tested in a BT474 cell line (Figure 4.18), with a final concentration of 100μg/ml selected for use in further analysis as it impeded growth by 40% compared to a control with no Trastuzumab therapy. Next the effect of treating the cells with 72hrs of 100μg/ml of Trastuzumab was compared across each cell in duplicate (Figure 4.19). The control and Trastuzumab treated cell lines were both harvested for analysis of microRNA expression levels.

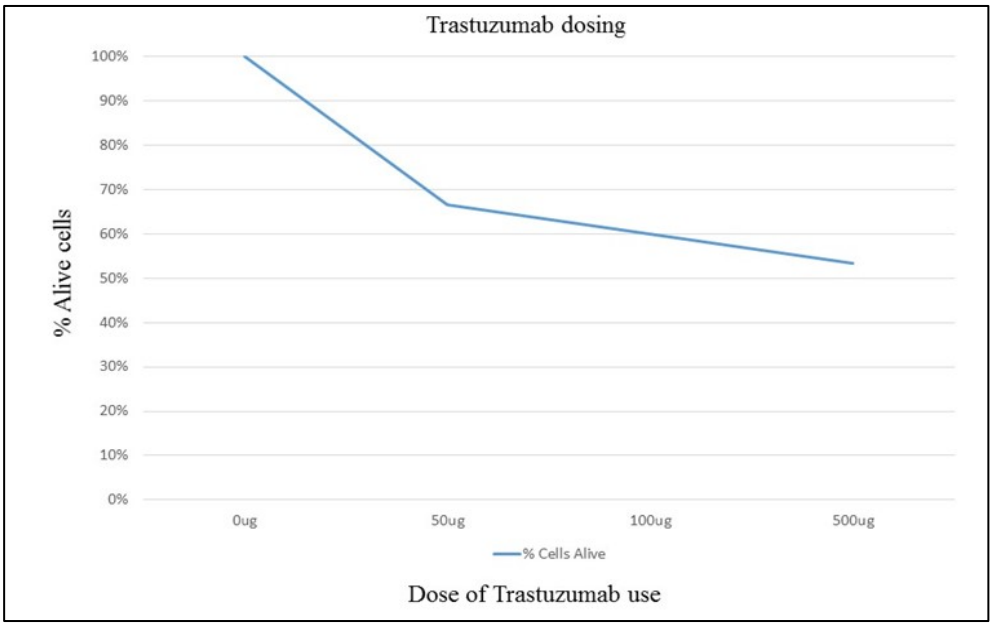


Figure 4.18 Impact of Trastuzumab dose on growth inhibition in BT474 cells.

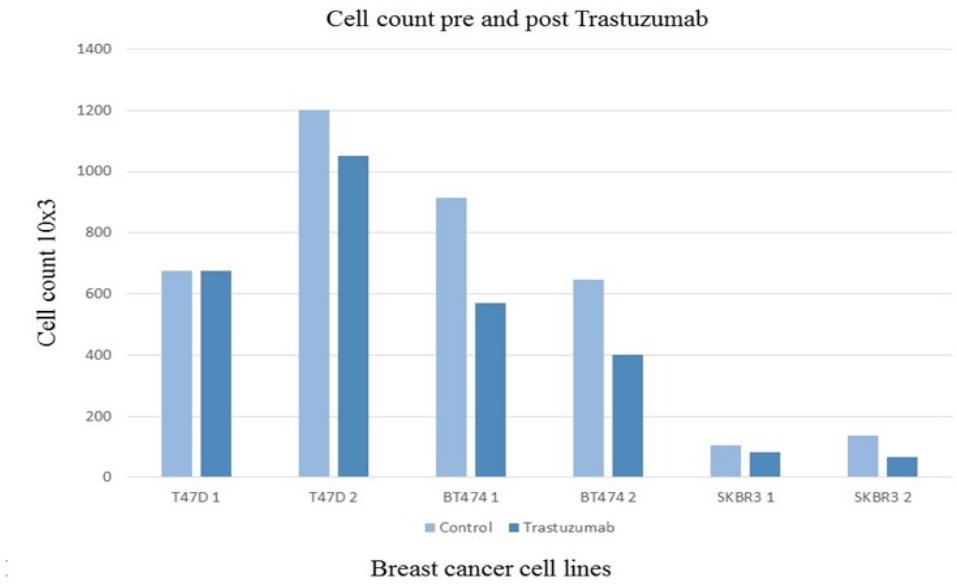


Figure 4.19 Impact of Trastuzumab on growth inhibition across breast cancer cell lines.

4.4.3.2 MicroRNA expression pre and post Trastuzumab therapy

Finally the expression of each of the ICORG target microRNA was tested across all three cell lines (Figures 4.20-24). Assessing microRNA Let 7a, no variation in expression was seen before and after Trastuzumab in either the T47D (p=0.144), BT474 (p=0.629) or SKBR3 (p=0.443) breast cancer cell lines. Similarly no difference in microRNA-21 expression was seen in the T47D (p=0.777), the BT474 (p=0.241) or the SKBR3 (p=0.535) cell lines before and after treatment. Comparing expression of microRNA-145 before and after Trastuzumab, a significant difference seen in the T47D cell lines (p=0.016) but not in the BT474 cell line (p=0.531), while no analysis was possible in SKBR3 cell lines as microRNA was not detected in the pretreatment group. For microRNA-155 no analysis was possible in the T47D or SKBR3 cell lines as expression was only detected in one of the two post Trastuzumab replicates. No significant difference in expression was seen in the BT474 cell line before and after Trastuzumab treatment (p=0.704). For microRNA-195, no difference in expression was seen in the T47D (p=0.145) or the BT474 (p=0.712) cell lines. However, a significantly lower expression is seen the SKBR3 cell line after treatment with Trastuzumab (p=0.042).

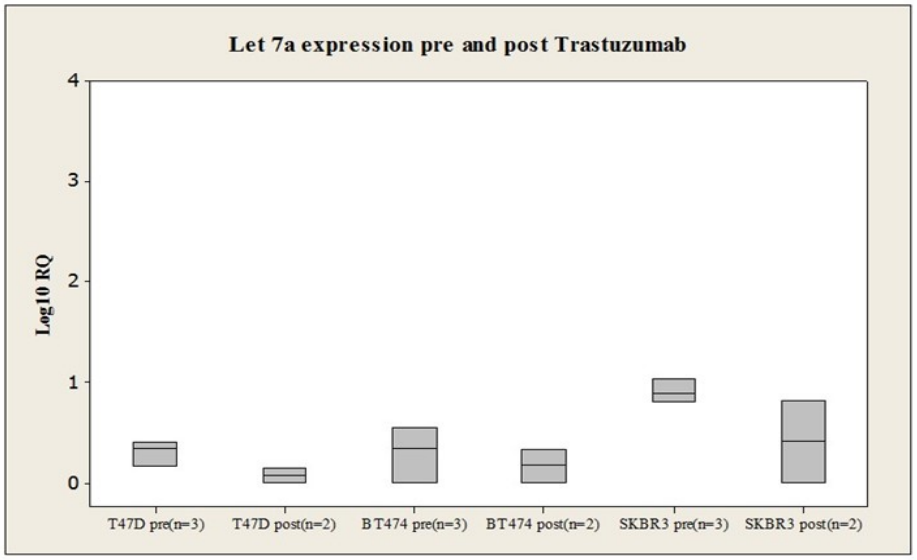


Figure 4.20 Impact of Trastuzumab on Let 7a expression across breast cancer cell lines.

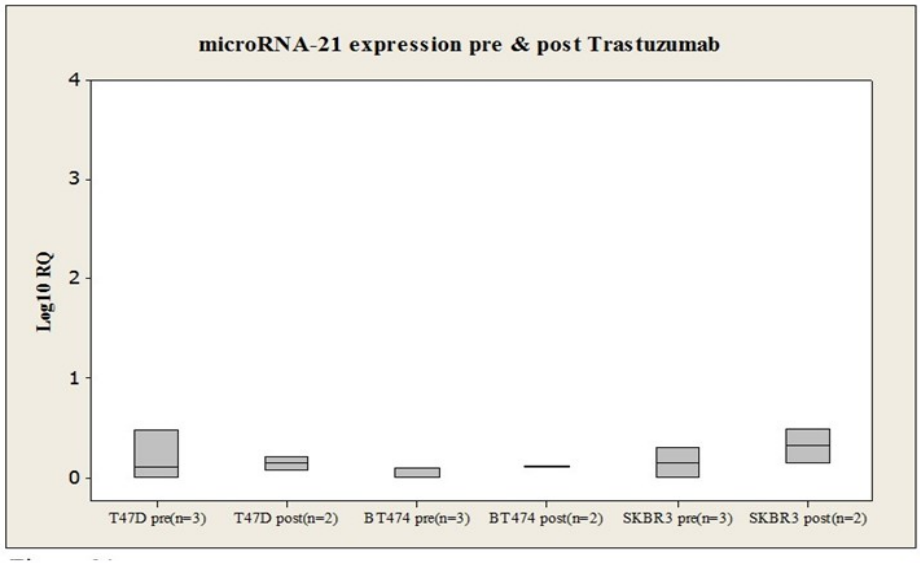


Figure 4.21 Impact of Trastuzumab on microRNA-21 expression across breast cancer cell lines.

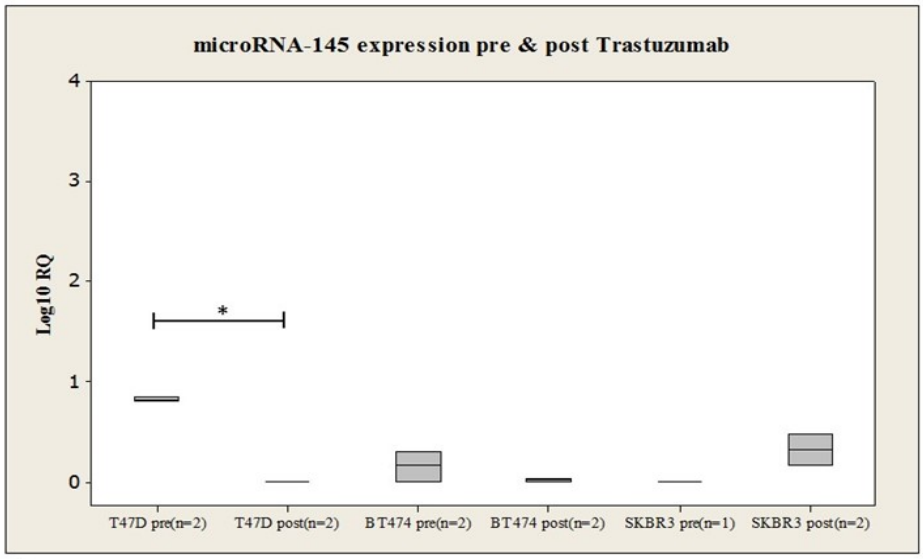


Figure 4.22 Impact of Trastuzumab on microRNA-145 expression across breast cancer cell lines.

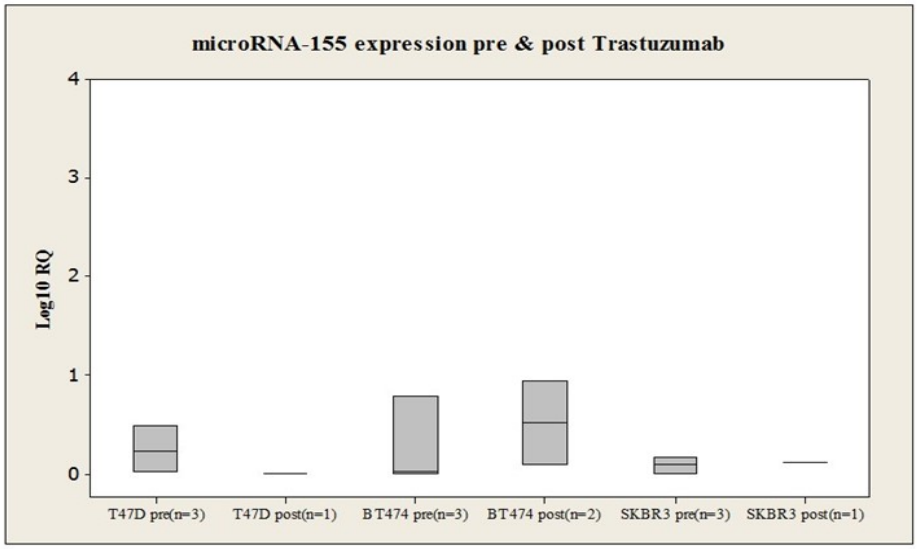


Figure 4.23 Impact of Trastuzumab on microRNA-155 expression across breast cancer cell lines.

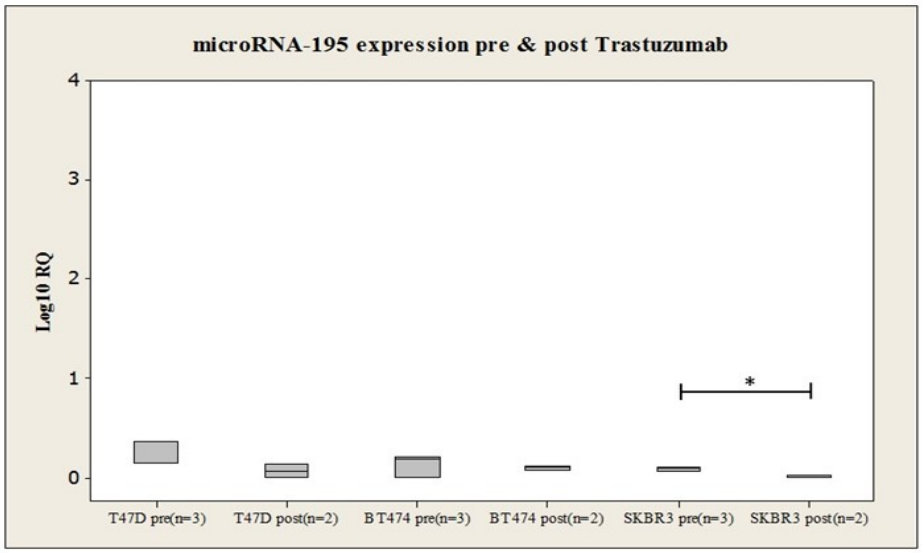


Figure 4.24 Impact of Trastuzumab on microRNA-195 expression across breast cancer cell lines.

4.5 Discussion

In this chapter, the role of microRNA as biomarkers for diagnosis and prognosis was explored. The main focus of this chapter was to identify a new potential biomarker for the HER2 receptor in breast cancer, and to identify a biomarker that could predict response to targeted HER2 receptor therapy. To do this a microarray of whole blood samples from controls and patients with breast cancer was analyzed to identify the highest ranking targets. Previously published potential microRNA biomarkers were also evaluated, comparing not only cancers to controls but also to assess if they could identify different breast cancer subtypes. Finally, the impact of Trastuzumab therapy on microRNA expression levels was assessed in both hormone positive and hormone negative HER2 receptor positive breast cancers.

A large number of papers have examined the role of microRNA as potential biomarkers in breast cancer [92, 107, 324, 325]. However, these initial studies focused on a small number of microRNA resulting in a large number of microRNA not being examined. The use of microarrays allows the analysis of thousands of microRNA in each sample, meaning the identification of an increasing number of potential biomarkers. A number of papers have looked at the use of microarrays to identify microRNA biomarkers for breast cancer [93, 131, 326, 327]. One of the first studies to run a microRNA expression microarray in breast cancer tissues, found that it could differentiate the different breast cancer phenotypes [131]. A pilot study using a circulating microRNA microarray found that expression of microRNAs varied significantly between patients with breast cancers and health control patients [326]. A variation between patients with breast cancer and healthy individuals was also seen in a further microarray analysis of circulating microRNA [327]. In our study, a microarray analysis of over 4000 circulating microRNAs between eleven healthy controls and twenty-six patients with either invasive breast cancer or ductal carcinoma in-situ (DCIS) found no significant difference between the control group and the cancer group, but this may be due to the low sample numbers.

A number of studies have looked at the potential of microRNA to identify HER2 receptor positive breast cancers, and if they could separate the two HER2 receptor positive breast cancers subtypes. Mattie et al found that clustering of microRNA could separate out HER2+/ER+, HER2+/ER- and HER2-/ER- breast cancers using

breast tissue sampling [131]. Another study of breast cancer tissue samples found that microRNA-342 was over-expressed in Luminal B HER2 breast cancers compared to other subtypes [93]. In our study we examined the potential of a number of microRNA to predictors HER2 receptor positive breast cancers. Using the results of a circulating microRNA microarray, the two highest ranking microRNA (microRNA-3188 & microRNA-4308) with differential expression between Luminal A and Luminal B HER2 breast cancers were chosen for further analysis to try identify a marker for HER2 receptor over-expression. However, on in vitro analysis, no significant difference in these microRNA expression was seen across the different breast cancer subtypes. A panel of microRNA (ICORG targets) previously shown to be altered in breast cancer patients was also assessed to see if they could separate out breast cancer subtypes. Comparing cancers to control cell lines, significant differences were found in three of the microRNAs (Let 7a, microRNA-21 and microRNA-145). Significant individual differences in microRNA expression were also observed between the subtypes, but no predictive marker for HER2 positive breast subtypes or other subtypes were identified.

In the final part of this study, the impact of Trastuzumab therapy on growth and selected microRNA expression in HER2 positive breast cancer cell lines was assessed.

Across the literature the impact of Trastuzumab on breast cancer cell line growth varies significantly, with different concentrations of Trastuzumab having varying influence on HER2 receptor positive cell lines. One study found that treating HER2 receptor positive breast cancers with 21 μg/ml of Trastuzumab for 48 hours could reduce growth by almost 60% in BT474 cells and over 25% in SKBR3 cell lines compared to controls [135]. Another study found that a dose as small as 10 μg/ml for 72 hours could reduce growth in a BT474 cell line, with increasing doses having no additional benefit [136]. Merlin et al, looked to identify the IC50 of Trastuzumab in a SKBR3 cell line and found that even at maximal concentration Trastuzumab cytotoxicity only reached 30% [328]. In our study an increased Trastuzumab concentration resulted in reduced cell growth, with a dose of 100 μg/ml of Trastuzumab over 72 hours resulted in up to a 40% reduction in cell growth. Our study also found variations in response to Trastuzumab in the two HER2 receptor

positive breast cancers, with a greater response seen in the BT474 breast cancer cell lines. This matches the results of a previous study, which found that only three (BT474, SKBR3 and ZR7530) of nine HER2-amplified cell lines had a robust response to Trastuzumab and a greater response was seen in the BT474 cell lines compared to the SKBR3 cell line [135].

A number of studies have looked at the role of microRNA in predicting response and resistance to Trastuzumab. To determine the effect of Trastuzumab on microRNAs, one study looked at the microRNA profile in BT474 and SKBR3 cell lines before and after treatment [329]. In this study a variation in expression levels was seen in a number of microRNA, but the only change observed in both cell lines was the up regulation of microRNA-194. None of the panel of ICORG target microRNAs had altered expression in this study in either cell line, matching the results in our study. However, another study of breast cancer tissue samples found that microRNA-21 was significantly reduced in patients with a pathological complete response compared to those with residual disease in patients treated with Trastuzumab. This study also assessed expression of microRNA-145 and microRNA-155 and found significant variation in expression in responders and non-responders. A study assessing the microRNA expression in patients that responded to neoadjuvant Trastuzumab, found that circulating microRNA-210 was significantly higher in patients with residual disease compared to those with a complete response [321]. This study also found that microRNA-210 was significantly raised in Trastuzumab resistant BT474 cell lines. In our study, none of the target microRNA had a variation in expression levels before and after treatment for both HER2 receptor positive cells lines. In the SKBR3 cell line, a significant reduction in expression of microRNA-195 was found after Trastuzumab treatment.

4.6 Conclusion

In this chapter the utility of microRNA as biomarkers was explored, explicitly addressing if microRNA could be a biomarker for diagnosis and prediction of response to treatment in HER2 receptor positive breast cancers. A microarray comparing microRNA expression of control healthy individuals against breast cancer patients found no significant variation in microRNA expression. This confirms one of the major issues in biomarker discovery, the lack of a consistent alteration in a

single biomarker associated with all breast cancers probably due to the heterogeneity seen in breast cancer patients. Due to this, there is a growing use of "big data" looking at multiple markers across thousands of patients to identify distinct patterns associated with early breast carcinoma to allow diagnosis or predict response.

One of the other main aims in breast cancer research is to examine the different breast cancer subtypes in order to identify subtype specific markers. In our study we endeavoured to identify a microRNA signature for HER2 receptor positive breast cancer by assessing variations in the circulation of patients and testing previously acknowledged microRNAs altered in breast cancer in the in vitro setting. While variations in microRNA expression were found between normal breast tissue cell lines and breast cancer cell lines, and also between the different breast cancer cell lines, no specific marker for HER2 receptor positive breast cancer was identified. Finally the effect of targeted anti-HER2 therapy Trastuzumab on microRNA expression was analysed. It was found that microRNA-195 had reduced expression post treatment in SKBR3 cell lines.

Overall this chapter has once again demonstrated the difficulties in identifying specific biomarkers for breast cancer. It was found that microRNA-195 expression levels were reduced by Trastuzumab treatment and further investigation is needed to evaluate if microRNA-195 alone or more likely in a combination with other markers can accurately predict which patients will respond to chemotherapy. To assess this accurately the target microRNAs would need to be analysed in the clinical setting. In the next chapter we addressed the ability of microRNA to identify different breast cancer subtypes and also determined whether circulating microRNA levels at diagnosis could predict which patients would respond to NACT.

Chapter 5

Clinical validation of circulating microRNA as breast cancer biomarker (ICORG 10-11 trial)

Manuscript under review
Prospective assessment of microRNA as markers of response to neoadjuvant chemotherapy in breast cancer
A McGuire, MC Casey, H Heneghan, O Kaliningrad, E Holian, R Waldron, A McDermott, AJ Lowery, J Newell, R Dwyer, N Miller, JAL Brown, M Keane, MJ Kerin.

5.1 Introduction

Adjuvant chemotherapy has been used in the management of breast cancer for several decades, and the current protocols are dependent on molecular subtype, disease stage and potential for response. Neoadjuvant chemotherapy is used in locally advanced breast cancer to reduce the tumour burden prior to surgery. This increases the number of patients suitable for breast conserving surgery, and also provides a unique opportunity for in-vivo assessment of how the tumour responds to chemotherapy. Pathological complete response is the complete eradication of the tumour post NACT. Studies have shown that pCR is associated with improved survival [4]. However, only about 18% of patients will achieve a pCR [4], with the majority of patients exhibiting only a partial or poor response to NACT, and currently there is no reliable clinically validated biomarker that can predict which patients will respond to NACT.

The introduction of molecular profiling, has resulted in the subdivision of breast cancer into multiple biologic subtypes which have prognostic and therapeutic significance [11, 313]. This insight into the molecular heterogeneity of breast cancer, has provided an explanation for the considerable variation in response to NACT. Molecular subtyping has been adopted into clinical practice to inform decision making in relation to therapeutic strategy; as comprehensive characterization of molecular subtypes requires whole genome profiling which is not routinely performed in the clinical setting, the expression of the estrogen receptor (ER), progesterone receptor (PR) and Her2/*neu* receptors (HER2+) which are routinely measured by immunohistochemistry (IHC) is frequently used as a practical, surrogate marker of breast cancer biologic subtype [330].

Response to NACT has been shown to vary by breast cancer subtype, with the highest complete response rates in the HER2+ (non-luminal) subtype [4]. Subtype specific therapy such as Trastuzumab, significantly increases the pCR rates in HER2 receptor positive breast cancers [70, 71]. Despite these advances in targeted/personalised therapy for breast cancer, to date there remains no accurate clinically validated way of assessing which patients will respond to NACT. Recently the use of non-invasive biomarker or “liquid biopsies” have been proposed as a possible way to not only distinguish breast cancer subtypes but also to predict

response to therapy. One of the most promising biomarkers in this regard are microRNAs.

