Novel Mechanisms for Neoadjuvant Chemotherapeutic Response Monitoring in Breast Cancer

A thesis submitted to the National University of Ireland as partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

By

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November 2016

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Acknowledgements

I would like thank my supervisor Dr James Brown. For inheriting me as your student, for all of your guidance and expertise and for your constant encouragement and approachability, thank you.

To Professor Michael Kerin, thank you for accepting me as a student and colleague, for your continual advice and direction and for all of the opportunities afforded to me while working in your laboratory and on your clinical service. I will be forever grateful.

I would like to thank Dr Róisín Dwyer. For stepping in when I was in need of help, for looking out for me when I was adrift and for always having an open door.

To Mr Karl Sweeney and Professor Martin Leahy I would like to express my sincere gratitude, for all of your ideas, advice, persistence and guidance.

To Grace Clarke, Emer Hennessy, Cathy Curran, Nicola Miller and all of the other researchers in the laboratory, I would like to extend my thanks, for your friendship and company, your knowledge and guidance, your collaboration and expertise.

I would like to thank Breast Cancer Research (formerly NBCRI) who funded this work.

Above all, I would like to thank my family. My parents, my sister, my brothers and my three nephews. Your constant love and support has seen me through many challenging times. You keep me grounded and give me perspective. You make it all possible.

Dedication

This work is dedicated to my family.

For always being there, for all your love and support.

Abbreviations

A&HCI	Arts & Humanities Citation Index
Ago2	Argonaute 2
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BAM	Bromoanisole
BKCI	Book Citation Index
CCR	Expanded Current Chemical Reactions
cDNA	Complementary DNA
CED	Central Domain
CMF	Cyclophosphamide, Methotrexate, Fluorouracil
CPCI	Conference Proceedings Citation Index
Ct	Cycle Threshold
CT	Computerised Tomography
DCE	Dynamic Contrast Enhanced
DCIS	Ductal Carcinoma in situ
DGCR8	DiGeorge Critical Region 8
dsRBD	Double-stranded RNA binding domain
dsRNA	Double-stranded RNA
DW	Diffusion weighted
E	Amplification Efficiency
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ER	Oestrogen Receptor
FBS	Foetal Bovine Serum
FDG	Fluorodeoxyglucose
GUH	Galway University Hospital
HBD	Heme-binding domain
HER2	Human Epidermal Growth Factor Receptor 2
IAC	Inter-Assay Control
IC	Index Chemicus
ICORG	All Ireland Co-Operative Oncology Research Group
IMS	Industrial methylated Spirits
LAF	Laminar Air Flow
LCIS	Lobular Carcinoma in situ
mRNA	Messenger RNA
miRISC	MicroRNA induced silencing complex
miRNA	MicroRNA
MRI	Magnetic Resonance Imaging

NAC	Neoadjuvant Chemotherapy
NCCTG	North Central Cancer Treatment Group
Nd:YAG	Neodymium-doped yttrium aluminium garnet
NIR	Near infra-red
NPI	Nottingham Prognostic Index
NSABP	National Surgical Adjuvant Breast and Bowel Project
NTC	No Target Control
PACT	Protein Kinase R Activator
PAI	Photoacoustic Imaging
PET	Positron Emission Tomography
Ran GTP	Ran guanosine triphosphate
ROI	Region of Interest
PBS	Phosphate Buffered Saline
pCR	Complete Pathological Response
piRNA	PIWI-interacting RNA
PR	Progesterone Receptor
R	Pearson Correlation Coefficient
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RQ-PCR	Real Time Quantitative Polymerase Chain Reaction
RTC	Reverse Transcription Control
SCI-EXPANDED	Science Citation Index Expanded
SERM	Selective Oestrogen Receptor Modulator
siRNA	Short interfering RNA
sO2	Oxygen Saturation
SSCI	Social Sciences Citation Index
TMA	Tissue Microarray
TNM	Tumour Node Metastasis staging system
TRBP	Transactivator Response RNA-Binding Protein
US	Ultrasonography
WoS	Web of Science
XPO5	Exportin-5

Communications arising from this work

First author presentations:

- **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ. *The potential role of photoacoustic tomography in breast cancer imaging*. College of Medicine, Nursing and Health Science Research Day, May 2014, NUI Galway

- **Casey MC,** Kerin MJ, Brown JAL, Sweeney KJ. *Happy 21st: microRNA comes of age*. Sir Peter Freyer Surgical Symposium, Sept 2014, Galway

- **Casey MC,** McDermott AM, Heneghan H, Brown JAL, Miller N, Kerin MJ. *Circulating microRNAs: Novel Breast Cancer Biomarkers and their use for guiding and monitoring response to therapy.* Circulating Biomarkers Conference, Oct 2014, Dundee, Scotland

- **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ. *Potential role of photoacoustic imaging in neoadjuvant chemotherapy response monitoring in Breast cancer.* Society of Academic and Surgical Research, Jan 2015, Durham, UK

- **Casey MC,** A McGuire, A Shalaby, M Webber, C Curran, G Callagy, E Bourke, RM Dwyer, JAL Brown, MJ Kerin. *Investigating the Role of AGO2 in Breast Cancer*. Irish Association for Cancer Research AGM, Feb 2015

- **Casey MC,** Kerin MJ, Brown JAL, Sweeney KJ. *Evolution of a research field: a microRNA example.* Sylvester O'Halloran Surgical Scientific Symposium, Mar 2015

- **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ. *Photoacoustic Imaging of Breast Tissue*. Microcirculation and Angiogenesis Symposium, NUI Galway, June 2015

- **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ. *Clinical Application of Photoacoustics in Breast Cancer Imaging.* BioPhotonics & Imaging Conference BioPIC 2015, July 2015, NUI Galway

- **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ. *Photoacoustic imaging in Breast Cancer*. Sir Peter Freyer Surgical Symposium, Sept 2015, Galway

- **Casey MC,** A McGuire, A Shalaby, M Webber, C Curran, G Callagy, E Bourke, RM Dwyer, JAL Brown, MJ Kerin. **Plenary Presentation**: *Assessing Ago-2 as a biomarker for breast cancer.* Sir Peter Freyer Surgical Symposium, Sept 2015, Galway

- **Casey MC,** A McGuire, A Shalaby, M Webber, C Curran, G Callagy, E Bourke, RM Dwyer, JAL Brown, MJ Kerin *Ago-2 in the further molecular stratification of breast cancer*. Society of Academic and Research Surgery, Jan 2016, London

First Author Publications:

- **Casey MC,** Kerin MJ, Brown JAL, Sweeney KJ. *Evolution of a research field – a microRNA example.* PeerJ (2015) 3:e829.

- **Casey MC**, Sweeney KJ, Brown JAL, Kerin MJ. *Exploring Circulating microRNAs in the Neoadjuvant treatment of Breast Cancer.* Int J Cancer 2016

Manuscripts in preparation:

- *The role of Ago2 in Breast Cancer.* **MC Casey,** A McGuire, A Shalaby, M Webber, C Curran, G Callagy, E Bourke, RM Dwyer, JAL Brown, MJ Kerin.

- *The potential role of photoacoustic imaging in neoadjuvant chemotherapeutic response monitoring in breast cancer.* **Casey MC**, Zafar H, Walsh S, Ennis R, Dwyer RM, Brown JAL, Sweeney KJ, Leahy MJ, Kerin MJ.

Awards

- Audience prize, best oral presentation: Threesis, Nov 2014, NUI Galway *"Now you see me"*

- Health Research Board (HRB) of Ireland Scholarship to attend the National Cancer Institute Summer Curriculum in Cancer Prevention, Washington, USA, August 3rd-7th 2015.

- Bursary to attend SARS meeting, London, January 2016

Summary of Contents

Breast cancer is the most frequently diagnosed female malignancy worldwide, causing the majority of cancer-related deaths amongst women. While the management of this disease has transformed dramatically in recent years, personalised breast cancer management has not yet been achieved. To further inform the development of precision medicine, this thesis investigated novel mechanisms for neoadjuvant chemotherapeutic response-monitoring.

With unprecedented access to scientific material, the field of microRNA (miRNA) research was analysed to enable the production of informed and targeted outputs. Recognising their known dysregulation in association with breast cancer, miRNAs were selected for analysis at the circulatory level as biomarkers of disease response to chemotherapy. Results outlined represent the initial *blinded* analysis of the first half of the national clinical trial that was undertaken to address this question.

Upstream of miRNAs, the potential role of the miRNA-binding protein Argonaute-2 (Ago2) was analysed. Differential expression of Ago2 protein between the molecular subtypes of breast cancer was detected *in-vitro,* and at the tissue level in clinical breast specimens. Ago2 protein was significantly less abundant in luminal cancers, displaying inverse association with the presence of luminal receptors and direct association with metastasis, supporting a potential prognostic or predictive role.

At the patient level, a novel imaging modality was introduced into the clinical setting to assess its ability to effectively visualise breast tissue. The system was optimised for clinical use, with individual breast pathologies displaying distinct photoacoustic signals. With the system optimised for clinical use and baseline parameters established, a study assessing early *in-vivo* tumour response detection to chemotherapy was developed and commenced.

By further informing mechanisms to tailor breast cancer management on an individualised basis, work presented here contributes to the development of personalised or precision medicine, pursuing tailored chemotherapeutic administration and improved patient outcomes from this disease.

Chapter 1

Introduction

PUBLISHED JANUARY 2016:

Casey MC, Sweeney KJ, Brown JA, Kerin MJ. Int J Cancer. 2016 Jan 12. doi: 10.1002/ijc.29985. Exploring circulating micro-RNA in the neoadjuvant treatment of breast cancer [Publication section].

Text modified for thesis format with addition of sections 1.2, 1.9-1.13.

1.1 Breast Cancer Burden

Breast cancer is the most frequently diagnosed cancer among women worldwide, accounting for 23% of total cancer cases [1]. In 2012, 1.7 million women globally were diagnosed with breast cancer, representing a >20% increase in incidence since 2008. Concurrently, worldwide >520 000 women died from breast cancer, representing a 14% increase in annual breast cancer related mortality and confirming breast cancer as the most common cause of cancer-related deaths amongst women [1]. Within developed countries the incidence of breast cancer continues to rise. This is likely due to the implementation of screening programs and improved imaging techniques, leading to many breast cancers being diagnosed at an earlier stage. Furthermore, recent improved molecular understanding of breast cancer and the use of increasingly effective chemotherapeutics has resulted in improved patient outcomes, with mortality decreasing by 2-3% per year in developed countries [2].

1.2 Breast Cancer Diagnosis and Screening

Breast cancer is diagnosed in two distinct patient groups; symptomatic patients and asymptomatic patients. Symptomatic patients refer to patients who notice a breast abnormality e.g. lump, nipple discharge, skin change, or patients whose general practitioners notice a breast abnormality. These patients are referred to symptomatic breast units nationwide to undergo investigation via 'triple assessment,' which comprises physical breast examination, radiological investigation, and breast biopsy, if indicated.

In patients who are asymptomatic, breast cancer is diagnosed via national screening programmes. It is internationally recognised that earlier detection of breast cancer leads to better patient outcomes from disease, and this is the basic principal that underlies breast cancer screening [3]. The Irish breast cancer screening programme '*BreastCheck'* is government-funded and

provides complimentary mammograms to eligible women, aged 50-69 years, on a two-yearly basis[1].

1.2.1 Mammography

Mammography remains the gold standard imaging technique for the detection of breast cancer, and has been shown to reduce mortality in national screening programmes [4, 5]. It is a relatively inexpensive, non-invasive imaging modality, with a specificity of 97% and sensitivity of 72-80% that increases with age [6, 7]. However, there are known limitations to mammography, including the use of ionizing radiation, a very limited sensitivity in dense breast tissue, and false positive results, producing unnecessary review appointments and breast biopsies [8-11].

1.2.2 Ultrasonography

Ultrasonography (US) is employed in several aspects of breast cancer diagnosis and management. It is utilised to characterise palpable breast masses e.g. cystic or solid, and to guide intervention e.g. biopsy. Further to this, US is used to assess the axillary lymph nodes for metastatic spread and to evaluate dense breast tissue. The use of US in conjunction with mammography in dense breast tissue has been shown to significantly improve breast cancer detection, with US capable of detecting mammographically-occult predominantly small, node-negative breast cancers [12, 13]. However, US is highly operator dependent, and for the purposes of whole breast screening, takes considerably longer to perform than mammography.

[1] http://www.breastcheck.ie/

1.2.3 Magnetic Resonance Imaging

In the context of breast screening, Magnetic Resonance Imaging (MRI) as an adjunct to mammography has recently been incorporated into the *American Cancer Society Guidelines* for patients with a high risk of breast cancer, in terms of a genetic mutation known to increase their risk of breast cancer e.g. BRCA, a history of radiation to the chest at ages 10-30 years, or an estimated lifetime risk of breast cancer of ≥ 20-25% [14]. Although highly sensitive for the detection of breast cancer, the main disadvantages of MRI include limited specificity, inability to detect calcifications, the requirement of intravenous contrast agents for functional imaging, extended length of scan time compared to mammography, and high cost [15].

1.2.4 Positron-Emission Tomography

Positron-Emission Tomography (PET) is a relatively newer system for breast imaging. In the evaluation of suspicious breast lesions, PET has a reported sensitivity and specificity of 80-90% and 71-95% respectively [16]. However, PET is not recommended for primary breast cancer diagnosis or staging as it has low sensitivity for small tumours and lobular carcinomas, and lacks evidence to support its superiority over other conventional imaging modalities [17]. Further to this, PET is expensive and requires the administration of an intravenous agent.

1.2.5 Computerised Tomography

Studies have shown that there is no significant difference between Computrised Tomography (CT) and mammography for distinguishing malignant from benign breast lesions [18]. Mammography however, is superior to CT for the visualisation of microcalcifications, and therefore is more sensitive and specific for breast cancer diagnosis [19]. Further to this, CT emits a much higher dose of radiation and is not cost effective for routine screening and diagnostic purposes [20]. For breast cancer staging however,

CT is the imaging modality of choice [21]. When there is specific concern regarding bony metastasis, isotope bone scans are utilised [22].

1.3 Breast Cancer Classification and Staging

The tumour node metastasis (TNM) staging system is internationally recognised and utilised to guide therapeutic decisions and stratify patients into prognostic groups. This system comprises factors including tumour size, lymph node status and presence of distant metastasis (Table 1.1). Prognostic groups based on TNM staging are outlined in Table 1.1.

1.4 Breast Cancer Pathology

Breast cancer encompasses a heterogeneous group of breast lesions that differ in terms of microscopic appearance and biologic behaviour. These lesions are classified as either *in situ* disease (*Ductal Carcinoma in Situ (DCIS) or Lobular Carcinoma in Situ (LCIS)),* where tumour cells show no evidence of surrounding tissue invasion, or *invasive disease*, where the tumour cells are seen to invade surrounding tissue, and thus have the potential to metastasize. There are several histologic subtypes of *invasive carcinoma*, namely infiltrating ductal carcinoma (76%), infiltrating lobular carcinoma (8%), Ductal/Lobular (7%), Mucinous/Colloid (3%), Tubular (2%), Medullary (1%) and Papillary (1%). These invasive tumours can metastasize via direct invasion, to the chest wall and skin, via lymphatic invasion to the axillary lymph nodes, or throughout the body by haematogenous spread, most commonly to the bones (32-55%), liver (15-27%), lung (12-27%) or brain (10-15%)[23].

Table 1.1 TNM Staging System with Prognosis

Tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ (DCIS/LCIS/Paget's)
T1	Tumour ≤2cm
T2	Tumour >2cm but <5cm
T3	Tumour >5cm
T4	Tumour of any size growing into chest wall or skin
Lymph Node Status	
Nx	Lymph nodes cannot be assessed
N0	Cancer has not spread to lymph nodes
N1	Cancer has spread to 1-3 axillary lymph nodes, or sentinel lymph node
N2	Cancer has spread to 4-9 axillary lymph nodes
N3	Cancer has spread to 10+ nodes/clavicular or supraclavicular nodes
Metastasis	
Mx	Presence of metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage	**TNM**	**Approximate 5yr Survival**[2]
Stage 0	Tis, N0, M0	95-99%
Stage 1	T1, N0, M0	90-94%
Stage 2a	T0/T1, N1, M0 or T2, N0, M0	
Stage 2b	T2, N1, M0 or T3, N0, M0	60-88%
Stage 3a	T0-T2, N2, M0 or T3, N1 or N2, M0	
Stage 3b	T4, N0-2, M0	42-55%
Stage 3c	T, N3, M0	
Stage 4	Any T, Any N, M1	15-18%

[2]http://www.obgyn.net/breast-cancer/breast-cancer-surgical-practice-guidelines/page/0/2

1.5 Breast Cancer Subtypes

In recent years, it is recognized that breast cancer is a heterogeneous disease characterised by discrete breast cancer subtypes [24]. While the exact number of subtypes remains to be elucidated, in a landmark paper, Sorlie et al. described *Luminal A*, *Luminal B*, *Basal* (also known as '*triple negative'* tumours) and *HER2* over-expressing (Table 1.2) [25]. More recently, 10 distinct breast cancer subtypes have been proposed, although this stratification is not yet applied clinically [26]. Currently utilized breast cancer subtypes have known distinct clinical behaviours and responses to therapy and are stratified according to presence or absence of the *oestrogen receptor* (ER), *progesterone receptor* (PR) and the *human epidermal growth factor receptor 2* (HER2) (Further discussed in section 1.7).

Table 1.2 Breast cancer subtypes with receptor status and prevalence[3]

Breast Cancer Subtype	ER	PR	HER2	Prevalence (%)
Luminal A	+	+/-	-	40
Luminal B	+	+/-	+	20
HER2	-	-	+	15-20
Basal/Triple negative	-	-	-	10-15

1.6 Breast Cancer Management

In recent years, the management of breast cancer has transformed dramatically. This is largely due to a greater understanding of the heterogeneity of this disease and the molecular characteristics of its distinct subtypes. Current techniques employed in the treatment of this disease include surgical resection, radiotherapy, hormonal therapy and chemotherapy.

[3] *Breast Cancer Facts & Figures 2013-2014.* American Cancer Society, 2013

1.6.1 Surgical Resection

Loco-regional control via surgical excision remains the most effective treatment modality for curative intent [27]. Operative intervention is becoming increasingly refined with a view towards breast conserving surgery. The radical '*Halsted procedure*,' involving the en bloc resection of the breast, pectoralis muscle and axillary contents, has long been replaced with targeted, radiologically-guided tumour excision or mastectomy, where breast conservation is not amenable e.g. multi-focal disease.

1.6.2 Radiotherapy

Radiotherapy is employed to achieve loco-regional disease control in breast cancer. Realisation that the combination of radiotherapy with breast conserving surgery had comparable disease-free and overall survival to mastectomy enabled breast conserving surgery to form the mainstay of surgical management of breast cancer [28, 29].

1.6.3 Hormonal Therapy

Hormone therapy is prescribed for patients who have confirmed hormone receptor positive disease. It functions to block activation of the hormone receptors by competitive inhibition of endogenous oestrogens (selective oestrogen receptor modulators (SERM) e.g. Tamoxifen) or by inhibiting oestrogen synthesis from adrenal androgens in post-menopausal women (Aromatase inhibitors e.g. Anastrozole). The use of approximately 5 years of adjuvant (post-surgery) Tamoxifen has been shown to reduce the annual rate of breast cancer death by 31% irrespective of the use of chemotherapy or other tumour characteristics [30]. With such a profound impact on patient survival, assessment of tumour hormone receptor status is mandated for all patients diagnosed with breast cancer worldwide [31].

1.6.4 Chemotherapy

The first successful chemotherapeutic regimen for operable breast cancer was described in 1976 by Bonadonna [32]. The combination adjuvant therapy with *cyclophosphamide, methotrexate* and *fluorouracil* (CMF) was shown to significantly reduce post-operative recurrence rates. By the early 1990's, *anthracycline*-containing regimens were introduced and are now recognized as superior to treatment with CMF alone [33]. Importantly, it was during this time that administration of chemotherapy in the pre-operative or neoadjuvant period began. The *National Surgical Adjuvant Breast and Bowel Project (NSABP) B-27* and the *Aberdeen trial* recognized the addition of a *taxane* to an *anthracycline*-based chemotherapy further reduced the risk of recurrence, and in the neoadjuvant setting improved the rates of complete pathological response and therefore overall outcome [34-36].

While no gold-standard chemotherapeutic regimen currently exists for breast cancer, it is accepted that an *anthracycline*-based regimen be utilized, with the addition of a *taxane* [37, 38]. The *Early Breast Cancer Trialists' Collaborative Group* (EBCTCG) report that the proportion of women who survive for 15 years following the diagnosis and treatment of breast cancer is higher in patients treated with *anthracycline*-based regimens rather than CMF [39]. For patients with breast cancer overexpressing the HER2 receptor, targeted therapy with the humanized monoclonal antibody *Trastuzumab* was shown to reduce the risk of death by 33% by the NSABP B-31 and the North Central Cancer Treatment Group (NCCTG) N9831 trials, resulting in the early cessation of enrolment and publication of study results [40] (Table 1.3).

It is recognised however, that there exists a requirement for novel chemotherapeutic agents, regimens and further targeted therapies with superior efficacy than those currently available [41, 42]. As breast cancer is a heterogenous disease, individual tumours respond variably to different therapies, necessitating the identification of optimal therapies for distinct breast cancer subtypes, and the validation of a mechanism to predict, monitor

and detect response, to ensure that the optimal chemotherapeutic regimen is administered for a given tumour.