MicroRNAs are non-coding RNA molecules, with a functional role in post transcriptional regulation of gene expression [204, 331]. The expression of microRNAs has been shown to be dysregulated in multiples cancers [332, 333]. Multiple microRNA, such as Let-7a, microRNA-21, microRNA-145, microRNA-155 and microRNA-195 have been shown to be dysregulated in the circulation and tissue between patients with breast cancer and health controls [91, 92, 121, 324, 334, 335]. There is also evidence that microRNA expression profiles can accurately classify breast cancers subtypes [93, 131, 324, 332]. Specific patterns of microRNA expression can identify hormone receptor and HER2 receptor status [93]. Further to this, it has been shown that microRNA expression can effect response or resistance to systemic chemotherapy in breast cancer [336].

The hypothesis is that a panel of microRNAs can predict which patients would respond to neoadjuvant chemotherapy. As microRNAs can also predict receptor status in breast cancer, it was also hypothesized that microRNAs could predict intrinsic breast cancer subtypes.

As discussed in previous chapters, hormone receptor status has a profound effect on survival and response to treatment in HER2 receptor positive breast cancer. However, to date little research has been done on the impact of hormone receptor status on microRNA expression. By comparing microRNA expression levels between Luminal B HER2 and HER2+ (non-luminal) breast cancer subtypes, we sought to determine the role of hormone receptor status on microRNA expression.

From this a number of objectives were set

a) Primary objectives

   - To identify specific combinations of miRNAs ('signatures') which are associated with breast cancer intrinsic subtypes, and thereby could aid in prognostication and treatment planning on an individual patient basis.
   - To identify if a panel of miRNAs, detectable in the circulation, which are altered in breast cancer patients can predict response to NACT.

b) Secondary objectives

   - To compare response to treatment, survival and discordance between the two HER2 receptor positive breast cancers subtypes.

5.2 Methods

5.2.1 Recruitment and time points for collection

Following ethical approval and informed patient consent, a single blinded, multicentre, prospective clinical trial was established (ICORG 10-11 study). Consecutive patients undergoing NACT for breast cancer were included. All patients included were aged 18 years or over, and gave written informed consent. Patients with distant metastatic disease at the time of presentation were excluded. Response to NACT was based on the Miller-Payne classification, with patients who had a complete response or >90% reduction in tumour size (Grade 4 & 5) categorized as "responders", while patients with <90% reduction in tumour size (Grades 1-3) categorized as "non-responders". Indication for chemotherapy was taken at individual unit level and were subtype and disease burden related and each patient was discussed at a breast multidisciplinary team meeting.

For this study, blood samples were collected in whole blood 4-8ml Ethylenediaminetetraacetic acid (EDTA) tubes at five specific time points (Figure 5.1)

- Blood Sample 1: at time of diagnosis prior to commencement of neoadjuvant chemotherapy
- Blood sample 2: mid-way through chemotherapy regimen (after 2nd cycle if they are enrolled in a 4 cycle regimen, or after 4th cycle if they are prescribed an 8 week regimen).
- Blood sample 3: post-chemotherapy (before surgical resection as applicable).
- Blood sample 4: 2 to 4 weeks post-surgical resection of tumour or 2 to 4 weeks post 3rd blood sampling if patients does not undergo surgery.
- Blood sample 5: once during follow-up, between 12-18 months post-surgical resection, or 12 to 18 months post 3rd blood sampling if patients does not undergo surgery.

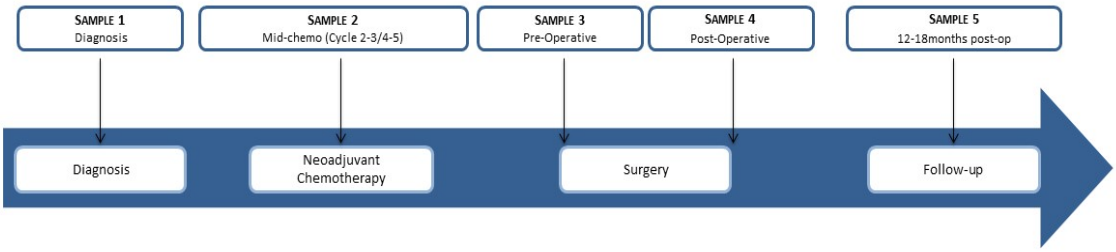


Figure 5.1 Timing of patient sampling

5.2.2 Patient demographics and data collection

On enrollment each patient was assigned a unique identifier, with patients clinicopathological details hidden from laboratory staff to keep the study blinded. At each time point a Case Report Form (CRF) form was also completed by research nurses. The CRF forms compiled contains vital patient and clinical data from each time point, such as the patient unique identifier, date of birth, histology & radiology results, along with the patients' treatment regimes. The information gathered on the CRF forms was then entered into a database.

5.2.3 Breast cancer subtypes

Breast cancer subtypes were defined using ER, PR and HER2 receptor status. Luminal cancer is defined as (ER and/or PR +ve, HER2 -ve), Luminal B HER2 defined as (ER and/or PR +ve, HER2 +ve), HER2+(non-luminal) as (ER and PR –ve, HER2 +ve) and Triple negative as (ER and PR -ve, HER2 -ve) according to standard clinical pathological guidelines [330]. As Ki67 was not routinely reported, the Luminal subtype could not be separated in to true Luminal A and Luminal B. As per ASCO guidelines (ALLRED score >2 or more than 1% stain positive), the ER and PR receptor status were determined independently by clinical pathologists using immunohistochemistry. The HER2 receptor status was identified by Herceptest^{TM} (DAKO Agilent pathology solutions), with a score of 3+ considered positive. Any 2+ inconclusive results were confirmed using a FISH testing as per ASCO guidelines, with a HER2/CEP17 ratio greater than two considered amplified.

5.2.3 Storage and logging of samples

Whole blood samples were stored at 4 degrees Celsius until transfer to the laboratory in the Dept of Surgery at NUI Galway for processing. On receiving the samples, the samples were coded into a data management system (Shire) and identified by a numeric code which links to the patients' unique identifier. The blood sample was then stored at 4 degrees Celsius again until ready for extraction.

5.2.4 MicroRNA targets and controls

A panel of five target microRNA and two control microRNA were selected for analysis (Table 5.1). Each target microRNA was selected based on work previously published from ours and other institutes, showing that these microRNA have altered expression in patients with breast cancer, and circulating levels could separate breast cancer from controls [91, 92, 121, 324, 334, 335]. Two control microRNA were selected as studies had found that they were reliable endogenous controls in human tissue and circulation [91, 337].

Table 5.1 Candidate microRNAs for investigation

MicroRNA	Previous association with breast cancer
Let 7a	Elevated in circulation in breast cancer
miR-21	Increased in breast tumour tissue
miR-145	Decreased in breast tumour tissue
miR-155	Increased in breast tumour tissue
miR-195	Increased circulation in breast cancer
miR-16	Reliable endogenous control in human breast circulation
miR-425	Reliable endogenous control in human breast circulation

5.2.5 Extraction of mRNA from peripheral blood samples

To extract mRNA from peripheral whole blood samples, the Trizol method of extraction was used. In a 5ml tube, 3ml of Trizol was mixed with 200uL of Bromoanisole (BAN). 1ml of whole blood taken from EDTA tube was pipetted into the Trizol/BAN solution and mixed until completely homogenous. This solution was then transferred into two 3ml round bottom tubes and left to stand at room temperature for 5 minutes. Follow this the blood/Trizol/BAN solution was spun in a centrifuge at 14000rpm for 15 minutes at 4 degree Celsius, to separate out the RNA precipitate. The upper 1ml of aqueous phase was removed from the collection tube and transferred to a fresh 2ml round bottom tube. The interphase (DNA) was then labelled and frozen at -20 degrees Celsius for future analysis. Next, 1ml of Isopropanol was added to the decanted aqueous phase and mixed by inversion, and left to stand at room temperature for 5 minutes. Following this it was spun in a centrifuge at 14000rpm at 18 degrees Celsius for 8 minutes. This forms a translucent RNA precipitate on the side and bottom of the tube. Taking care not to disturb the pellet, the supernatant was removed and disposed. The pellet is then washed to improve the 260/280 ratio, 1ml of 75% ethanol was carefully added to the RNA pellet, mixed by vortexing and spun at 14000rpm at 18 degrees Celsius for 5 minutes. Following this, the ethanol was removed and disposed, with the ethanol washing step repeated. After removal of the ethanol the pellet was left to air dry at room temperature for 5 minutes. Then the pellet was dissolved in 30uL of nuclease free water, left at room temperature for 5 minutes, vortexed and centrifuged at 14000rpm for 15 seconds. The two sample were then combined back together

(representing one patient blood sample) into a single RNA storage tube, giving a total of 60uL of RNA, which was then stored at -80 degrees Celsius.

5.2.6 Complementary DNA synthesis for microRNA

The dTNP mix/RT buffer/stem loop primer(for each target)/multiscribe/Rnase inhibitor, along with each sample were taken out of freezer and allow to defrost on ice. Using NanoDrop 1000 spectrophotometer (NanoDrop ND-1000 Technologies Inc., DE, USA), the mRNA concentration and purity for each sample was assessed. A 1uL aliquot of RNA was pipetted onto the apparatus pedestal, with a RNA absorbance ratio at 260 and 280 nm between 1.8 to 2.2 deemed indicative of pure RNA. Using the miRNA concentration, the total/miRNA needed for each 5uL sample was calculated. The volume of each reagent needed to make 10uL premix per target was also calculated, and made up for each target microRNA, each premix was then vortexed to mix and spun down. Next 10uL of premix was added to each sample. Following this, the calculated 5uL mix (total/miRNA+NFW) was added to each sample. To out rule contamination of any of the premix components a RTC was also made for each target, containing 10uL of target premix and 5uL of NFW. The samples were then spun again in a mini centrifuge and loaded into an Applied Biosystems GeneAmp PCR system 9700 and the cDNA generation program was run, samples were heated to 16 degrees for 30 minutes, 42 degrees for 30 minutes, 85 degrees for 5 minutes and then cooled to 4 degrees. Following completion of cDNA generation, samples were then transferred into sealed tube and diluted with 30uL of NFW to get a final total of 45uL of cDNA to measure. The cDNA is then stored at -20 C.

5.2.7 Real time quantitative polymerase chain reaction

To show there was no variation between PCR plates, an interassay control (IAC) was added to every PCR plate. MicroRNA 26b was chosen as the IAC, with cDNA from a control sample made up as above along with an RTC. For each plate of PCR, 1uL of cDNA of the IAC was run in triplicate. Consecutive PCR plates could then be linked, by having the average CT of the IAC within 0.3 standard deviations on each plate.

The cDNA for each sample, along with each target microRNA PDAR was slowly defrosted on ice. Premix for each sample and the RTC for each target was made up using fast mastermix, NFW and PDAR for each target microRNA. Following this each premix was vortexed, down and using an electronic pipette, premix was added to the PCR plate. Next 1uL of cDNA for each sample was added in triplicate to the PCR plate. To out rule contamination of the PCR plate, a NTC was run on each plate, which contains premix for each target and 1uL of NFW in triplicate. After cDNA for each sample, RTC, IAC and NFW for the NTC were added for each sample, the plate was then sealed using an optical adhesive cover. Next the plate was spun down using a centrifuge for one minute at 2000RPM. Amplification of the cDNA was done using a 7900HT fast-time PCR system. Using SDS 2.4 software, the samples and target microRNA were assigned as per PCR plate set up and saved on the system. Next the PCR plate was added to the machine and PCR was amplified by the plate being heated up to 95 degrees for 20 seconds and them cooled to 60 degrees for 20 seconds and this process was repeated for 40 cycles. Following completion of the run, the cycle threshold for detection of each target microRNA was analyzed using RQ manager software. The results were then saved and then converted to Microsoft excel for further analysis. The average CT value of the three replicates of each sample was calculated, along with the standard deviation between the 3 replicates. Any samples outside of 0.3 standard deviations of each other was excluded and the sample was repeated. Samples were also excluded if either the RTC or NTC for the target microRNA were contaminated, new cDNA was made for these samples, and the PCR was repeated.

5.2.8 Biogazelle Q base

To account for inter user variation, each operator performing microRNA analysis was required to calculate efficiencies for each of the eight microRNA. To do this as part of the protocol for the study, it was required that the Biogazelle Q base software be used. Efficiencies were calculated by making serial dilutions of a control sample for each of the target microRNA. Using excel a scatter plot was created using the average cycle threshold (CT) value for each dilution and the slope was calculated using the equation y=mx+c. After calculating the slope the efficiency was determined using the equation $E= (10^{-1/slope-1}) \times 100$, with only efficiencies between 90-110% used.

Using Biogazelle Q base software, each operator added in their efficiency for each of the target microRNA. Next each individual plate data was uploaded for all target microRNA for each individual sample. Next all failing replicates (>0.3 standard deviations apart) were removed, until no failing targets remained. For analysis of the results, microRNA 16 & 425 were set as controls, with the results of the target microRNAs normalized to the controls. The results were then exported and sent off to the ICORG statisticians for the final analysis.

5.2.9 Statistical analysis

Data were analyzed using R statistical software version 3.2.3. Non-Parametric statistics were used due to evidence of non-normally distributed data and non-ignorable outliers. The two-sample Wilcoxon Rank Sum test was used for all two sample comparisons and the Kruskal-Wallis test, followed by Wilcoxon Rank Sum test for pairwise comparisons with Bonferroni adjustment, to compare median response between the levels of factors of interest. Results with a p-value < 0.05 were considered statistically significant.

5.3 Results

5.3.1 Patient demographics

From May 2011 to April 2014, 124 patients were recruited from 8 centres across Ireland. The clinicopathological details are shown (Table 5.2), median age of patients included was 55 years old (range 25-76). The Luminal subtype was the most common subtype 61 (49.2%), followed by Triple negative 25 (20.2%), Luminal B HER2 22 (17.7%), with HER2+(non-luminal) the least common 16 (12.9%). Following NACT, 56 (45.2%) of patients were found to be responders, with a complete pathological response seen in 39 (31.5%) of patients. As expected the highest response rates were seen in the HER2+ (non-luminal) breast cancer subtype (68.8%), follow by Triple negative (64%), Luminal B HER2 (59.1%) and Luminal (26.2%) respectively.

Table 5.2 Clinicopathogical details

Total No.	124
Median age (range)	55 (25-76)
Grade: No. (%)	
1	1 (0.8)
2	64 (51.6)
3	57 (46.0)
NA	2 (1.6)
Lymph node (pre op): No. (%)	
Positive	77 (62.1)
Negative	47 (37.9)
Surgery: No. (%)	
WLE	69 (55.2)
Mastectomy	55 (44.8)
Subtype: No. (%)	
Luminal	61 (48.8)
Luminal HER2	22 (17.6)
HER2+	16 (12.8)
Triple negative	25 (20)
Pathological complete response: No. (%)	
Yes	32 (25.8)
No	92 (74.2)

5.3.2 MicroRNA detection at time of diagnosis in the circulation

All five target microRNAs were detected in the circulation, along with both control microRNA. Ten individuals were removed from the microRNA analysis, due to insufficient sampling, or no detection of any of the five target microRNAs on PCR, leaving 114 patients for the final analysis.

5.3.3 Relationship of circulating microRNA expression to clinicopathogical parameters

Associations between the five target microRNAs and clinicopathogical parameters including grade, lymph node status at diagnosis, hormone receptor status and HER2 receptor status are shown (Table 5.3). For four of the target microRNA (Let-7a, microRNA-21, microRNA-145 and microRNA-155), no significant variation in expression was seen across any of the variables. MicroRNA-195 had significantly higher expression in grade 2 compared to grade 3 breast cancers ($p=0.016$). Increased microRNA-195 expression was also significantly associated with ER

positive breast cancers (p=0.014), while no significant variation in microRNA-195 expression was seen in relation to PR or HER2 receptor status.

Micro RNA	Grade		Lymph node status		ER status		PR status		HER2 Status	
	2 n=62	3 n=50	+Ve n=70	-Ve n=13	+Ve n=75	-Ve n=39	+Ve n=60	-Ve n=54	+Ve n=34	-Ve n=80
7a	0.112		0.620		0.242		0.545		0.407	
21	0.124		0.732		0.090		0.164		0.783	
145	0.868		0.153		0.406		0.063		0.877	
155	0.217		0.477		0.483		0.986		0.593	
195	0.016		0.224		0.014		0.580		0.477	

The expression of the five target microRNA in the four breast cancer subtypes was analysed (Figure 5.2). No significant variation in microRNA expression was seen between the breast cancer subtypes for any of the target microRNA overall or between individual subtypes, (Let-7a p=0.670, microRNA-21 p=0.287, microRNA-145 p=0.910, microRNA-155 p=0.913. However, microRNA-195 approached significance (p=0.087), with increased expression seen in Luminal tumours compared to the HER2+(non-luminal) and Triple negative subtypes.

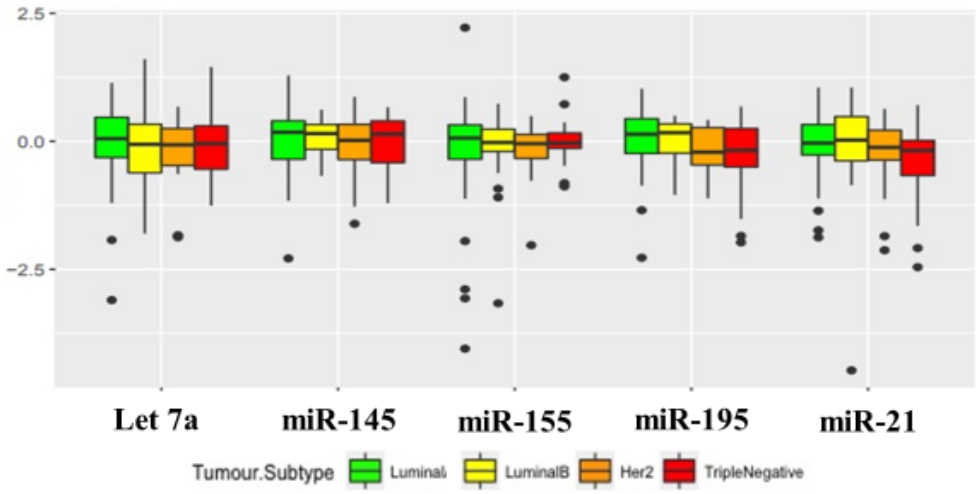


Figure 5.2 MicroRNA expression by subtype.

5.3.4 Relationship of circulating microRNA in responders versus non-responders

The relationship between the target microRNAs expression and tumour bed response to NACT in responders compared to non-responders was assessed (Figure 5.3). A significantly lower expression of microRNA-21 was seen in the responders compared to non-responders (p=0.036). On univariate analysis of microRNA-21, it was found to be an independent predictor of responders (OR 0.538, 95%CI 0.308-0.943, p>0.05) (Figure 5.4). For every unit increase microRNA-21 the odds ratio of being non-responder relative to responder is 1.86 times higher. For the microRNA-195, a significant difference in expression is seen between responders and non-responders (p=0.017) (Figure 5.3). On univariate analysis, microRNA-195 was not found to be an independent predictor for responders (OR 0.561, 95%CI 0.285-1.104, p<0.1) (Figure 5.5). However, for every unit increase in microRNA-195 expression the odds ratio of being non-responder relative to responder is 1.78 times higher.

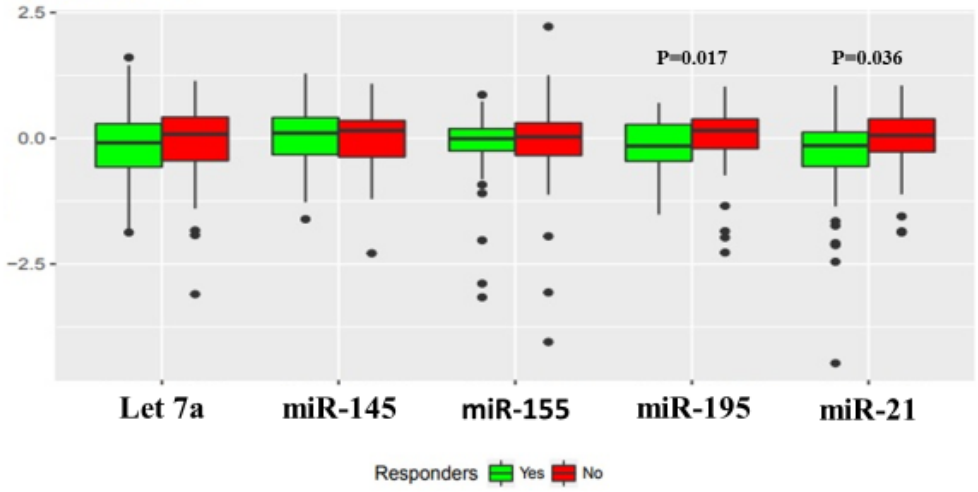
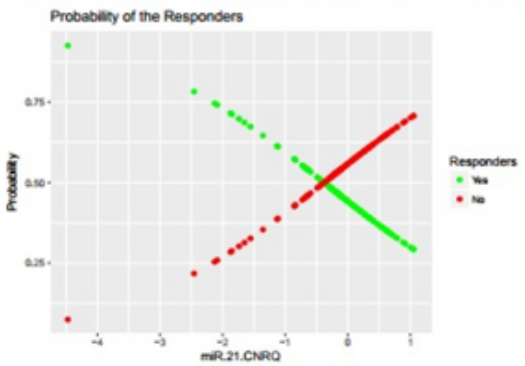


Figure 5.3 MicroRNA expression by Responders vs non-responders.

Odds Ratio (95 CI)

	Dependent variable:
	Responders
miR.21.CNRQ	0.539** (0.308, 0.943)
Constant	0.791 (0.534, 1.174)
Observations	109
R^2	0.066
χ^2	5.545** (df = 1)
Note:	*p<0.1; **p<0.05; ***p<0.01

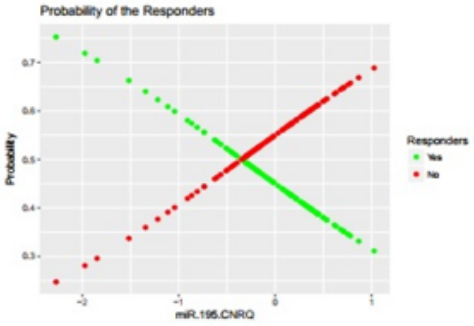


Every unit increase microRNA-21 the odds ratio of being non-responder relative to responder is 1.86 (1/0.538) times higher

Figure 5.4 Univariate analysis microRNA-21. Left; table assessing odds ratio. Right; figure of probability of being responder by increase in microRNA level.

Odds Ratio (95 CI)

	Dependent variable:
	Responders
miR.195.CNRQ	0.561* (0.285, 1.104)
Constant	0.818 (0.557, 1.201)
Observations	109
R^2	0.036
χ^2	2.965* (df = 1)
Note:	*p<0.1; **p<0.05; ***p<0.01



Every unit increase microRNA-195 the odds ratio of being non-responder relative to responder is 1.78 (1/0.561) times higher

Figure 5.5 Univariate analysis microRNA-195. Left; table assessing odds ratio. Right; figure of probability of being responder by increase in microRNA level.

On multivariate analysis, adjusting for age, subtype, grade and nodal status, microRNA-21 was not found to be an independent predictor of response (OR 0.768, 95%CI 0.294-2.009). Only a higher tumour grade was found to be a significant predictor of response on multivariate analysis (OR 5.598, 95%CI 1.242- 25.236, p>0.05).

5.3.5 Relationship of individual target microRNA response to NACT in different breast cancer subtypes

The variation in expression of each target microRNA by response to NACT was assessed in the four breast cancer subtypes. In microRNA-21 there was a

significantly decreased expression seen in responders compared to non-responder in the Luminal subtype (p=0.048) (Figure 5.6). For microRNA-145, there was a significantly lower expression in responder compared to non-responders in Luminal breast cancers (p=0.033) (Figure 5.7). In the HER2+(non-luminal) subtype, a low microRNA-145 expression was significantly associated with not having a complete response to NACT (0.019). No significant variation in microRNA-145 expression is seen based on response to NACT in either the Luminal B HER2 or the Triple negative subtype.