Table 1.3. Chemotherapeutics used to treat breast cancer

Drug Class	Mechanism of Action	Example	Reference
Anthracyclines	Inhibit DNA & RNA synthesis Disrupt DNA damage response Inhibit Topoisomerase II	Doxorubicin Epirubicin Mitoxantrone	[43]
Taxanes	Disrupts microtubule function	Docetaxel Paclitaxel	[44]
Alkylating Agent: Nitrogen Mustard	Interfere with DNA replication	Cyclophosphamide	[34]
Anti-metabolites	Prevent folate use for DNA generation	Methotrexate Fluorouracil Capecitabine	[45]
Anti-HER2/EGFR	Inhibit Tyrosine kinase Arrest cell cycle Suppress angiogenesis	Trastuzumab Pertuzumab Lapatinib	[46]

1.6.4.1 Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy (NAC) refers to the use of chemotherapy prior to any surgical intervention. Traditionally, NAC was reserved for locally advanced breast cancers, converting technically inoperable tumours into candidates for mastectomy. With the increasing trend toward breast conserving surgery however, the use of preoperative systemic therapy was extended to include patients with invasive, early-stage operable tumours. Currently, the use of NAC is established as a therapeutic avenue for selected high-risk breast cancers, tumours ≥2 cm and for locally advanced disease [47]. The advantages of preoperative chemotherapy include tumour burden reduction, more conservative surgical resection and the unique and novel opportunity to assess *in-vivo* tumour responsiveness to chemotherapeutics [48]. While the NSABP B18 trial, among others, failed to identify any improvement in disease-free and overall survival between neoadjuvant and adjuvant therapies, it was established that patients achieving *complete pathological response* (pCR) to NAC experienced improved outcomes, while unresponsive patients or patients with progressive disease during NAC

experienced worse outcomes from their disease [36, 49-51]. Supporting this, it was identified that the early response to neoadjuvant treatment can predict pCR and therefore may serve as a predictor of long-term outcome [52, 53].

The ability to monitor and detect individual patient tumour response to systemic therapy could drastically alter the current management of breast cancer. This could enable tailored treatment plans, whereby patients found to have unresponsive or progressive disease could have their chemotherapeutic regimen altered or potentially discontinued with the provision of expedited surgery. Unfortunately, no reliable clinically validated means of predicting chemotherapeutic responders from non-responders currently exists. As a result, certain cohorts of patients are being exposed to the potential deleterious effects of chemotherapy, without any guarantee of benefit. The identification of a mechanism to effectively distinguish chemotherapeutic responders from non-responders could confer multiple benefits, including a reduction in chemotherapy-induced morbidity and mortality, more effective administration of chemotherapeutics and potentially, the individualisation of patient care.

1.6.4.2 Tailoring Chemotherapeutic administration

In recent years, significant strides have been made in pursuit of the individualisation of chemotherapeutic administration. *Oncotype Dx* is a 21-gene reverse transcription assay that was clinically validated in a prospective manner in the NSABP B-14 and B-20 trials [54, 55]. This assay generated a recurrence score between 0 and 100 that correlated to the likelihood of disease recurrence within 10 years of diagnosis. Patients were categorised as either low, intermediate or high risk of breast cancer recurrence based on their score. The authors concluded that patients at low risk of recurrence could be treated with endocrine therapy alone, sparing these patients from receiving systemic chemotherapy that would not be of benefit [56]. Within the intermediate risk group, the benefit was not so clear, which resulted in the subsequent TAILORx trial. This study was devised to further address the 'intermediate risk' group, by randomising patients with an intermediate risk score to receive

either hormonal therapy alone or combination chemotherapy and hormonal therapy. While initial study results corroborate the findings of NSABP B-14 and B-20, long term follow-up of patients in the intermediate group is ongoing. These landmark studies have greatly enhanced the individualisation of chemotherapeutic administration by providing physicians with a clinically validated indicator of disease recurrence.

In the neoadjuvant setting, I-SPY 2[4] is a clinical trial that is currently recruiting patients in an attempt to identify which drug agents are most effective within which breast cancer subtypes and which early indicators of response are predictors of treatment success (e.g. MRI measurements, blood and tissue samples). This is following on from the I-SPY trial, which identified the ability of pCR to predict outcome was substantially improved when analyzed within tumor subsets. The findings of this study are eagerly anticipated as the identification of further means of patient stratification into responders and non-responders, in conjunction with the identification of novel effective chemotherapeutic agents, will hopefully bring us ever closer to personalized breast cancer management.

1.7 Current Molecular Markers for Breast Cancer

Current gold standard molecular markers established for use in breast cancer are the ER, PR and HER2 receptors, and the proliferation marker *Ki67*. The *American Society of Clinical Oncology* guidelines mandate that the immunohistochemical markers ER, PR and HER2 be assessed in all cases of invasive breast carcinoma, to guide management decisions, including choice of chemotherapeutic regimen [31].

[4] www.clinicaltrials.gov

1.7.1 ER/PR Receptor

ER/PR expression, as identified by immunohistochemistry, provides an index for sensitivity to endocrine treatment and acts as a marker of chemosensitivity. Approximately two thirds of tumours display ER-positivity, correlating with improved responsiveness to endocrine therapy and improved patient outcomes. PR expression is strongly dependent on ER expression, with less than 1% of breast cancers displaying sole PR-positivity. In this instance, limited benefits from endocrine therapy have been described [57]. Associating chemotherapeutic treatments and ER/PR status, ER positivity correlates with poor tumour response [58]. However, ER-negative tumours are more likely to achieve complete pathological remission and thus experience improved outcomes with chemotherapy [59].

1.7.2 HER2 (ERBB2)

HER2 is a membrane tyrosine kinase receptor that upon activation affects cell proliferation and survival [60]. It is located on chromosome 17q12 and is an oncogene, amplified in ~15-20% of breast cancer cases. Initially identified as a prognostic marker, HER2 over-expression is associated with increased relapse rates, increased incidence of metastases and worse overall survival [61, 62]. However, the development of therapies specifically targeting HER2 has resulted in significant improvements in outcomes for patients with HER2 positive breast cancer [63]. Risk of relapse and death are reduced by approximately 50% and 30% respectively, improving disease-free and overall survival [64]. In 2013 the addition of Trastuzumab to neoadjuvant chemotherapy in patients with HER2 positive tumours was found to double complete pathological remission rates (compared to chemotherapy alone) and was associated with a longer event free survival [65]. Most recently, use of neoadjuvant followed by adjuvant HER2 has demonstrated sustained benefit in event-free survival and a strong association with complete pathological remission [66].

1.7.3 Basal/Triple Negative Breast Cancers

'Basal' and 'triple negative (TNBC)' breast cancer subtypes overlap greatly in terms of their immunophenotype (ER, PR & HER2 negative), aggressive clinical behavior and increased prevalence in younger, African-American patients. However, they are not synonymous, as not all basal cancers determined by gene expression analysis lack ER, PR and HER2 and not all triple-negative cancers show a basal phenotype by expression array analysis [67]. Due to a more aggressive clinical pathology, both of these subtypes are associated with a higher risk of mortality. Both subtypes lack all known effective biomarkers and therefore targeted therapies. However, both subtypes are highly sensitive to neoadjuvant chemotherapy [68-70]. The highest rates of complete pathological remission have been achieved in TNBC tumours utilising a neoadjuvant regimen of docetaxel, doxorubicin and cyclophosphamide, while the addition of bevacizumab is expected to increase this rate further [65].

1.7.4 Ki67

Ki67 is a nuclear non-histone protein utilized as a marker of proliferation, as it is absent in quiescent cells, yet universally expressed among proliferating cells. Immunohistochemical staining of Ki67 expression levels is associated with the percentage of tumour cell nuclei positively stained and are used to determine a Ki67 score. A score of less than 10% is considered low, 10-20% borderline, and high if more than 20% [71]. In early and advanced breast cancer the Ki67 score can predict the response to chemotherapy [72]. A high pre-treatment score is associated with a good chance of complete pathological remission to therapy and therefore improved long-term outcome [73]. Lee et al. [74] describe a significant decrease in Ki67 index following neoadjuvant chemotherapy, a finding that is recognized as a strong predictor of recurrence-free and overall survival. Of concern regarding the use of Ki67 is the lack of standardization of analytical practice, with laboratories utilising differing cut-off points to differentiate between 'high' and 'low' Ki67. Further to this, as with all of the above described markers, determination of expression levels

requires tumour tissue, mandating invasive sampling techniques, which further emphasizes the need for a non-invasive breast cancer biomarker.

1.7.5 Requirement of further biomarkers

While currently utilised breast cancer biomarkers are ubiquitously employed, they are far from ideal. Invasive tissue sampling is required to assess for the presence of the ER, PR and HER2 receptors and to determine Ki67. These markers cannot be utilised for the early detection of breast cancer, nor can they be employed as markers of tumour progression, metastasis or recurrence. Further biomarkers are required to enable early breast cancer diagnosis, improve patient prognostication and to facilitate treatment optimization and individualisation. Such biomarkers could confer multiple benefits, including tailored patient care programs and potentially, the development of effective new therapies. Individualisation of patient care could reduce the morbidity and potential mortality associated with chemotherapy or radiotherapy and potentially improve patient outcomes from this disease. An ideal biomarker would be non- or minimally-invasive and have the ability to detect a disease at an early stage, provide prognostic information, be utilised to monitor response to treatment and to detect disease progression or recurrence.

1.8 Introduction to MicroRNAs

MicroRNAs (miRNA) are a naturally-occurring class of short, non-coding RNA molecules ~19-25 nucleotides in length. MiRNAs have been demonstrated to regulate gene expression at the post-transcriptional level, via binding primarily to 3' or 5' untranslated regions of target messenger RNAs (mRNA), leading to inhibition of translation or mRNA degradation [75]. Interestingly in addition to their inhibitory role, miRNAs have recently been demonstrated to facilitate increases in transcript levels, under certain conditions [76, 77].

Since their discovery in 1993, knowledge of the role of miRNAs in regulating gene expression across a spectrum of pathological processes has grown exponentially [78]. It is now recognized that certain miRNAs are highly specific for tissue and developmental stages, exerting a regulatory effect on a myriad of cellular processes including cell development, differentiation, proliferation and apoptosis [75, 77, 79]. Many miRNA are expressed in tissue- and disease-specific patterns and are known to correlate with clinicopathological features and prognostic indices across a spectrum of pathologies [80-85].

1.8.1 MiRNA Biogenesis and Function

MiRNA generation is a complex process that commences in the nucleus (Figure 1.1). It involves multiple miRNA machinery proteins, including Drosha, diGeorge critical region 8 (DGCR8), Dicer1, Exportin-5 (XPO5), transactivating response (TAR) RNA-binding protein (TRBP), Protein Kinase R (PKR) activator (PACT) and Argonaute (Ago). MiRNA genes are transcribed by RNA polymerase II/III as primary miRNAs (pri-miRNAs). These pri-miRNAs are processed by the Drosha-DGCR8 complex, to become pre-miRNAs. Pre-miRNAs are transported into the cell cytoplasm by the nuclear receptor export protein XPO5. Here, pre-miRNAs are cleaved by the RNase III enzyme Dicer into a mature duplex miRNA, with TRBP and PACT increasing Dicer's stability and processing activity. One strand of the duplex represents a mature miRNA and is incorporated into the RNA-induced silencing complex (RISC) to form miRISC. RISC is formed by the association of Dicer, TRBP, PACT, Ago-2 (most commonly) and GW182, a protein that stabilises Ago-2. The miRNA:RISC complex (miRISC) then targets mRNA containing complementary sequences to the mature miRNA, to inhibit translation or induce mRNA degradation via the Ago component (Figure 1.1)[75, 79, 86, 87].

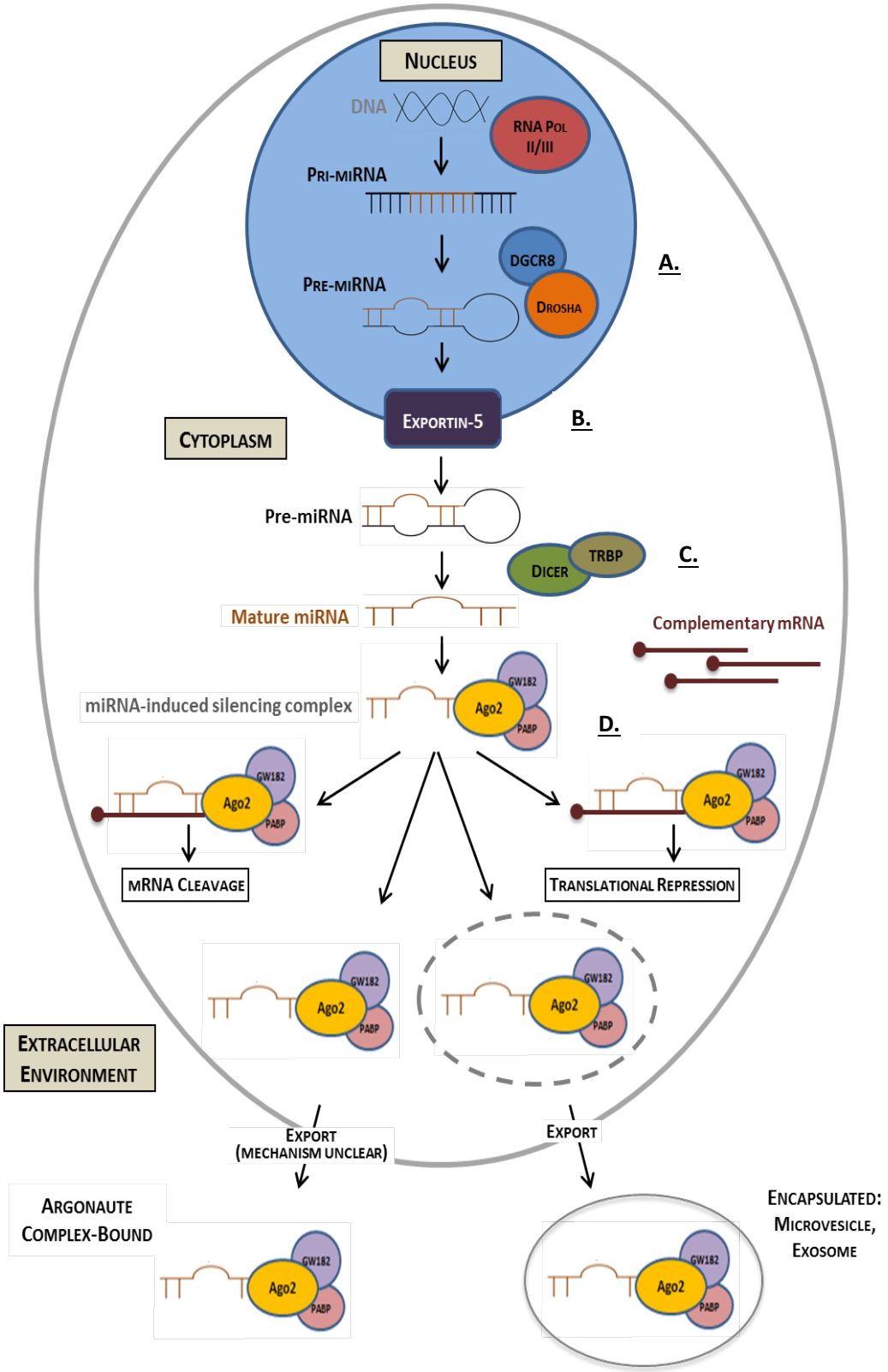


Figure 1.1 MiRNA Biogenesis and Mechanism of Action [88]. A. DGCR8 and Drosha. See Section 1.9.1. B. Exportin-5. See Section 1.9.2 C. Dicer and TRBP. See Section 1.9.3. D. Ago2. See Section 1.9.4.

1.8.2 MiRNA in Circulation

Further to their intracellular function, it has been recognized that miRNAs function at an intercellular level, transmitting information from one cell population to another and inducing changes through this novel extracellular signalling mechanism [89, 90]. Although small RNAs were detected in the circulation as early as 2004 [91], it was in 2008 that the presence of miRNAs in the circulation was confirmed, with significant differences in expression patterns detectable between patients with cancer compared to controls [92, 93]. These circulating miRNAs were found to be present with remarkable stability, indicating that they must be protected from the digestive action of circulatory RNases. Recent studies have confirmed that miRNAs are transported by a variety of mechanisms that shield them from this RNase degradation, including packaged into membrane-derived vesicles, such as exosomes, bound to lipoproteins and as part of ribonucleoprotein complexes [89, 90, 94-96]. It has been demonstrated that an estimated ≥90% of circulating miRNA are bound to Argonaute-2 (Ago2) containing complexes, with only a minority being transported packaged in vesicles [95, 97]. While some mechanisms regulating the packaging and export of membrane-bound miRNA are understood [98-100], the process of non-membrane-bound miRNA export from cells remains unclear [101, 102].

While the exact sources of all circulating miRNAs remain to be elucidated, it appears there are two, complimentary, sources of circulating miRNA: miRNA released passively into the bloodstream following tissue injury and cell death and miRNA actively exported from cells into the bloodstream. However, in both cases, miRNA could be either protein bound "un-encapsulated" or miRNA protected inside membrane coated vesicles (such as exosomes). Controversy remains regarding the proportions of free and membrane-bound circulating miRNA, due to a lack of standardization of sample processing techniques, preventing data from individual studies from being directly compared.

1.8.3 MiRNA and Breast Cancer

In 2005, genome-wide miRNA expression analysis enabled identification of miRNAs that were differentially expressed in breast cancer tissue [103]. The panel of 29 miRNAs identified differentiated tumours from normal tissues with an accuracy of 100%. Importantly, miRNA expression correlated with distinct tumour phenotypes, ER and PR expression and tumour stage. More recently, a miRNA expression pattern of 31 miRNAs evaluated in 93 tumour samples was found to predict hormone receptor status, and thus classify tumours by genetic subtype [104]. These findings were independently corroborated by Lowery, Miller [83] who profiled 453 miRNAs in 29 early-stage breast cancer specimens, identifying a distinct panel of miRNAs corresponding to expression of ER, PR and HER2. Translating these tissue findings into the circulation, it was found that miR-195 expression was significantly elevated in breast cancer patients (n=148) compared to controls (n=44), and that these levels reduced post-operatively [105]. Furthermore, high levels of circulating miR-21 and miR-10b were found to be associated with ER negativity, thus a poorer prognosis [105]. In a further study, elevated miR-155 expression was associated with PR positivity [106].

The ability of miRNA expression profiles to classify breast tumours by biopathologic variables currently utilized to determine responsiveness to neoadjuvant chemotherapy highlights the potential of miRNA signatures as novel predictive and prognostic biomarkers that could allow individualization of breast cancer treatment and improved selection of patients for neoadjuvant chemotherapy.

1.8.4 MiRNA as novel biomarkers of NAC Response

The role of miRNA in neoadjuvant chemotherapeutic response prediction and monitoring has been investigated across a variety of pathologies. In colorectal carcinoma, a distinct miRNA expression signature that could effectively predict colorectal cancer response to neoadjuvant chemoradiotherapy was identified [107]. In human gastric cancer, decreased let-7i expression was

found to have a significant association with a poorer response to chemotherapy and shorter overall survival [108]. In the breast cancer setting, the ability of a panel of miRNAs to predict response of triple negative breast carcinoma to neoadjuvant chemotherapy has been investigated [109]. Although study numbers were limited (11 patients), results indicated higher miR-200b-3p and miR-190a expression and lower miR-512-5p expression was associated with a better pathologic response to chemotherapy. As biomarkers are ideally non-invasive, blood-borne or circulating miRNA expression has also been investigated in this setting. A two-gene signature of miR-375 and miR-122 was identified in the sera of patients with stage II-III locally advanced and inflammatory breast carcinoma with the ability to predict breast cancer metastatic disease relapse with a sensitivity of 80% and specificity of 100% [110]. Patients relapsing following neoadjuvant chemotherapy were found to have significantly up-regulated expression of miR-122 while patients with higher circulating miR-375 experienced a good clinical outcome.

It is recognized that specific miRNA expression signatures are associated with resistance to all forms of breast cancer treatment, including chemotherapy, anti-endocrine therapy and radiotherapy [111-115]. Regarding chemotherapeutic resistance, several recent reports have revealed the key regulatory role of miRNAs affecting drug resistance proteins and targeting proteins involved in apoptosis (Table 1.4).

In circulatory studies, an association between serum miR-125b levels from patients with invasive ductal breast carcinoma receiving NAC and chemoresistance was described [116]. Increased expression of miR-125b was found to have a significant association ($p=0.008$) with non-response to chemotherapy. Further to this, forced miR-125b over-expression in breast cancer cells *in-vitro* increased chemotherapeutic resistance, with subsequent reduction in miR-125b levels sensitizing the cells to chemotherapy once more. Similar results were observed regarding miR-210, whereby increased plasma miR-210 levels correlated with a reduced sensitivity of HER2 breast carcinoma to *Trastuzumab* therapy. High pre-treatment circulating mir-210

was found to be associated with lower pCR rates and lymph node metastasis. Recently the radiological and clinical response of breast cancer to either neoadjuvant chemotherapy or hormonal therapy was assessed using a low-density miRNA array. Significantly increased Let-7a was found in the plasma of patients achieving a radiological response following neoadjuvant chemotherapy, but not hormonal therapy [117].

The effect of chemotherapy on miRNA expression has also been assessed. Fluctuation within a panel of miRNA was also found in patients with primary operable or locally advanced breast cancer receiving neoadjuvant chemotherapy [118]. Of a panel of eight miRNAs, miR-221, miR-195 and miR-21 were noted to decrease most significantly with the administration of chemotherapeutics, although correlation with response to systemic therapy was not conducted. A further study recognized expression of two particular miRNAs to be induced by treatment with chemotherapeutics, namely miR-34a and miR-122. Elevated expression of these miRNAs was detected both in tumour tissue and serum, and was particularly associated with anthracycline-based regimens in patients achieving partial response to neoadjuvant chemotherapy [119].

These findings highlight the potential of miRNA to act as minimally-invasive biomarkers of NAC response. To date, no large scale clinical trial has been conducted to assess the potential for a panel of circulating miRNA to predict or detect tumour response to neoadjuvant chemotherapy in breast cancer.

Table 1.4 MiRNAs with validated involvement in neoadjuvant breast cancer chemotherapeutic resistance

MIRNA	EXPRESSION	TARGET(S)	DRUG ASS^{N}	SOURCE	REF
miR-7	Decreased	MDR-1	Cisplatin	Cell Line	[120]
miR-19	Increased	MDR-1, MRP-1 & BCRP via PTEN	Paclitaxel Mitoxantrone VP-16	Cell Line	[121]
miR-21	Increased	PTEN PTEN	Doxorubicin Trastuzumab	Cell Line	[122, 123]
miR-25	Increased	ABCG2 ULK1	Epirubicin	Cell Line	[124]
miR-30c	Decreased Decreased	YWHAZ TWF1, IL-11	Doxorubicin Paclitaxel Doxorubicin	Cell Line Human Breast Tissue (n=51) Cell Lines	[125] [126]
miR-34a	Increased Decreased Decreased	BCL-2, Cyclin D1 Notch-1 E2F3, PXR	Docetaxel Adriamycin Doxorubicin	Cell Line Cell Line Cell Line	[127] [128] [129]
miR-125b	Increased Increased	E2F3 Bak 1	5-Fluorouracil Paclitaxel	Blood Serum Cell Line	[116] [130]
miR-137	Decreased	P-glycoprotein, via YB-1	Vincristine Doxorubicin Paclitaxel	Cell Line	[131]
miR-149	Decreased	NDST1	Adriamycin	Cell Line	[132]
miR-155	Increased	FOXO3a	Doxorubicin VP-16 Paclitaxel	Human Breast Tissue (n=126)	[133]
miR-200c	Decreased Decreased	P-glycoprotein MDR mRNA TrkB, Bmi1	Doxorubicin Doxorubicin	Human Breast Tissue (n=39) Cell Line Cell Line	[134] [135]
miR-210	Increased	Not studied	Trastuzumab	Blood Serum (n=43) Cell Lines	[112]
miR-221	Increased	Not studied	Adriamycin	Blood Plasma (n=125)	[136]
miR-288	Decreased	MDR-1 P-glycoprotein	Doxorubicin	Cell Line	[137]
miR-320a	Decreased	TRPC5, NFATC3	Adriamycin Paclitaxel	Cell Line	[138]
miR-345	Decreased	MDR-1	Cisplatin	Cell Line	[120]
miR-451	Decreased	P-glycoprotein, MDR-1	Doxorubicin	Cell Line	[113]
miR-489	Decreased	Smad3	Adriamycin	Cell Line	[139]
miR-663	Increased	HSPG2	Adriamycin	Cell Line	[140]

HSPG2: Heparin Sulfate Proteoglycan 2; Smad3: Mothers against decapentaplegic homolog 3; NDST1: GlcNAc N-deacetylase/N-sulfotransferase-1; TRPC5: Transient Receptor Potential Channel C5; NFATC3: Nuclear Factor of Activated T-cells Isoform C3; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; PXR: Pregnane X Receptor; BCL-2: B-cell lymphoma 2; YB-1: Y-box binding protein-1; MRP-1: Multidrug Resistance-associated Protein-1; BCRP: Breast Cancer Resistance Protein; ABCG2:*ATP-binding cassette (ABC) protein;* ULK1:*protein kinase gene; TWF1: Twinfilin 1; IL-1: Interleukin-1; TrkB: Tyrosine Receptor Kinase type 2; Bmi1: B-cell-specific Moloney murine leukemia virus integration site 1; Bak 1: Bcl-2 antagonist killer 1.*

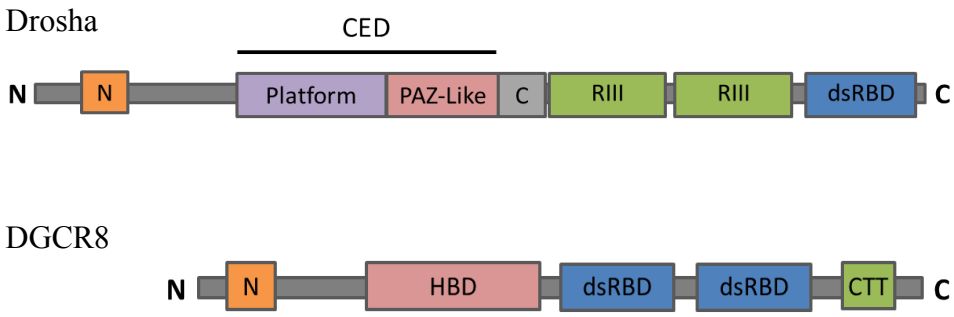
1.9 Introduction to miRNA Machinery

While miRNA have a known regulatory role in various forms of cancer, as tumour suppressors or oncogenes, dysregulation of the miRNA machinery can also play a crucial role in cancer initiation and progression, highlighting their potential for use as disease biomarkers [87, 104].

1.9.1 Drosha and DGCR8

Drosha is a class 2 ribonuclease III enzyme encoded by the Drosha gene. The critical domains of Drosha include the central domain (CED), which is highly conserved and essential for cleavage activity, and a C-terminal consisting of two tandem RNaseIII catalytic domains and a double-stranded RNA-binding domain (dsRBD). For Drosha to function, it must interact with the protein DGCR8 to form the complex known as *Microprocessor*, which consists of a heterotrimer of one Drosha with two DGCR8 molecules [141, 142]. Within this complex, Drosha forms the catalytic subunit, cleaving the 3' and 5' strands of stem-loops in pri-miRNAs to release hairpin-shaped pre-miRNA for processing by Dicer in the cytoplasm (Figure 1.1 A.). Drosha also functions to determine cleavage sites, by measuring approximately 11 base pairs from the basal junction of the dsRNA [141, 143]. The dsRBD of Drosha has weak RNA-binding capacity however, thus DGCR8 is required to increase the affinity for RNA binding and to stabilise Drosha [144]. DGCR8 is also responsible for the recognition of the substrate pri-miRNA. DGCR8 proteins contain an N-terminal region for nuclear localization, a central heme-binding domain (HBD), two dsRBDs and a C-terminal tail that is responsible for binding with Drosha [145].

A.



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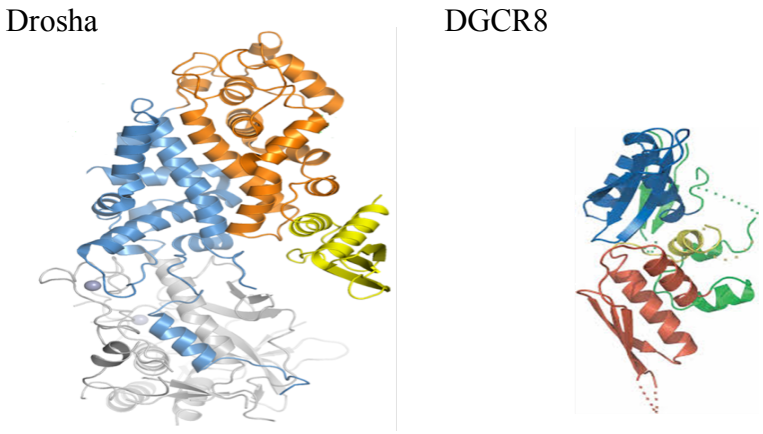


Figure 1.2 A. Schematic representation of the structure of Drosha and DGCR8. CED, Central Domain; N, N-terminal; PAZ, PIWI Argonaute Zwille; C, Connector; RIII, RNase III; HBD, Heme-binding domain; dsRBD, double-stranded RNA-binding domain; CTT, C-terminal tail. B. Crystal structure of Drosha and DGCR8. Drosha: Blue and Orange, RNase III domains; Yellow, double-stranded RNA-binding domain; Gray, Central Domain. DGCR8: Red and Blue, double-stranded RNA-binding domains; Green, Heme-binding domain.

1.9.2 Exportin-5

Pre-miRNA are transported from the nucleus to the cytoplasm by Exportin-5 (XPO5), a member of the karyopherin family of nucleocytoplasmic transport factors (Figure 1.1 B.) [146]. XPO5 not only transport pre-miRNA, but also protect them from digestion by nucleases [147]. The binding of pre-miRNA to XPO5 is Ran guanosine triphosphate (RanGTP)–dependent. XPO5 bound to RanGTP recognises and binds the 2-nucleotide 3-prime overhang structure and the double-stranded stem of the pre-miRNA. This complex of pre-miRNA, XPO5 and RanGTP then migrates into the cytoplasm by direct binding with nucleoporins, where pre-miRNA are released in response to the hydrolysis of RanGTP to RanGDP, stimulated by RanGAP and other required cofactors [148, 149]. Directionality of complex movement is dictated by the gradient produced by RanGTP being predominantly located within the nucleus and RanGDP predominantly within the cytoplasm. Once the pre-miRNA is released, XPO5 diffuses back through the nuclear pore complex to participate in another round of transport.

A.

B.

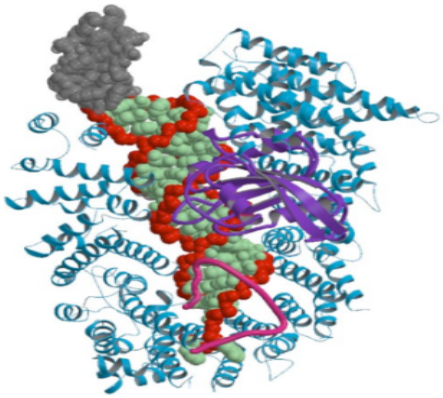
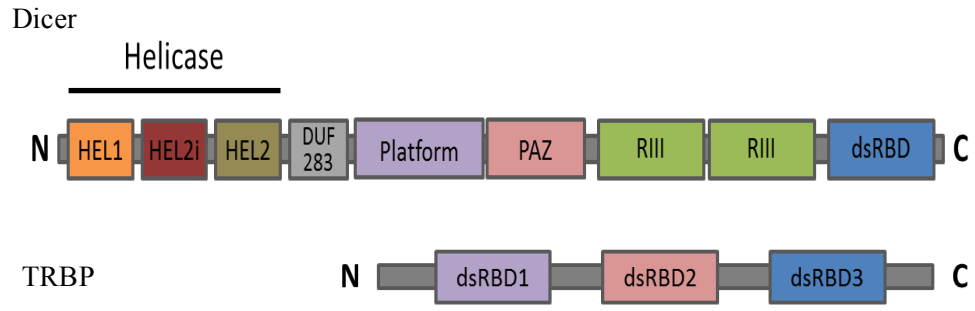


Figure 1.3 A. Schematic representation of the structure and domain of Exportin-5. B. Crystal structure of Exportin-5:RanGTP complex bound to pre-miRNA. Blue, Exportin-5; Purple, RanGTP; Green, Red and Grey, pre-miRNA complex.

1.9.3 Dicer and TRBP

Dicer is a class 3 ribonuclease II enzyme encoded by the Dicer1 gene. Once pre-miRNAs have arrived in the cytoplasm, the PAZ domain directly mediates dsRNA end recognition, and Dicer cleaves the substrate at approximately 22 base pairs from their open helical ends to produce mature miRNAs (Figure 1.1 C.) [150]. Further to this, Dicer can also generate short-interfering RNAs (siRNAs) by cleaving long double-stranded RNA (dsRNA) precursors [151]. In association with Dicer, TRBP contributes to RNA substrate binding, product length determination and the assembly of RNA-induced silencing complexes (RISCs) [152].

A.



B.

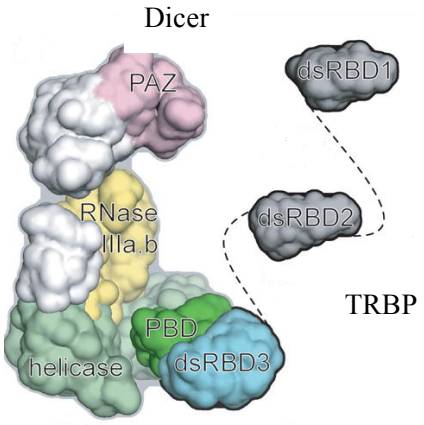


Figure 1.4 A. Schematic representation of the structure and domains of Dicer (Adapted from Lau, Guiley [153])and TRBP. HEL1, HEL2i and HEL2, three predicted globular domains; PAZ, PIWI Argonaute Zwille; RIII, RNase III; dsRBD, double-stranded RNA binding domain. B. Crystal Structure of Dicer and TRBP[154].

1.9.4 Argonaute Proteins

Argonaute (Ago) proteins play an essential role in the regulation of gene expression via small non-coding RNAs, including siRNAs, PIWI-interacting RNAs (piRNAs) and miRNAs [155-158]. These small RNA molecules directly bind to an Ago protein, becoming incorporated into Ago-containing complexes, to exact their respective functions (Figure 1.1 D.). Named due to resemblance to the tentacles of the octopus *Argonauta argo* [159], Ago proteins can be divided into two phylogenetic subclasses, the Ago subfamily and the Piwi subfamily. While Piwi proteins are mainly expressed in germline cells, binding piRNAs to silence transposable genetic elements, Ago proteins mainly interact with miRNAs and siRNAs to exact post-transcriptional gene-silencing [156, 157, 160]. This gene-silencing is achieved either by destabilization of the mRNA or by translational repression [161].

The human subfamily of Ago proteins are ubiquitously expressed, comprising AGO1, AGO2, AGO3 and AGO4. They have been detected within the cytoplasm, localised to processing bodies (P-bodies) and to the nucleus [162]. The genes of AGO1, AGO3 and AGO4 are clustered on chromosome 1, while the AGO2 gene is located on chromosome 8. Ago proteins have a molecular weight of approximately 100 kDa and are characterised by the PAZ domain, the MID domain (middle) and the PIWI domain (Figure 1.5). The PAZ domain forms a specific binding-site for the 3' protruding end of the small RNA with which it associates, while the MID domain specifically anchors the 5'end [163]. The N domain assists in the loading and unwinding of the small RNA duplex, while the PIWI domain contains the catalytic triad responsible for RNA cleavage [164]. Ago2 is the only mammalian Ago protein with the ability to exact small-RNA-directed endonucleolytic cleavage of target mRNA. While Ago3 displays a catalytic triad like Ago2, it is non-functional. Ago1 and Ago4 lack this catalytic triad altogether [165].

A.

B.

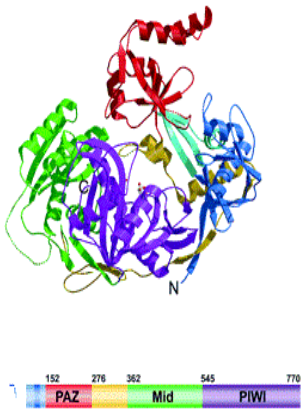


Figure 1.5A. Schematic representation of structure and domains of Argonaute. N, N-Terminal; PAZ, PIWI Argonaute Zwille; MID, Middle domain; PIWI, P-element Induced WImpy testis domain. B. Crystal structure of Argonaute. Green, Mid domain; Red, Paz domain; Blue, N-Terminal; Purple, PIWI.

1.9.5 MiRNA Machinery as Breast Cancer Biomarkers

The abnormal expression of miRNA machinery genes Drosha, DGCR8, XPO5, Dicer, TRBP and Ago2 has been reported in various cancers, as outlined in Table 1.5. With regard to breast cancer, all of the miRNA machinery genes have shown either up- or down-regulation in association with this disease. Down-regulation of Drosha and Dicer has been associated with more aggressive subtypes, while increased expression of XPO5 has been associated with increased breast cancer susceptibility [166-168]. Up-regulated TRBP has been associated with an unfavourable prognosis from breast cancer, with increased DGCR8 expression reportedly involved in breast cancer tumourigenesis and aggressiveness [169, 170]. Increased expression of the Ago2 gene has been reported in association with more aggressive breast cancer subtypes, while single-nucleotide polymorphisms of

Ago2 have been associated with disease free and overall survival in breast cancer [171, 172].

The dysregulation of the miRNA machinery genes is known to play a pivotal role in the initiation and progression of malignancies [87]. However, the molecular and cellular pathways regulating these genes and controlling miRNA biogenesis remains largely unknown [173].The use of these genes as biomarkers is still in its infancy, with further investigation required to fully elucidate mechanisms of miRNA maturation, miRNA-machinery regulation and the cancer-specific functions of these genes and their resultant proteins.

Table 1.5. MiRNA machinery gene dysregulation and associated cancer type

MiRNA Machinery Gene	Protein	Gene	Expression	Cancer Type	Ref
Drosha	√	√	Increased	Skin	[174]
	√	0	Decreased	Ovarian	[175]
	0	√		Endometrial	[176]
	0	√		*Breast*	[168]
	0	√		Neuroblastoma	[177]
	0	√		Gallbladder	[178]
DGCR8	0	√	Increased	Colorectal	[179]
	√	√		Ovarian	[180]
	0	√		Skin	[181]
	0	√		*Breast*	[169]
XPO5	0	√	Increased	Urothelial	[182]
	0	√		*Breast*	[166]
Dicer	√	√	Increased	Skin	[174]
	0	√	Decreased	Neuroblastoma	[177]
	0	√		Endometrial	[176]
	√	0		*Breast*	[168, 183]
	0	√		Gallbladder	[178]
	√	√		transitional cell carcinoma	[184]
TRBP	0	√	Increased	Prostate,	[185]
	0	√		Diffuse Large B-Cell Lymphoma,	[186]
	√	0		*Breast*	[170]
Ago2	0	√	Increased	Prostate	[185]
	0	√		Hepatocellular	[187]
	0	√	Decreased	Gastric	[188]
	√	√		*Breast*	[171, 172]
	√	√		Melanoma	[189]
	0	√		Lung	[190]

1.10 Further mechanisms for Monitoring Disease Response

In addition to the requirement of biological markers of disease response, further non-invasive mechanisms of tumour detection and *in-vivo* therapeutic response monitoring are necessary. Radiological evaluation of breast cancer is the current gold-standard technique for disease response monitoring, however, the ideal imaging modality and disease response parameter has yet to be defined (Section 1.11)[191, 192].

1.11 Current Radiological Methods for Disease Response Monitoring

No guideline currently exists regarding the ideal assessment strategy for NAC response monitoring. It has been established that clinical examination and conventional breast imaging modalities, such as mammography and ultrasonography, cannot adequately delineate the extent of disease response or residual breast disease following the administration of NAC [193, 194]. While MRI is currently considered the most accurate imaging technique for the assessment of residual disease following NAC, it is widely established that it is not ideal in this setting [41, 195]. Overestimation of residual disease can result in excessive surgical resection of breast tissue, while underestimation may result in positive margins, mandating re-operative intervention. In this post treatment setting, longest diameter and volumetric measurements have proven the most accurate means of assessing residual disease [196, 197]. For the detection of early response to NAC however, conflicting reports regarding the utility of MRI exist, with multiple studies and clinical trials ongoing[5] [198-201].

1.12 Requirement of Functional Imaging

There remains much controversy regarding radiologically-evaluated *in-vivo* tumour response to systemic therapy, defined as *complete response, partial*

[5] www.clinicaltrials.gov

response, stable disease and *progressive disease* [202]. While these definitions rely solely on the measurement of tumour size/volume reduction, it is recognised that not all disease response manifests this way [202-204]. To overcome this deficit, functional tissue imaging to detect novel endpoints of tumour response to therapy, such as changes to metabolism or tumour hypoxia, is being pursued. The use of Fluorodeoxyglucose (FDG) Positron Emission Tomography (PET) to measure response in this setting has been proposed [204]. In breast cancers responding to systemic therapy, a qualitative decline in FDG tracer uptake was identified, that antedated a reduction in tumour size [205]. However, FDG-PET is not routinely employed in disease response monitoring due to high cost, the requirement of intravenous contrast and a lack of standardised response criteria. Diffusion-weighted (DW) and dynamic contrast-enhanced (DCE) MRI have also been evaluated in this setting. DW-MRI is non-invasive and detects functional tumour response to chemotherapy by measuring changes in water diffusion following cell lysis. When a tumour is responding to therapy, cell death with cell lysis occurs, increasing the mean tumour diffusion value [206]. DCE-MRI assesses tissue vasculature and vascular permeability by measuring temporal changes in contrast enhancement within the intravascular and extravascular tissue compartments [207]. In the breast cancer setting it has been proposed that different contrast uptake patterns exist for completely responding and partially responding tumours [208]. The use of DW and DCE-MRI for NAC response monitoring in breast cancer is not routine however, due to the requirement of an intravenous contrast agent and a lack of standardisation in imaging technique and protocol [209].

There exists a requirement for a novel imaging technique with the ability to effectively detect early *in vivo* tumour response to therapy by analysing anatomical and functional parameters. This ability could enable the more accurate detection of response to therapy, potentially supporting the development of individualised patient care programmes, tailored to the characteristics of each patient's tumour. Treatment strategies could be adjusted and personalised e.g. the discontinuation of systemic therapy in

tumours displaying lack of response or progression, preventing prolonged patient exposure to the deleterious effects of chemotherapy and expediting surgical intervention.

1.13 Photoacoustic imaging

Photoacoustic (PA) imaging (PAI) represents an exciting and novel approach for detailed visualisation of breast tissue. Based on light excitation and ultrasound detection, this non-ionizing imaging modality enables real-time molecular imaging without the requirement for exogenous contrast agents [210]. The combination and co-registration of optical contrast with the high spatial resolution and deep tissue penetration of ultrasound, enables localisation of anatomical tissue structures and evaluation of the tumour microenvironment [211]. It offers a unique scalability of spatial resolution and depth penetration not obtainable with conventional imaging techniques such as mammography, ultrasonography, magnetic resonance imaging or positron emission tomography [210]. Co-registered with an ultrasound system, the unit is ideal for bedside clinical imaging.

Figure 1.6 VisualSonics Vevo® LAZR Photoacoustic Imaging System

1.13.1 PA Imaging Principles

1.13.1.1 Thermoelastic Expansion

PAI obtains optical contrast from biological tissues via thermoelastic expansion [211]. Illumination with a tunable laser causes photons to propagate diffusely within tissues. The absorption of these photons produces a miniscule localised heating effect, inducing thermoelastic expansion. This phenomenon generates pressure or ultrasound waves, which are emitted and detected by broadband multi-element linear-array transducer probes and converted into images.