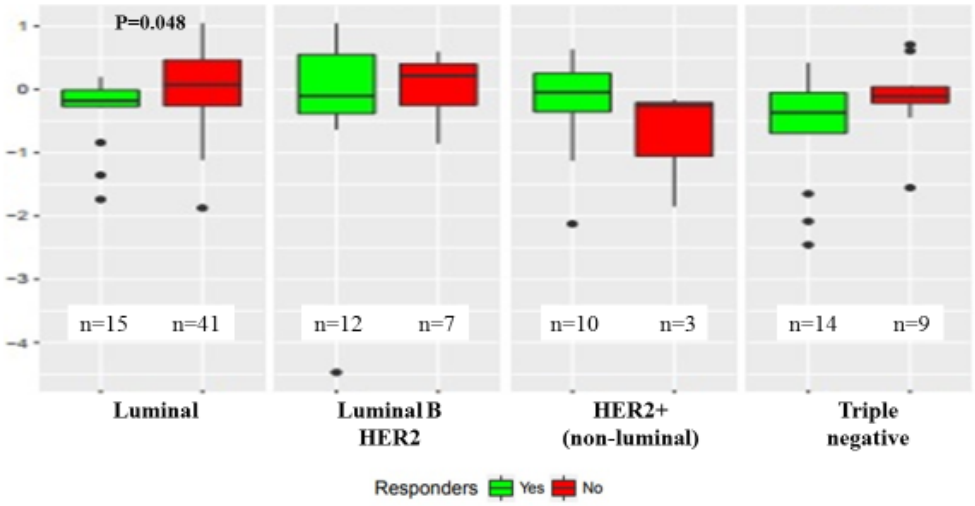


Figure 5.6 MicroRNA-21 responders by subtype.

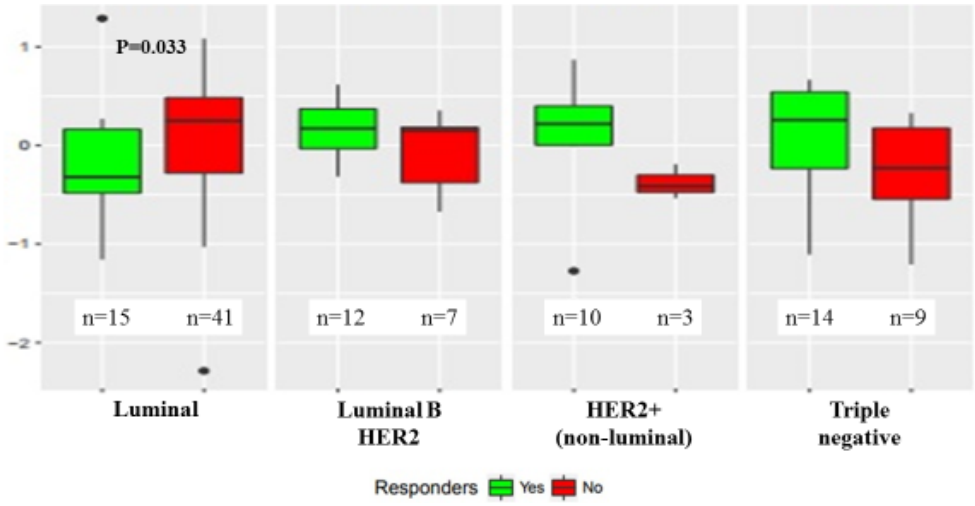


Figure 5.7 MicroRNA-145 responders by subtype.

In microRNA-195, although significant differences in expression were seen between responders and non-responders overall, no significant variation was seen in any of the individual subtypes (Figure 5.8).

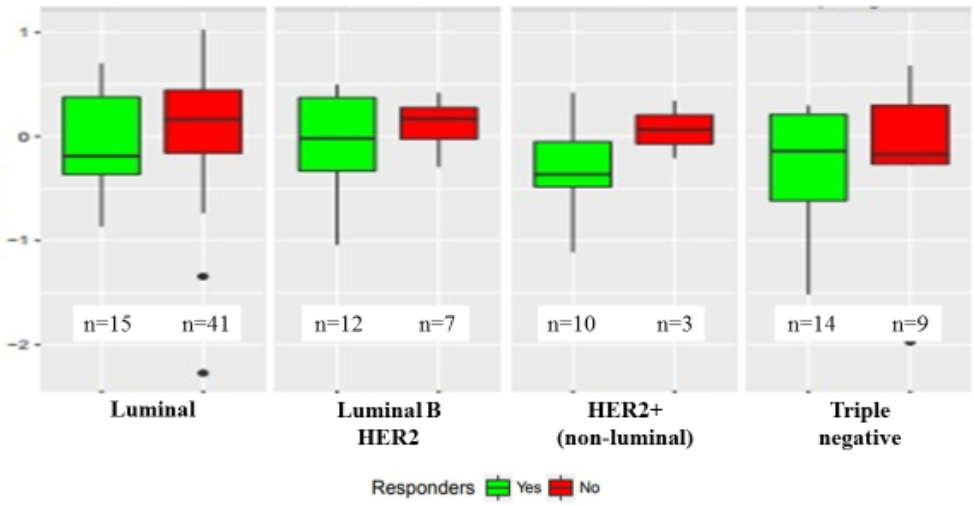


Figure 5.8 MicroRNA-195 responders by subtype.

5.3.6 HER2 receptor positive cohort

In total 38 patients included in the trial were HER2 receptor positive, 22 (57.9%) were Luminal B HER2 and 16 (42.1%) were HER2+(non-luminal). The median age, similar to the overall cohort was 55 (range 34-71), with a higher proportion of patients being node positive 27 (71.1%).

5.3.7 Pathological complete response in HER2 receptor positive breast cancer subtypes

As expected there was a higher pCR in the HER2 receptor positive cohort compared to the overall cohort, with 15 (39.5%) of patients having a pCR compared to the overall rate of 25.8%. As found in the previous chapter, a significantly higher pCR of 56.3% was seen in the HER2+(non-luminal), when compared to the Luminal B HER2 27.3%.

5.3.8 Disease free survival, recurrence rates and discordance rates

For overall cohort disease free survival data was only available for 101 patients. There was a high 3 year DFS of 90.1%, with only 10 patients in total having a

recurrence. Discordance in subtype occurred in three patients, two patients losing receptors status (One Luminal to Triple negative and one Luminal B to HER2+(non-luminal)), and only one patient gained a receptor status (HER2+(non-luminal) to Luminal B).

When comparing the 3 year DFS between the two HER2 receptor positive subtypes, although number are small in each group a difference is still seen. The 3 year DFS for the Luminal B HER breast cancer subtype was 100%, while the 3 year DFS for the HER2+(non-luminal) breast cancer subtype was only 75%.

5.4 Discussion

In this blinded, multicentre, prospective clinical trial the ability of circulating microRNA to predict response to NACT was assessed. In this analysis, the expression levels of five target miRNAs were assessed at the time of diagnosis, prior to NACT, and evaluated in relation to tumour characteristics, biologic features and response to NACT. We have demonstrated that expression levels of microRNA-21 and microRNA-195 at presentation were altered between responders and non-responders. On univariate analysis, microRNA-21 was found to be an independent predictor of having a response to NACT. MicroRNA-21 and microRNA-145 expression levels had significant variation in expression between responders compared to non-responders in the Luminal breast cancer subtype. There was no significant association seen between any of the five target miRNAs and any of the breast cancer subtypes, although microRNA-195 was significantly elevated in ER positive compared to ER negative breast cancers. Response to NACT has a major impact on risk of recurrence and survival, with patients that have a complete response having over 90% 5 year overall survival [4]. Although the role of chemotherapy in breast cancer is accepted, a majority of patients who get this treatment derive only a partial or no benefit, with non- responders having very poor outcomes [4, 69]. With such a low benefit to toxicity ratio, the addition of microRNAs and particularly the ability to predict increased response rates would be very helpful.

MicroRNAs have been shown as potential diagnostic biomarkers in breast cancer, with all selected targets in this study having been shown to be altered in breast

cancer patients compared to controls. For both microRNA-21 and microRNA-195, it has been established that a higher expression is associated with breast cancer and poor outcome [91, 107, 335]. Our study not only adds to the growing evidence of the use of microRNA as biomarkers, but is the first to demonstrate that both microRNA-21 and microRNA-195 could be potential predictors of response to NACT. Previous studies have also examined the role of microRNA in predicting response to NACT, and have found that microRNA could be potential biomarkers to predict response. In one study, a group of circulating miRNA were found to exhibit strong correlation with response to NACT [338]. In another study assessing the microRNA expression before and after NACT, discovered that a significant variation in microRNA-34a was seen between patients with a partial response, compared to patients with a complete response in HER2+ (non-luminal) and Triple negative breast cancer subtypes [339]. Overall this highlights the potential of microRNAs alone, or with additional markers to predict which patients will respond to NACT.

In our study, a breast cancer subtype specific response to NACT was also identified, with elevated microRNA-21 and microRNA-145 expression significantly associated with non-responders compared to responders in the Luminal breast cancer subtype. From in-vitro studies, an elevated microRNA-21 expression has been shown to be associated with chemoresistance in a Luminal breast cancer cell line [340]. In another study, the ability of circulating micoRNA to predict response to NACT in Luminal breast cancers was assessed, and it was found that circulating microRNA-19a and microRNA-205 in serum may predict chemosensitivity versus chemoresistance in Luminal breast cancer subtype [341]. The Luminal breast cancer subtype is known to have the lowest levels of response to NACT, although there are small numbers in our study. We have shown the possibility of using microRNA for identifying which Luminal breast cancers will respond to NACT and further validation with a larger cohort of patients is warranted.

Predefined microRNA were used as targets for this study based on publications at the time of the study design. The use of predefined microRNA may have limited the potential of this study, with multiple new potential target microRNA identified since that could not be assessed in this study. A recent study found up to 48 publications on circulating microRNA in breast cancer alone [342]. Adding in more recently

discovered microRNA and potentially those that are subtype specific may have real advantages. It has been shown that outcome after a pCR to NACT is dependent on tumour biology, with the lowest rates of recurrence seen in Luminal cancers that have a pCR [343]. By combining microRNAs with clinical and pathological markers this may increase specificity, and using a combination of markers, it may be possible to make a predictive score for response to treatment resulting in a more personalised approach to treatment.

5.5 Conclusion

This study has shown again that microRNA are readily detectable in the circulation of breast cancer patients and for the first time, their potential as biomarkers in predicting response to NACT. Using a blinded, multicentre, prospective clinical trial, the ability of a panel of five target microRNAs to predict response to NACT was assessed. The microRNA-21 and microRNA-195 were shown to be significantly reduced levels in patients that responded to NACT, while a subtype specific significant variation in microRNA-21 and microRNA-145 expression was found to be a predictor of response to NACT. Using a combination of microRNAs alone, or in addition with other clinicopathological factors may provide an accurate test to assess which patients will benefit from NACT. In this analysis, the microRNA expression at time of diagnosis was used to assess response to NACT, and may provide a way in the future to select appropriate therapy for patients.

Chapter 6

Conclusions

&

Future Perspectives

The overall aim of this study was to assess the factors that influence outcome in patients with HER2 receptor positive breast cancers. It is known that HER2 receptor positive breast cancer has a poor prognosis compared to Luminal cancers, however, the development of targeted therapy such as Trastuzumab has significantly reduced recurrence and improved survival. Despite this improvement, there were still a large proportion of patients that have only a partial or no response at all to Trastuzumab. Furthermore, the use of Trastuzumab can had both major economic impacts, and serious clinical side effects, such as cardiac failure. This has led to the development of additional targeted therapies for HER2 receptor positive breast cancers, such as Laptinib and Pertuzumab. However, to date the clinical use of these new therapeutics has not resulted in further significant improvements in survival. This highlights the need for further research into factors that can influence survival in HER2 positive breast cancers, and shows the need for the development of new markers to predict which patients would benefit from a targeted anti-HER2 therapy.

A patients age at diagnosis has a major influence on the management of breast cancer. By performing a review using recently published literature we have shown that age impacts every aspect of treatment. Breast cancer diagnosed at an earlier age tends to me more aggressive, and is more likely to be genetically inherited. The identification of mutations to the BRCA genes as a driver of breast cancer has had a major impact on breast cancer screening, due to the carriers higher lifetime risk of developing breast cancer. At the other end of the spectrum, breast cancer occurring later in life has a tendency to be Luminal, and has a high survival rate. Another major factor influencing survival is the introduction of screening programs, which has resulted in a larger proportion of patients being diagnosed, and at an earlier stage. This increased detection rate has resulted in the development of genomic tests, such as Oncotype Dx for early stage Luminal A breast cancer, to predict if selected patient groups will not have any increased survival benefit from adjuvant chemotherapy. Improved tests, such as molecular testing or genetic screening, will allow better patient stratification and lead to the better use of resources, and importantly spare patients unnecessary and costly treatments. The molecular separation of breast cancers, demonstrated by the widespread stratification and targeted therapies utilising hormone or HER2 receptors, has resulted in a more individualised management of breast cancer patients in the recent past. However,

further improvements are needed, such as increased stratification, understanding factors that influence breast cancer risk, survival and treatment response. Incorporating these new risk factors will play a key role in improving future decision making and breast cancer management.

As shown in previous studies, patients with hormone receptor positive Luminal B HER2 breast cancers had a reduced recurrence rate and improved overall survival, when compared to the hormone receptor negative HER2+(non-luminal) breast cancer subtype. Interestingly, by assessing the impact of Trastuzumab on these two subtypes, a greater response was found in the Luminal B HER2 breast cancer subtype, a very novel finding with implications in disease management and the understanding the molecular mechanisms at play in each subtype. A key interesting observation was the impact of Trastuzumab on distant metastasis patterns, with the greatest reduction observed in bone metastasis in the Luminal B HER2 breast cancer subtype. As bone metastasis is most commonly seen in Luminal cancers, this raises the question: What impact does Trastuzumab therapy have on hormone receptor status? Based on previous studies that show cross-talk between the HER2 receptor and hormone receptors may cause resistance to hormone therapy, we hypothesise that Trastuzumab therapy can prevent this cross-talk, and reduce resistance to hormone therapy. Further investigation is needed to assess if blocking cross talk between HER2 and hormone receptors could provide an exciting new therapeutic strategy.

In a systematic review of LRR rates across the different breast cancer subtypes since the Trastuzumab era, a significant variation in LRR was found between subtypes. A significant lower rate of LRR was seen in the Luminal B HER2 subtype (3.3%) compared to the HER2+(non-luminal) subtype (5.7%) overall. This work showed that LRR is no longer common in breast cancer, with only 3.44% of patients having a LRR, compared to rate of 7.9% seen in a study performed prior to the introduction of Trastuzumab. Further to this, in a subset analysis of studies that recorded LRR after breast conserving surgery, a lower rate of LRR was seen compared to the overall group. This shows that current guidelines are accurately predicting which patients are suitable for breast conserving surgery. Our study again highlights the continued need for investigation into the differences between the HER2 receptor

positive breast cancer subtypes, in an effort to reduce LRR in the HER2+(non-luminal) breast cancer subtype, down to the levels seen in the Luminal cancers.

Investigating the rate of breast cancer discordance, we found almost a quarter of all of recurrences have a change in subtype between the primary breast cancer and recurrence sites. Assessing discordance by hormone and HER2 receptor status, overall there was a significantly higher proportion gaining receptor status compared to losing receptor status. A higher discordance seen in hormone receptor status, compared to the HER2 receptor, which had only 3% of cases showing discordance. As expected when comparing the two HER2 receptor positive subtypes this resulted in twice as many Luminal B HER2 breast cancers showing discordance, compared to the HER2+(non-luminal) breast cancers. However, the reasons for discordance have not yet be determined. Discordance may be due to a heterogeneity in the primary tumour, and further research is needed to find ways to identify this heterogeneity in pathology samples. This may also be due to cancers evolving due to therapeutics pressures, however much work will be needed to determine if either, both, or another mechanism is responsible. An improved understanding and testing for discordance will lead to more tailored and individualised therapeutic strategies.

With the increasing cost of new therapeutic agents, the need to investigate their cost effectiveness remains a significant factor in treatment availability and is performed by health boards all over the world. The use of adjuvant Trastuzumab to treat HER2 positive breast cancers has been shown to be cost effective in the literature, but to date no study has assessed the cost of effectiveness of neoadjuvant Trastuzumab therapy. To exploring the cost effectiveness of neoadjuvant Trastuzumab therapy, the cost per QALY were compared to adjuvant Trastuzumab therapy. Assessing the mean cost, as expected a recurrence was found to have substantial bearing on the overall cost. When comparing patients in the adjuvant Trastuzumab cohort and the neoadjuvant Trastuzumab cohort, no significant difference was seen in mean cost, QALYs or cost/QALY. On multivariate analysis of the factors that impacted cost, treatment with neoadjuvant Trastuzumab was found to be significant, along with a later stage of diagnosis and age. While there is a clear and continued need to develop new therapeutic agents, a better understanding of the impact on healthcare costs of both current and new treatments needs further careful consideration.

As demonstrated (both here and previously by many others), over-expression of the HER2 receptor has a major impact on treatment and survival. Currently, diagnosis of HER2 over-expression in both primary tumour and recurrence requires an invasive biopsy, which has led to increasing research investigating non-invasive biomarkers to diagnose breast cancer using factors circulating in the blood. As a large variation in response to targeted anti-HER2 therapies is seen, a major aim of breast cancer research is discovering biomarkers that can predict which patients will benefit from chemotherapy, identifying responders from non-responders. Using a microarray analysis of circulating microRNA levels, possible microRNA biomarkers for HER2 receptor positive breast cancer were identified comparing Luminal A and Luminal B HER2 breast cancers. The two highest ranking microRNA targets (microRNA-3188 and microRNA-4308) were investigated further *in vitro*. However, validation in breast cancer cell lines was unsuccessful, as no significant variation was found. Fortunately, the study testing additional microRNA candidates, previously published in the literature (Let 7a, microRNA-10b, microRNA-21, microRNA-145, microRNA-155, and microRNA-195) was positive. This work, investigated if a subtype specific response could be found using these miRNA candidates. Comparing breast cancer cell lines to the normal non-tumourigenic breast cell line found that Let 7a, microRNA-21 and microRNA-145 had significantly different expression levels. Investigating microRNA expression levels across the cancer cell lines representing different breast cancer subtypes, individual variation between subtypes was seen, but no subtype specific or HER2 receptor specific microRNAs were identified. These results highlight the difficulty and complexity of translational research, where in vitro or in vivo findings may not be clearly or easily corroborated. To combat this the emphasis needs to be placed on increasing prospective clinical trials, to not only identify future possible biomarkers, but to increase the chances of fast and practical incorporation of results into clinical practice.

To directly address this, a multicentred, prospective, blinded clinical trial was established (ICORG10-11 clinical trial) to assess the ability of selected circulating microRNA to predict a clinical response to NACT. In this clinical trial the expression of six target microRNA (Let 7a, microRNA-10b, microRNA-21, microRNA-145, microRNA-155, and microRNA-195) were assessed at time of diagnosis, prior to any treatment, and compared to response to NACT to identify if

these microRNA could predict responders versus non-responders. Two of the targets (microRNA-21 and microRNA-195) had significantly higher expression levels in the non-responders compared to the responders to NACT. On univariate analysis microRNA-21 was found to be an independent predictor of response to NACT. Assessing if there was a breast cancer subtype specific response to NACT in the targets, microRNA-21 and microRNA-145 expression levels were found to be significantly raised in Luminal A breast cancer in non-responders, compared to responders. This is one of the first prospective clinical trials to show the potential of microRNA as biomarkers for predicting response to NACT. As only the initial time point (taken at diagnosis) was analysed using treatment response in this study, further analysis of the microRNA expression at other key time points, such as during and after chemotherapy, may further validate and enhance the current results. Further work combining microRNAs levels with other clinical and pathological factors such as receptor status may further improve the sensitivity and specificity of predicting which patients will respond to chemotherapy, one day resulting in personalised treatment plans.

In the majority of studies investigating factors affecting HER2 receptor positive breast cancers, the role of hormone receptor status is rarely assessed. This work presented here has demonstrated that hormone receptor status has a major influence, correlating with variations in response to treatment, recurrence rates and recurrence patterns. Further investigating the variations in response to Trastuzumab treatment in the Luminal B HER2 and HER2+(non-luminal) breast cancer subtypes, may identify further possible treatment avenues or improve current treatment choices. Understanding the molecular difference between responses seen in these subtypes may also provide insight into the variations in response to NACT seen and help identify which patients will benefit from chemotherapy. An interesting path for future research is the role and choice of biomarkers in breast cancer diagnosis and management. We demonstrated the use of microRNA in predicting the response to NACT in breast cancer. However, this was only using the blood sample taken at diagnosis. The next step for investigation is to assess the variation of microRNA expression along the various key points in the timeline of NACT treatment, to determine if any other patterns of microRNA expression correlate with response to NACT. Connecting microRNA expression at diagnosis with expression after NACT

may provide further key evidence needed to bring microRNA testing into clinical practice as a biomarker for breast cancer, allowing improvements in breast cancer management and most importantly in patient outcomes.

The work discussed here explored the differential effects of treatment, responses and biomarkers on individual breast cancer subtypes. This work highlights the fundamental molecular and clinical differences between related HER2 receptor positive breast cancer subtypes, and demonstrates that more work is needed to fully discover and utilize these differences to effect changes in breast cancer treatment or diagnosis.

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Appendix 1: Ethical Approval form for the conduct of ICORG 10-11

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

Merlin Park University Hospital
Ospidéal na h-Ollscoile, Páirc Mheirlinne
GALWAY UNIVERSITY HOSPITALS

Clinical Research Ethics Committee
Unit 4
Merlin Park Hospital
Galway.

22nd October, 2010.

HAEM/ONC CLINICAL TRIALS UNIT

Professor Michael J. Kerin
Professor of Surgery
University College Hospital
Galway.

Ref: C.A. 485 – Circulating miRNAs: Novel breast cancer biomarkers and their use for guiding and monitoring response to neoadjuvant chemotherapy

Dear Professor Kerin,

I have considered the above project, and I wish to confirm Chairman's approval to proceed.

Yours sincerely,

Dr. Shaun T.O'Keeffe
Chairman Clinical Research Ethics Committee.

c.c. Olive Forde/Helen O'Reilly, Clinical Trials Nurse Co-Ordinators, Clinical Trials Office, University College Hospital, Galway.

Appendix 2: Patient Information Leaflet and Consent Form ICORG 10-11

Study Title: Circulating miRNAs: Novel breast cancer biomarkers and their use for guiding and monitoring responses to chemotherapy

Study Number/ICORG Number 10-11

Investigator Name: <Name>

Investigator Address: <Address>

Sponsor/Supporter Name and Address: ICORG- the All Ireland Cooperative Oncology Research Group (ICORG), 60 Fitzwilliam Square North, Dublin 2, Ireland.

Introduction:

You are being invited to take part in a Research Study. In order to decide whether or not you should agree to be part of this study, it is important for you to understand why the research is being done, what it will involve, as well as the possible risks, benefits and discomforts. This process is known as Informed Consent.

This Patient Information Leaflet gives detailed information about the Research Study that your study doctor will discuss with you. Please take time to read the following information carefully and make sure you fully understand it. If you would like to know more about something mentioned in this leaflet, or have any questions about this research study, please be sure to ask your study doctor or nurse.

Thank you for reading this information leaflet. Please take your time to decide whether or not you wish to take part.

What is the purpose of the study?

The aim of the study is to identify patients who respond to treatment. Unfortunately there is no readily available clinical test, at present, which accurately differentiates responders from non-responders, thus a number of women are prescribed treatment with potentially severe toxicity without knowing whether they will benefit from it.

Who is organising the research?

This study is organized and sponsored by ICORG, the All-Ireland Cooperative Oncology Research Group.

What will happen during the study?

If you take part in this study an extra sample of blood will be taken at 5 time points during and after your treatment regimen. You will be having routine bloods taken at these time points so we will just take an extra sample at those times.

During biopsy and surgery, if possible, we would like to send a very small sample from your tumour to the research laboratory for molecular profiling in order to correlate molecular expression profiles from the tumour and the blood. (GUH only)

How many people will take part in the study?

About 125 patients will take part in the study.

What do I have to do?

If you decide to take part we will ask you to give an extra blood sample at 5 time points during and after your treatment *and to agree that we will use a small piece of your tumour tissue for research* (GUH only).