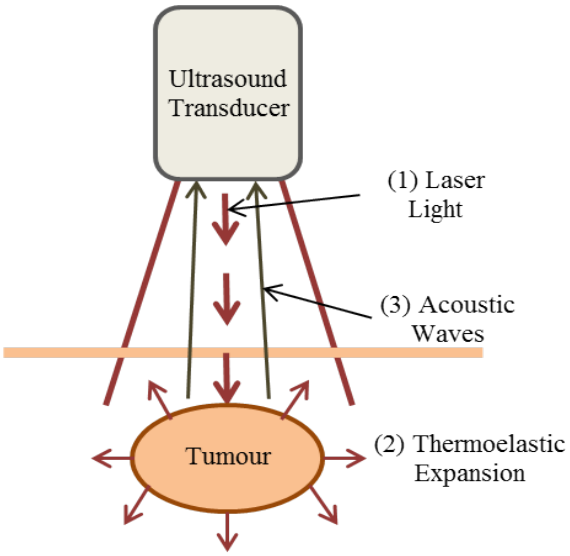


Figure 1.7 PAI mechanism. (1) Laser light is emitted from the ultrasound transducer. (2) Thermoelastic expansion occurs in response to tissue heating. (3) Acoustic waves are produced by the thermoelastic expansion and are detected by the transducer.

1.13.1.2 Gain

As light and sound travel through the tissues being imaged, their signal becomes attenuated as depth penetration increases. This attenuation, or energy loss, is due to absorption, reflection and scattering of the light and sound waves by the tissue. If not corrected, this would result in low intensity images that appear lighter in superficial layers and darker in deeper layers. To compensate for this attenuation, signal intensity of the returning sound waves can be amplified. The degree of this receiver amplification is called the gain. Increasing this gain will amplify only the returning sound wave signal, increasing the brightness of the entire image. To selectively amplify the weaker signal returning from deeper tissues, time gain compensation can be adjusted.

Different tissues display different attenuation coefficients, i.e. they attenuate light differently (calculated by decibels per centimetre of tissue). For the clinical application of PAI, evaluation of breast tissue attenuation was required in conjunction with the calculation of optimised gain to achieve effective imaging.

1.13.1.3 Contrast Agents

The main endogenous chromophores, or light-absorbing tissue components, are haemoglobin (oxy and deoxy), lipid, water and melanin. These different chromophores have known unique optical scattering and absorption properties at different wavelengths [212]. Using known coefficients of haemoglobin at defined wavelengths enables the calculation of oxygenated and deoxygenated haemoglobin and the estimation of haemoglobin concentration (Figure 1.8). Using these natural tissue components as endogenous contrast, PAI allows for molecular imaging without the requirement of exogenous contrast agents.

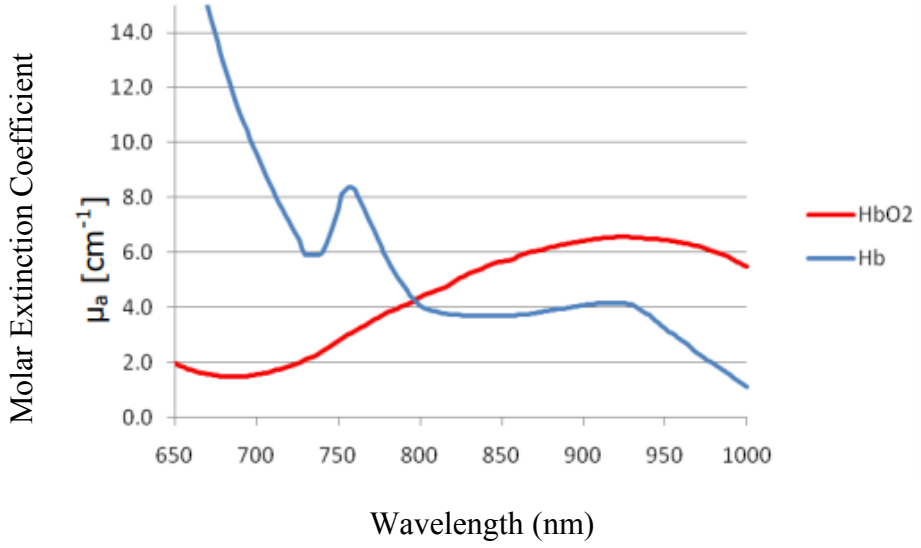


Figure 1.8 Absorption spectrum of haemoglobin (Hb) in the spectral range relevant to the VisualSonics laser system [210]. HbO2, haemoglobin oxygenated to 100%; Hb, fully deoxygenated haemoglobin.

While exogenous contrast agents are not essential for PAI, their use can greatly enhance PAI contrast [213]. The use of targeted exogenous agents, such as specific proteins or enzymes, can provide specific information regarding a molecular or cellular process of interest [214]. Small-molecule dyes can also be utilised for generalised or targeted molecular imaging, such as the conjugation of dye to a targeting antibody, such as Trastuzumab in HER2 imaging for breast cancer [215]. This study highlighted the ability to non-invasively detect HER2 overexpression in cell lines *in-vitro* by conducting PAI using fluorescent dye-tagged Trastuzumab as a contrast agent. The use of nanoparticles (plasmonic e.g. gold, silver, and non-plasmonic e.g. single-walled carbon nanotubes) as exogenous contrast agents for PAI has also been investigated. While advantageous in terms of a high tunability (absorption and scattering properties) and ease of chemical modification (e.g. dye conjugation), their long-term safety in clinical applications is of concern due to the risk of toxicity [216, 217].

1.13.2 Clinical Applicability of PAI in breast

To date, a range of pre-clinical PAI systems have been evaluated in a variety of organ systems, including ophthalmology [218], brain [219], dermatology [220], haematology [221], urology [222], gynaecology [223] and breast [224]. Regarding breast imaging, the clinical applicability of a range of PA systems has been evaluated, with some very promising results.

The ability of light-based or optoacoustic imaging systems to detect breast cancer distinct from healthy tissue has been outlined in a number of studies. The Twente Photoacoustic Mammoscope (PAM) detected high PA contrast in association with tumour tissue compared to healthy breast tissue [225]. However, technical issues with this system resulted in an appreciable volume of patient measurements not being analysed and system depth restrictions prevented lesions close to the chest wall from being imaged [225-227]. A laser-based optoacoustic system was assessed in 27 patients with suspicious breast lesions identified on conventional mammogram or ultrasound. The ability of this system to detect breast cancer was revealed, with the successful detection of 18 of the 20 lesions with confirmed malignancy [228]. In the remaining two cases, breast cancer was not visualised by the system. Further to this, diffuse optical spectroscopy and diffuse optical imaging identified distinct parameters of tumour tissue compared to healthy surrounding breast tissue [229]. These studies confirm the ability of light-based imaging techniques to detect tumour distinct form healthy surrounding tissue, highlighting their potential clinical application. However, system-specific technical issues, poor sensitivity and minimal patient numbers have limited their application in clinical breast imaging to date. The identification of a light-based imaging system that can generate standardised, reproducible images of tumours distinct from non-tumour tissue could potentially enable the expedited diagnosis of breast cancer, or be utilised for chemotherapeutic response monitoring.

1.13.3 PAI in NAC response monitoring

In conjunction with the anatomical localisation of tissue pathology, by exploiting the endogenous haem signal, PAI permits longitudinal studies of tumour progression, regression or treatment response by evaluating the vascular dynamics within the tumour microenvironment [230]. By measuring blood concentration changes within tissue and quantifying networks of vasculature formation, tumour development and treatment response can potentially be monitored [231]. A hypoxic microenvironment is known to strongly correlate with tumour growth, malignant progression and resistance to therapy, whereas an increase in tumour oxygen concentration has been reported to correlate with tumour response to systemic therapy [232, 233]. Studies assessing the potential for light-based or optoacoustic imaging in this setting have shown much promise. Upon imaging 23 patients undergoing neoadjuvant chemotherapy with diffuse optical spectroscopic imaging, a significant association was identified between an increase or flare in oxyhaemoglobin in partially responding tumours and patients achieving complete pathological response, compared to non-responsive tumours [233]. Further to this, US-guided near-infrared tomography of breast tumours in 32 women undergoing neoadjuvant chemotherapy, reported pre-treatment tumour haemoglobin content as a strong predictor of pathologic response, in conjunction with percentage change in total haemoglobin [234].

The ability of light-based imaging techniques to detect breast pathology distinct from healthy breast tissue, and to measure molecular parameters indicative of tumour response or resistance to systemic therapy, highlights an exciting potential for this form of imaging as a valuable tool in clinical practice. As a light-based imaging modality, the VisualSonics Vevo® LAZR PAI system, at the time of writing, had not previously been investigated in the clinical breast imaging setting.

1.14 Thesis Hypothesis and Aims

Hypothesis: The identification of further prognostic and predictive biomarkers for breast cancer, and novel mechanisms for detecting *in-vivo* tumour response to neoadjuvant chemotherapy could enable the individualisation of breast cancer management, and improved patient outcomes from this disease.

Aims:

- To analyse the field of miRNA research from point of discovery to present to guide current investigative goals (Chapter 2) and enable the production of targeted scientific outputs.
- To assess the fluctuation in a panel of circulating miRNAs over the course of neoadjuvant chemotherapy for breast cancer (Chapter 3).
- To determine if systemic miRNA analysis could be used to predict and monitor response to neoadjuvant chemotherapy in breast cancer (Chapter 3).
- To investigate the potential of Argoanute-2 levels to stratify breast cancer (Chapter 4).
- To assess association between Ago2 levels in clinical patient specimens with clinicopathological parameters to determine potential as a further prognostic or predictive biomarker for breast cancer (Chapter 4).
- To apply the VisualSonics Vevo® LAZR Photoacoustic Imaging system in the clinical setting to image real-time patient breast tissue (Chapter 5).
- To optimise the VisualSonics Vevo® LAZR Photoacoustic Imaging system for breast imaging (Chapter 5).
- To develop the VisualSonics Vevo® LAZR Photoacoustic Imaging system for use in the evaluation of neoadjuvant chemotherapeutic response monitoring (Chapter 5).

Chapter 2

BIBLIOMETRIC ANALYSIS OF MICRORNA

PUBLISHED MARCH 2015:

Casey M, Sweeney KJ, Brown JA, Kerin MJ. (2015) Evolution of a research field—a micro (RNA) example. *PeerJ* 3:e829 https://doi.org/10.7717/peerj.829

Featured Article in *Lab Times Online*, April 2015,
http://www.labtimes.org/editorial/e_599.lasso

2.1 Introduction

With expanding scientific production and unprecedented access to information, a means of independently assessing and analysing research output becomes apparent and essential. Consequently, various data analysis tools and internet-based search engines have been devised to enable the processing and organisation of this scientific output into a manageable form. Bibliometric parameters are one such tool utilised for this assessment. First described by Paul Otlet in 1934 [235], bibliometrics refers to the quantitative statistical analysis of publications to enable activity and dynamics within research fields to be mapped.

The onset of the digital age revolutionised the manner in which scientific knowledge is produced and distributed. Gibbons et al provide an account of this fundamental change in their concept of '*Mode 2*' knowledge production [236]. This refers to the development of a highly interactive and transdisciplinary research system that is socially distributed. Nowotny et al elaborated this concept still further, highlighting novel and contemporary scientific practices with an increasing range of '*knowledge producers*' [237]. Various theories of science evolution have been proposed, with some authors analogising the progression of research to the evolution of living organisms: the introduction of new concepts, development of novel research directions and the emergence and loss of hypotheses [238].

The field of microRNA (miRNA) research presents an exceptional opportunity to observe the progression of a novel area of scientific investigation from point of discovery to rapidly maturing field, using bibliometrics. The term microRNA was coined by Gary Ruvkun in 2001, to refer to a naturally-occurring class of short, non-coding RNA molecule between 19 and 21 nucleotides long [239]. Lee et al discovered the first miRNA in 1993, isolating *Lin-4* from the nematode *Caenorhabditis elegans* [240]. It took seven years before a second miRNA, *Let-7,* was discovered by Pasquinelli et al [241]. The revelation that *Let-7* sequence, expression and function were conserved across animal phylogeny [241], from nematodes to

humans, resulted in a research revolution. Subsequently, thousands of miRNAs have been identified in eukaryotes, including plants, fish, fungi and mammals. In humans alone, at the time of writing, approximately 2,555 unique mature miRNAs have been identified[6]. While the exact function of all recognized miRNAs remains to be fully elucidated, they are known to regulate gene expression via binding target messenger RNA (mRNA), inhibiting translation or triggering mRNA degradation. Importantly, it has been demonstrated that in addition to their inhibitory role miRNAs can function to induce or activate transcript levels [76, 77]. Through these mechanisms miRNAs fulfil a regulatory role in various cellular processes, including cell development, differentiation, proliferation and apoptosis [75, 77, 79].

Deregulated miRNA expression patterns have been noted across all organisms, encompassing a wide spectrum of pathological processes, from immunological defects in fish, altered developmental phase transition and flowering time in plants, and neurodegeneration, cardiovascular disease and cancer in humans [242, 243]. Discovery of deregulated miRNA expression led to the hypothesis that miRNAs could potentially be used as diagnostic or prognostic markers of disease. Furthermore, miRNAs are attractive therapeutic targets for the treatment of various conditions, including cancer. The novel role of miRNAs and their importance in many different processes has led to an explosion into the scientific enquiry of miRNA function. Prior to conducting research in this field, analysing its developmental stage and key milestones could provide direction and guidance for investigative goals to enable informed, targeted scientific outputs.

[6] http://www.mirbase.org/

2.2 Aim

The aim of this study was to utilise one data analysis tool, Web of Science™, in conjunction with current theories of research evolution, to quantitatively analyse a novel field of investigation, miRNAs, from point of discovery to outline progression of the field and to potentially predict its future course.

2.3 Materials and Methods

2.3.1 Database

Citation data was retrieved from the *Web of Science*™ (WoS) *Core Collection database*[7], produced by Thomson Reuters (see section 3.1). Search results from WoS encompassed entries from the *WoS Core Collection*, comprising *Science Citation Index Expanded* (SCI-EXPANDED), *Social Sciences Citation Index* (SSCI), *Arts & Humanities Citation Index* (A&HCI), *Book Citation Index* (BKCI), *Conference Proceedings Citation Index* (CPCI), *Current Chemical Reactions* (CCR Expanded)and *Index Chemicus* (IC)[8].

2.3.2 Search Terms and Methods

The WoS database was searched utilising the terms "miRNA" and "microRNA" with the Boolean operator "OR." Upon analysis of initial search findings, conflicting results were identified, namely publications containing the following: *mirna estuary, mirna bay, mirna river, mirna equation, Mirna A (author)* and *mirna SC*. As such, the Boolean operator "NOT" was utilised to exclude these findings and refine search results. Further to this, the research category of "*Agriculture*" was excluded from the search, as several inaccurate search results were identified within this classification.

Although recognised as the first miRNAs discovered, the initial publications pertaining *Lin-4* and *Let-7* did not utilise the terms microRNA or miRNA (coined in 2001), as such these papers did not feature in the search results. To account for this, the *Web of Science Core Collection* database was searched for *Lin-4* and *Let-7* in isolation, utilising the Boolean Operator "NOT" to

[7] http://apps.webofknowledge.com/
[8] http://images.webofknowledge.com/WOKRS513R8.1/help/WOS/hp_database.html

exclude miRNA and microRNA, so as to prevent overlap with original search results.

The publication timeframe analysed encompassed January 1993 to December 2013. Publications of all languages were accepted, comprising all peer-reviewed articles, including reviews, letters to the editor and editorials.

2.3.3 Data Analysis

WoS data toolswere utilised to perform certain elements of result analysis e.g. generating *Journal Citation Reports*. Additional data analysis was performed using *Microsoft Excel 2010*© and *Minitab* version 16®.

N.B. The results returned from WoS upon searching study criteria were found to increase with the passage of time. This is thought to be due to delayed indexing of journals among other factors. As a result, some figures containing quantitative numbers may differ slightly among sections of this manuscript, when totalled.

2.4 Results

2.4.1 Database

Several research platforms currently exist for examining bibliometrics, including the Web of Science™, Highwire© and PubMed®. Prior to commencing this study, the suitability of these databases was individually analysed to allow selection of the optimal data resource. Ultimately, WoS was chosen for the purpose of this study due to superior journal coverage, approximately 9,300 compared to HighWire (1,700) and PubMed (5,669). An additional significant factor was the return of a substantially higher proportion of results using the defined study search criteria (see section 2.3.2)

2.4.2 Publication Distribution

The number of published items identified pertaining to miRNAs, as catalogued in the Web of Science™ Core Collection database (1993-2013), totaled 26,177 publications. The first publication occurred in 1993, with minimal additional publications (35) until 2003 (Figure 2.1). 62% of all miRNA literature (16,348 publications) was published within the period 2011-2013 inclusive. Currently, the highest output occurred in 2013, with 25% of total miRNA publications (6,560). Of total publications identified, 99.2% of items were published in English (25,980 publications), with the remainder of articles in French (49 publications), Chinese (46 publications), German (38 publications), Spanish (17 publications), Polish (14 publications), Japanese (9 publications), Czech (2 publications) and Hungarian (2 publications) (Figure 2.2).

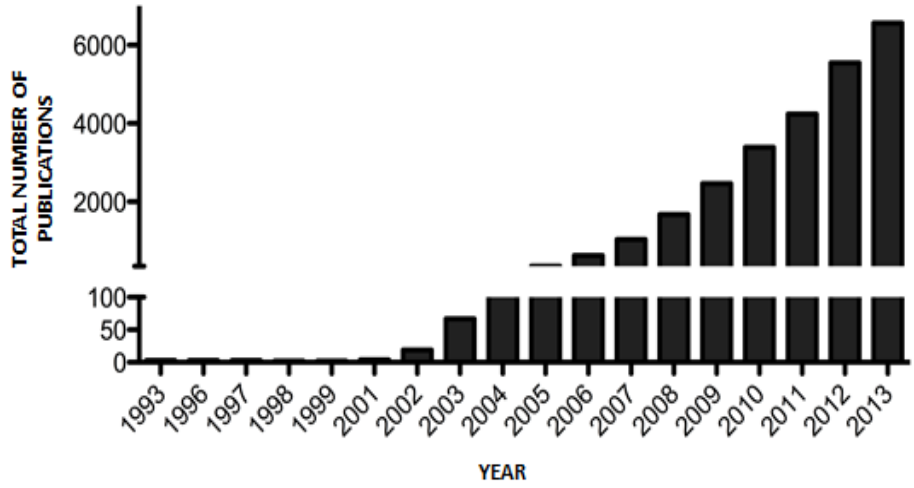


Figure 2.1 Number of miRNA publications per year

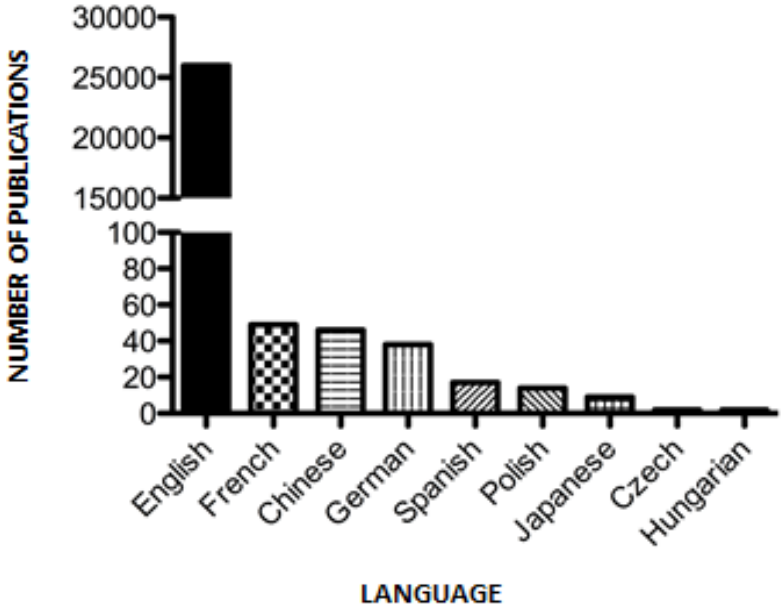


Figure 2.2 Language of miRNA publications

2.4.3 Publications by Country

To further analyse miRNA-related literature, the distribution of publications by country was determined. In total, 84 countries contributed to the miRNA literature, as outlined in Table2.1 (Top 25 countries). The five most prolific countries accounted for 83% of total publications (21,652). The USA was responsible for 42% of all miRNA literature (11,056 publications) followed by the Peoples' Republic of China 21% (5,584 publications), Germany 8% (2,083 publications), the United Kingdom (1,527 publications) and Japan 6% (1,474 publications). Further nations featuring within the top 10 most prolific countries included Italy 6% (1,455 publications), Canada (904 publications), France (848 publications), Australia (650 publications) and South Korea (645 publications) (Figure 2.3). Interestingly, separating the United Kingdom into its constituent countries, England alone would rank 6th (1,239 publications) behind the USA, the Peoples' Republic of China, Germany, Japan and Italy.

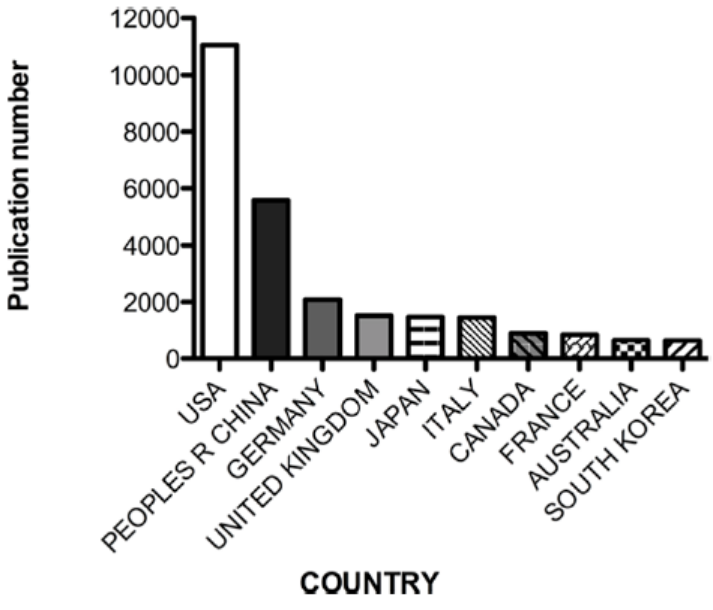


Figure 2.3 Number of Publications per Country

Table 2.1 Rank of 50 countries publishing miRNA material most prolifically with cumulative number of publications per country.