How long will I be on the study?

You will be in the study for 15-21 month depending on the time point of the last blood sampling.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign the attached consent form and given a copy of this information leaflet to keep.

If you decide to take part but later change your mind, you are free to withdraw at any time without giving a reason. This will not affect the standard of care you receive. Likewise, your study doctor may decide to withdraw you from the study if it is in your best interest.

What are the possible risks of taking part?

Some known risks, although rare, are associated with placing a needle into a vein or under the skin. Patients may feel faint, or experience mild pain, bruising, irritation or redness at the site of puncture. In rare cases, inflammation of the vein or infection may occur. Care will be taken to avoid these complications.

For more information about risks and side effects, ask your study doctor.

What are the possible benefits of taking part?

You may not benefit from participating in this study. The information we get from this study may help future patients with breast cancer, as we may be able to use the information derived from this study to predict those patients who are most likely to respond to neoadjuvant or adjuvant chemotherapy, and also those who are unlikely to respond to these treatments. This latter group could then be spared the severe toxicities associated with these treatments, from which they would derive no benefit.

What about future use of my sample for research?

We would like to keep some of your blood *and tissue sample* (GUH only) in case other tests become available in the future. The research that is being done with your sample is not designed to specifically help you and will not affect your care. It might help people who have breast cancer in the future. These samples will be stored in a biological resource bank (biobank) to be used for our current research and for our future research. We will not be able to contact you to ask your permission for each individual study, but ask you now for your overall permission to use your donated samples for research purposes.

Prior to any such research taking place, ethics approval will be sought.

Where will my samples be stored?

Samples will be stored at the Department of Surgery, Surgical Laboratory, Clinical Science Institute, NUI Galway, in a laboratory freezer at -80°C.

How long will samples be stored for?

Your samples will be kept indefinitely in this Biobank.

Who will have access to my sample?

Only researchers at the National Breast Cancer Research Institute and their collaborators or affiliated research groups, will have access to these samples.

Will my taking part in this study be kept confidential?

When you donate a sample, it will be given an individual identification number. All samples donated will be stored labelled with this number. Names, addresses and hospital numbers will never appear on the sample. Therefore researchers will be unable to identify you from the sample.

If information from this study is published or presented at scientific meetings, your name and other personal information will not be used.

If you consent to take part in the study any of your medical records may be inspected and/or copied for purposes of quality assurance, and data analysis by the company organising (ICORG) the research and the following organisations:

- Department of Health and Children (DoHC)
- Research Ethics Committee (*Enter name here)*

However, strict confidentiality will be maintained at all times.

Can I stop being in the study?

Yes, you can decide to stop at any time.

If you withdraw from the study your unused samples will stay in the biobank unless otherwise authorised by you.

Can anyone else stop me from being in the study?

The study doctor may stop you from taking part in this study at any time if

- it is in the best interest for your health
- you do not follow your responsibilities for taking part in the study
- you need treatment not allowed in the study
- the study is stopped by the sponsor
- Administrative reasons require your withdrawal.

What happens if I am injured because I took part in this study?

It is important to note that nothing said in this consent form alters your legal rights to seek to recover damages should injury be suffered as a result of participation in this study.

Every reasonable precaution will be taken to ensure your safety during the course of the study.

Participation in this study is covered by an approved policy of insurance in the name of ICORG [Sponsor]. In addition, the medical practitioners involved in this study have current medical malpractice insurance coverage *under the current Clinical Indemnity Scheme.*(to be removed for private hospitals). The Sponsor [ICORG] will comply with Irish Law (statutory and otherwise) in the unlikely event of your becoming ill or injured as a result of participation in this clinical study. The amount of any compensation paid may, however, be reduced if you have not complied with the instructions issued for the study.

It is important that you tell your study doctor, _____, if you feel that you have been injured because of taking part in this study. You can tell the study doctor in person or call him/her at _____.

What are the costs of taking part in this study?

You will not be charged for the cost of tests done for the purpose of this study. You will not be paid for your participation in this study.

Contact for further information

You <u>will not</u> be able to obtain any results/information on tests carried out on your sample.

If you have any questions concerning the procedures of this study, or if any problems arise during the study, you should contact the following people:

Study Doctor name: _____ Telephone: _____

For questions about your rights or if you wish to make a complaint while taking part in this study, call the _____ at _____

INFORMED CONSENT FORM

Study Title:

Study Doctor Name: _____ Hospital Name: _____

1. I confirm that I have been given a copy of the Patient Information Leaflet and Consent Form ICORG Version X, dd/mmm/yyyy, Hospital Name Version X, dd/mmm/yyyy. I have read the patient information leaflet and consent form or it has been read to me. This information was explained to me and my questions were answered.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without my medical care or legal rights being affected.

3. I understand that relevant parts of my medical records may be seen by ICORG, Ethics Committees, and all organisations as listed in this Patient Information Leaflet provided they agree not to disclose my name.

4. I understand that data related to me collected during this study will be processed and analysed as is required by this clinical study and according to the Data Protection Act.

5. I understand that my sample may be used for research as described in the Patient Information Leaflet.

6. I agree to take part in the above study.

_____	_____	_____
Name of Patient (Print)	Signature of Patient	Date
_____	_____	_____
Name of Witness (Print) (IF APPLICABLE)	Signature of Witness	Date
_____	_____	_____
Name of Investigator (Print)	Signature of Investigator	Date
_____	_____	_____
Name of Research Nurse (Print) (IF APPLICABLE)	Signature of Research Nurse	Date

Appendix 3: Publications arising from this work

Cancer Metastasis Rev (2015) 34:145–155
DOI 10.1007/s10555-015-9551-7

CLINICAL

Metastatic breast cancer: the potential of miRNA for diagnosis and treatment monitoring

Andrew McGuire · James A. L. Brown · Michael J. Kerin

Published online: 27 February 2015

Abstract Breast cancer affects approximately 12 % women worldwide and results in 14 % of all cancer-related fatalities. Breast cancer is commonly categorized into one of four main subtypes (luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) positive and basal), indicating molecular characteristics and informing treatment regimes. The most severe form of breast cancer is metastasis, when the tumour spreads from the breast tissue to other parts of the body. Significantly, the primary tumour subtype affects rates and sites of metastasis. Currently, up to 5 % of patients present with incurable metastasis, with an additional 10–15 % of patients going on to develop metastasis within 3 years of diagnosis. MicroRNAs (miRNAs) are short 21–25 long nucleotides that have been shown to significantly affect gene expression. Currently, >2000 miRNAs have been identified and significantly, specific miRNAs have been found associated with diseases states. Importantly, miRNAs are found circulating in the blood, presenting an opportunity to use these circulating disease-related miRNAs as biomarkers. Clearly, the identification of circulating miRNA specific to metastatic breast cancer presents a unique opportunity for early disease identification and for monitoring disease burden. Currently however, few groups have identified miRNA associated with metastatic breast cancer. Here, we review the literature surrounding the identification of metastatic miRNA in breast cancer patients, highlighting key areas where miRNA biomarker discovery could be beneficial, identifying key concepts, recognizing critical areas requiring further research and discussing potential problems.

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Keywords miRNA · Breast cancer · Metastatic · Biomarker · Metastatic sites · Metastatic rates · Subtype

1 Introduction

Breast cancer is the second most common cancer diagnosed worldwide, affecting approximately one in eight women during their lifetime [1]. It affects 1.3 million women each year and accounts for 23 % of all cancer cases and 14 % (465,000) of all cancer-related deaths [2]. The most severe form of breast cancer occurs when the cancer spreads from the breast tissue to other regions of the body (metastasis), significantly increasing the tumour burden and often resulting in a fatal diagnosis. Breast cancer metastasis follows a cascade starting with local invasion of the surrounding tissue, spreading into the blood or lymphatic vessels and ending with dissemination of tumour cells to distal organs [3, 4] (Fig. 1, left). Despite modern treatments, metastatic breast cancer (MBC) is often incurable, with up to 5 % of patients presenting with distal metastases at time of diagnosis [2]. Currently, distal metastasis (M1) occurs in 10–15 % of patients within the first 3 years. Furthermore, approximately one third of women who have breast cancer with no lymph node involvement at time of diagnosis will develop distal metastases [5]. Significantly, the rate and site of metastasis can vary largely and is thought to be dependent on primary tumour subtype. Clearly, further knowledge is needed to both diagnose and treat metastatic breast cancer. Recently, microRNAs (miRNAs) have shown promise as new biomarkers for many cancers, including metastatic breast cancer [6–8]. Importantly, miRNAs have been linked to all stages along the metastatic cascade in breast cancer [9–15] (Fig. 1, right). Here we examine studies using circulating miRNAs as biomarkers for metastases, markers for tumour

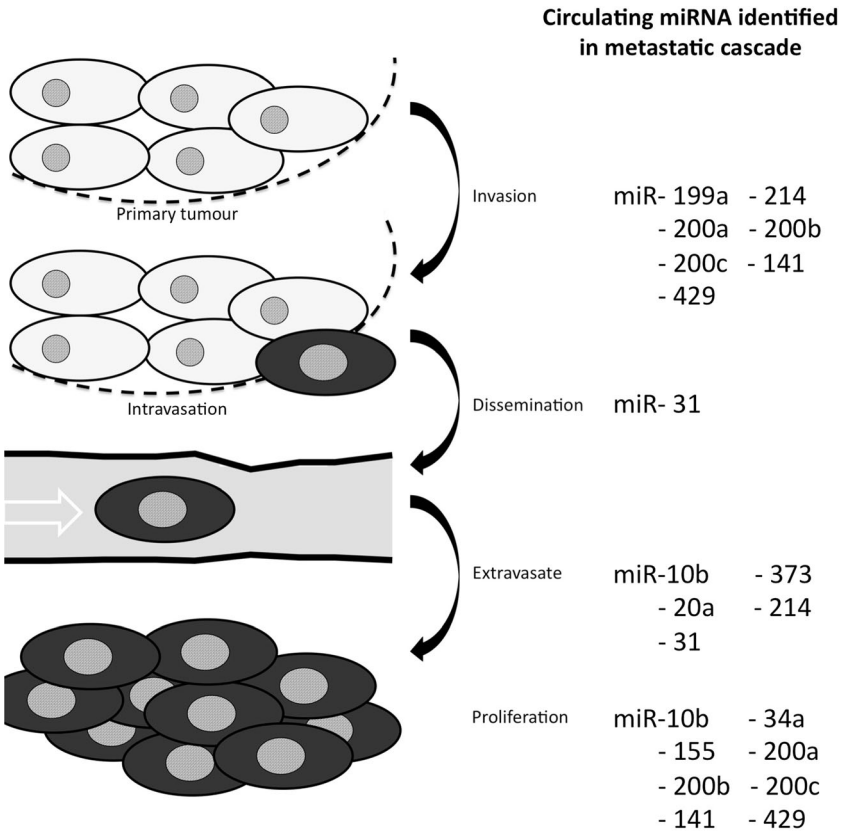


Fig. 1 Stages of breast cancer metastasis. *Left*: Order of process resulting in breast cancer metastasis. *Right*: Circulating miRNA associated with key steps in the metastatic cascade

recurrence and response to clinical treatments. We likewise discuss the potential applications of miRNA for therapeutic metastatic breast cancer diagnosis, treatment and basic research.

2 MicroRNA

MicroRNAs (or miRNAs) were originally discovered in the early nineties in Caenorhabditis Elegans [16]. MicroRNAs are a 21–25 long class of small non-protein coding RNA that function as gene regulators by inhibiting the degradation of their target mRNAs and inhibiting translation (Fig. 2). miRNAs have been demonstrated to be involved in cell development, differentiation, proliferation and apoptosis [16]. The first human, disease-related miRNA characterized was from chronic lymphocytic leukemia [17] and subsequently, circulating miRNA were identified in patients with diffuse large B-cell lymphoma [18]. Consequently, miRNAs were linked to many other diseases and cancers [19, 20]. Since then, >2000 miRNAs have been identified in humans and these miRNAs regulate an estimated 30 % of all human genes [21]. miRNA can exert their action in cancers through both tumour suppression and oncogenic mechanisms (as oncomirs) [16, 22]. Fragile sites and genomic regions involved in oncogenic rearrangements in cancer are similarly thought to

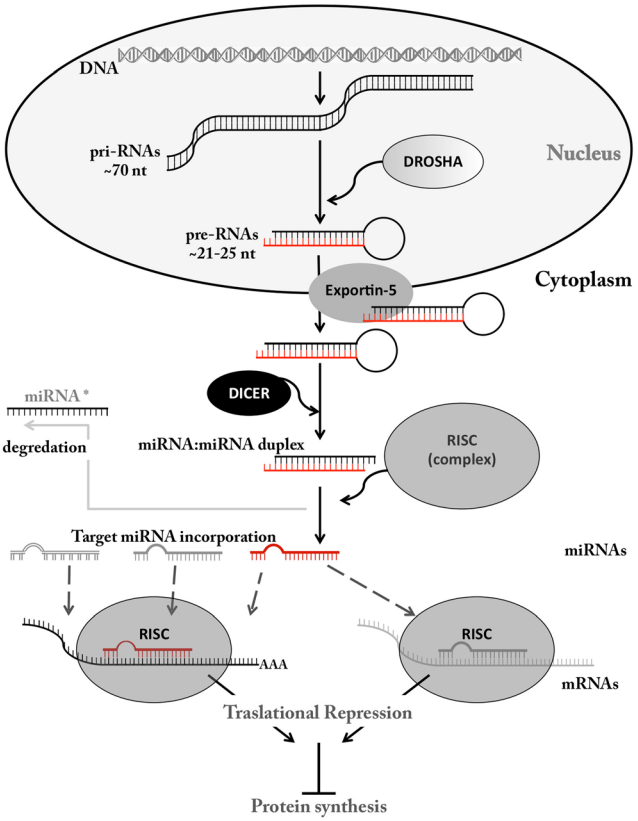


Fig. 2 miRNA biogenesis and mechanism of action

influence the production of cancer-related miRNA [23]. Furthermore, as a proof-of-principle for any potential therapeutic application of miRNA, circulating miRNAs have been identified which correlate with breast cancer subtypes (Table 1) [24–27].

2.1 miRNA biogenesis and action

miRNAs are formed from precursors called pri-miRNAs that are processed in the nucleus by Drosha, an RNA III type nuclease. These pri-miRNAs are transported to the cytoplasm by exportin-5, where they are cleaved by Dicer, another RNAse III enzyme, forming an asymmetric duplex (miRNA:miRNA). This miRNA duplex is then separated, and the mature target miRNA molecule is incorporated into the RNA-induced silenced complex (RISC) where it binds a member of the Argonaute (Ago) protein family [the other miRNA molecule (miRNA*) is normally degraded] [16, 28, 29]. The active RISC complex is then able to target mRNA transcripts with a sequence complementary to the mature incorporated miRNA molecule, leading to inhibition of protein expression (Fig. 2). miRNA can be exported from cells packaged in membrane-bound extracellular compartments (exosomes) or bound to RNA binding proteins [30]. Exosomes provide another method of extracellular signalling as they are able to bind and merge with other cells, thus influencing their environment. Furthermore, exosomes have been directly implicated in cancer [31]. Significantly, miRNAs are differentially secreted or selectively packaged into exosomes, with different cell and tumour types displaying distinctive miRNA profiles [32].

2.2 Breast cancer diagnosis

Currently, breast cancer can be subcategorized based on the status (+/−) of the hormone receptors oestrogen receptor (ER) and progesterone receptor (PR) and the Receptor tyrosine-protein kinase erbB-2 (ERBB2 or HER2). Furthermore, recent genetic testing has enabled the molecular subtyping of breast cancers [33, 34]. Presently, there are four major molecular subtypes: luminal A, ~50–60 % of breast cancers; luminal B, 10–20 %; HER2+ve, 15–20 %, with the remaining 10–20 % considered Basal subtype [35]. Further subcategorizing the common molecular breast cancer subtypes has allowed clinicians to tailor treatments to each individual patients cancer [36]. In particular, the Oncotype DX test evaluates 16 cancer-related genes and 5 reference genes, with the results used to estimate the likely reoccurrence in patients and diagnostically to determine if a patient should receive chemotherapy [37]. Significantly, as miRNAs have been implicated in cancer metastasis, miRNA signatures are being pursued as novel clinical diagnostic targets to allow further subtyping of breast cancer and for predicting metastasis or therapeutic resistance [38–41]. The potential of miRNA as biomarker targets is facilitated by their stability in blood and their ability to withstand repeated freezing and thawing cycles [42].

Table 1 Breast cancer molecular subtypes

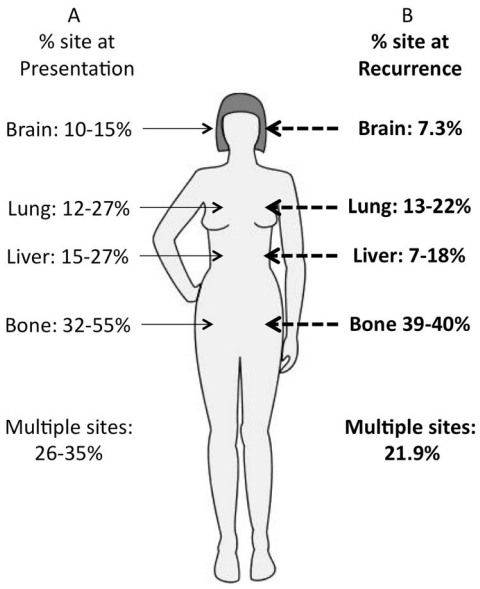
Breast cancer subtype	Molecular subtypes			Subtype-specific circulating miRNA	Ref
	ER	PR	HER2		
Luminal A	+	+	−	miR-29a, miR-181a, miR-652	[24]
Luminal B	+	+	+	miR-342	[25]
HER2+ve	+	−	+	miR-10b, miR-21	[26]
Basal (TNBC)	−	−	−	miR-210	[27]

2.3 miRNA in metastatic breast cancer tissue

Identifying and categorizing miRNAs expressed during the different stages of metastases will accelerate any therapeutic potential of these biomarkers, while illuminating the underlying mechanisms of cancer [43]. Currently, a number of studies have investigated miRNA expression profiles (upregulation or downregulation) of metastatic breast cancer tissue, providing insights into the processes of breast cancer initiation, progression and maintenance [44–47]. Significantly, it has been demonstrated that restoring the expression of individual miRNA observed to be lost in breast cancer models (such as miR-31, miR-126 or miR-335) can suppress metastasizes *in vivo* [48, 49]. Additionally, it has been suggested that cancer stem cells may influence metastasis [50–52], which would further contribute to any breast cancer miRNA profile. It is hoped that identifying breast cancer-specific miRNA and their functional relevance will lead to improvements in the early detection and treatment of tumours, particularly in younger patients.

2.4 Metastases

Breast cancer metastasises though the lymphatic system or via the circulatory system and is the overwhelming cause of mortality in patients with malignancies, causing 90 % of deaths in solid tumours [53]. Metastasis in breast cancer is characterized by a distinctive spread via regional lymph nodes to the lungs, liver, brain and bones [54]. Importantly, the rates and sites of distal metastasis can vary depending on age and stage of diagnosis [55, 56]. The most common site of metastases is the bone, often the first site of distal metastases in up to 50 % of patients [57], with lungs and liver as the second and third most common metastatic sites (respectively) (Fig. 3a, left). Significantly, 10–15 % of metastatic breast cancer patients will develop brain metastases, making breast cancer the second most common source of brain metastasis [58]. A similar distribution



C

Expression in metastastic disease	MicroRNA	Ref
Up regulated	miRNA-105, miRNA-21, miRNA-141, miRNA-200a, miRNA-200b, miRNA-200c, miRNA-203, miRNA-210, miRNA-375, miRNA-801, miRNA-20a, miRNA-214, miRNA-10b, miRNA-373, miRNA-34a	[7][103,104] [108-110]
Down regulated	miRNA-299-5p, miRNA-411, miRNA-452, miRNA-17, miRNA-155	[7][107][111]

Fig. 3 **a** Sites of metastatic breast cancer at presentation/diagnosis. **b** Recurrence sites of metastatic breast cancer. **c** Summary of published miRNA-associated breast cancer

of metastasis is seen following relapse (post-treatment), with ~22 % of patients having multiple sites of metastasis (Fig. 3b, right) [59, 60].

Further examining metastasis by molecular breast cancer subtypes, distinctive patterns of metastasis sites are observed (Table 2). Bone metastases remain the most common metastatic site in luminal A, B and HER2+ve breast cancers [59, 61, 62]. However, basal cancers were found to primarily metastasize to the lungs [63]. Interestingly, luminal cancers also tend to have a lower rate of brain metastases. Significantly, brain metastasis for the HER2+ve subtype is high [64], despite the implementation of trastuzumab-based treatments for HER2+ve breast cancers in the late 1990s. As Herceptin is not expected to cross the blood brain barrier, it is not believed to have influenced these rates. Currently, a number of circulating miRNA have been identified that are dysregulated (up or down regulated) in breast cancer metastasis (Fig. 3c). Identifying circulating miRNA associated with distinct metastatic sites could provide another powerful diagnostic tool for clinicians to evaluate disease stage and monitor progression.

Table 2 Sites of breast cancer metastasis by molecular subtypes

Metastasis sites	Breast cancer subtype			
	Luminal A	Luminal B	HER2+ve	Basal
Brain	6.6 %	8.2 %	23.3 %	18.1 %
Lungs	25.1 %	29.2 %	32.4 %	35.4 %
Bone	62.1 %	64.5 %	47.7 %	32.2 %
Liver	25.1 %	26 %	39.9 %	23.8 %

2.5 Subtype and metastasis stage

Currently, the severity of a person's breast cancer is based on the TNM staging, where T describes the tumour size, N defines the lymph node status (+/−) and M relates to any distant metastases (0/1). In addition to TNM staging, breast cancer can be also divided into groups, stages I–V, depending on size and metastatic spread. Significantly, this combined staging is used clinically to inform the choice of treatment regime. However, TNM staging has its limitations and drawbacks, including overtesting and uncertainties in staging due to limits in sampling auxiliary lymph nodes. Furthermore, the four breast cancer molecular subtypes contain different disease progression, survival and relapse rates. Luminal subtypes tending to have slower metastatic spread, lower reoccurrence rates and better outcomes than HER2+ve or basal subtypes [61, 65–69]. This difference is independent of histological subtype or time of detection, with the majority of basal carcinomas detected in the early stages of breast cancer. When comparing median survival (from time of first distal metastasis), luminal A and B subtypes display longer overall survival (2.2 and 1.6 years) compared to HER2+ve subtype (1.3 years). However, the basal subtype has the worst overall survival rate (0.7 years). This is reflected in the presentation rates of the metastatic disease, stratified by molecular subtype (Table 3).

Significantly, the relapse rates vary considerably by subtype, with HER2+ve the highest (51.4 %), followed by luminal B (42.9 %), basal (35.1 %) and luminal A (27.8 %). Interestingly, in addition to the lowest recurrence rate, luminal A relapse also occurs later than the other subtypes [61]. Clinically, HER2+ve cancers have a poor prognosis; however, following development of anti-HER2 treatments, there has been an improvement in disease-free survival (from 72.2 to 78.6 %) [70, 71]. Bone remains the most common primary metastatic site, while luminal B has a higher rate of metastases to other visceral organs (such as liver), compared to luminal

Table 3 Metastasis of breast cancer molecular subtypes (approximates)

Breast cancer subtype	% Metastasis at presentation	% Metastasis at recurrence	MBC median survival (years)
Luminal A	2–2.6	27.8	2.2
Luminal B	1–2.5	42.9	1.6
HER2	5–6	51.4	1.3
Basal	4–5	35.1	0.7

A. The basal subtype often presents with a younger onset, larger mean tumour size and higher grade, with the lowest overall survival [72, 73].

MicroRNAs have the potential to provide an additional mechanism for classifying breast cancer subtypes and tracking disease progression. A number of studies have investigated microRNAs in tissue as a means for identifying the main molecular breast cancer subtypes [24–26, 74–76]. Importantly, recent studies have found that microRNAs (miR-210, miR-328, miR-484 and miR-874) have the potential to predict prognosis or risk of recurrence [26, 77, 78]. Furthermore, it has been shown that microRNAs may be able to identify a subtype-specific response to treatment [27, 79, 80].