Rank	Country	No. publications
1	United States	11,056
2	People's Republic of China	5,584
3	Germany	2,083
4	United Kingdom	1,527
5	Japan	1,474
6	Italy	1,455
7	Canada	904
8	France	848
9	Australia	650
10	South Korea	645
11	Netherlands	625
12	Spain	621
13	Switzerland	492
14	Denmark	420
15	Taiwan	411
16	India	371
17	Israel	348
18	Sweden	335
19	Belgium	292
20	Ireland	254
21	Singapore	239
22	Austria	203
23	Brazil	201
24	Poland	184
25	Greece	158

2.4.4 Research Categories

Analysing the ontological research categories by which miRNA publications were classified in the WoS database, the top 50% of miRNA publications identified were categorised into medicine and health sciences, comprising Biochemistry Molecular biology (23.5%, 6,163 publications), Oncology

(15.5%, 4,070 publications), Cell biology (15%, 3,923 publications) and Genetics heredity (10%, 2,554 publications) (Table 2.2). Further ontological categories of miRNA research listed included horticulture, marine science and entomology.

Table 2.2 Rank of 25 research categories featuring miRNA publications most frequently, with number of publications per research category, and percentage of overall publications.

Rank	Category	No. Publications	Percent of Total Publications (%)
1	Biochemistry Molecular Biology	6163	23.53
2	Oncology	4070	15.54
3	Cell Biology	3923	14.98
4	Genetics Heredity	2554	9.75
5	Science Technology Other Topics	2474	9.45
6	Biotechnology Applied Microbiology	1634	6.24
7	Research Experimental Medicine	1623	6.19
8	Haematology	1131	4.32
9	Cardiovascular System Cardiology	1068	4.077
10	Neurosciences Neurology	1057	4.04
11	Pharmacology Pharmacy	935	3.57
12	Gastroenterology Hepatology	906	3.46
13	Pathology	895	3.42
14	Biophysics	893	3.40
15	Plant Sciences	745	2.84
16	Immunology	701	2.68
17	Developmental Biology	696	2.66
18	Chemistry	575	2.19
19	Endocrinology Metabolism	573	2.19
20	Life Sciences Biomedicine Other Topics	516	1.97
21	Virology	482	1.84
22	Mathematical Computational Biology	411	1.57
23	General Internal Medicine	344	1.31
24	Surgery	333	1.27
25	Physiology	308	1.18

2.4.5 Peer-Reviewed Publications

The categories of research, as defined in Table 2.2, were comprised of miRNA publications that featured in various international peer-reviewed journals. Table 2.3 outlines the 50 most prolific journals that published miRNA research during the study period. In this 20 year period, the youngest journal *Public Library Of Science ONE* (*PLoS ONE)* delivered the largest number of miRNA-related publications at 1,589 (6% of total), with *Nucleic Acids Research* responsible for 489 publications (2% of total) and *Proceedings of the National Academy of Science of the USA (PNAS)* 451 publications (2% of total). It was found that the top 50 journals were responsible for 38% of total miRNA publications, of which the top 10 journals represented 18% of total miRNA publications identified.

Table 2.3 Rank of 50 journals that published miRNA material most frequently, with cumulative number of publications per journal.

Rank	Journal	No. Publications	Rank	Journal	No. Publications
1	PLoS ONE	1589	26	Circulation Research	135
2	Nucleic Acids Res.	489	27	Clinical Cancer Res.	134
3	PNAS	451	28	Molecular Cell	125
4	BLOOD	432	29	Gene	123
5	Journal of Biological Chemistry	346	30	Carcinogenesis	120
6	Biochem Biophys Res Communications	329	31	British Journal of Cancer	116
7	RNA a Publication of the RNA Society	303	32	Int Journal of Mol Sciences	114
8	Cancer Research	302	33	Bioinformatics	109
9	BMC Genomics	296	34	Journal of Immunology	107
10	Hepatology	252	35	BMC Bioinformatics	102
11	Circulation	246	36	Plant Cell	102
12	Oncogene	211	37	International Journal of Oncology	101
13	Faseb Journal	210	38	Neuro Oncology	99
14	Cell Cycle	200	39	Mol Biology Rep	97
15	Febs Letter	180	40	Science	96
16	Modern Pathology	179	41	European Journal of Cancer	95
17	Gastroenterology	176	42	Febs Journal	93
18	Laboratory Investig.	171	43	Molecular Therapy	93
19	Genes Development	167	44	Genome Research	92
20	Journal of Virology	162	45	Development	91
21	Cell	159	46	American J Respir Critical Care Med	90
22	RNA Biology	146	47	Current Biology	90
23	International Journal of Cancer	136	48	EMBO Journal	90
24	Nature	136	49	PLoS Genetics	90
25	Oncology Reports	136	50	Cancer Letters	89

2.4.6 Document Type

MiRNA publications were comprised of various document types including original articles, review articles, news items, editorial material, corrections, reprints, database reviews, proceedings papers, letters and meeting abstracts. Of the 26,177 publications identified, 69% of all documents were original research articles (18,111 publications). In addition to original articles, 14% of published items comprised meeting abstracts (3,659 publications), 13% review articles (3,314 publications) and 3% were editorial material (647 publications). The remaining publications comprised of a minimal number of article corrections, news items and reprints. Table 2.4 shows an analysis of published document types for the top 10 journals which published miRNA-related documents. Constant with the overall trend, articles featured most prominently, comprising 88% of publications for these top 10 journals (4,789 publications), followed by meetings abstracts 8% (393 publications), reviews 1.5% (73 publications), editorial material 0.6% (28 publications) and corrections 0.4% (20 publications). Proceedings papers, letters and database reviews comprise the remainder, providing minimal input.

Table 2.4 Analysis of document type featuring in the 10 journals publishing miRNA literature most prolifically

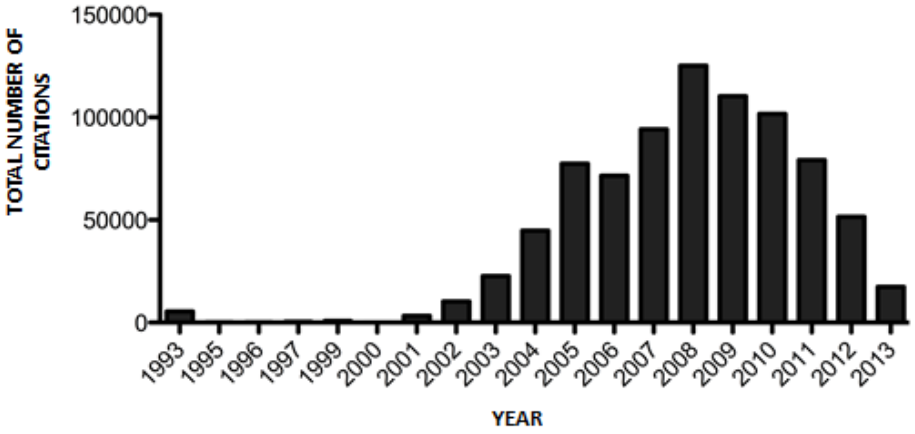
Rank	Journal	Article	Review	Correction	Editorial Material	Letter	Meeting Abstract	Proceeding Paper	Database Review
1	PLOS ONE	1587	2	2	2	-	-	-	-
2	Nucleic Acids Research	485	3	3	3	-	-	-	-
3	PNAS	438	-	-	-	1	-	-	-
4	Blood	170	13	13	13	1	239	1	
5	Journal of Biological Chemistry	315	6	6	6	-	-	1	-
6	Biochem Biophy Res Comm	310	17	17	17	-	-	-	-
7	RNA a Publication of the RNA Soc	283	13	13	13	2	-	-	-
8	Cancer Research	267	17	17	17	2	10	-	-
9	BMC Genomics	293	-	-				19	1
10	Hepatology	93	2	2	2	11	144	-	-

2.4.7 Publication Citations

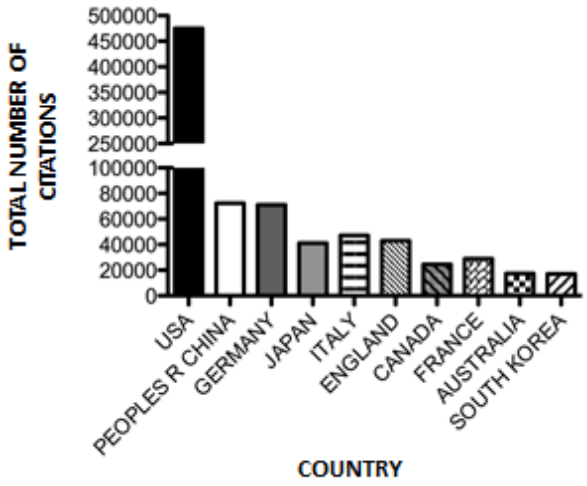
837,898 citations, for all miRNA related publications, were found by querying the *WoS Core Collection* Database. In concordance with publication numbers, citations per year increased exponentially peaking in 2008, at which point citation rate decreased (Figure 2.4A). Ranking the total number of citations by country, publications originating from the USA received the highest total number of citations (n=475,300) followed by China (n=72,265), Germany (n=71,051), Italy (n=47,084) and England (n=42,970) (Figure 2.4B). Analysing the average citation per item for the top 10 countries publishing miRNA material, the USA retained the first position (44.3 citations per item). However, the remaining positions changed with the second position now held by England (35.9 citations per item) followed by Germany (35.4 citations per item), France (34.4 citations per item) and Italy (34.2 citations per item). Interestingly, of the top ten countries China now displays the least number of citations per item (n=14.5) (Figure 2.4C).

Examining the citations per item from the top 20 countries (as described in Table 1), the rankings change considerably with Switzerland (originally 13th) now displaying the highest number of citations per item at 55.1, followed by USA (44.3 citations per item), then the Netherlands (39.6 citations per item), England (35.9 citations per item) and Sweden (35.8 citations per item) (Figure 2.5).

A.



B.



C.

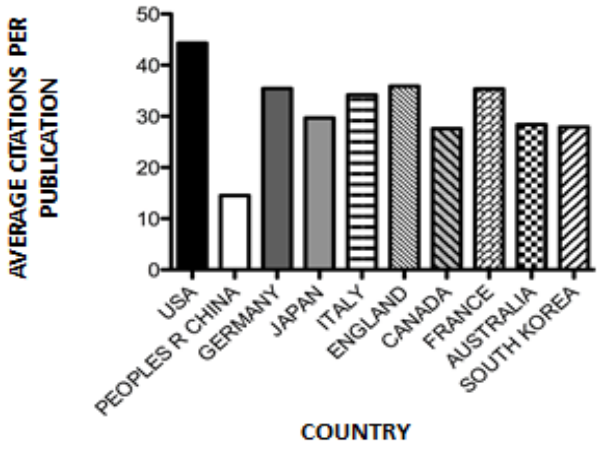


Figure 2.4 **A.** Number of citations per yearly publication. **B.** Number of cumulative citations per country. **C.** Average citation per publication per country.

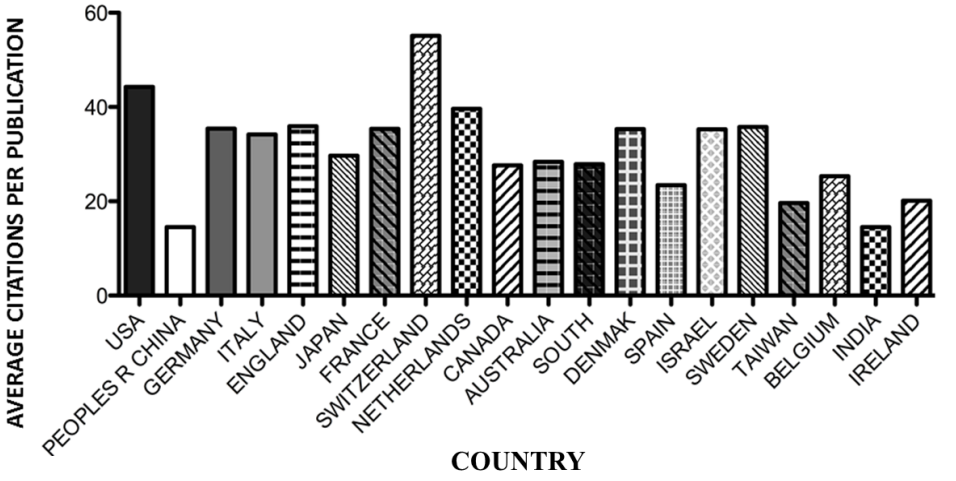


Figure 2.5 Average average citation per item publication per top 20 countries publishing miRNA material

To further analyse the citation pattern, we investigated citations of the top 10 journals publishing miRNA material (Figure 2.6). *Proceedings of the National Academy of Science of the USA (PNAS)* was the most frequently cited with 6% of the total citations (46,112 citations), followed by *Cancer Research Nucleic Acids Research* and *PLoS ONE* each with 3% of total citations (26,763, 26,118 and 20,242 citations respectively). The final entry in this list is *RNA A Publication of the RNA Society* with 2% of the total citations (19,217 citations).

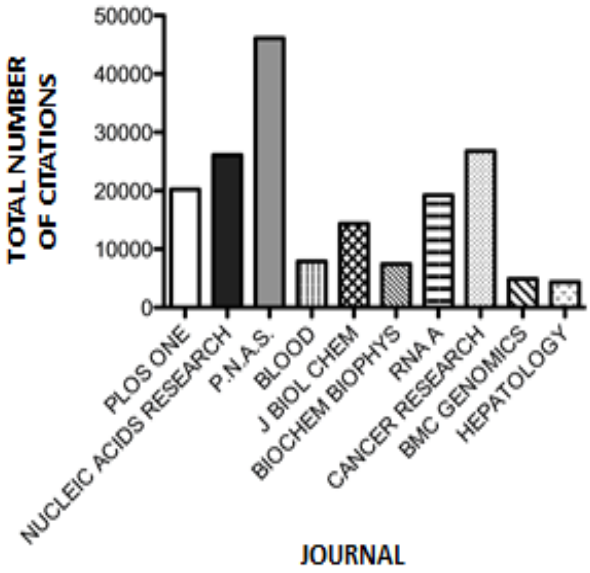


Figure 2.6 Number of citations per journal for the 10 journals publishing miRNA material most prolifically.

Considering citations per publication, Table 2.5 outlines the 10 most cited miRNA publications since the discovery of this research field. The top 3 publications cited featured in the journal *Cell*, with an accumulative total of 11,581 citations (1.4% total citations). 6 of the top 10 publications cited featured in *Nature, Nature Reviews Cancer or Nature Methods,* with one publications featuring in *PNAS.* The first, seminal, miRNA publication by Lee et al ranks third (3,671 citations) and the second key miRNA publication by Pasquinelli et al [241] features 28th (931 citations). The top 10 cited papers feature over a 16 year period, with three publications in 2005, three in 2006 and one publication in each of the years 1993, 2004, 2008 and 2009. Of this top 10, four items are review articles.

Table 2.5 Ten most cited miRNA publications since the discovery of this research field

Rank	Citation	Title	Author	Journal	Year
1	4,167	Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets	Lewis BP, et al	Cell	2005
2	3,743	MicroRNAs: Target Recognition and Regulatory Functions	Bartel DP, et al	Cell	2009
3	3,671	The C-Elegans heterochronic gene Lin-4 encodes small RNAs with antisense complementarity to Lin-14	Lee RC, et al	Cell	1993
4	3,512	MicroRNA expression profiles classify human cancers	Lu, J et al	Nature	2005
5	2,976	The functions of animal microRNAs	Ambros V.	Nature	2004
6	2,391	Oncomirs – microRNAs with a role in cancer	Esquela-Derscher A, et al	Nature Rev Cancer	2006
7	2,337	Mapping and quantifying mammalian transcriptomes by RNA-Seq	Mortazavi A, et al	Nature Methods	2008
8	2,291	A microRNA expression signature of human solid tumors defines cancer gene targets	Volinia S, et al	PNAS	2006
9	2,196	MicroRNA signatures in human cancers	Calin GA, et al	Nature Rev Cancer	2006
10	2,168	Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs	Lim LP, et al	Nature	2005

To further examine significant miRNA publications, the top 10 most cited primary research miRNA publications were identified (Table 2.6). *Cell* was still seen to contribute 3 of the top 10, with *Nature, Nature Genetics* and *Nature Methods* contributing 5 publications and *PNAS* one publication, with the addition of one publication by *Science.*

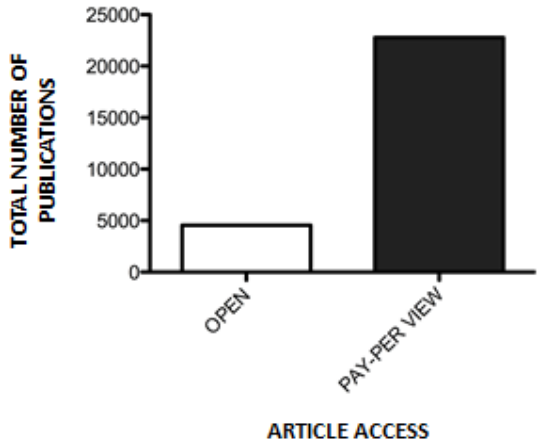
Table 2.6 Ten most cited primary research miRNA publications since the discovery of this research field

Rank	Citations	Title	Author	Journal	Year
1	4,167	Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets	Lewis BP, et al.	Cell	2005
2	3,671	The C-Elegans heterochronic gene Lin-4 encodes small RNAs with antisense complementarity to Lin-14	Lee RC, et al.	Cell	1993
3	3,512	MicroRNA expression profiles classify human cancers	Lu J, et al.	Nature	2005
4	2,337	Mapping and quantifying mammalian transcriptomes by RNA-Seq	Mortazavi A, et al.	Nature Methods	2008
5	2,291	A microRNA expression signature of human solid tumors defines cancer gene targets	Volinia S, et al.	PNAS	2006
6	2,168	Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs	Lim LP, et al.	Nature	2005
7	2,151	Prediction of mammalian microRNA targets	Lewis BP et al.	Cell	2003
8	2,009	Identification of novel genes coding for small expressed RNAs	Lagos-Quintana M et al.	Science	2001
9	1,954	Combinatorial microRNA target predictions	Krek A et al.	Nature Genetics	2005
10	1,851	The nuclear RNase III drosha initiates microRNA processing	Lee Y et al.	Nature	2003

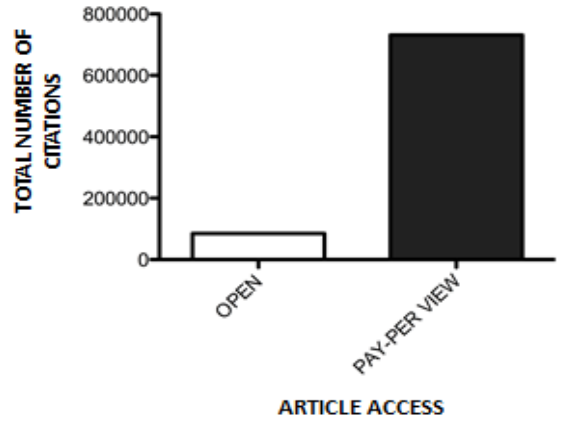
2.4.8 Open Access Vs Pay-Per-View

Of the miRNA related publications identified, 17% of publications were open access (n=4,560), with 83% of publications pay-per-view access (n=22,788) (Figure 2.7A). Analysing the citations of these two categories of publication, open access items were cited 84,864 times, representing 10% of overall citations. However, pay-per-view publications were cited 731,470 times, which represented 90% of overall citations (Figure 2.7B). Average citation per publication reveals 32.1 citations per pay-per-view publication, compared to 18.6 citations per item for open access publications (Figure 2.7C).

A.



B.



C.

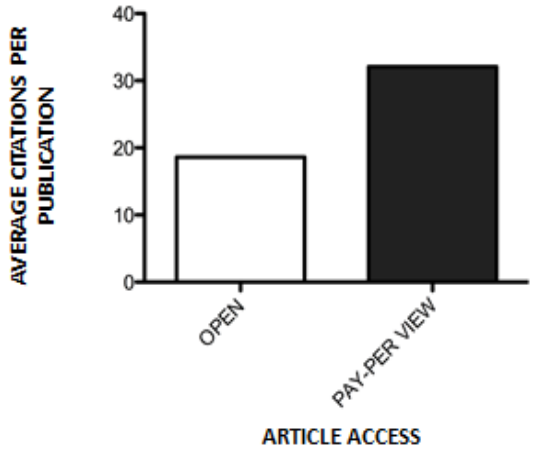


Figure 2.7. A. Number of open access versus pay-per-view publications.
B. Number of cumulative citations by open versus non-open access publications.
C. Average number of citations per open and pay-per-view publication.

2.4.9 Hallmarks of miRNA research

Analysing the bibliometric data retrieved, we identified the key discoveries in the field of miRNA research. The seminal miRNA publication, outlining the discovery of these short RNA molecules, by Lee et al [240] is certainly the first hallmark of miRNA research. Subsequent to this, recognition of conservation of miRNA sequence expression across animal phylogeny from nematodes to humans by Pasquinelli et al [241], with the identification of further miRNAs, also represents a significant key advancement in this field. As previously discussed (section 2.3.7), these papers feature third and twenty-eighth respectively in the most cited miRNA publications, highlighting their visibility and influence.

Following these crucial findings, discovery of the regulatory roles of miRNAs in various cellular processes, from differentiation to apoptosis, should be considered highly significant in furthering the understanding of the functionality of these short RNA molecules [75, 77, 79]. The next, related key event in the miRNA field was the discovery of deregulation of miRNA expression associated with human diseases, such as cancer [242-244]. This discovery raises a potential use of miRNA, as both predictive and prognostic biomarkers of disease. At present, multiple clinical trials are currently registered with ClinicalTrials.gov, investigating the ability of miRNA to function as biomarkers of disease and response to current therapies.

Another key event in the evolution of the field has been the discovery that miRNA are capable of extra cellular signalling [89]. This novel finding significantly added to knowledge of the mechanisms that can be employed for cell-cell signalling and communication. The most recent development in the field that should be considered significant is the therapeutic use of miRNAs as targeted therapies to modulate disease [245]. The attainment of personalised disease management via the use of miRNAs is highly appealing, though many obstacles currently remain, including identification of optimal delivery methods, off-target effects and safety.

2.5 Discussion

This work represents the first bibliometric analysis of the miRNA field. Analysis revealed an exponential increase in research output, with yearly publications more than quadrupling between 2005 (n=356) and 2008 (n=1672), and increasing eighteen-fold by 2013 (n=6,560) (Figure 2.1). In describing phylomemetic patterns of science evolution, Chavalarias and Cointet (2013) outline the importance of '*special events*' in the progression of a research field, with scientific output increasing significantly during the subsequent time period. '*Special events'* pertaining to the miRNA field can be identified by analysing the most cited of all primary research miRNA publications since the discovery of this research field (Table 2.4). The timeline covered by the 10 most cited primary research miRNA publications spans from 1993 to 2008, highlighting the continual key discoveries being made in the miRNA field. Of these ten publications, three were published in the journal *Cell,* with five published in *Nature* or one of its subsidiary journals, one publication in *PNAS* and one publication in *Science* (Table 2.6). The average *journal impact factor* of the ten most cited primary research miRNA publications is 27.8, highlighting their visibility and influence as a driving force behind the exponential increase in yearly miRNA publications. Currently, one key event in the evolution of the field was recognised that was not identified in this bibliometric analysis, and that is miRNA involvement in extracellular signalling [89]. This publication did not make it into the top 10 most cited papers (although it has >1200 citations), possibly due to its relatively recent publication date (2008). However, it is anticipated that in the future, this publication will enter the top 10 most cited papers in the field. The trend of miRNA publications therefore, adheres to Chavalarias and Cointet's association, with output increasing exponentially in concordance with hallmarks of their brief history – discovery [240], recognition of deregulation in cancer [242], cardiovascular disease [243], autoimmune and neurodegenerative disease [244, 246], potential use as disease biomarker [247], expression in serum/plasma [247] and use of anti-miR's as targeted therapy [245].

While 84 countries have contributed to miRNA literature to date, five countries dominate scientific production in this research field (Table 2.1). The USA, China, Germany, the United Kingdom and Japan are collectively responsible for more than 80% of all current miRNA literature with 96% of total citations. The distribution of citations per country differs considerably when considering average citations per publication of the 20 most prolific countries (Figure2.5). Switzerland exhibits the highest number of citations per item, with the USA featuring second, followed by a grouping of the Netherlands, England, Sweden and Germany. While China features second in the top 10 countries publishing miRNA material, it exhibits the lowest number of citations per publication (n=14.5), reflecting a large portion of uncited literature (Figure 2.5).

Further analysing citations by journal access type (open compared to pay-per-view), it was observed that pay-wall restricted journals, which represented the majority of publications (83%), accounted for 90% of total citations. Interestingly, the average citation per publication was 1.7 times higher for pay-wall restricted journals compared to open journals (Figure 2.7C). While this adheres to currently observed citation patterns in the literature, due to the growing popularity of open access journals and open-access publication requirements from funding agencies, it is proposed that this discrepancy will no longer be apparent in future years, with open-access publications and citation numbers currently increasing [248].

Of the miRNA publications identified in the WoS database, it is interesting to note that 69% of all documents were original articles, reflecting the relative youth of this novel field of investigation, with only ~10 years of sustained multi-group research efforts. In an analysis of the progression of a field of science, Bonaccorsi (2008) outlines increasing diversity within research paradigms as an instrumental factor, attributing this to various scientific hypotheses and the investigative techniques applied to examine them [249]. With the discovery of a definitive role for miRNA in the pathogenesis of multiple disease processes, the predominance of medicine and health sciences becomes apparent (Table 2.2). Categories of published research span the

gamut of medical domains, from immunology to oncology, haematology to virology and neuroscience to surgery. While further areas of investigation feature such as entomology and agriculture, their contribution to overall research output is currently negligible.

In an analysis of the dynamic interest in research topics within the biomedical scientific community, Michon et al [250] identified trends that are exemplified by this analysis of miRNAs. When novel research is initially published, it generally appears in high impact journals, followed by a lag in scientific output, prior to subsequent progression of publications. The initial miRNA publication outlining the discovery of lin-4 appeared in 1993 in the journal '*Cell'* which then brandished an impact factor of 37.2. Subsequent to this however, miRNA output did not significantly progress further until 2003. While miRNA publications began to escalate, the average journal impact factor of the top 10 publishing journals was 13.6. Five years later, with miRNA output increasing still further, the equivalent impact factor decreased to 9, with a further drop to 7.4 by 2012. With increasing scientific output, a shift towards lower impact journals is seen, producing a *long-tail distribution* of publishing when viewed by host journal impact factor. Originally described by Vilfredo Pareto, a social economist, the *long-tail distribution* can refer to a number of observable phenomena. In this context, it is used to describe a publishing pattern whereby high and medium impact factor journals feature in the minority, with the majority of journals having minimal citation impact [250]. Presence of this distribution is recognised as a sign of acceptance of a research topic as valid within the scientific community [250]. Reaching this stage of publication saturation, Pfeiffer et al [251] advocate the development of novel research directions within a given field as particularly advantageous, with pioneering work potentiating publication in high impact journals, and thus returning the cycle to the beginning of the *long-tail distribution* once more.

2.6 Conclusion

The current unprecedented access to scientific material and bibliometric information provides an opportunity to analyse the dynamics of scientific landscapes, enabling the production of informed, targeted scientific outputs. When we consider the ongoing remodelling of scientific production, this analysis of publication trends, citations and distribution patterns was very informative. Recognising the developmental stage of a particular research field provides researchers with direction and guidance, both in current and future investigative goals. The requirement of novel miRNA research directions was identified in this study, which prompted recognition of a novel research query; miRNA as disease response markers in breast cancer neoadjuvant chemotherapy. This question was pursued in the ICORG 10-11 National Clinical Trial.

Chapter 3

CIRCULATING MIRNAS: NOVEL BREAST CANCER BIOMARKERS AND THEIR USE FOR GUIDING AND MONITORING RESPONSE TO THERAPY

NATIONAL CLINICAL TRIAL.

PROTOCOL NUMBER: ICORG 10-11

3.1 Introduction

Breast cancer is a heterogeneous disease characterised by discrete molecular breast cancer subtypes. Each subtype has known distinct clinical behaviours and responses to therapy. As a result of this, the management of breast cancer has transformed considerably in recent years, including the administration of chemotherapy in the neoadjuvant setting.

Historically, neoadjuvant chemotherapy (NAC) was reserved for locally advanced breast carcinoma, converting technically inoperable tumours into candidates for mastectomy. With the increasing trend toward breast conserving surgery however, the use of primary systemic therapy was extended to include patients with invasive, early-stage operable tumours. The adoption of NAC has led to increasing rates of breast conserving surgery and provides an opportunity to assess *in-vivo* tumour responsiveness to chemotherapeutics [48]. Although previous studies failed to identify any improvement in disease free and overall survival between neoadjuvant and adjuvant therapies, it has been established that patients achieving *complete pathological response* (pCR) to NAC therapy experience improved outcomes, while unresponsive patients or patients with progressive disease during NAC experience worse outcomes [36, 49, 50]. Supporting this, it has been shown that the early response to neoadjuvant treatment can predict pCR and therefore may serve as a predictor of long-term outcome [52, 53].

Unfortunately, at present, there is no reliable method for predicting neoadjuvant chemotherapeutic responders from non-responders. While the likelihood of achieving pCR varies greatly by breast cancer subtype (from 7.5% in luminal cancers to 45% in HER2/Triple negative cancer [252]) many patients are exposed to the potential morbidity and mortality associated with chemotherapy, without any certainty of benefit from treatment [253]. This has resulted in global efforts to discover breast cancer biomarkers that can predict and detect response to neoadjuvant therapy. Such biomarkers could confer multiple benefits, including tailored patient-care programs, reduced chemotherapy-induced morbidity or mortality and potentially expedite the

identification of effective new therapies for the treatment of breast carcinoma. At present, circulating microRNAs (miRNAs) represent a forerunner in the search for a non-invasive biomarker for breast cancer.

In a seminal 2005 study, genome-wide miRNA expression analysis enabled identification of miRNAs that were differentially expressed in breast cancer tissue compared to healthy controls [103]. Importantly, miRNA expression correlated with distinct tumour phenotypes, ER and PR expression and tumour stage. Previous work published by our group independently corroborated these findings. In whole blood samples, our research group found that miR-195 expression was significantly elevated in breast cancer patients compared to controls, and that these levels reduced post-operatively. Furthermore, high levels of circulating miR-21 and miR-10b were found to be associated with ER negativity, thus a poorer prognosis [105].
The ability of miRNA expression profiles to classify breast tumours by biopathologic variables currently utilized to determine responsiveness to neoadjuvant chemotherapy highlights the potential of miRNA signatures as novel predictive and prognostic biomarkers that could allow individualization of breast cancer treatment and improved selection of patients for neoadjuvant chemotherapy.

Cancer Trials Ireland (formerly ICORG, the All-Ireland Co-operative Oncology Research Group ICORG)[9] was founded in 1996 by a group of consultant physicians specializing in cancer management. It is a not-for-profit registered charity that is funded by the Irish Cancer Society and the Health Research Board. The aim of this group was to create a formal structure for clinical research in Ireland, making it a more attractive location for international cancer research groups and the pharmaceutical industry.
At present, over 95% of consultants treating cancer in Ireland are members of Cancer Trials Ireland, comprising consultants specializing in medicine, surgery, radiation oncology and haematology, as well as research specialists

[9] http://www.cancertrials.ie

including oncology research nurses and translational scientists. Since incorporation, Cancer Trials Ireland and has opened 350 research trials, involving more than 15,000 cancer patients. Under the auspices of Cancer Trials Ireland, the ICORG 10—11 National Clinical trial was initiated to investigate the potential of a panel of circulating miRNA to serve as non-invasive predictive and prognostic biomarkers for breast cancer.

3.2 Aim

The stated aims of the study protocol were to

- Investigate correlation between the expression of a panel of circulating miRNA with patient clinicopathological parameters including breast cancer subtype, tumour stage and grade, nodal status etc.
- Investigate if systemic miRNA analysis can be used as a biomarker for monitoring breast cancer response to neoadjuvant chemotherapy.

As per the approved study protocol, and given temporal restraints on this thesis, outlined hereafter is the blinded interim analysis of the samples collected from the initial 50% of patients enrolled to the ICORG 10-11 National Clinical trial (n=239). The aims of the present chapter were to

- Complete data collection and analysis so that the interim power calculation could be conducted (as per study protocol).
- Assess the fluctuation in a panel of circulating miRNAs over the course of neoadjuvant chemotherapy for breast cancer.
- Determine if systemic miRNA analysis could be used to predict and monitor response to neoadjuvant chemotherapy in breast cancer.

3.3 Materials and Methodology

3.3.1 Ethical Approval

Ethical approval to conduct the ICORG 10-11 Clinical Trial was sought and granted from the Clinical Research and Ethics Committee, Galway University Hospital (Appendix 1).

3.3.2 Study Centres

As ICORG is a national body, patient accrual was open to multiple centres throughout Ireland, including Galway University Hospital, St. James' Hospital, Beaumont Hospital, Bons Secours Hospital Cork, Sligo General Hospital, Waterford Regional Hospital and Letterkenny General Hospital.

3.3.3 Patient selection, enrolment and anonymization

Patients who satisfied the following inclusion criteria were invited to participate in this prospective trial:

1. Newly diagnosed with breast cancer, without metastatic disease and scheduled to undergo neoadjuvant chemotherapy.
2. Aged 18 years or over.
3. Capable of providing written informed consent.

Written informed consent was obtained from each eligible patient (Appendix 2). Independent ICORG appointed investigators then completed a Patient Registration Form, with each patient being assigned a study number so that effective anonymization could be achieved. All Case Report Forms and study communication identified patients by initials and assigned study number only, ensuring adequate blinding of data. All patient clinicopathological and

demographic data was retained at ICORG headquarters (Dublin), ensuring blinded data analysis at our centre[10].

Patients could withdraw from the trial voluntarily at any point as per EU directive and ICH-GCP guidelines. An Off-Study Form was completed, and if requested patient samples would be destroyed.

3.3.4 Sample size

ICORG appointed study biostatisticians conducted a preliminary power calculation using miR-195 expression data from previous work conducted by this research group. A total of 122 patients would be required to achieve 80% power to detect differences in miRNA expression levels as small as 0.136 RQ units (figure 3.1). Stipulated in the study protocol was the requirement to revisit the power calculation once 50% of samples had been collected and analysed, so that minimal group size could be re-estimated.

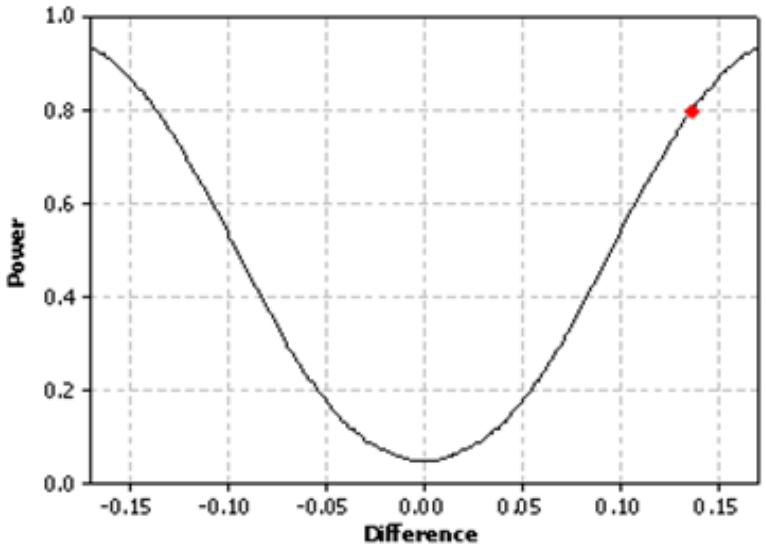


Figure 3.1 Power calculation for ICORG 10-11

[10]https://clinicaltrials.gov/ct2/show/NCT01722851?term=circulating+miRNA&rank=1

3.3.5 Blood Sample Collection

Whole blood was collected in Vacutainer® K2 EDTA tubes (Becton, Dickinson and Company) and stored at 4°C until transported to the laboratory in the Department of Surgery at NUI Galway for processing. The timepoints at which blood samples were collected are illustrated in figure 3.2. Samples were collected at a time at which each patient was providing routine blood samples for laboratory analysis.

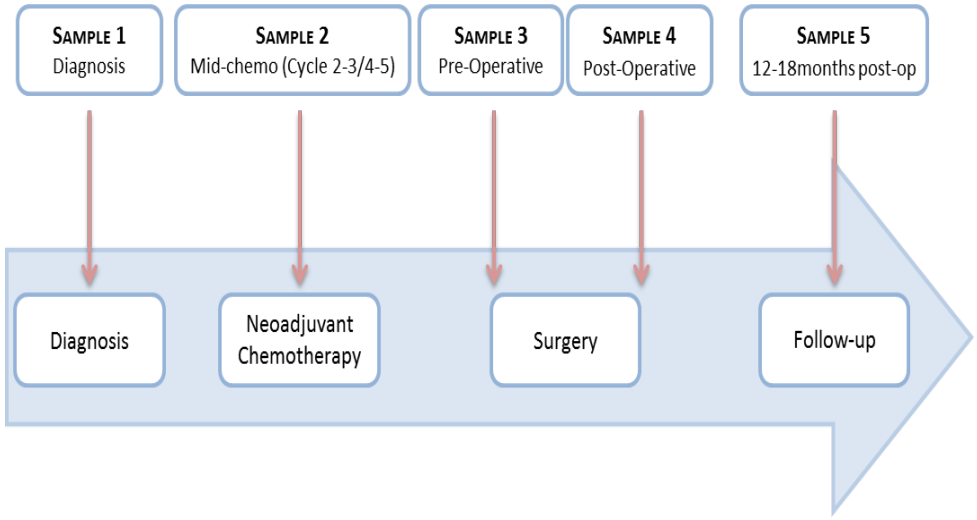


Figure 3.2 Timeline of blood sample collection

3.3.6 Candidate miRNA and Endogenous Control selection

The panel of miRNAs chosen for analysis in this clinical trial is outlined in Table 3.1. These miRNAs were selected based on documented association with breast cancer (up- or down regulation, association with metastasis etc).

The endogenous controls were selected based on previous work published from this laboratory, whereby the combination of miR-16 and miR-425 was found to generate more reliable RQ-PCR results than the use of either miRNA in isolation or the use of previously documented endogenous controls [254].

Table 3.1. Candidate miRNAs and Endogenous Controls

Candidate miRNAs and Endogenous Controls		Ref
Let-7a	Increased in circulation of patients with breast cancer compared to control	[105]
miR-10b	Decreased expression in breast tumour tissue compared to controls	[255]
miR-21	Increased expression in circulation and breast tumour tissue in association with metastasis	[256]
miR-145	Decreased expression in breast tumour tissue compared to controls	[103]
miR-155	Increased expression in breast tumour tissue compared to controls	[103]
miR-195	Circulation levels reflect breast tumour tissue levels Increased expression in breast tumour compared to controls	[105]
miR-16	Reliable endogenous control combination for analysis of miRNA expression by RQ-PCR in blood.	[257]
miR-425		

3.3.7 Study Cohort

As the timing of the fifth blood sample was 12-18 months following patient surgery (or in the case of a patient not going for surgery, 12-18 months following sample 3), the anticipated patient enrolment period for this study was 4 years. As such, for the purposes of this thesis, a blinded analysis of all blood samples collected from the initial 61 patients enrolled to the ICORG 10-11 Clinical trial (50% total samples).

3.3.8 Whole Blood miRNA Extraction

A modification of the Tri Reagent® BD (Molecular Research Center) co-purification protocol was then performed to extract miRNA. To achieve sample homogenization and phase separation, 200µl of Bromoanisole (BAN) and 3 ml of Trizol were mixed thoroughly with 1 ml of whole blood in a 5 ml tube. The homogenous lysate was then divided into two 2 ml tubes and left to stand at room temperature for 5 minutes. The solution was then spun at 14000 rpm at 4°C for 15 minutes. 1 ml of the clear aqueous phase from each tube was transferred into respective fresh 2 ml tubes, and the RNA contained within precipitated by adding 1ml of Isopropanol. The tubes were again left at room temperature for 5minutes, before centrifuging at 14000 rpm at 18°C for 8 minutes, to pellet the RNA. Once the spin was complete, the supernatant was discarded and 1ml of 75% ethanol added to each pellet. Each tube was vortexed briefly and spun at 14000 rpm at 18^{o}C for 5 minutes before discarding the ethanol. To improve the purity of the extracted RNA (as indicated by an improved 260/280 ratio on nano–spectrophotometry) this ethanol wash step was repeated. Once the ethanol from the second wash was discarded, each RNA pellet was left to air dry (tube lids left open) for 5 minutes before 30 µL of NFW was added to each. Tubes were vortexed briefly and then left to stand at room temperature for 5 minutes to allow the RNA pellets to solubilise. After a brief centrifuge (10-20 seconds), corresponding 2 ml tubes were combined (each pair of 2 ml tubes representing 1 patient blood sample) to provide a finished yield of 60 µl of total RNA per

ml of blood extracted. Extracted RNA was then transferred into storage tubes prior to analysis of RNA concentration and integrity and storage at -80^{o}C.

3.3.9 MiRNA Analysis of Concentration and Integrity

MiRNA concentration and purity was assessed using the NanoDrop 1000™ spectrophotometer. This system uses a pulsed xenon lamp as the light source and a spectrometer utilizing a linear CCD array (charged-coupled device) to analyse the light that has passed through the sample. Spectral measurements are made with a tightly controlled pathlength of 0.1 cm and RNA concentration is automatically calculated using the formula

RNA Concentration (ng/ µl) = *(A260 x e)/b*

A260 = absorbance at 260nm, e = extinction coefficient (ng–cm/ ml) and b = path-length (cm)

The instrument is controlled by PC based software, activated by selecting the desktop icon. Once activated, the system requires a 'blank' nuclease-free water (NFW) sample to initialise the system. With the sampling arm open, a 1 µl aliquot of NFW was pipetted onto the apparatus pedestal, the sampling arm closed and the '*ok*' option on the PC software selected. A reference 'blank' sample is next required by the system. After wiping both pedestals of the apparatus with a laboratory wipe, a 1 µl sample of NFW was pipetted onto the pedestal, the sampling arm closed and *'Blank'* selected on the PC software. Once this reference reading was recorded, the system was prepared to analyse samples.

A 1µl aliquot of sample RNA was pipetted onto the apparatus pedestal and the sampling arm closed. On the PC software, to optimise settings for small RNA analysis, '*Other*' was selected as the sample type and an extinction coefficient of '*33*' was manually entered before selecting '*Measure*'. RNA with an absorbance ratio at 260 nm and 280 nm (A260/A280) between 1.8

and 2.2 was considered pure, with lower ratios indicating the presence of protein or phenol. Similarly, absorbance ratio at 260 nm and 230 nm (A260/A230) between 1.8 and 2.2 was considered acceptable, with lower ratios indicating the carry-over of guanidinium salts.