2.6 Breast cancer treatments

Currently, surgery is the primary treatment for early stage breast cancer. However, the use of chemotherapy, radiotherapy and hormone therapy has vastly improved survival rates [81–83]. For the treatment of metastatic breast cancer, chemotherapy and radiotherapy are used in the neoadjuvant setting, before breast conservative surgery or mastectomy and axillary node clearance. In progressive disease (stage IV), chemotherapy and radiotherapy are the principal treatments, along with hormone therapy. Defining metastatic specific miRNA has the potential to categorize breast cancer and inform and improve treatment choices. Furthermore, the use of specific miRNA as therapeutics has the potential to one day become a valid treatment option [84–86]. Indeed, there are current clinical trials investigating the efficacy of using miRNA to treat cancer [87].

2.7 Chemotherapy and miRNA

Chemotherapy usually involves a combination of drugs and is the leading treatment, often combined with hormone therapy, in metastatic breast cancer. The most common chemotherapeutics used are anthracyclines (doxorubicin and epirubicin), taxanes (paclitaxel and docetaxel), fluorouracil (5-FU) and cyclophosphamide. Currently, there is no evidence of benefit of one regime over another. However, a meta-analysis has indicated a benefit of adding taxanes to an anthracycline-based regime, demonstrating a 5 year risk reduction of 5 % in disease-free survival and 3 % in overall survival [88]. Significantly, HER2+ve patients treated with trastuzumab in combination with chemotherapy had increased median survival rates from 20.3 to 25.1 months [89]. Despite these treatment advancements, a large proportion of patients do not respond to traditional chemotherapy or hormone therapy [90]. In this context, circulating miRNAs have been explored as potential biomarkers, to predict treatment response [91–96]. Currently, only a few recent studies have explored the relationship of miRNAs with subtype-specific treatment [94, 97, 98] (Table 4). The early identification of circulating miRNA that can diagnose disease and/or chemotherapeutic responses will greatly facilitate improved treatments, leading to better outcomes for patients.

3 Diagnosing metastatic disease

Mammography is the gold standard for breast cancer screening, but it is mainly used for detection of local disease and is unreliable for diagnosing metastatic disease, with a false positive rate of ~50 % (7–9 % of these patients require a biopsy) [99]. Sentinel lymph nodes are the first lymph node in a tumour bed that receives lymphatic drainage from the tumour tissues, and sentinel lymph node biopsy (SLNB) currently provides the most accurate diagnosis for metastatic disease [100]. Currently, SLNB is recommended for early breast cancer, without any clinical evidence of nodal involvement [101]. However, SLNB only diagnoses regional metastasis. If distal metastases are suspected, SLNB needs to be combined with additional techniques, such as imaging. The development of an accurate biomarker, such as circulating miRNA, to diagnose or predict metastatic spread, could negate/reduce the need for many patients to undergo invasive procedures or surgery.

3.1 Circulating miRNA as biomarkers in metastatic breast cancer

Identifying circulating miRNA to use as biomarkers for metastatic breast cancer is currently a key priority for many research groups (Table 5). The first miRNA shown to be highly expressed in metastatic breast cancer was miR-10b (using mouse and human cells), with a clinical correlation in primary breast carcinomas [111]. A subsequent study confirmed this, finding elevated miR-10b, miR-34a and miR-155 levels in patients with metastatic breast cancer [7]. Further supporting this, it was recently shown that miR-10b and miR-373 were increased in lymph node positive breast cancer [108]. Excitingly, a significant increase in circulating miR-10b and miR-373 was demonstrated in lymph node positive patients, compared to patients with no nodal involvement or healthy controls. Differences in miRNA levels in lymph node positive patients were also observed in a subsequent study [106], where higher levels of miR-20a and miR-214 were found in lymph node positive patients, compared to lymph node negative patients. miR-210 was also identified as a potential marker for lymph node metastasis, however only in a small cohort [94]. Interestingly, miR-10b was identified as a potential biomarker for brain [109] and bone [110] metastases in breast cancer. Together however, these independent results cast doubt on the use of miR-10b as a metastatic specific marker. Furthermore, miR-299-5p and miR-411 were found to have significant differences in metastatic breast cancer patients,

Table 4 Metastatic breast cancer treatment regimes by subtype

Subtype	Circulating miRNA	Menopause	Node negative	Node positive
Luminal A	miR-19a, miR-205	Pre	Tamoxifen±chemotherapy	Chemotherapy+tamoxifen±ovarian ablation
		Post	Aromatase inhibitor (AI)+tamoxifen±chemotherapy	Chemotherapy+AI with tamoxifen
Luminal B	N.D	Pre	Tamoxifen+Herceptin±chemotherapy	Chemotherapy+Herceptin+tamoxifen
		Post	AI with tamoxifen+herceptin± chemotherapy	Chemotherapy+herceptin+AI with tamoxifen
HER2	miR-210	Pre	Herceptin+chemotherapy	Herceptin+chemotherapy
		Post	Herceptin+chemotherapy	Herceptin+chemotherapy
Basal	miR-27a, miR-30e, miR-155, miR-493	Pre	±Chemotherapy	Chemotherapy
		Post	±Chemotherapy	Chemotherapy

Bone disease adds denosumab, zoledronic acid or pamidronate to chemotherapy regime

with the additional miRs miR-215 and miR-452 of interest, without reaching statistical significance [105]. Additionally, miR-21 has also been identified as a marker for breast cancer and predictor of stage [103]. Recently, eight miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375 and miR-801) were found to be significantly higher in patients with circulating tumour cells (CTC) [104]. In another study, higher levels of miR-105 were found in early onset breast cancers that metastasized, compared to cancer that did not [102]. This study also found that overexpression of miR-105 promoted metastasis *in vivo*. The miRNAs miR-17 and miR-155 have been identified as potential differentiators between metastatic and non-metastatic breast cancer [107]. Supporting those studies, a number of these miRNAs have previously been identified as markers in metastatic triple negative breast cancer samples [79]. As the above publications did not specifically state the sites of metastases, this may partially account for the diverse miRNA identified. Importantly, these studies highlight the importance and potential application of circulating miRNA as biomarkers that can discriminate non-metastatic from metastatic breast cancer.

Additionally, miRNAs have been identified in metastatic breast cancer tissue samples [9–11, 48, 112–114]. While there is some correlation between circulating and tissue microRNAs, again a large diversity of miRNAs were identified. The number of individual non-overlapping miRNAs identified highlights the complexity of metastasis, staging and breast cancer subtype definition. This emphasizes the need for further comprehensive investigations using similar comparable experimental methodology with more defined/improved breast cancer typing criteria.

4 Circulating miRNA in other metastatic tumours

While the identification of circulating miRNA in breast cancer is progressing rapidly, exploring miRNAs investigated in other (often related) metastatic cancers may inform current work in the breast cancer field. Significantly, many of the circulating miRNA observed in other metastatic diseases have likewise been observed in metastatic breast cancer studies.

4.1 miRNA in metastatic colon cancer

In metastatic colon cancer, a significant increase in miR-29a in early liver metastasis was found [117]. In addition, high serum levels of miR-200b showed significant correlation with lymph node and distal metastatic disease in colorectal cancers [118]. Interestingly, miR-200b was identified as an independent predictor of tumour reoccurrence in colon cancer.

Furthermore, tumour reoccurrence in colon cancer was found to be predicted by a panel of six miRNAs (miR-15a, miR-103, miR-148a, miR-320a, miR-451 and miR-596) [115]. Recently in metastatic colon cancer, three miRNAs (miR-106a, miR-130b and miR-484) were found to be significantly overexpressed in patients not responding to first-line chemotherapy [116].

4.2 miRNA in metastatic cervical cancer

In cervical squamous cell carcinoma, a group of six miRNAs (miR-20a, miR-1246, miR-2392, miR-3147, miR-3162-5p, miR-4484) were found to identify lymph node metastasis [124]. Supporting this, miR-20a was found to be significantly increased in patients with lymph node positive cervical cancer, compared to both controls and patients with lymph node negative disease [122].

4.3 miRNA in metastatic gastric cancer

A recent study identified six miRNAs significantly increased in lymph node metastases of gastric cancer: miR-21, miR-27a, miR-106b, miR-146a, miR-148a and miR-223 [123]. In

Table 5 MicroRNA in metastatic tumours

MicroRNA	Tumour	Ref.	Cohort (*N*)
miRNA-105	Breast (serum)	[102]	38 Patients
miRNA-21	Breast (serum)	[103]	102 Patients, 20 controls
miRNA-141, miRNA-200a, miRNA-200b, miRNA-200c, miRNA-203, miRNA-210, miRNA-375, miRNA-801	Breast (serum)	[104]	61 Patients, 76 controls
miRNA-215, miRNA-299-5p, miRNA-411, miRNA-452	Breast (serum)	[105]	75 Patients, 20 controls
miRNA-20a, miRNA-214	Breast (serum)	[106]	48 Patients, 54 controls
miRNA-210	Breast (serum)	[94]	8 Patients, 31 controls
miRNA-17, miRNA-155	Breast (serum)	[107]	72 Patients, 40 controls
miRNA-10b, miRNA-373	Breast (serum)	[108]	35 Patients, 10 controls
miRNA-10b, miRNA-34a, miRNA-155	Breast (serum)	[7]	30 Patients, 29 controls
miRNA-10b	Breast (serum)	[109]	20 Patients, 10 controls
miRNA-10b	Breast (serum)	[110]	122 Patients, 59 controls
miRNA-10b	Breast (serum)	[111]	23 Patients
miRNA-126, miRNA-335	Breast (tissue)	[48]	11 Patients
miRNA-21, miRNA-139-5p, miRNA-486-5p	Breast (tissue)	[9]	6 Patients
Let 7i, miRNA-16, miRNA-26a, miRNA-27a, miRNA-143, miRNA-196a, miRNA-375, miRNA-503, miRNA-519a, miRNA-519b-3q, miRNA-361-5p	Breast (tissue)	[10]	48 Patients
miRNA-27b-3q, miRNA-107, miRNA-103a-3p	Breast (tissue)	[11]	58 Patients
miRNA-22	Breast (tissue)	[112]	108 Patients
miRNA-373	Breast (tissue)	[113]	11 Patients
miRNA-21	Breast (tissue)	[114]	113 Patients
miRNA-15a, miRNA-103, miRNA-148a, miRNA-320a, miRNA-451, miRNA-596	Colon (serum)	[115]	30 Patients
miRNA-27b, miRNA-158a, miRNA-326	Colon (serum)	[116]	150 Patients
miRNA-29a	Colon (serum)	[117]	20 Patients
miRNA-200c	Colon (serum)	[118]	182 Patients
miRNA-141	Prostate (serum)	[119]	21 Patients
miRNA-141	Prostate (serum)	[120]	56 Patients
miRNA-141, miRNA-375, miRNA-378	Prostate (serum)	[121]	84 Patients
miRNA-20a, miRNA-203	Cervical (serum)	[122]	80 Patients
miRNA-21, miRNA-27a, miRNA-106b, miRNA-146a, miRNA-148a, miRNA-223	Gastric (serum)	[123]	20 Controls, 16 patients

addition, a significant increase in levels of miR-21, miR-146a and miR-148a were found to correlate with increased spread in the lymph node.

4.4 miRNA in metastatic prostate cancer

Recent work in metastatic prostate cancer found miR-141 to accurately predict treatment response, compared to standard markers such as prostate-specific antigen (PSA), lactate dehydrogenase and circulating tumour cells [119]. Interestingly, levels of miRA-141 were found to be elevated in bone metastatic prostate cancer [120]. Importantly, miR-141 expression levels were found to correlate to alkaline phosphatase but not to PSA. A further study looking at metastatic castration resistant prostate cancer again found miR-141, plus miR-375 and miR-378 to be overexpressed compared to low-risk localized patients [121].

5 Conclusions

Our understanding of metastatic breast cancer has advanced considerably over the last number of years, yet metastasis remains the major cause of morbidity and mortality in breast cancer. Up to one in three breast cancer patients diagnosed will develop metastatic breast cancer, and despite current treatments, 78 % of these will die within 5 years. Clearly, there is an urgent need to find new clinically relevant biomarkers and tests to allow the early detection of metastatic breast cancer and for the monitoring of treatment response.

The defining of different molecular breast cancer subtypes has significantly aided the treatment of breast cancer, allowing more tailored individual treatment regimes. Significantly, treatment using hormone therapies and Herceptin has increased survival in luminal and HER2 positive breast cancers. However, basal (triple negative) breast cancers continue to have poorer outcomes. Chemotherapy remains the major treatment for metastatic breast cancer, yet similar regimes are given for all subtypes. Additional research is needed to define further subtypes and identify new markers that predict their response to chemotherapy. MicroRNAs have emerged as one such potential marker for predicting metastatic disease and response to treatment. Recently, a small number of miRNAs have shown increased expression in the circulation of metastatic breast cancer patients. In particular, miR-10b has been highlighted across five studies and has been linked to specific sites of distal metastasis. Despite the differences in the rate of metastasis across the breast cancer subtypes, to our knowledge, only one study (Dai et al.) has assessed the miRNA profiles associated with each molecular subtype [125]. Importantly, this study used tumour samples, not circulating miRNA, and did not include metastatic disease. A study investigating circulating miRNA profiles in patients with different molecular subtypes and metastatic disease is greatly needed. Furthermore, the use of different extraction methods and starting material may explain the lack of consensus between microRNAs currently identified in the indicated studies. However, another possible reason may be the diversity of sites of distal metastasis in each study. Only two studies have explored metastatic site-specific miRNAs, Ahmad et al. [109] (brain) and Zhao et al. [110] (bone), with both studies identifying elevated miR-10b. The identification of truly site- or subtype-specific metastatic miRNA may provide the diagnostic tool required to improve personalized metastatic breast cancer treatments. Interestingly, many circulating miRNAs identified in metastatic breast cancer were also found in studies of other metastatic cancers. This may indicate that the identified miRNAs are indicative of the sites of metastasis or that the miRNAs correspond to common underlying mechanisms of cancer metastasis.

Here, we highlighted the current knowledge and potential of microRNAs as biomarkers for improving the diagnosis and treatment of metastatic breast cancer. However, significant further directed research is needed to identify and confirm miRNA that can predict site-specific metastasis disease outcome or patient response to treatments.

Acknowledgments We would like to thank all the members of Prof Kerins' group for stimulating discussion. AM, JALB and MK are funded by the National Breast Cancer Research Institute (NBCRI).

Conflict of interest The authors declare that they have no conflict of interest.

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Breast Cancer Res Treat (2017) 164:221–229
DOI 10.1007/s10549-017-4225-5

BRIEF REPORT

Differential impact of hormone receptor status on survival and recurrence for HER2 receptor-positive breast cancers treated with Trastuzumab

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Received: 15 February 2017 / Accepted: 29 March 2017 / Published online: 4 April 2017

Abstract

Introduction Hormone receptor status has major implications for treatment and survival of breast cancer. Yet the impact of hormone receptor status on outcome after Trastuzumab has received little attention. The objective here was to explore any differential effects of Trastuzumab treatment (Trast +ve) on Luminal B HER2 or HER2+(ER−) breast cancer subtypes.

Methods A cohort of 469 HER2 receptor-positive breast cancers was categorised by molecular subtype and Trastuzumab treatment. Effects of Trastuzumab treatment on survival, locoregional recurrence and distant metastasis were investigated by subtype, using univariate and multivariate analysis.

Results Trast +ve Luminal B HER2 patients had significant improvements in 5-year DFS ($p < 0.001$) and OS ($p < 0.001$), while Trast +ve HER2+(ER−) patients had significant improvements in 5-year DFS ($p = 0.012$) alone. Only Trast +ve Luminal B HER2 cancers displayed a significant reduction in LRR rates ($p < 0.001$). A significant reduction in distant metastasis rates was seen in Trast +ve Luminal B HER2 ($p < 0.001$) and HER2+(ER−) ($p = 0.009$) cancers. Interestingly, bone metastasis rates in Trast +ve Luminal B HER2 cancers demonstrated the greatest reduction (36.2–6.7%). Multivariate analysis of Trast +ve patients found no difference in distant metastasis rates ($p = 0.96$) between subtypes. Significantly, lower LRR rates were seen in Trast +ve Luminal B HER2 cancers, compared to Trast +ve HER2+(ER−) ($p = 0.018$).

Conclusion An enhanced response to Trastuzumab was seen in Luminal B HER2 cancers. We highlight how Trastuzumab treatment changed the natural history of the HER2 receptor-positive breast cancer, demonstrating improved efficacy in changing the outcome of hormone receptor-positive patients.

Keywords HER2 · Trastuzumab · Luminal B · Luminal B HER2 · HER2 · HER2+(ER−) · Breast cancer · Survival · Metastasis · Distant

Introduction

Advances in molecular profiling have allowed breast cancer to be categorised into clinically relevant molecular subtypes. [1–3]. In approximately 20–30% of breast cancers, the HER2 receptor is over expressed [4, 5], resulting in increased cell signalling, uncontrolled cellular proliferation and poor clinical prognosis. Half of these are hormone receptor-positive Luminal B HER2 and half are hormone receptor-negative HER2+(ER−). Differences in survival and outcome occur between the two HER2 receptor-positive subtypes [6, 7]. However, some studies have shown no significant difference when assessing long-term survival between the two subtypes [8–10]. Clinically, the two subtypes present with distinct patterns of

Electronic supplementary material The online version of this article (doi:10.1007/s10549-017-4225-5) contains supplementary material, which is available to authorized users.

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recurrence. In Luminal B HER2 subtype bone is the most common distant metastasis site, similar to Luminal A breast cancers [8, 11]. HER2+(ER−) cancers have the highest rates of locoregional recurrence (LLR) overall and tend to initially metastasise to visceral organs, such as the lung [8, 12].

Trastuzumab is a monoclonal antibody that binds to the HER2 receptor and interferes with the HER2-mediated signalling cascade, preventing proliferation and eventually leading to cell death [13]. Trastuzumab was originally used to treat metastatic breast cancers and was shown to significantly improve median survival from 20.3 to 25.1 months [14]. Multiple studies have shown that Trastuzumab used in the adjuvant setting reduces recurrences and can increase survival in HER2-positive patients by up to 33–39% [15–18]. More recently, several studies have shown that when used in the neo-adjuvant setting Trastuzumab significantly increases pathological complete response (pCR) rates [19, 20].

While improvements in survival in HER2 receptor-positive cancers have been demonstrated in multiple studies, few studies have examined if Trastuzumab treatment has a varied response between Luminal B HER2 and HER2+(ER−) breast cancers. The aim of this study was to assess the impact of Trastuzumab therapy on the survival or outcome on the two HER2 receptor-positive breast cancer subtypes, before and after the introduction of Trastuzumab as part of the adjuvant treatment regime.

Methods

Patient cohort

This study group consists of all patients with HER2 receptor-positive breast cancers treated at a tertiary referral unit entered into a prospectively maintained database from 1991 to 2014. Only patients with a definitive HER2 receptor-positive subtype were included. All clinical pathological details and treatment regimes were analysed. Hormone receptor-positive patients received hormone therapy, as per standard clinical treatment protocols at the time of diagnosis. Clinically, testing for HER2 receptor status began at our centre in 1999. In order to find HER2 receptor-positive patients not treated with Trastuzumab, we used a cohort of patients identified using retrospective testing of pathology samples. A cohort of HER2 receptor-positive patients who received no Trastuzumab treatment was provided by retrospective testing of HER2 receptor status on patients included on a prospectively collected tissue microarray (samples from 1994 to 2001). Historically in our program, Trastuzumab therapy was introduced as adjuvant therapy in 2006, prior to this it was available only to patients recruited on clinical trials. Patients were categorised as received adjuvant/neo-adjuvant Trastuzumab (Trast +ve) or no Trastuzumab treatment (Trast −ve).

Subtypes definitions

Breast cancer subtypes were defined using ER, PR and HER2 receptor status. Luminal B HER2 was defined as (ER and/or PR +ve, HER2 +ve) and HER2+(ER−) as (ER and PR –ve, HER2 +ve) according to standard clinical pathological guidelines. The ER and PR receptor status were determined independently by clinical pathologists using immunohistochemistry as per ASCO guidelines (ALLRED score >2 or more than 1% stain positive). The HER2 receptor status was identified by Herceptest, as part of the routine clinical evaluation, with a score of 3+ considered positive. Any +2 inconclusive results were confirmed using a FISH testing as per ASCO guidelines, with a HER2/CEP17 ratio greater than two considered amplified.

Survival

Overall survival (OS), disease free survival (DFS), and patterns of recurrence were determined. The 5-year DFS & OS were determined and only patients who had completed 5 years of follow-up were included in the analysis.

Recurrence

Breast cancer recurrence was defined as a return of cancer after treatment and after a disease free period. Only stage I–III breast cancers were included for this section of the analysis. Recurrence was divided into LRR and distant metastasis. LRR is defined as recurrence at the same site of the primary cancer or the regional lymph nodes, while distant metastasis is recurrence at a distant site from the primary cancer.

Statistics

Statistical analysis was performed using R statistical software version 3.2.3. A p value of less than 0.05 was considered statistically significant. The Kaplan–Meier method was used to determine survival distributions. The log rank was used to determine any statistically significant differences in survival between the indicated groups. Cox regression was used for multivariate analysis, with logistic regression used to analyse categorical data.

Ethics, consent and permissions

This study was conducted in accordance with the granted National University of Ireland Galway and University

College Hospital Galway ethical approval. Informed consent was obtained from all patients. All patients had histologically confirmed breast cancer and all relevant clinicopathological and demographic data were obtained from a prospective breast cancer database.

Results

Cohort description

The study consisted of 468 HER2 receptor-positive patients eligible for this study, treated in our institute between 1991 and 2014. From these, 287 (61%) were found to be Luminal B HER2, with the remaining 181 (39%) patients HER2+(ER−). The median age of patients was 63 and the median follow-up was 49 months. The majority of the overall cohort was recruited after Trastuzumab received approval for adjuvant treatment in 2006 (Table 1). For the purpose of this analysis, patients were categorised as either Trast +ve (received adjuvant/neo-adjuvant Trastuzumab) or Trast −ve (no Trastuzumab treatment).

Overall, 299 (63.9%) patients were treated with Trastuzumab (Trast +ve). The clinical pathological details of the cohort are listed in Table 1, demonstrating the two subtypes are relatively matched for age, stage and treatment. The only statistical significant difference between the Luminal B HER2 and HER2+(ER−) was observed in the grade category, where the HER2+(ER−) cohort had a higher proportion of grade 3 cancers (49.8 vs. 79.3% $p < 0.001$). Furthermore, 263 (91.6%) of the Luminal B HER2 cancers received adjuvant hormone therapy. In the series recurrence occurred in 94 (20.1%) patients, of which 15 (3.2%) had LRR alone. 54 (11.5%) patients had distant metastasis alone and 25 (5.3%) patients had both LRR and distant metastasis. There was no significant difference in the distribution of age, stage or treatment of cancers between the two subtypes (Table 1).

Trastuzumab treatment and breast cancer subtype significantly affects survival

Univariate analysis of survival

Survival was similar in Luminal B HER2 compared to HER2+(ER−) subtypes (Figure S1) and Trastuzumab treatment significantly improved overall survival in both subtypes (Figure S2). No difference was seen in survival between the two subtypes in either the Trast –ve or Trast +ve patients. Next to assess the impact of Trastuzumab introduction on each subtype, the 5-year DFS and OS were compared between the Trast –ve and Trast +ve groups in both subtypes. Analysing the DFS and OS by subtype, an increased survival rate is seen for Trast +ve patients in both Luminal B HER2 and HER2+(ER−) patients. However, a greater improvement was seen in Luminal B HER2 patients. Luminal B HER2 cancers had a statistically significant improvement in both 5-year DFS ($p < 0.001$) and OS ($p < 0.001$) (Fig. 1a, b), while the HER2+(ER−) only had a significant improvement in DFS ($p = 0.012$) but not OS ($p = 0.135$) (Fig. 1c, d).