3.3.10 Reverse Transcription

MicroRNA extracted from whole blood samples (Section 3.3.8) was reverse transcribed to complementary DNA (cDNA) for each miRNA being analysed. Starting concentrations of 100 ng/µL of each sample were utilised, requiring that some miRNA be diluted prior to use. The total volume of sample to be added to each reaction equaled 5 µL therefore the volume of NFW to be added to each diluted sample was calculated also. Dilutions were calculated using the spectrophotometer miRNA concentration result (obtained by using the extinction coefficient of '*33*' as described in Section 3.3.9) and calculated as per the following example:

					5µl of sample	
Sample identifier	Nano. Conc.	Dilution	Dilution	ng/µl of dilution	ng of dilution	ng of NFW
ABC123	290.94	1:3	1+2	96.98	1.03	3.94

Target-specific stem-loop primers were used to prime the miRNA and MultiScribe™ reverse transcriptase (Life technologies) used to synthesise a complimentary cDNA strand. Each sample reaction contained the following:

Reverse Transcription:	
dNTP mix (100mM)	0.17µl
10X RT Buffer	1.65 µl
NFW	4.57 µl
RNase Inhibitor (20U/µl)	0.21 µl
Stem-loop Primer (50nM)	3.1 µl
MultiScribe RT (50U/µl)	1.1 µl
Sample RNA (100ng/µl)	5µl

These reagents were combined and vortexed before being loaded into the GeneAmp® 9700 thermal cycler (Applied biosystems) in 200 µL tubes. To ensure the integrity of the results obtained, for each batch of samples analysed, a reverse transcription control (RTC) was included containing all reagents listed previously, with 5 µL NFW in place of sample RNA. Samples were incubated at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes, before being cooled to 4°C. Once this cycle was complete, the resultant 15 µL of cDNA produced was transferred into storage tubes and stored until required at -20°C.

3.3.11 Real-time Quantitative Polymerase Chain Reaction (RQ-PCR)

RQ-PCR was used for relative quantification of targets of interest. This process allows for synchronous amplification, detection and measurement of PCR products as the reaction progresses i.e. in real time. The reaction consists of two phases, an exponential phase, in which the quantity of amplified product approximately doubles during each cycle of denaturation, primer annealing and template extension, and a plateau phase during which reduced reagents limit further reaction. The cycle threshold (Ct) is reached once enough product has been amplified to produce a detectable fluorescent signal.

Where sequences were available, TaqMan assays were obtained (pre-formulated forward/reverse primer and MGB probe for miR-425, miR-195, miR-155, miR-145, miR-21 and miR-16, Life Sciences). For each sample to be analysed the following reagents and volumes were required:

ABI miRNA Kit:	
TaqMan Fast Mastermix	5 µl
NFW	3.8 µl
TaqMan Assay (PDAR)	0.5 µl
cDNA	0.7µl
Total Volume	**10 µl**

Where assays were compiled using individual probes and forward/reverse primers (miR-10b, miR-26b and Let-7a, MWG Biotech), reagents and volumes required per sample were as follows:

MWG/ABI Mix:	
TaqMan Fast Mastermix	5 µl
NFW	1.6 µl
Probe (0.2µM)	0.5µl
F Prime (1.5µM)	1.5µl
R Primer (0.7µM)	0.7µl
cDNA	0.7µl
Total Volume	**10 µl**

Each reaction was run on a 96-well plate, with each well containing 10 µl of solution (as above). Each sample being analysed was run in triplicate to determine intra-assay variation. Each plate also contained a 'No Target Control' (NTC) and an RTC (as described in Section 3.3.10) to ensure the purity of the RQ-PCR and reverse transcription processes. These wells contained target-specific premix (as per tables 1.1 or 1.2) and 0.7 µl of NFW or target-specific RTC respectively. Further to this, each plate contained an

inter-assay control (IAC) to account for inter-assay variability (threshold standard deviation for inter-assay replicate variability was 0.3).

Once premix and samples were loaded into desired wells, each plate was sealed with MicroAmp optical adhesive film before being spun in a large centrifuge at 2000 rpm for 1 minute. The plate was then loaded into the AB7900HT instrument and standard fast thermal cycling conditions were used: heating to 95^{o}C for 15 seconds x 40 cycles to open the DNA double helix, followed by 60^{o}C for 60 seconds to allow annealing to take place.

3.3.12 PCR Amplification Efficiencies

In an optimal PCR reaction, where primer and reagent conditions and concentrations are ideal, the exponential phase should produce an amplification efficiency of 100% i.e. a doubling of amplification of PCR product with each cycle. However, amplification efficiencies may fluctuate, therefore prior to conducting miRNA expression analysis, amplification efficiencies were calculated for each target RQ-PCR assay. Serial dilutions of cDNA template were performed (neat to 10^{-6}), and each was amplified using the standard conditions used for subsequent gene expression analysis as described in 3.3.11 (with the exception of using absolute quantification (standard curve) assays). A dilution curve was constructed by plotting the cycle threshold (Ct) against the dilution factor of the cDNA. The slope of the dilution curve was then used to calculate the amplification efficiency (E) via the following equation:

$$E = (10\text{-}1/\text{slope}\text{-}1) \times 100$$

Amplification efficiencies from 90-110% indicate a robust and reproducible RQ-PCR assay.

3.3.13 Relative Quantification

To produce reliable RQ-PCR data miRNA expression levels require normalization to those of endogenous controls. To achieve this, raw fluorescence data from RQ-PCR was exported into the software package qBase plus. This software system scales data to an internally defined calibrator and endogenous control gene(s) to generate relative quantities using the following formula:

$$\Delta Ct = \text{Average Ct of test sample} - \text{Average Ct of calibrator}$$

The ΔCt values obtained were then converted into a linear form using the amplification efficiency (E) determined in section 3.3.12 and the equation $E^{-\Delta Ct}$. To correct for any potential non-biological variation in gene expression data, miR-16 and miR-425 were used as endogenous controls to normalise the data via the comparative Ct method (ΔΔ Ct) as follows:

$$\Delta\Delta Ct = [(\text{Ct target gene}) - (\text{Ct EC})] - [(\text{Ct reference gene}) - (\text{Ct EC})]$$

ΔΔCt values were then converted into linear form using $E^{-\Delta\Delta Ct}$.

3.3.14 Statistical Analysis

Gene expression values obtained from RQ-PCR were exported from qBase to Minitab (v17) statistical software for analysis. As this miRNA expression data was not normally distributed, all results were log transformed for the analysis. One-way ANOVA was used to test for significance between miRNA expression values and neoadjuvant chemotherapeutic timepoint. Pearson correlation coefficient was used to assess the presence and strength of relationship between miRNA expression in each timepoint grouping.

Table 3.2. Pearson Correlation Coefficient Interpretation

Pearson Coefficient (R)	Result
0	No linear relationship
≤ 0.3(-)	A weak positive (negative) relationship
0.3-0.7(-)	Moderate positive (negative) relationship
≥ 0.7 (-)	Strong positive (negative) relationship
1 (-)	Perfectly linear positive (negative) relationship

3.4 Results

3.4.1 Trend of circulating miRNAs

A total of 239 blood samples were received prospectively from the 61 patients involved in the preliminary blinded trial analysis, representing 50% of the total sample number to be accrued for this trial. RNA was successfully extracted from each sample, and miRNA expression effectively conducted via target-specific cDNA synthesis and RQ-PCR. The panel of six study miRNAs was investigated (Let-7a, miR-10b, miR-21, miR-145, miR-155 and miR-195) using endogenous controls miR-16 and miR-425. As this clinical trial is still ongoing, unblinding of patient clinicopathological data was not possible. As such, this results section presents a blinded analysis of the expression data of a panel of miRNAs in the sequential blood samples of 61 patients receiving neoadjuvant chemotherapy for breast cancer.

3.4.2 Centres and Sample Collection

Regarding study accrual centres, of the 61 patients included in the preliminary analysis, 82% of patients (n=50) were recruited from Galway University Hospital (GUH), with 18% of patients (n=11) enrolled from St James' University Hospital, Dublin. Of these 11 patients, 2 withdrew from the trial, 1 due to adverse chemotherapeutic reaction, and 1 decided not to continue treatment. Of a total of 239 blood samples collected for these patients, 205 samples (86%) were collected from GUH, with an average of 4 blood samples collected per patient. From the single distant site, a total of 34 blood samples were collected (n=14%), with an average of 3 blood samples per patient (Table 3.3).

Table 3.3. Samples accrued per patient.'√' indicates samples received and 'o' absent samples.

Patient Number	Baseline	Peri-Neoadjuvant	Pre-op	Post Op	Follow-up
1	√	√	√	√	√
2	√	√	√	√	√
3	√	√	√	√	√
4	√	o	√	√	√
5	√	√	√	√	√
6	o	o	o	o	o
7	o	o	√	√	√
8	o	o	o	√	√
9	√	√	√	√	√
10	√	√	o	o	√
11	√	√	√	o	√
12	√	√	√	√	√
13	√	√	o	o	√
14	√	√	√	√	√
15	√	√	√	√	√
16	√	√	√	o	√
17	√	√	√	o	√
18	√	√	o	o	o
19	√	√	√	√	√
20	√	√	√	√	o
21	√	√	√	√	√
22	√	√	√	√	o
23	√	√	o	o	√
24	√	o	√	√	√
25	√	√	√	√	√
26	√	√	√	√	√
27	√	√	√	√	√
28	√	√	o	√	o
29	√	o	√	o	√
30	√	√	√	√	√
31	√	o	√	√	√
32	√	√	√	√	√
33	√	√	√	√	√
34	√	√	o	√	√
35	√	√	√	√	√
36	√	√	√	√	√
37	√	√	√	√	√
38	√	√	√	√	o
39	√	Withdrew from study			
40	√	√	√	√	o
41	√	√	√	o	√
42	o	√	√	√	√
43	√	√	√	√	√
44	√	o	√	√	o
45	√	√	√	√	o
46	√	√	√	o	o
47	√	√	o	√	o
48	√	√	√	o	o
49	√	√	√	√	√
50	√	√	o	√	√
51	√	√	√	√	o
52	√	√	o	√	o
53	√	√	√	√	o
54	√	Withdrew from study			
55	√	√	√	√	o
56	√	√	√	√	o
57	√	√	√	√	√
58	o	√	√	√	o
59	√	√	√	√	√
60	√	√	√	√	√
61	√	√	√	o	o

Regarding sample yield by timeline, as each patients' neoadjuvant chemotherapy progressed, sample retrieval was seen to decline (Figure 3.3), with the majority of samples collected at baseline, and least collected at follow-up.

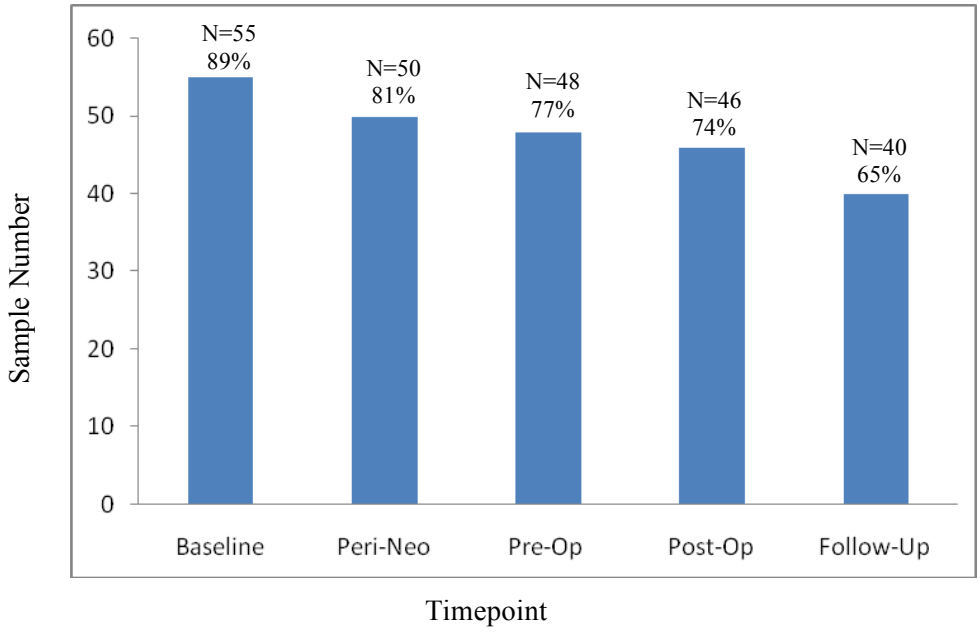


Figure 3.3. Sample number collected by Timepoint

3.4.3 RNA yields from Blood Sample Extraction

MiRNA yield was sufficient from each sample processed. Table 3.4 provides a breakdown of RNA extraction yield with associated 260/280 purity ratios.

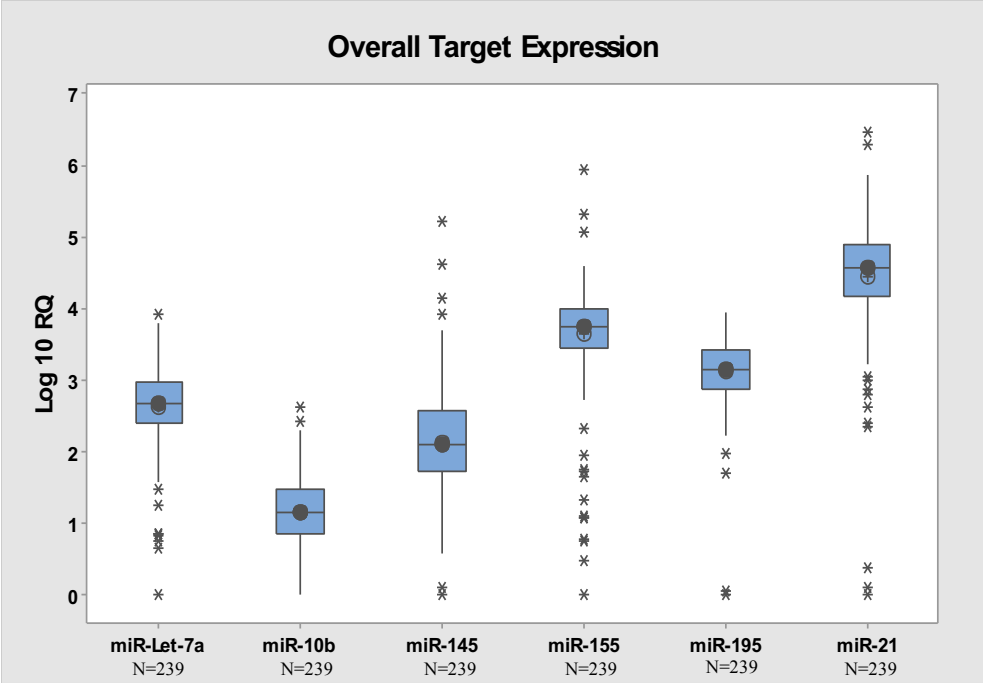
Table 3.4. MiRNA extraction yield data.

Timepoint	Sample No.	Mean miRNA yield	SD	Min Yield	Max Yield	Mean 260/280	Min 260/280	Max 260/280
Baseline	55	313.3	343.8	37.5	1987.4	1.76	1.5	2.25
PeriNeo	50	327.3	255.9	30.8	1242.4	1.78	1.32	2.18
Pre-Op	48	232.4	212.9	28.1	889.2	1.78	1.33	2.23
Post-Op	46	201.0	150.3	18.7	573.7	1.77	1.32	2.44
Follow-Up	40	168.4	162.8	28.8	753.3	1.74	1.37	1.87

3.4.4 Circulating miRNA expression

Overall expression of each target-miRNA is displayed in Figure 3.4A, with mean expression of each target miRNA at each timepoint displayed in Figure 3.4B. MiRNAs with known down-regulation in association with breast cancer, miR-10b and miR-145, were expressed at lowest concentrations, with miRNAs having known up-regulation in association with breast cancer, miR-21, miR-155, miR-195 and Let-7a, expressing at higher levels. As the aim of this trial was to examine miRNA trends in breast cancer patients undergoing neoadjuvant chemotherapy, results have not been analysed in conjunction with unaffected controls, as such the significance of this up or down-regulation cannot be assessed.

A)



B)

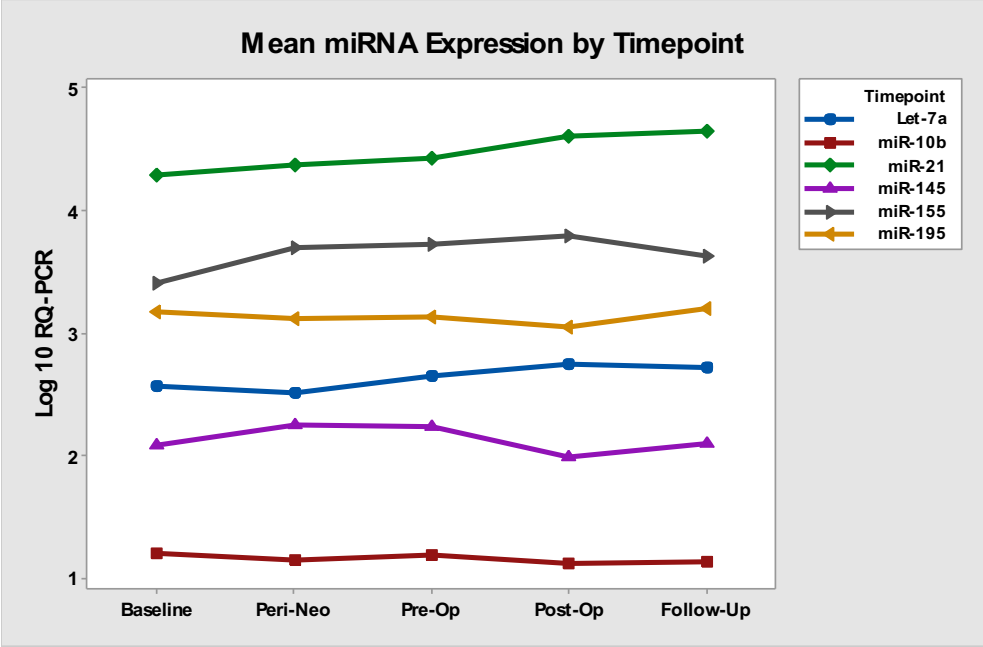


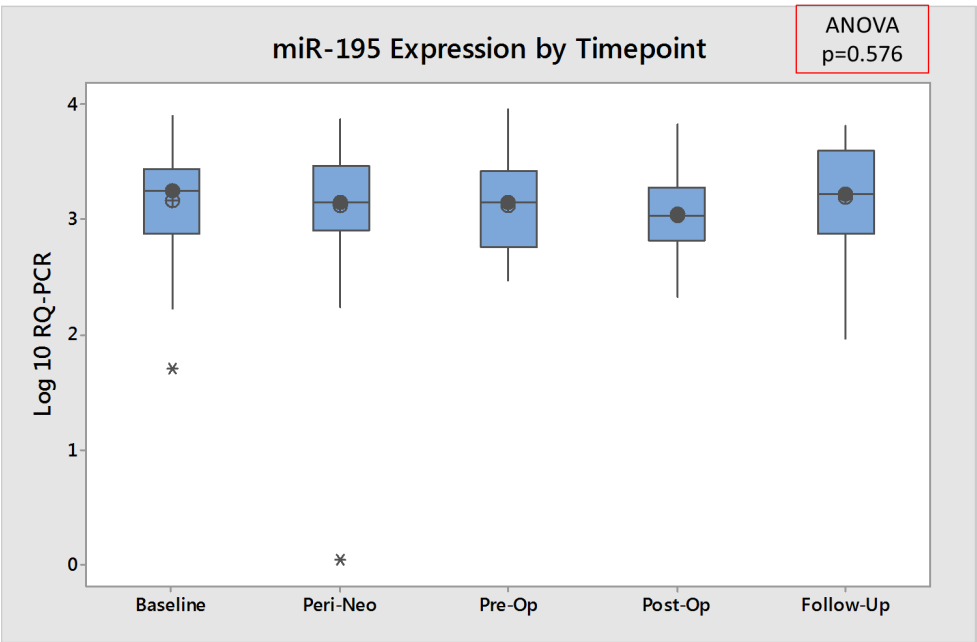
Figure 3.4 A) Overall miRNA expression by timepoint B) Mean miRNA expression by timepoint

3.4.5 Circulating miR-195 expression

No significant alteration in expression pattern of miR-195 was identified during neoadjuvant chemotherapy or in response to surgery (ANOVA p=0.576) (Figure 3.5A).

Mean miR-195 expression by timeline was examined to assess the overall trend of expression (Figure 3.5B). A trend towards a decrease in expression post-operatively with a subsequent increase in expression upon follow-up (12-18 months later) can be appreciated. While this trend did not achieve significance (p= 0.109 and 0.774 respectively) this finding is in keeping with previous trends in miR-195 expression identified by our laboratory group.