Multivariate analysis of survival

No significant increased risk was seen in the HER2+(ER−) subtype when compared to Luminal B HER2 cancers for either 5-year DFS (HR 1.31, 95% CI 0.42–4.1) or 5-year OS (HR 2.18, 95% CI 0.79–6.03) (Table S1). Analysing risk factors for survival, higher grade was not associated with worse outcome but Trastuzumab given in the neo-adjuvant setting was associated with a significant improvement in 5-year DFS (HR 0.16, 95% CI 0.04–0.63). A multivariate cox proportional hazard model analysis of survival was performed, where the model was controlled for age, stage, grade and chemotherapy treatment (Fig. 2). A similar outcome is seen to the univariate analysis, with a greater improvement in survival seen in Luminal B HER2 patients treated with Trastuzumab. In Luminal B HER2 cancers, a significantly increased hazard ratio is seen in Trast –ve patients: DFS (HR 3.82, 95% CI 1.5–9.4; $p = 0.004$) and OS (HR 2.49, 95% CI 1.1–5.6; $p = 0.03$). However, no significant increase in hazard ratio was seen in Trast –ve HER2+(ER−) cancers compared to the Trast +ve group in 5-year DFS (HR 2000.18, 95% CI 0.63–7.52; $p = 0.598$) or OS (HR 1.36, 95% CI 0.39–4.72 $p = 0.962$).

Effects of Trastuzumab treatment on recurrence rates

Univariate analysis of recurrence

Recurrences occurred in 52 (18.1%) of Luminal B HER2 breast cancers and 42 (23.2%) of HER2+(ER−) breast cancers overall. A significant reduction in recurrence rates in Trast +ve patients was observed in both Luminal B HER2 (38.3 vs. 8.5%, $p < 0.001$) and in HER2+(ER−) (36.7 vs. 18.3%, $p = 0.009$) (Table 2). Luminal B HER2 cancers displayed a significant reduction in LRR (16 vs. 1.8%, $p < 0.001$); however, there was no significant reduction in HER2+(ER−) breast cancers (16.7 vs. 10.6%, $p = 0.261$). Trastuzumab treatment induced a significant reduction in distant metastasis rates in both subtypes, with a greater reduction observed in Luminal B HER2 (36.2 vs. 6.7%, $p < 0.001$) compared to HER2+(ER−) (31.7 vs. 12.5%, $p = 0.03$).

Table 1 Demographics

	Luminal B HER2 ($n = 287$) *N* (%)	HER2+(ER−) ($n = 181$) *N* (%)	*p* Value
Age: mean, Years±SD	64.62 ± SD	62.83 ± SD	0.160
Age category: *N* (%)	14.81	12.33	0.602
0–50	53 (18.5)	30 (16.6)	
50+	234 (81.5)	151(83.4)	
Grade: *N* (%)			**<0.001**
1,2	137 (50.2)	34 (20.7)	
3	136 (49.8)	130 (79.3)	
NA	14	17	
TNM Stage: *N* (%)			0.623
0	14 (4.9)	15 (8.2)	
1	55 (21.8)	40(26.3)	
2	103(40.9)	53 (34.9)	
3	68 (27)	43 (28.3)	
4	26 (10.3)	16 (10.5)	
NA	21	14	
Surgery: *N* (%)			0.062
Mastectomy	123 (50.8)	85(60.7)	
Wide local excision	119 (49.2)	55(39.3)	
NA	45	41	
Radiotherapy: *N* (%)			0.338
No	56 (22.1)	41 (26.3)	
Yes	197 (77.9)	115 (73.7)	
NA	34	25	
Adjuvant chemotherapy: *N* (%)			0.891
No	97 (36.2)	59 (35.5)	
Yes	171 (63.8)	107 (64.5)	
NA	19	15	
Neo-adjuvant chemotherapy: *N* (%)			0.387
No	195 (78.3)	105 (74.5)	
Yes	54 (21.7)	36 (25.5)	
NA	38	40	
Trastuzumab: *N* (%)			0.844
No	100 (35.2)	65 (36.1)	
Yes	184 (64.8)	115 (3.9)	
NA	3	1	
Neo-adjuvant Trastuzumab	38 (13.2)	32 (17.7)	0.261
pCR	10 (26.3)	13 (40.6)	0.368
Total	52 (18.1)	42 (23.2)	0.181
LRR	18 (6.3)	22 (12.2)	**0.027**
Distant	47 (16.4)	32 (17.7)	0.714

Bold values indicate significant *p* value

Analysis of the effects of Trastuzumab treatment on distant metastasis by site of recurrence and subtype was performed (Table 3). For Trast –ve patients, bone was the most common site of metastasis for Luminal B HER2 cancers, while lung was the most common site in HER2+(ER−). Following Trastuzumab treatment in the Luminal B HER2

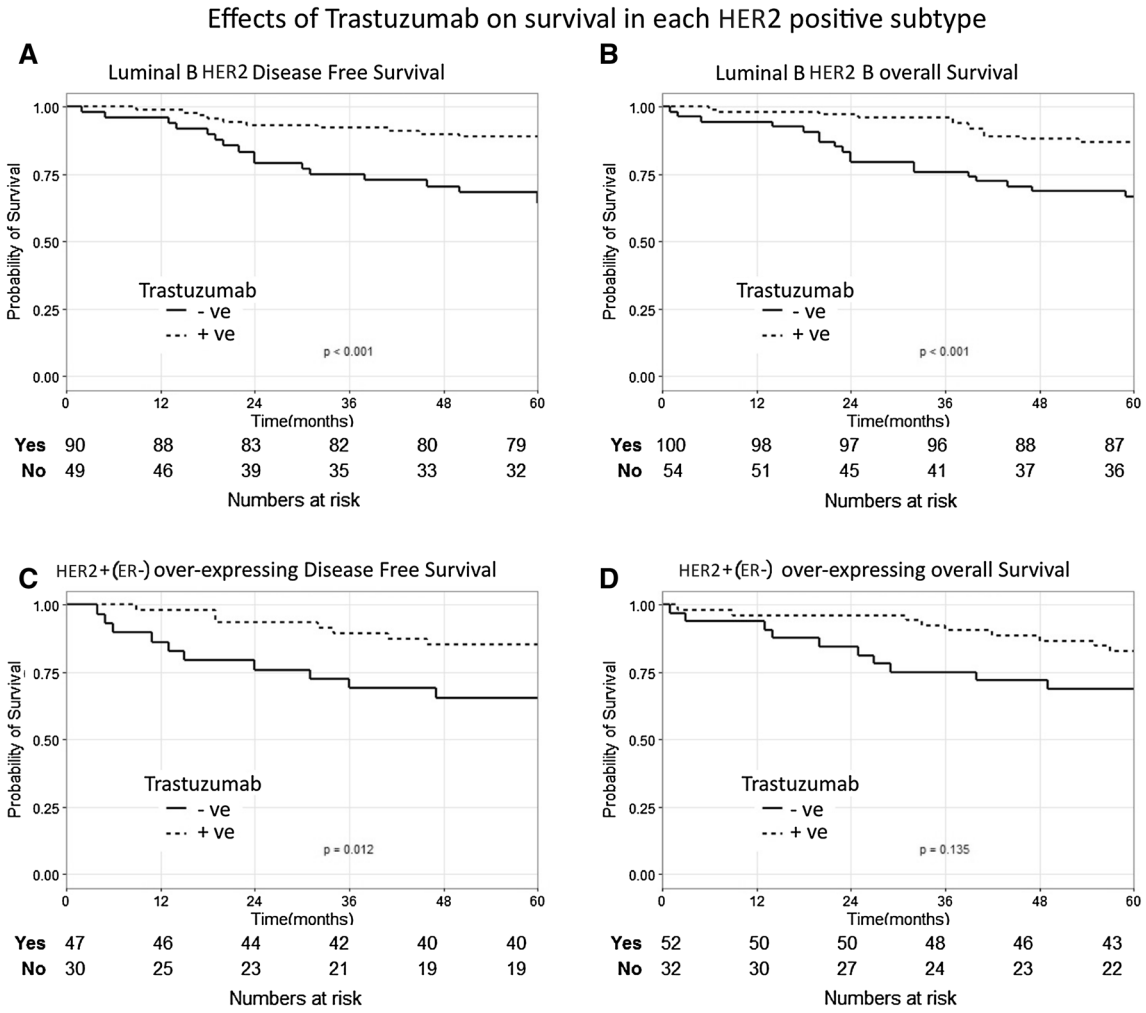


Fig. 1 Kaplan–Meier curves of individual HER2 receptor-positive breast cancer subtypes. **a**, **b** Luminal B HER2 DFS and OS (respectively). **c**, **d** HER2+(ER−) DFS and OS (respectively). *DFS* Disease-Free Survival, *OS* Overall survival, Trast +ve: Patients treated with Trastuzumab, Trats −ve +: Patients who did not receive Trastuzumab

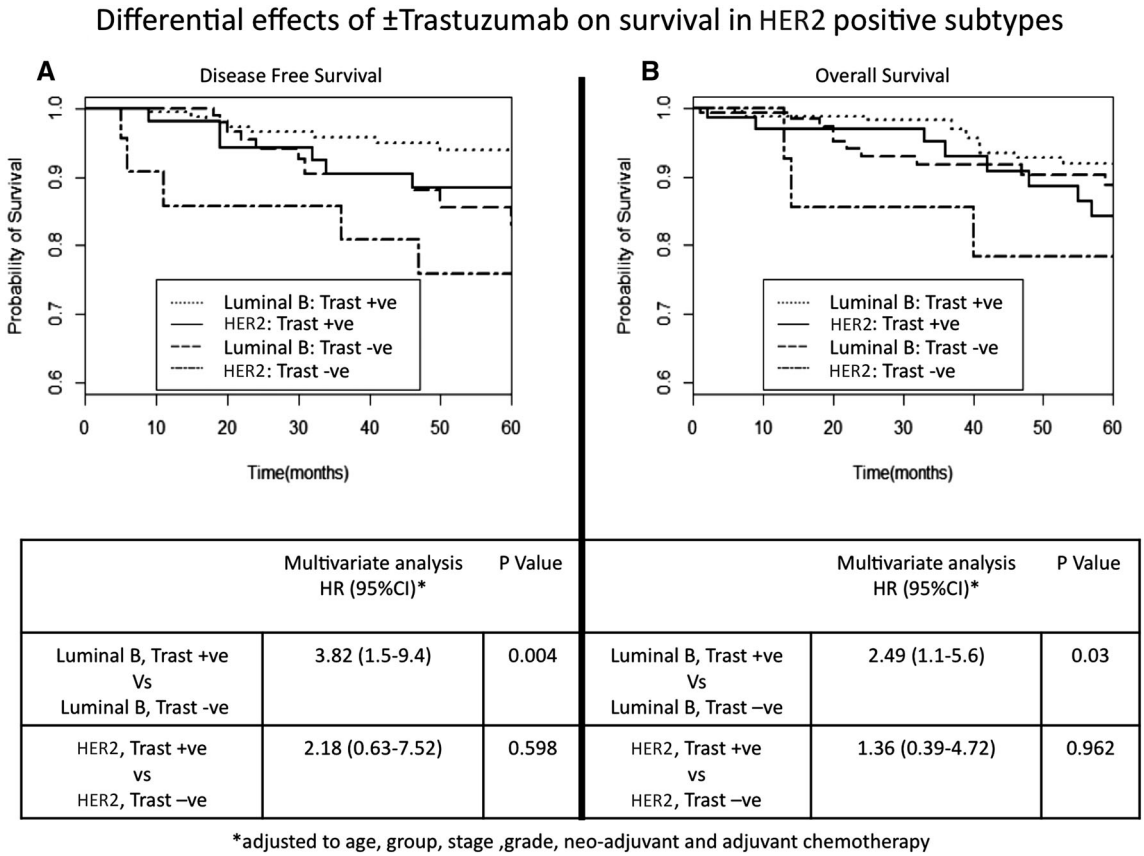
cancers, a significant reduction was seen for all distant sites of metastasis (except brain). The site with the greatest reduction in metastasis, due to Trastuzumab treatment, was in bone (22.9 vs. 3.8%, $p < 0.001$). HER2+(ER−) breast cancers did show decreases in all metastatic sites except for brain in Trast +ve patients; however, these reductions only approached significance in bone ($p = 0.075$), lung ($p = 0.086$) and liver ($p = 0.075$).

Multivariate analysis of recurrence

Performing a multivariate analysis of recurrence risk by treatment, metastatic site and subtype (Table 4), no difference was seen for LRR between Luminal B HER2 and HER2+(ER−) cancer in Trast –ve patients (OR 1.39, 95% CI 0.47–4.5: $p = 0.557$). Importantly in Trast +ve patients, a significantly lower odds ratio for LRR was seen in Luminal B HER2 cancers compared to HER2+(ER−) (OR 0.13, 95% CI 0.02–0.59; $p = 0.018$). Distant metastasis rates to bone were the only significant difference between the two subtypes, in the no Trastuzumab group (OR 4.63, 95% CI 1.53–17.5; $p = 0.012$). Following Trastuzumab treatment, no difference was seen between subtypes in bone metastasis (OR 0.958, 95% CI 0.17–5.60; $p = 0.96$) but a significantly lower risk in brain metastasis is seen in the Luminal B HER2 cancers (OR 0.19, 95% CI 0.03–0.85; $p = 0.041$).

Discussion

The impact of Trastuzumab treatment has been well established, greatly improving survival and significantly reducing recurrence in HER2 receptor-positive breast cancers [15, 21, 22]. It has also been shown to improve pathological complete response rates, and in our study pCR



	Multivariate analysis HR (95%CI)*	P Value		Multivariate analysis HR (95%CI)*	P Value
Luminal B, Trast +ve Vs Luminal B, Trast -ve	3.82 (1.5-9.4)	0.004	Luminal B, Trast +ve Vs Luminal B, Trast –ve	2.49 (1.1-5.6)	0.03
HER2, Trast +ve vs HER2, Trast –ve	2.18 (0.63-7.52)	0.598	HER2, Trast +ve vs HER2, Trast –ve	1.36 (0.39-4.72)	0.962

*adjusted to age, group, stage ,grade, neo-adjuvant and adjuvant chemotherapy

Fig. 2 Cox proportional analysis of DFS and OS for patients treated with Trastuzumab. **a** Comparing DFS of Luminal B HER2 to HER2+(ER−) subtypes, Yes and No Trastuzumab treatment. **b** Comparing OS of Luminal B HER2 to HER2+(ER−) subtypes, Yes and No Trastuzumab treatment. *DFS* Disease-Free Survival, *OS* Overall survival, Trast +ve: Patients treated with Trastuzumab, Trats −ve +: Patients who did not receive Trastuzumab

Table 2 Recurrence rates (stage I–III breast cancers)

	Trastuzumab	Luminal B ($n = 259$) *N* (%)	HER2 ($n = 164$) *N* (%)
Total Recurrence: *n* (%)	No	36/94 (38.3)	22/60 (36.7)
	Yes	14/165 (8.5)	19/104 (18.3)
		$p < 0.001$	**$p = 0.009$**
LRR: *n* (%)	No	15/94 (16)	10/60 (16.7)
	Yes	3/165 (1.8)	11/104 (10.6)
		$p < 0.001$	$p = 0.261$
Distant: *n* (%)	No	34/94(36.2)	19/60 (31.7)
	Yes	11/165(6.7)	13/104 (12.5)
		$p < 0.001$	**$p = 0.003$**

Bold values indicate significant *p* value

LRR Local Regional Recurrence, *Distant* distant metastasis

was associated with an improved DFS. A pooled analysis of 11,955 patients in neo-adjuvant chemotherapy trials found that, overall, patients achieving pCR had a higher level DFS than those with residual cancer [23]. However, few studies have investigated the differential effects of Trastuzumab treatment on Luminal B HER2 and HER2+(ER−) breast cancers. Our analysis revealed that in the Trastuzumab era, Luminal B HER2 cancers specifically had a greater improvement in overall survival. Interestingly, while Trastuzumab treatment resulted in a significant reduction in LRR rates for Luminal B HER2 cancers, only a modest improvement was seen for the HER2+(ER−) subtype. In both subtypes, Trastuzumab treatment resulted in significant reductions in overall rates of distant

Table 3 Distant metastasis rates

Metastasis site	Luminal B		*p* Value	HER2		*p* Value
	No Trastuzumab $N = 86$	Trastuzumab $N = 173$		No Trastuzumab $N = 60$	Trastuzumab $N = 104$	
Bone: *n* (%)	24 (25.5)	5 (3)	**<0.001**	6 (10)	3 (2.1)	0.075
Brain: *n* (%)	4 (4.3)	4 (2.4)	0.466	5 (8.3)	9(8.7)	0.999
Lung: *n* (%)	20 (21.3)	3 (1.8)	**<0.001**	9 (15)	7 (6.7)	0.086
Liver: *n* (%)	13 (13.8)	7 (4.2)	**0.005**	6 (10)	3 (2.9)	0.075

Bold values indicate significant *p* value

Table 4 Logistic regression analysis (site of recurrence)

	Unadjusted OR# No Trastuzumab Luminal B versus HER2	*p* Value	Adjusted OR# No Trastuzumab* Luminal B versus HER2	*p* Value	Unadjusted OR# Trastuzumab Luminal B versus HER2	*p* Value	Adjusted OR# Trastuzumab* Luminal B versus HER2	*p* Value
LRR	0.95 (0.399,2.338)	0.907	1.39 (0.471,4.50)	0.557	0.16 (0.04,0.51)	**<0.001**	0.13 (0.02,0.59)	**0.018**
Distant	1.22 (0.618,2.459)	0.566	1.21 (0.508,2.95)	0.660	0.50 (0.211,1.16)	0.107	0.51 (0.17,1.44)	0.214
Bone	3.09 (1.246,8.805)	**0.022**	4.63 (1.53,17.50)	**0.012**	1.05 (0.253,5.22)	0.945	0.958 (0.17,5.60)	0.960
Brain	0.49 (0.117,1.923)	0.301	0.41 (0.09,1.76)	0.231	0.26 (0.07,0.829)	**0.029**	0.19 (0.03,0.85)	**0.041**
Lung	1.53 (0.661,3.786)	0.333	2.15 (0.78,6.688)	0.161	0.26 (0.05,0.946)	0.05	0.36 (0.06,1.57)	0.198
Liver	1.44 (0.536,4.324)	0.483	1.03 (0.33,3.39)	0.958	1.49 (0.405,7.04)	0.569	1.03 (0.22,5.64)	0.971

Bold values indicate significant *p* value

95% CI

* Adjusted to stage and grade

LRR Local Regional Recurrence, *DFS* Disease-Free Survival, *OS* Overall survival

metastasis at all sites, except for brain metastases. Furthermore, a greater reduction was seen in Luminal B HER2 breast cancers, with the largest reduction in overall metastasis rates seen in the bone metastasis.

Few previous studies have compared survival or recurrence rates between the two HER2 receptor-positive breast cancers since the introduction of Trastuzumab. Romond et al. compared survival between the two HER2 receptor-positive breast cancers and found at 4-year follow-up that hormone receptor status minimally influenced the response to Trastuzumab, although hormone receptor status was reported as a significant predictor in DFS [15]. Our findings support previous findings where Trastuzumab treatment led to a reduction in LRR only in Luminal B HER2 cancers [24]. We demonstrate a statically significant reduction in LRR rates observed in Luminal B HER2 patients, while no significant difference was seen in the HER2+(ER−) subtype.

Previous studies demonstrated that bone was the most common recurrence site in Luminal B HER2 breast cancers and lung was most common for HER2+(ER−) [8, 9]. We show Trastuzumab treatment resulted in a reduction in metastasis to all sites, except the brain which is explained by the fact that Trastuzumab does not cross the blood/brain barrier [25, 26]. Surprisingly, Luminal B HER2 cancers treated with Trastuzumab showed the greatest reduction in distant metastasis to the bone. While Trastuzumab treatment did not lead to significant variations in brain

metastasis rates, it did result in the Luminal B HER2 cancers having a significantly reduced odds ratio of brain metastasis. This correlates with previous studies which show a higher brain metastasis rates in hormone receptor-negative tumours [26].

A potential reason for variation between subtypes could be that HER2 receptor over expression reduces the response to hormone therapy. Studies have observed increased hormone resistance rates in Luminal B HER2 cancers compared to Luminal A cancers [27, 28]. Cross talk between HER2 receptors and hormone receptors results in activation of the hormone receptor, even in the presence of hormone treatment [29]. Clinical trials have shown that the addition of Trastuzumab to hormone treatment improves survival in metastatic breast cancer [30]. In our study, the introduction of Trastuzumab significantly reduced distant metastasis, especially in bone metastasis rates. By reducing the activity of HER2 receptors, Trastuzumab may restore the response to hormone therapy in Luminal B HER2 cancers [31]. In the neo-adjuvant setting, this may provide an explanation as to why lower levels of pCR are seen in Luminal B HER2 cancers compared to HER2+(ER−) cancers [23].

Another potential difference between subtypes could be the increased number of grade 3 cancers seen in the HER2+(ER−) group. It has been shown that both HER2+(ER−) and triple-negative breast cancers have worse outcomes and present with higher-grade cancer than Luminal cancers [6]. Although, in a pooled analysis of neo-adjuvant Trastuzumab trials, grade 3 cancers had a higher pCR rate than grade 1 and 2 cancers [23]. Importantly, our study revealed that on multivariate analysis of patients treated with Trastuzumab, grade 3 cancers did not have a lower DFS or OS risk when compared to grade 1 and 2 breast cancers.

In patients receiving neo-adjuvant Trastuzumab chemotherapy, around 40% of patients will have a complete pathological response [20, 32]. This shows that a large proportion of patients only have a partial or no response to Trastuzumab treatment. This resulted in the development of new anti-HER2 receptor treatments targeting different pathways such as Pertuzumab, which has been shown to increase response [33]. Our study highlights the improved prognosis associated with anti-HER2 receptor therapy; it also demonstrates that a large proportion of patients survived despite not being treated with Trastuzumab. We believe this clearly indicates that Trastuzumab treatment is not required for all HER2 receptor-positive breast cancers. There is a clear need to develop a molecular or genetic scoring system to identify which patients will benefit from Trastuzumab treatment and those that will not.

This study once again shows the benefit of Trastuzumab treatment in HER2 receptor-positive breast cancers, demonstrating the effects on both survival and recurrence rates. It also highlights how a targeted therapy has altered responses in related breast cancer subtypes, emphasising their molecular differences. Demonstrating a more positive impact of Trastuzumab treatment on Luminal B HER2 cancers supports the need to further characterise the mechanism of action of Trastuzumab in each subtype, suggesting that key differences remain to be defined. This work highlights the need to fully understand the subtype-specific effects and mechanisms of action of Trastuzumab therapy, which will allow truly individualised breast cancer management regimes to be implemented.

Funding This work is supported by funding from Breast Cancer Research.

Author contributions AM, JALB and MJK performed data analysis and wrote the manuscript; AM, OK and EH performed the statistical analysis; CM, RM, AL, CC and MJK participated clinically in patient data provision and in manuscript preparation.

Compliance with ethical standards

Conflict of interest The authors declare that there are not conflicts of interest.

Competing interests The authors declare that they have no competing interests.

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Ann Surg Oncol (2017) 24:3124–3132
DOI 10.1245/s10434-017-6021-1

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – BREAST ONCOLOGY

Locoregional Recurrence Following Breast Cancer Surgery in the Trastuzumab Era: A Systematic Review by Subtype

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ABSTRACT

Introduction. Increasing evidence suggests that molecular subtype influences locoregional recurrence (LRR) of breast cancer. Previous systematic reviews that evaluated the quantitative influence of subtype on LRR predated the use of Trastuzumab. This study assessed the impact of subtype on LRR in a contemporary treatment era.

Methods. A comprehensive search for all published studies assessing LRR according to breast cancer subtype was performed. Only studies with patients treated with Trastuzumab were included. Relevant data were extracted from each study for systematic review. Primary outcome was LRR related to breast cancer subtype.

Results. In total, 11,219 patients were identified from seven studies. Overall LRR rate was 3.44%. The lowest LRR rates were in luminal A (1.7%), and the highest rates were in triple-negative (7.4%) subtypes. There were significantly lower risks of LRR in patients with luminal A subtype compared with luminal B [odds ratio (OR) 0.54, 95% confidence interval (CI) 0.38–0.76; $p < 0.0004$], HER2/neu-overexpressing (OR 0.32, 95% CI 0.24–0.45; $p < 0.0001$) and triple-negative breast cancers (OR 0.25, 95% CI 0.19–0.32; $p < 0.0001$). There were significant differences in LRR between the luminal B and HER2/neu-overexpressing breast cancers (OR 0.61, 95% CI 0.41–0.89; $p = 0.0145$). The reduced risk in HER2/neu overexpressing compared with triple-negative breast cancers approached statistical significance (OR 0.75, 95% CI 0.55–1.03; $p = 0.0933$).

Conclusions. Significant variations in LRR occur across breast cancer subtypes, with lowest rates in luminal cancers and highest rates in triple-negative breast cancers. Low levels of LRR highlight advances in breast cancer management in the contemporary era.

Breast cancer is a heterogeneous disease, with varying survival and outcomes. Through molecular profiling, breast cancer can be subdivided into different subtypes.[1,2] This has resulted in more individualized treatment of breast cancer, with targeted therapy emerging for the varying subtypes resulting in improved survival.[3–5]

There remains, however, a proportion of patients who will have a recurrence of their disease despite optimum treatment.[6] The prevention of locoregional recurrence (LRR) is important, as one breast cancer death can be prevented over the next 15 years for every four local recurrences avoided.[7] Our institute has previously undertaken a systematic review on LRR rates and found LRR varies across breast cancer subtypes.[8] In this study, surprisingly the highest LRR rates occurred in HER2/neu-overexpressing tumours. At the time the studies analyzed were conducted, a targeted therapy for HER2+ receptor cancers was being introduced called Trastuzumab. Trastuzumab is monoclonal antibody that binds to the HER2 receptor, reducing cell signaling and resulting in immune system activation causing increased antibody-dependent cytotoxicity.[9] It was first used for metastatic breast cancer, where it was shown to prolong survival.[10] Later studies found that adjuvant treatment with Trastuzumab could reduce risk of mortality by up to 39%.[5,11,12] Recently,

Electronic supplementary material The online version of this article (doi:10.1245/s10434-017-6021-1) contains supplementary material, which is available to authorized users.

First Received: 15 February 2017;
Published Online: 28 July 2017

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neoadjuvant chemotherapy studies have shown that the addition of Trastuzumab increases pathological complete response rates.[13–15] However, despite increased survival and improved outcomes, 8.2% of patients will still have a breast cancer recurrence.[16]

To date, the impact of Trastuzumab on LRR rates has yet to be compared across breast cancer subtypes in a systematic review. The purpose of this study was to compare the LRR rates across the different breast cancer subtypes in the Trastuzumab era.

METHODS

An electronic-based search was performed of Medline using the following search algorithms: (1) "Breast cancer" AND ("breast conserving surgery" OR "breast conservation" OR "wide local excision" OR "WLE" OR "lumpectomy" OR "quadrantectomy") AND ("recurrence" OR "outcome"); and (2) "Breast cancer" AND ("mastectomy" OR "modified radical mastectomy") AND ("recurrence" OR "outcome"). English was chosen as a language restriction. Only publications after 2006 were included unless treatment with Trastuzumab was specifically mentioned. All abstracts and full texts were independently examined by two authors (A.M. and A.L.) for determination of eligibility.

Retrospective and prospective studies were included which reported LRR in operable breast cancer patients. Only studies with subtype-specific LRR rates were included. Studies failing to report rates in all subtypes were excluded. Treatment with adjuvant Trastuzumab was a further inclusion criterion, unless all included patients were treated after 2006 when Trastuzumab would have been part of standard treatment regime. Any study that had less than 50% of the HER2+ receptor patients treated with Trastuzumab was excluded. A cutoff point of 50% was chosen as it resulted in a majority of HER2+ receptor patients being treated with Trastuzumab.

From the eligible studies, authors, country of origin, year of publication, journal, and surgery type were extracted. Treatment regimes where accessible, such as chemotherapy, radiotherapy, and hormone therapy, were recorded. Different criteria for subtype were used across the different studies, with the Ki-67 not reported in a number of studies. Due to this, the four traditional subtypes based on hormone receptor and HER2 receptor status were used (Supplement Table 1): luminal A (ER/PR+, HER2−), luminal B (ER/PR+, HER2+), HER2/neu overexpressing (ER/PR−, HER2+), and triple-negative (ER/PR−, HER2−).[2] The primary outcome was LRR defined as recurrence in the ipsilateral breast or chest wall or ipsilateral draining lymph nodes.

To investigate the effect of breast-conserving therapy (BCT), i.e., breast-conserving surgery plus whole breast radiotherapy on LRR, a subgroup analysis was performed. A further subset analysis was performed to compare the luminal cancers (luminal A and luminal B) to HER2/neu-overexpressing and triple-negative breast cancers. This was done to compare results from a previous study from this institute.

RESULTS

In total, 95 eligible studies were identified that reported LRR rates by breast cancer subtype. From these studies, 77 were excluded, because patients in the studies were not treated with Trastuzumab. Eight were excluded, because the LRR rates for all four subtypes could not be extracted. Three were excluded, because less than half of the HER2+ receptor patients were treated with Trastuzumab. Therefore, seven studies were included in the final analysis (Fig. 1).

In these seven studies, data were extracted for 11,219 patients (Table 1).[17–23] Radiotherapy was given to all patients who underwent BCT. Radiotherapy rates after mastectomy could not be extracted across all the studies. Hormone receptor-positive patients received hormone therapy as per standard guidelines. Chemotherapy was given to 82.1% of patients; 8.6% received neoadjuvant chemotherapy. The median follow-up was 53 months (range 44–84).

There were 6540 (58.3%) luminal A, 1469 (13.1%) luminal B, 1040 (9.3%) HER2/neu-overexpressing, and 2170 (19.3%) triple-negative breast cancers. The total LRR rate was 3.44% across all studies; in patients who were treated with BCT, this was lower at 2.8%. The lowest rates of recurrence were in luminal A cancers 1.7%. Luminal B had 3.3%, HER2/neu-overexpressing rates were 5.7%, and the highest rates were in triple-negative cancers at 7.4%.

First, we analysed the overall data results (Figs. 2, 3). There was a significantly lower risk of LRR in patients with luminal A subtype of breast cancer compared with luminal B [odds ratio (OR) 0.54, 95% confidence interval (CI) 0.38–0.76; $p < 0.0004$], HER2/neu-overexpressing (OR 0.32, 95% CI 0.24–0.45; $p < 0.0001$), and triple-negative breast cancers (OR 0.25, 95% CI 0.19–0.32; $p < 0.0001$; Fig. 2a–c). When comparing the luminal B cancers to the HER2/neu-overexpressing and triple-negative cancers, a significantly lower risk of LRR is observed (OR 0.61, 95% CI 0.41–0.89; $p = 0.0145$) and (OR 0.47, 95% CI 0.33–0.65; $p < 0.0001$), respectively (Fig. 3a, b). There also was a trend towards a significantly lower risk in the HER2/neu-overexpressing compared with triple-negative

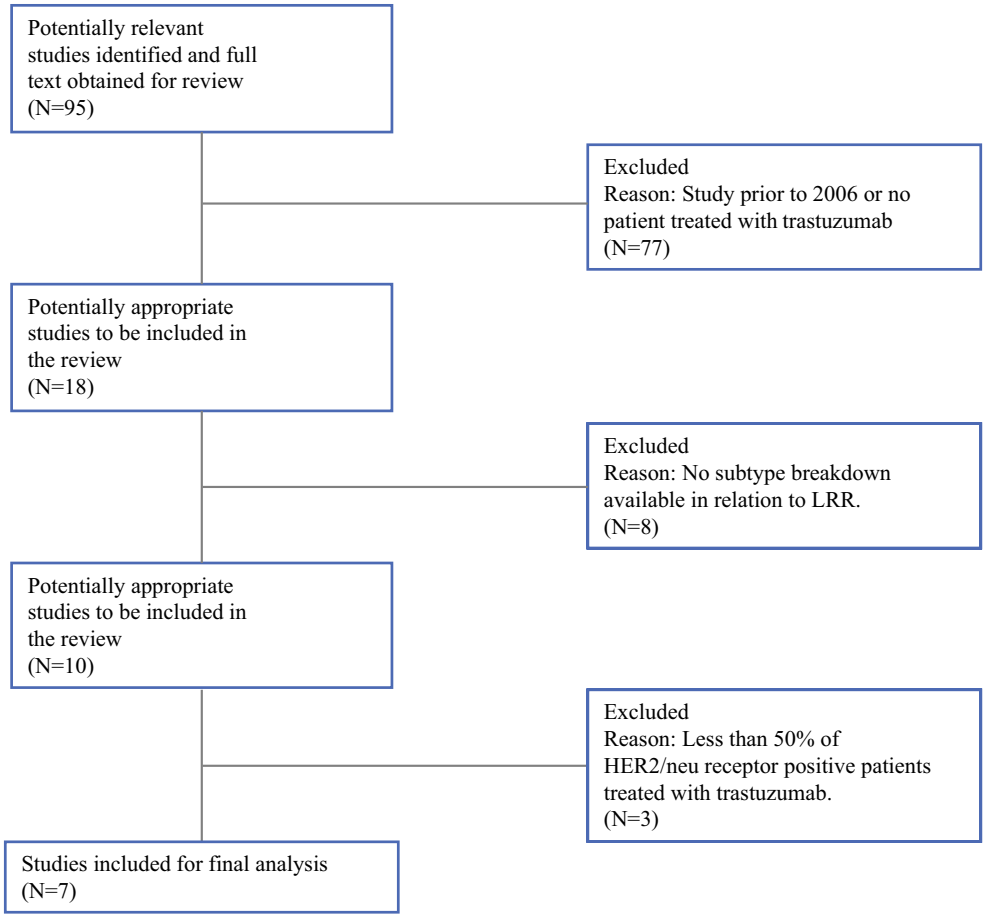

FIG. 1 Eligible studies; quality of reporting of meta-analyses (QUOROM) statement flow diagram

TABLE 1 Details of eligible studies

Authors references	Country of origin	Years	Study duration	No. of patients analysed	Luminal A	Luminal B	HER2/neu	Triple negative	Follow up (months)
Mazouni et al.[17]	France	2013	2004–2010	791	631	50	22	88	60
Yamazaki et al.[18]	Japan	2015	2002–2011	217	96	43	27	51	84
Mersin et al.[19]	Turkey	2011	2004–2008	1101	667	246	82	106	44
Kim et al.[20]	US	2012	2000–2008	5683	3218	636	549	1280	52
Shim et al.[21]	South Korea	2014	2008–2012	1244	699	227	145	173	48
Lee et al.[22]	South Korea	2015	2003–2011	1432	860	162	157	253	53
Swisher et al.[23]	US	2015	2005–2012	751	369	105	58	219	75.6

breast cancers (OR 0.75, 95% CI 0.55–1.03; $p = 0.0933$; Fig. 3c).

In total, three studies with 1759 patients included [Mazouni et al. ($n = 791$), Yamazaki et al. ($n = 217$), and Swisher et al. ($n = 751$)] were suitable for subgroup analysis for LRR in patients undergoing BCT (Fig. 4). A similar trend in LRR rates are observed in comparison to the combined data with the lowest rates in luminal A (1.3%), luminal B rates (2.5%), HER2/neu-overexpressing (5.6%), and the highest rates were in triple-negative breast cancers (6.4%). Interestingly, lower rates of LRR are seen in all four subtypes compared with overall results. No significant difference is seen between luminal A cancers and luminal B cancers (OR 0.77, 95% CI 0.28–2.09; $p = 0.8123$) or HER2/neu-overexpressing (OR 0.37, 95% CI 0.14–0.97; $p = 0.0794$; Fig. 4a, b). Only triple-negative breast cancers showed a significantly higher risk of LRR compared with luminal A cancers (OR 0.31, 95% CI

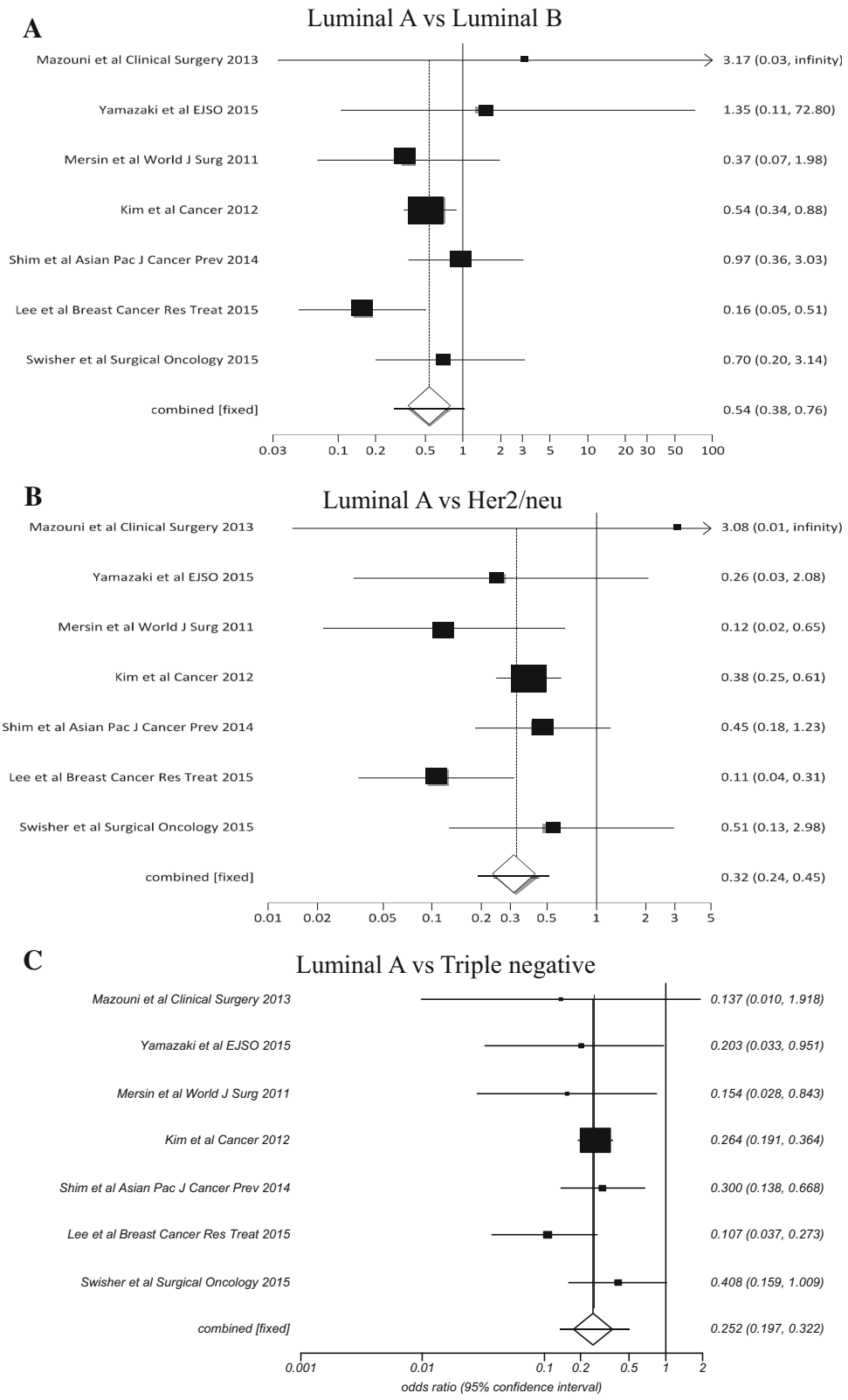

FIG. 2 Forest plots comparing LRR rates between the luminal A with the other breast cancer subtypes overall. In each *panel*, each study is shown by the point estimate of the odds ratio (OR) and 95% CI for the OR (*extending lines*); the combined OR and 95% CI by random effects calculations are shown by *diamonds*

0.16–0.6; $p = 0.0007$; Fig. 4c). There also was no significant difference between luminal B cancers and either HER2/neu-overexpressing (OR 0.46, 95% CI 0.13–1.56; $p = 0.349$) or triple-negative cancers (OR 0.46, 95% CI 0.13–1.56; $p = 0.349$; Fig. 4d, e). Once again, no difference was seen between HER2/neu-overexpressing breast

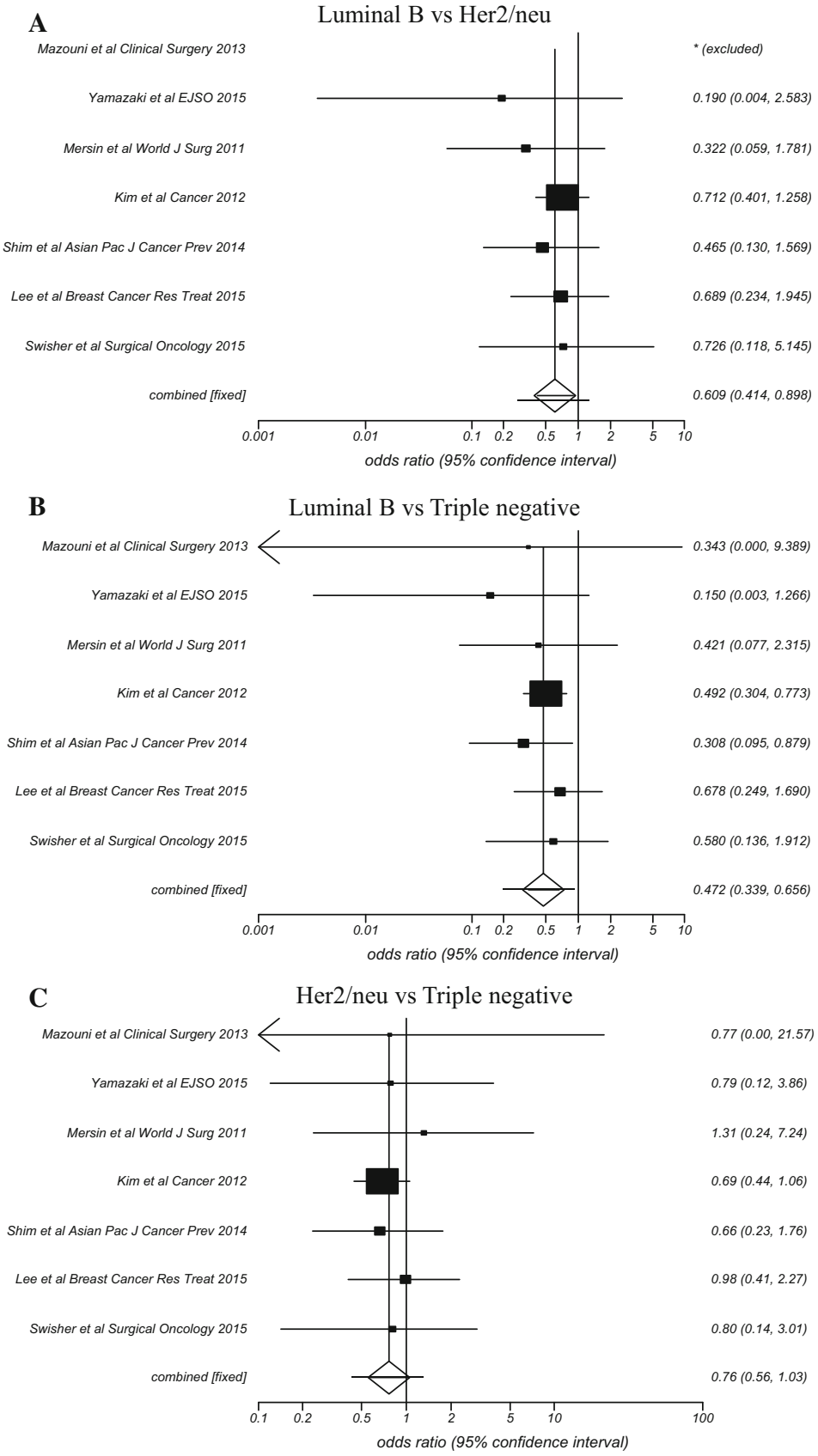

FIG. 3 Forest plots comparing LRR rates between the non-luminal A breast cancer subtypes overall. In each *panel*, each study is shown by the point estimate of the odds ratio (OR) and 95% CI for the OR (*extending lines*); the combined OR and 95% CI by random effects calculations are shown by *diamonds*

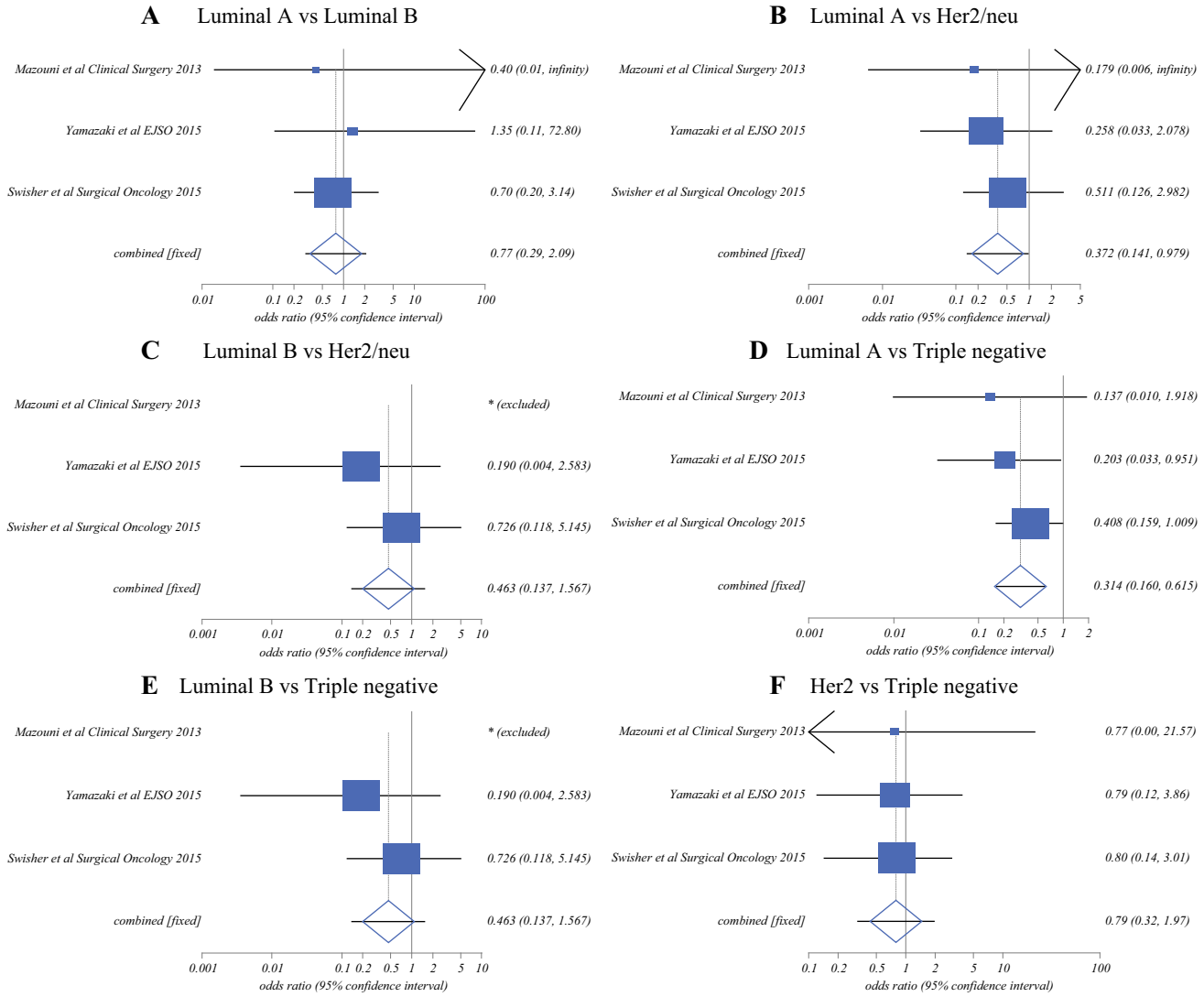

FIG. 4 Forest plots comparing LRR rates between the different breast cancer subtypes in the breast conservative therapy cohort. In each *panel*, each study is shown by the point estimate of the odds ratio (OR) and 95% CI for the OR (*extending lines*); the combined OR and 95% CI by random effects calculations are shown by *diamonds*

cancers and triple-negative (OR 0.79, 95% CI 0.31–1.97; $p = 0.7785$; Fig. 4f).

Next, we sought to compare luminal cancers to nonluminal cancers (Supplemental Fig. 1). In the combined group, luminal cancers had significantly reduced LRR rates compared with HER2/neu-overexpressing (OR 0.37, 95% CI 0.27–0.50; $p < 0.0001$; Fig. S1A) and triple-negative breast cancers (OR 0.29, 95% CI 0.23–0.36; $p < 0.0001$; Fig. S1B). In the BCT subgroup, no significant difference is seen between the luminal cancers and the HER2/neu-overexpressing cancers (OR 0.38, 95% CI 0.15–0.96; $p = 0.0719$; Fig. S1C). However, a significantly lower LRR rate is seen when compared to the triple-negative (OR 0.33, 95% CI 0.17–0.61; $p = 0.0004$; Fig. S1D).

DISCUSSION

Previous work by this group has shown significant variations in LRR rates across breast cancer subtypes.[8] This review was undertaken before the routine availability of Trastuzumab, with less than 6% of HER2+ receptor patients in the study receiving Trastuzumab. The introduction of Trastuzumab treatment has been shown to improve survival significantly in HER2+ receptor breast cancers. However, the impact of Trastuzumab introduction on LRR across the different breast cancer subtypes has yet to be explored. In this systematic review of 11,219 patients, the impact of the introduction of the targeted therapy Trastuzumab on LRR is demonstrated.

When comparing results with the previous systematic review, a reduction in LRR is seen across all subtypes.[8] The overall LRR rates decreased from 7.9 to 3.44%, with LRR rates post BCT reducing from 7.12 to 2.8%. The reduction in LRR across the two studies highlights the effect of multiple changes in breast cancer management over the past two decades. An early study reporting LRR rates from 1986 to 1992 reported overall rates as high as 18.5% and rates post BCS at 16%,[24] whereas an overview of all randomized radiotherapy trials beginning before 1995 found that the 5-year risk LRR post BCT was 7%.[7] Improvement in imaging, earlier diagnosis, surgical planning, and adjuvant therapy has resulted in significant improvement in survival and outcomes for breast cancer patients. This reduction in LRR is seen across all the breast cancer subtypes, with the greatest reduction in HER2/neu-overexpressing breast cancer post BCT: rates reduced from 15.7 to 5.6%. The highest rates of LRR are seen in triple-negative breast cancers both in the overall analysis and post BCT. Post BCT there is no longer any significant difference seen between luminal B and HER2/neu-overexpressing subtypes, although a significant difference is seen overall.

In our study, luminal A breast cancers had the lowest rate of LRR both overall and post BCT compared with the other three subtypes. The introduction of hormone therapy has resulted in a significant reduction in recurrence rates in luminal breast cancers.[25,26] Surprisingly, in the overall results despite the introduction of Trastuzumab, there was still a significant difference in LRR between luminal A and luminal B breast cancers. This could be explained by the large proportion of luminal B patients that have only a partial or no response to Trastuzumab therapy. Neoadjuvant Trastuzumab trials found that only 30.9% of luminal B breast cancer had a complete pathological response.[27]

Multiple studies have examined the effect of Trastuzumab on LRR rates in HER2+ receptor breast cancers.[11,13,28–30] Reduced LRR are seen across all these studies, but the rate of reduction varies from 1 to 7%. One study reported approximately a 40% reduction in the number of LRR cases, but this only resulted in a 1.8% reduction in LRR overall.[11] Only Keiss et al. showed a significant reduction in LRR rates after the introduction of Trastuzumab. The largest study with more than 3000 patients treated with 1 year of adjuvant Trastuzumab showed little improvement in LRR, with rates reducing only from 4 to 3%.[30] However, in a study that compared reduction in LRR in both of the HER2+ receptor subtypes, a difference is observed between these subtypes.[20] In luminal B cancers, LRR rates dropped from 6 to 3% following the introduction of Trastuzumab, whereas HER2/neu-overexpressing breast cancers had the same rate of 6% before and after treatment. This matches the result from this systematic review, where a significantly lower risk of LRR is seen in luminal B breast cancers compared with HER2/neu-overexpressing.

Triple-negative breast cancers had the highest rates of LRR in both the combined results and the BCT subgroup overall. Triple-negative breast cancers are known to be aggressive and to have the lowest survival rates of the breast cancer subtypes. This is highlighted in neoadjuvant chemotherapy studies, where it was found that despite triple-negative breast cancers having the highest pathological complete response rate, these cancers have the poorest survival rates.[31,32] This is due to the aggressive nature of these cancers, whereby the patients who are not responding to treatment have poorer outcomes. Triple-negative breast cancers also have been linked to genetically inherited cancers, such as BRCA 1 and 2, which are known to be more aggressive and tend to occur at an earlier age.[33,34]

Our analysis of luminal versus nonluminal cancers has highlighted that there are significantly higher LRR rates in nonluminal breast cancers, indicating a possible need for more aggressive local therapy. Multiple randomized, controlled trials have shown equivocal survival in stages 1 and 2 breast cancer patients undergoing BCT as with mastectomy.[35,36] Furthermore, for stages 1 and 2 breast cancers undergoing BCT, wider margins are not indicated based on tumour subtype under current recommendations.[37] This is based on a number of studies, which showed lower LRR rates in patients undergoing BCT compared with mastectomy in triple-negative breast cancers.[38,39] Comparing the results of our subset analysis of patients who had BCT, lower LRR rates are seen in all subtypes compared with the combined results. This further highlights the safety of BCT.

Although the benefit of radiotherapy as a component of BCT in reducing LRR has been shown, the use of postmastectomy radiotherapy (PMRT) remains a topic of debate. Significant improvement in LRR for high-risk patients having PMRT are seen in long-term survival studies.[40,41] However, the use of PMRT in intermediate-risk patients is still unclear, with studies showing varying benefit.[42,43] Current guidelines for patients with one to three positive lymph nodes recommend patients undergo PMRT.[43] In our study, the significant variation in LRR between luminal and nonluminal subtypes may provide a way to stratify intermediate-risk patients that would benefit from PMRT. One study looked at LRR rates before and after the introduction of PMRT in the four subtypes.[44] In this study, whereas the greatest reduction in LRR was seen in luminal cancers, there was still more than 50% reduction in LRR in HER2/neu-overexpressing and triple-negative breast cancers. A retrospective study comparing LRR free survival between patients with triple-negative breast

cancers found that the highest rates of LRR were in post-mastectomy patients without adjuvant radiotherapy.[38] This highlights the need for a prospective study to analyze PMRT in triple-negative breast cancers to assess what cohorts may derive benefit.

There are some limitations in this review that must be acknowledged. The breast cancer subtypes were not standardized across the series and were assigned based on hormone receptor and HER2 receptor status. The use of Ki-67 to stratify further the luminal cancer subtypes may provide further information on differences in LRR between the breast cancer subtypes. A potential bias is the varied follow-up between the studies, which varied from 44 to 84 months, with LRR in luminal breast cancers tending to reoccur at a later stage. Important risk factors, such as patient age, tumour stage, and tumour grade, could not be independently assessed and further analysis accounting for these risk factors may provide a more accurate analysis. Furthermore, radiotherapy post BCT is reported as whole breast radiotherapy. Ideally, radiotherapy rates would be separated into local and regional radiotherapy. Finally, LRR after different surgery types was not reported in four of the studies, and only one study (Mersin et al.) reported LRR rates after mastectomy. Due to this, direct analysis of LRR rates post BCT cannot be compared with post mastectomy rates.

CONCLUSIONS

Trastuzumab introduction has had an impact on LRR rates in HER2+ receptor breast cancers. Compared with previous systematic reviews, the largest reduction in LRR was seen in HER2/neu-overexpressing breast cancer patients post BCT. Despite the reductions in LRR rates in HER2+ receptor breast cancers, significant differences remain between breast cancer subtypes. Overall, this systematic review has demonstrated vast improvements in reducing LRR across all breast cancer subtypes over the past few decades. Variations in LRR rates remain between the four breast cancer subtypes; the lowest rates are seen in luminal A breast cancers, and the highest rates are found in triple-negative breast cancer. These variations also are seen in a subset analysis of patients who had BCT. The lower levels of LRR seen post BCT shows that even in the more aggressive HER2/neu-overexpressing and triple-negative breast cancers, BCT is equivocal to mastectomy in reducing LRR. Further investigation is needed to assess the benefits of PMRT in higher-risk breast cancer subtypes.

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McAnena et al. BMC Cancer (2018) 18:203
https://doi.org/10.1186/s12885-018-4101-7

BMC Cancer

RESEARCH ARTICLE Open Access

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

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Abstract

Background: Recent studies have shown that breast cancer subtype can change from the primary tumour to the recurrence. Discordance between primary and recurrent breast cancer has implications for further treatment and ultimately prognosis. The aim of the study was to determine the rate of change between primary and recurrence of breast cancer and to assess the impact of these changes on survival and potential treatment options.

Methods: Patient demographics were collected on those who underwent surgery for breast cancer between 2001 and 2014 and had a recurrence with biopsy results and pathology scoring of both the primary and recurrence.

Results: One hundred thirty two consecutive patients were included. There were 31 (23.5%) changes in subtype. Discordance occurred most frequently in luminal A breast cancer ($n = 20$), followed by triple negative ($n = 4$), luminal B ($n = 3$) and HER2 (n = 3). Patients who changed from luminal A to triple negative ($n = 18$) had a significantly worse post-recurrence survival ($p < 0.05$) with overall survival approaching significance ($p = 0.064$) compared to concordant luminal A cases ($n = 46$). Overall receptor discordance rates were: estrogen receptor 20.4% ($n = 27$), progesterone receptor 37.7% ($n = 50$) and HER2 3% (n = 4). Loss of estrogen receptor and progesterone receptor was more common than gain (21 vs. 6 ($p = 0.04$) and 44 vs. 6 ($p = 0.01$) respectively). Nine patients (6.8%) gained receptor status potentially impacting treatment options.

Conclusion: Discordance in subtype and receptor status occurs between primary and recurrent breast cancer, ultimately affecting survival and potentially impacting treatment options.

Keywords: Breast cancer, Subtype, Discordance, Post-recurrence survival, Triple negative

Background

Breast cancer is the second most common cancer worldwide and the most common cancer among women with an estimated 1.67 million women diagnosed annually, and the fifth leading cause of death from cancer overall [1]. Risk of recurrence and outcome in breast cancer have conventionally been stratified according to the tumour size, grade, nodal status and especially tumour subtype [2]. 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) [3]. Each subtype exhibits distinct prognoses, rates of recurrence and different treatment strategies [4]. 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 [5]. Traditionally, recurrent tumours have been have been assumed to be biologically similar (the

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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 [6]. This can impact prognosis with loss of receptor status associated with a poorer prognosis [7, 8]. 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 [9, 10].

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.

Methods

Case selection

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. PAS software was used to access pathology records with MOSAIQ software used to determine patient pathways and treatment.

Pathology

Analysis of all samples was performed at the Pathology Laboratory, University Hospital Galway independently by clinical pathologists. 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. The ER and PR receptor status were determined independently by clinical pathologists using immunohistochemistry [11] as per ASCO guidelines (ALLRED score > 2 or more than 1% stain positive). The HER2 receptor status was identified by Herceptest [12] as part of the routine clinical evaluation, with a score of 3+ considered positive. Any + 2 inconclusive results were confirmed using FISH testing [13] as per ASCO guidelines, with a HER2/CEP17 ratio greater than two considered amplified.

Statistical analysis

Data analysis was performed using SPSS Version 21 (SPSS Inc., Chicago, IL). 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. Statistical significance was accepted for $p < 0.05$.

Ethics, consent and permissions

This study was conducted in accordance with the granted National University of Ireland Galway and University College Hospital Galway ethical approval. All patients had histologically confirmed breast cancer and all relevant clinic-pathological and demographic data were obtained from a prospectively maintained breast cancer database. This study used retrospectively collected, de-identified data, and no patients were involved.

Results

Patient demographics

One hundred thirty two patients were included. Mean age at diagnosis was 53.3 year (range 21–84). 58 patients (44%) had a loco-regional recurrence while 74 (56%) had a distant recurrence (Table 1). Bone was the most common distant recurrence ($n = 27$), followed by liver ($n = 22$) and lung ($n = 16$) (Table 2). 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 (range 2–144 months) (Table 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). 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 3).

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).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

Table 1 Cohort description

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)
Original subtype	
Luminal A	67 (50.7%)
Luminal B	10 (7.5%)
HER2	15 (11.3%)
Triple negative	40 (30.5%)
Recurrence subtype	
Luminal A	54 (40.9%)
Luminal B	9 (6.9%)
HER2	16 (12.1%)
Triple negative	53 (40.1%)

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, $p = 0.096$). In terms of HER2 receptor, there was a significant difference between the discordant (n = 4) and

Table 2 Distant recurrence location & change in subtype

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 3 Primary tumour features

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 Receptor discordance

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%)

concordant ($n = 128$) groups in OS and PRS (OS 157 vs. 57 months, $p < 0.05$; PRS 60.7 vs. 19.5 months, $p < 0.05$). However, the very low numbers in the discordant group limit the value of this result. 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%), $p = 0.01$). Of the four HER2 receptor discordant cases, two gained and two lost receptor status, however these numbers are too low to draw statistical significance.

Table 5 Impact of subtype change and gain in receptor status on survival

Patient Details	Total	Change subtype	Gain of Receptor
	(n = 132)	(n = 31) 23.5%	(*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)

Subtype discordance & survival

There were 31 patients (23.5%) who had a different subtype on recurrence, 17 were loco-regional recurrences and 14 were distant (Fig. 1). 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 5). 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) (Additional file 1: Table S1). 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, $p = 0.064$) (Fig. 2A). Importantly, there was a statistically significant shorter 5 year PRS between the two groups, (8.6 vs. 22.5 months,

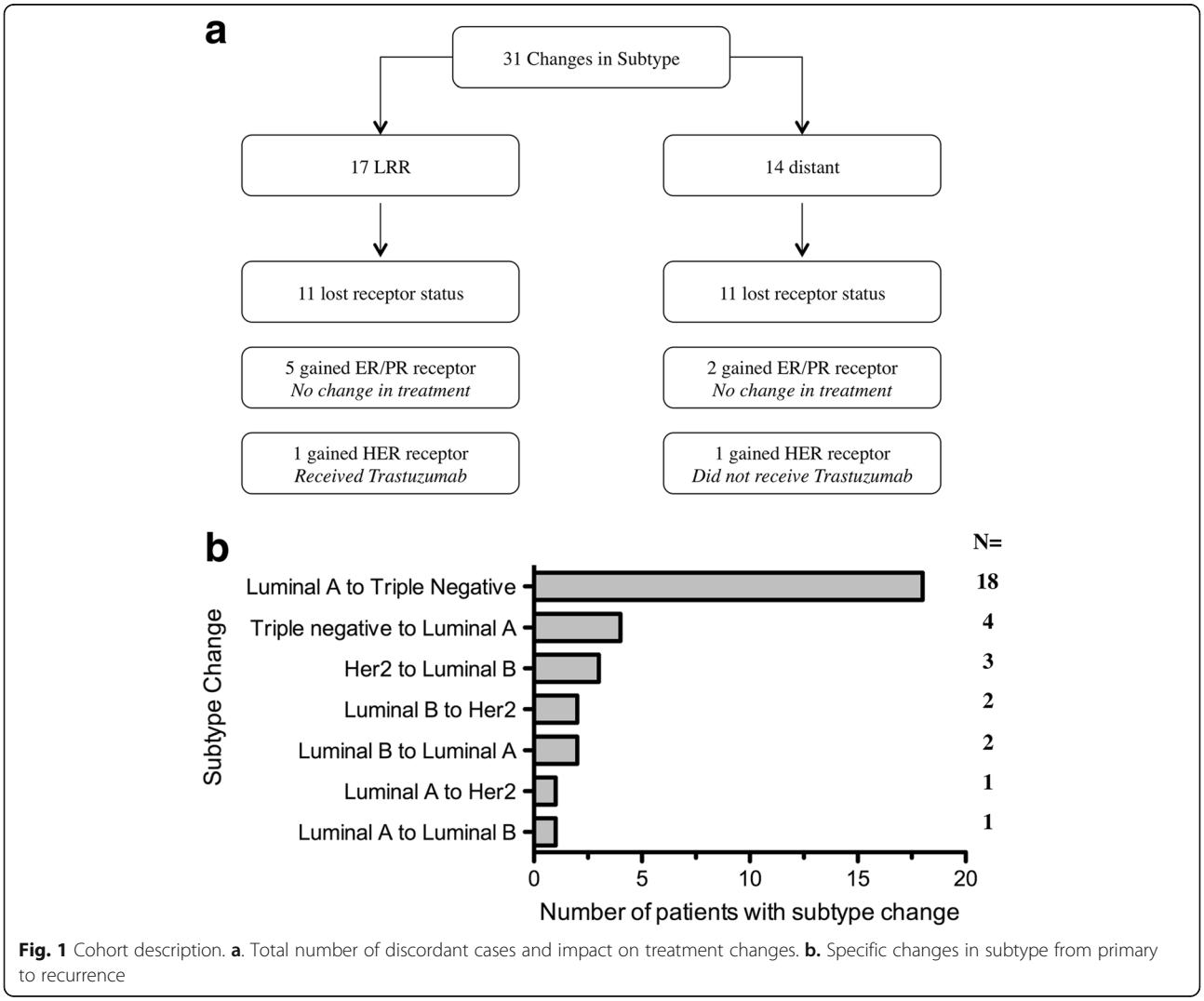

Fig. 1 Cohort description. **a**. Total number of discordant cases and impact on treatment changes. **b.** Specific changes in subtype from primary to recurrence

$p < 0.05$) (Fig. 2B). 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, $p = 0.919$) (Additional file 2: Figure S1).

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 patients 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 patient 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 of the recurrence, with both subsequently testing

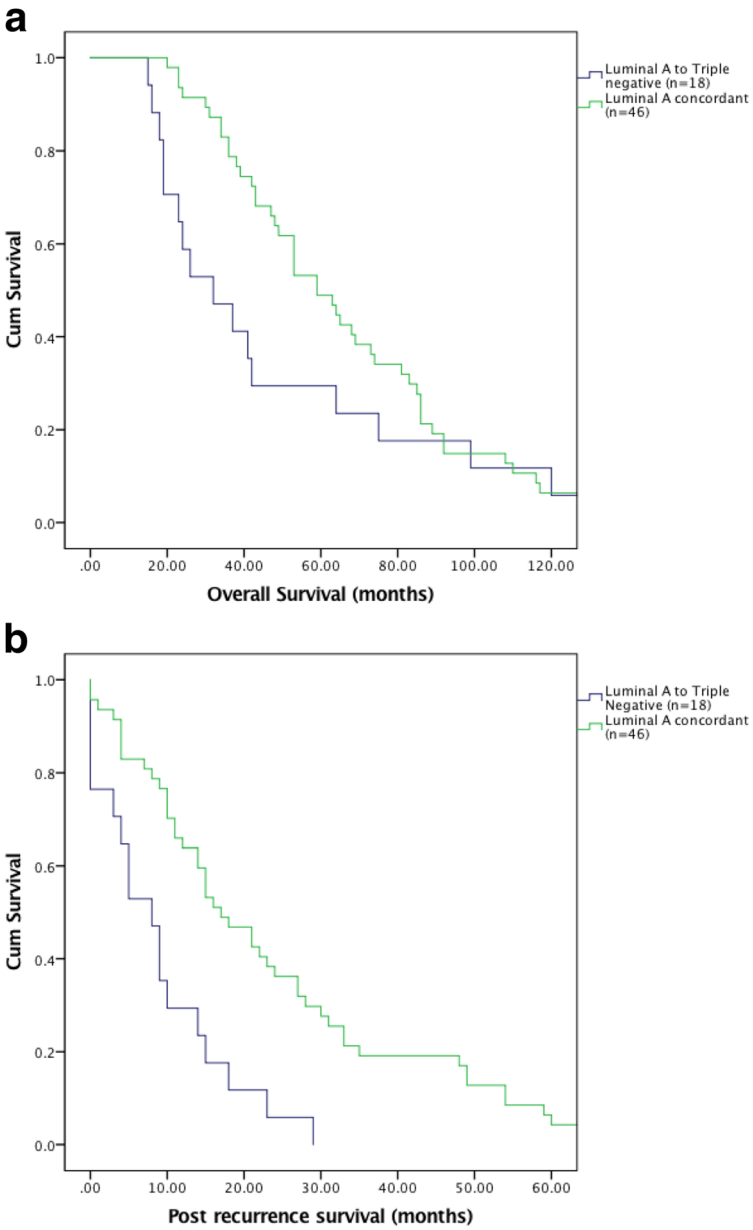

Fig. 2 Luminal (**a**) to triple negative ($n = 18$) vs. triple negative concordant ($n = 46$)**.** A 10 year overall survival ($p = 0.064$). **b** 5 year post-recurrence survival ($p < 0.05$)

positive on FISH. One patient had a distant recurrence in bone, and was enrolled in the TRIO 022 trial [14], 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 1 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.

Discussion

In our single-centre analysis the rate of subtype change of was 23.5%, supporting previously published figs. [15, 16]. 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 [16–20].

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 [6]. HER2 receptor exhibits the lowest rate of discordance between primary and recurrence [21]. 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 [22].

There are a number of possible aetiologies for receptor discordance. Firstly, variability exists in the reproducibility and accuracy of immunohistochemical staining [23]. 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 [6, 24, 25] and the clone with the more aggressive phenotype could potentially initiate the micro-metastatic process [26]. 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 [27]. Genuine switches in biology of the cancer appear to be a rare event based on currently available gene expression data [28, 29], however this does not exclude the potential for smaller scale genomic alterations and mutations [30]. 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 [31]. 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 [32, 33].

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 [7, 18, 34, 35]. Two prospective studies aimed to address these limitations - the BRITS study [36] in the United Kingdom which was carried out at 20 secondary care sites, and the DESTINY study [10] 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 [37]. 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 [15, 35, 38].

Current guidelines by the American Society of Clinical Oncology (ASCO) [39] 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 [40], 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 [41, 42]. 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.

Conclusions

In summary, our study demonstrates the discordance of receptor and subtype between primary and recurrent breast cancer at our institution. 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, multicentre trial collecting data on patients who experience recurrence (including routine biopsy's 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.

Additional files

Additional file 1: Table S1. Impact of Location/Neoadjuvant chemotherapy/Surgery on discordance. Quantifying: Recurrence location, Neoadjuvant Chemo Rx, Surgery, Change subtype, Gain of Receptor. (DOCX 24 kb)

Additional file 2: Figure S1. Triple negative to Luminal A ($n = 4$) vs. triple negative concordant ($n = 35$). A 10 year overall survival ($p = 0.378$). B 5 year post recurrence survival ($p = 0.919$). (TIFF 542 kb)

Abbreviations

CDK: Cyclin dependent kinase;; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; HER2: Her2 over-expressing breast cancer; Her2: Human epidermal growth factor receptor 2; HR: Hormone receptor; HR: Hormone receptor; IHC: Immunhistochemical; OS: Overall survival; PR: Progesterone receptor; PRS: Post-recurrence survival; TN: Triple Negative

Acknowledgements

The authors would like to thank the translational Breast Cancer research group for critical discussion of this manuscript.

Funding

This work is supported by funding from Breast Cancer Research. The funding body was not involved in the design of the study, collection, analysis, interpretation or writing of the manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PFM, AM, AR, CC, CM, RM, BK collected and analysed the data. PFM, JALB, MK wrote the manuscript. JALB, MK final approval of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

This study was conducted in accordance with the granted National University of Ireland Galway and University College Hospital Galway ethical approval. All patients had histologically confirmed breast cancer and all relevant clinic-pathological and demographic data were obtained from a prospectively maintained breast cancer database.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 12 July 2017 Accepted: 5 February 2018
Published online: 20 February 2018

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