A)



B)

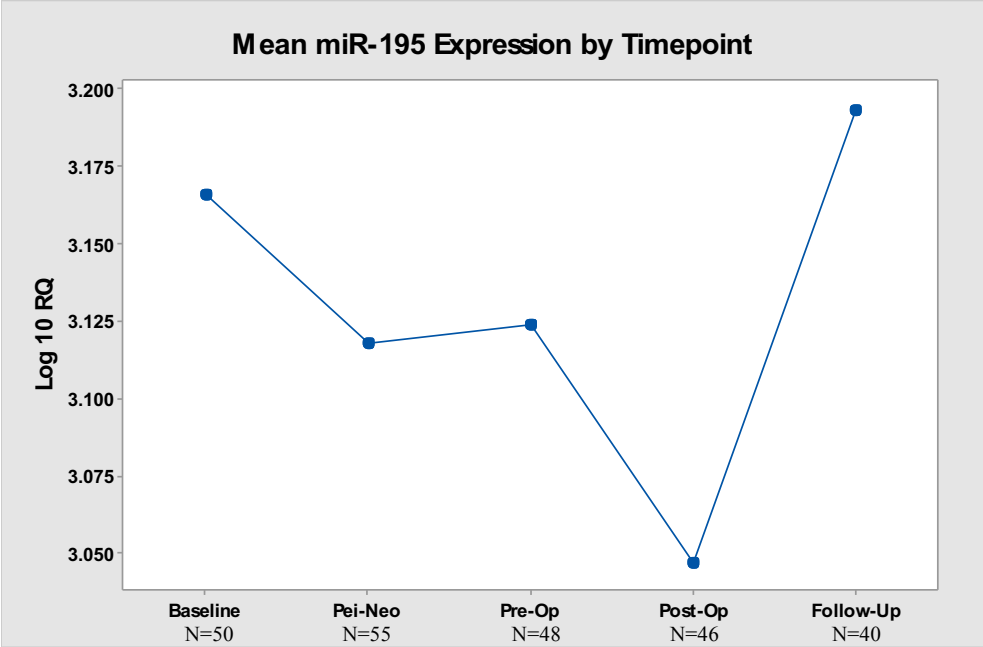


Figure 3.5A) Overall miR-195 expression by timepoint B) Mean miR-195 expression by timepoint.

Positive correlation was identified between baseline miR-195 expression and expression during neoadjuvant chemotherapy.

Table 3.5. Correlation between miR-195 expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.606 *0.000				R p-value
Pre-Op	0.110 0.489	0.136 0.398			R p-value
Post-Op	0.107 0.483	0.186 0.221	0.299 0.064		R p-value
Follow-Up	0.269 0.124	0.263 0.140	0.409 0.027	0.196 0.291	R p-value

3.4.6 Circulating miR-145 expression

No significant alteration in miR-145 expression pattern was identified in response to chemotherapy or surgery (ANOVA p=0.322).

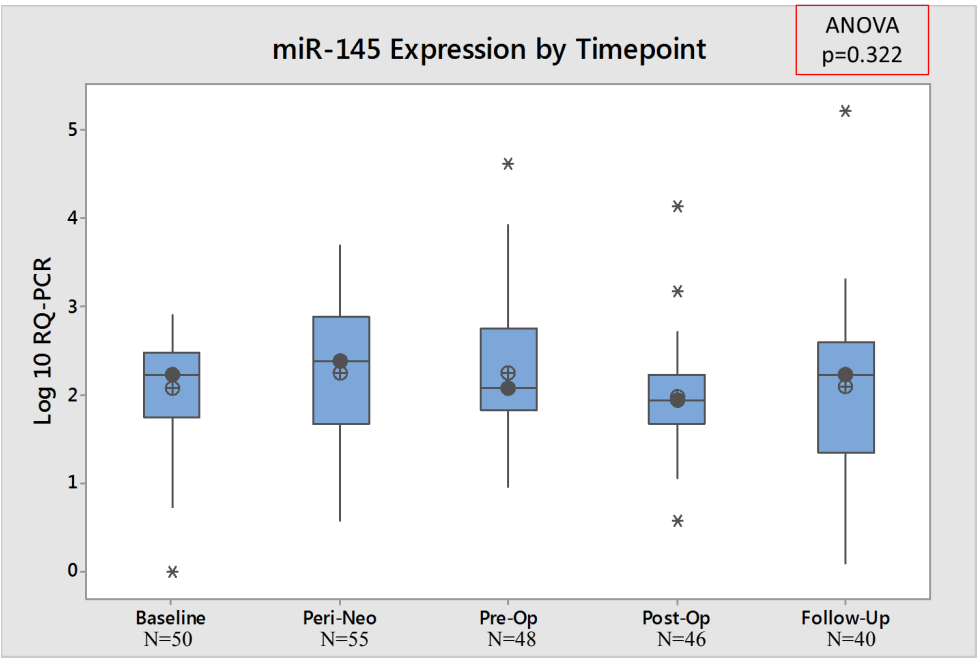


Figure 3.6. MiR-145 Expression by Timepoint

Statistically significant positive correlation was identified between miR-145 expression at various timepoints. Baseline, peri-neoadjuvant and post-operative miR-145 expression displayed moderate positive correlation, with peri-neoadjuvant and follow-up miR-145 levels displaying strong positive correlation.

Table 3.6. Correlation between miR-145 expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.549 *0.000				R p-value
Pre-Op	0.321 0.046	0.651 *0.000			R p-value
Post-Op	0.434 *0.005	0.313 0.056	0.342 *0.038		R p-value
Follow-Up	0.516 *0.003	0.714 *0.000	0.348 0.075	0.504 *0.005	R p-value

3.4.7 Circulating miR-155 expression

No significant alteration in expression pattern in response to chemotherapy or surgery (ANOVA p=0.139).

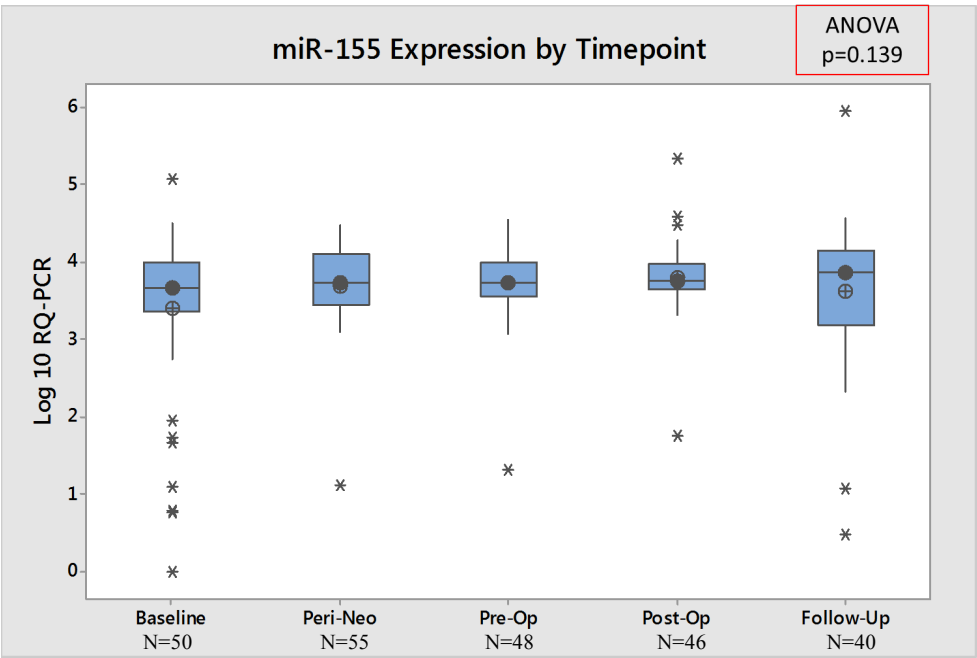


Figure 3.7. MiR-155 expression by Timepoint

Multiple significant positive correlations were identified in miR-155 expression at various timepoints. Baseline, peri-neoadjuvant and pre-operative iR-155 expression was found to have moderate correlation, with peri-neoadjuvant and follow-up nmiR-155 displaying strongly positive correlation.

Table 3.7. Correlation between miR-155 expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.642 *0.000				R p-value
Pre-Op	0.434 *0.005	0.581 *0.000			R p-value
Post-Op	0.413 *0.008	-0.040 0.815	0.126 0.472		R p-value
Follow-Up	0.314 0.075	0.732 *0.000	0.356 0.063	-0.127 0.512	R p-value

3.4.8 Circulating miR-21 expression

No significant alteration in expression pattern was identified in response to chemotherapy or surgery (ANOVA p=0.139).

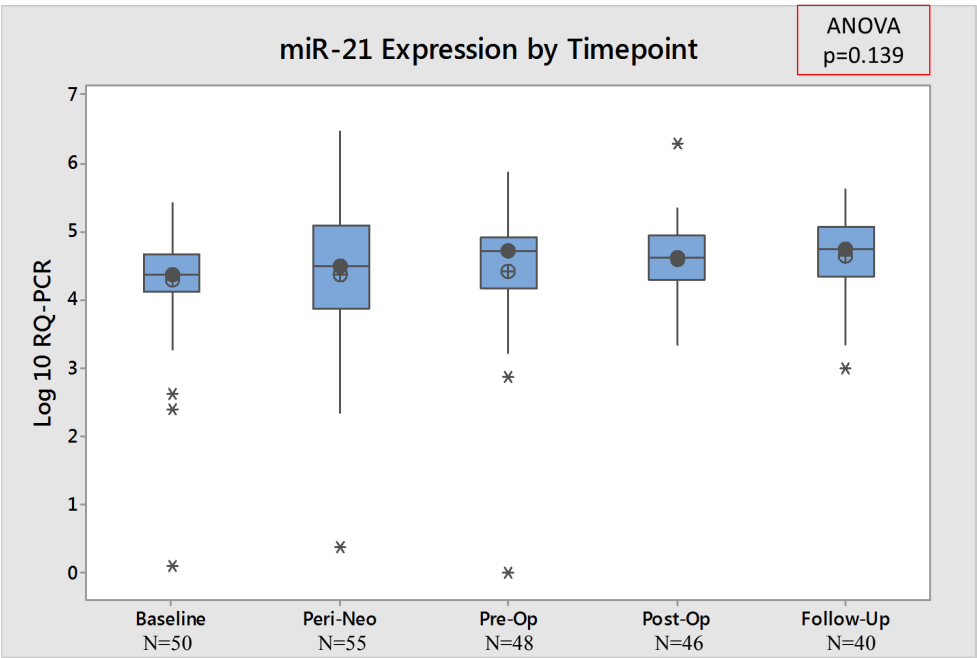


Figure 3.8. MiR-21 expression by Timepoint

Significant positive correlation was identified between post-operative miR-21 expression and follow-up.

Table 3.8. Correlation between miR-21 expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.136 0.352				R p-value
Pre-Op	-0.181 0.259	0.121 0.464			R p-value
Post-Op	0.127 0.418	0.379 1.015	0.299 0.068		R p-value
Follow-Up	-0.079 0.662	0.023 0.904	0.280 0.141	0.674 0.000*	R p-value

3.4.9 Circulating miR-10b expression

No significant alteration in expression pattern was identified in response to chemotherapy or surgery (ANOVA p=0.942). Mir-10b was expressed at such low levels that it was not detectable in all patient samples.

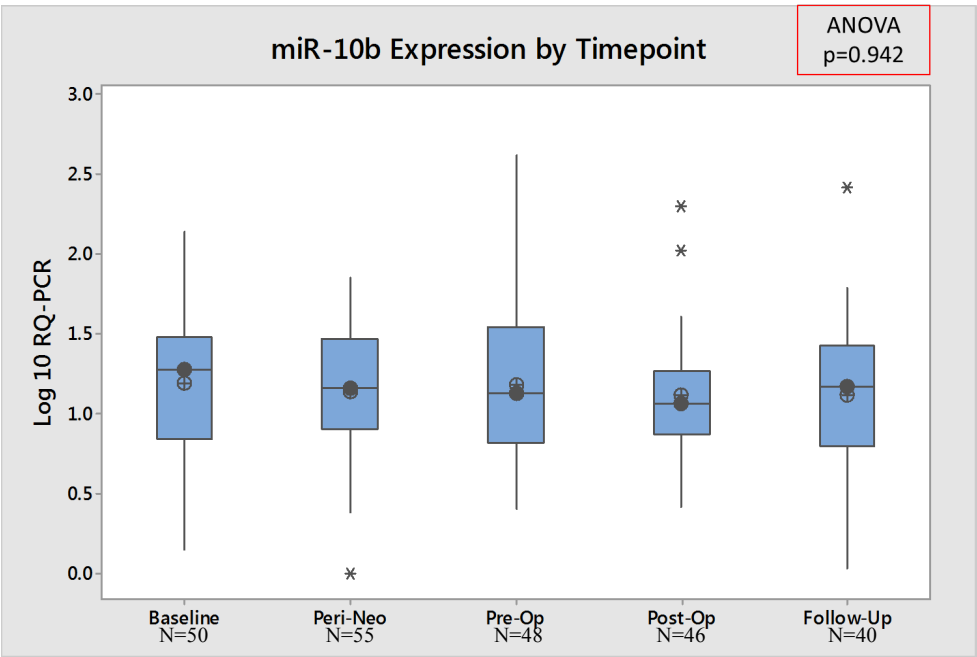


Figure 3.9. MiR-10b expression by Timepoint

A significant strong positive correlation (R) was identified between miR-10b at baseline and expression during neoadjuvant chemotherapy, with high baseline expression indicating high peri-neoadjuvant expression.

Table 3.9. Correlation between miR-10b expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.724 0.000*				R p-value
Pre-Op	-0.216 0.374	-0.072 0.791			R p-value
Post-Op	0.269 0.281	0.372 0.141	-0.166 0.538		R p-value
Follow-Up	-0.006 0.982	0.036 0.916	-0.162 0.565	0.061 0.830	R p-value

3.4.10 Circulating Let-7a expression

No significant alteration in expression pattern in response to chemotherapy or surgery was identified (ANOVA p=0.156).

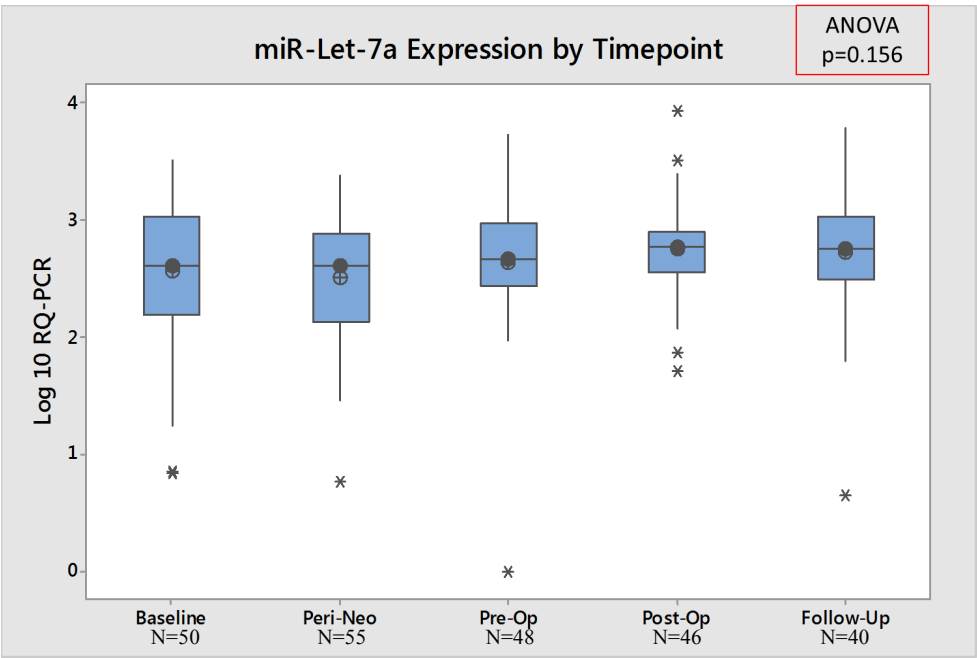


Figure 3.10. Let-7a expression by Timepoint

A moderate positive correlation was identified between baseline Let-7a expression and post-operative Let-7a expression.

Table 3.10. Correlation between Let-7a expression and study timepoints. R=Pearson Correlation Coefficient

Timepoint	Baseline	Peri-Neoadjuvant	Pre-Op	Post-Op	
Baseline					
Peri-Neo	0.245 0.105				R p-value
Pre-Op	-0.138 0.396	0.001 0.995			R p-value
Post-Op	0.369 *0.019	0.116 0.494	-0.014 0.934		R p-value
Follow-Up	-0.053 0.776	-0.173 0.389	0.050 0.798	0.119 0.538	R p-value

3.4.11 Power Recalculation

The initial power calculation for ICORG 10-11 was based on previous work conducted by our laboratory. To ensure the accuracy of this power calculation, an interim analysis was stipulated in the study research proposal, whereby the power analysis would be recalculated using miR-195 expression data from the initial 50% of study samples obtained. MiRNA expression data from the sequential samples collected from the initial 61 patients enrolled onto the ICORG 10-11 trial confirmed the findings of the initial power calculation. The assessment of 122 patients would achieve statistical power of 80%. As a result, no adjustment to patient enrolment was required.

3.5 Discussion

The management of breast cancer, in terms of diagnosis, chemotherapeutics and surgical intervention, continues to adapt in line with translational research and clinical evidence. Whilst the use of chemotherapy in the neoadjuvant setting is now acceptable for any patient considered a candidate for adjuvant therapy [258], there currently exists no clinically validated means of differentiating chemotherapeutic responders from non-responders. To assess the potential for circulating miRNA to act as minimally-invasive biomarkers in this setting, the ICORG 10-11 clinical trial was implemented. The primary objective of this study is to assess the ability of a panel of miRNA to detect or monitor tumour response to neoadjuvant chemotherapy. A further objective is to assess association between miRNA and patient clinicopathological parameters, including patient demographic data e.g. age, menopausal status, and clinicopathological data e.g. tumour size, tumour grade, hormone receptor status, metastasis. An association between miRNA expression patterns and these parameters could support a potential prognostic role for miRNA expression analysis.

At present, the ICORG 10-11 trial is closed to accrual due to full patient complement. At the time of writing, 125 patients were enrolled nationwide with sample collection nearing. Compilation of patient clinicopathologic and demographic data continues by independent ICORG appointed investigators, maintaining effective study blinding.

Though one of the strengths of this trial, blinded sample analysis represented a significant challenge in terms of temporal restrictions and thesis preparation. Ideally, a completed interim analysis would have been conducted, assessing not only the trend of each patients' miRNA expression, but any association between this trend and patient clinicopathological details and chemotherapeutic response. As this is an ongoing national clinical trial, unblinding of patient details and access to neoadjuvnat chemotherapeutic response data is prohibited until such time as all study participants have completed neoadjuvant chemotherapy and definitive surgery, and all blood

samples obtained by the laboratory have been analysed for miRNA expression.

A further challenge was presented by the sheer volume of patient samples and target miRNA to be analysed within a restricted time. Upon receipt, each sample was logged onto a laboratory database (for registration and storage purposes), before RNA was manually extracted. For each individual sample, cDNA for each of the eight miRNA targets of interest was reverse transcribed, before PCR was conducted in triplicate, for each of the eight miRNA targets, for each of the 239 blood samples. All PCR data produced was then manually checked to ensure effective controls, and mean expression and standard deviation were calculated before relative quantification was assessed using qBase software. This workload within a restricted timeframe curtailed further involvement in seeing this trial through to its conclusion. For future trials, it is recommended that a dedicated laboratory technician be assigned the duty of sample analysis, rather than researchers pursuing higher degrees. The expected conclusion date for this trial is Spring 2017, at which point final analysis of primary and secondary study objectives will have been achieved.

While overall study objectives could not be realized during the scope of this thesis, lessons were learned in conducting the interim analysis, particularly with regards to blood sample collection and storage. While miRNA was effectively extracted from each blood sample collected, upon analyzing miRNA yield in section 3.4.3, a large variance in sample yield is appreciable from yield standard deviation. This could be attributable to sample factors (such as clotted samples, insufficient volume, lag time between being placed at $4^{o}C$ and transport between centres or between receipt and extraction) as well as researcher factors, in terms of extraction technique. The 260/280 ratio is used as an indicator of sample integrity and purity, with a target result of 1.8 to 2. While the minimum 260/280 for each sample timepoint is at least greater than 1.3, the mean 260/280 confirms that the majority of samples reached optimal purity. It was recognised from this data that blood collection and storage could be optimized, resulting in the laboratory converting from the use of Vacutainer® K2 EDTAtubes to the use of PAXgene tubes (PreAnalytiX) for future trials. These blood collection tubes provide

immediate stabilization of sample RNA, allowing for more effective sample transport and storage, and the optimisation of miRNA yields. For future trials, this should allow for more robust sample analysis and study findings.

Regarding miRNA expression, while only trends within cancer patients could be observed, the pattern of expression of each miRNA is interesting given the known association between each target miRNA and breast cancer. MiR-10b and miR-145 are known to be down-regulated in association with breast cancer [103, 255]. From this analysis, we can see that both of these miRNAs are expressed at much lower levels than the remaining miRNAs, all of which have known up-regulation in association with breast cancer (Figure 3.4a). The significance of up or down regulation cannot be assessed however, due to the lack of control samples outlining normal expression levels. The analysis of this miRNA expression data in conjunction with patient neoadjuvant chemotherapeutic response and clinicopathological parameters is eagerly anticipated.

To summarise, while this blinded, interim, clinical trial analysis cannot determine overall study endpoints, some notable observations were made.

- Although the trial was open to nationwide accrual, the principal investigating centre was responsible for recruiting the vast majority of patients.
- Detection of all study miRNA was successfully achieved in sequential blood samples in 61patients.
- Re-evaluation of study power using miR-195 expression data from patient samples confirmed the preliminary power calculation and trial enrolment numbers required to achieve 80% power were maintained.
- Analysis of miR-195 expression revealed a trend towards a decrease post-operatively with a subsequent increase in expression upon follow-up (12-18 months later). Although not significant, this finding is in keeping with previous trends in miR-195 expression identified by our laboratory group.
- Positive correlation was identified at various timepoints for each miRNA analysed.

- Study miRNAs with known down-regulation in association with breast cancer (miR-10b and miR-145) were expressed at much lower levels than study miRNAs with known up-regulation in association with breast cancer (Let-7a, miR-21, miR-155 and miR-195).

Further to this, lessons were learned that will enable enhanced design of future clinical trials.

- Considerable sample mRNA yield variance was noted using Vacutainer® K2 EDTA storage tubes. Our laboratory group recognised that an improvement in this area would enable the production of more robust and reliable extraction results. This finding, in conjunction with further research conducted in our laboratory, resulted in our group changing to the use of Paxgene tubes (PreAnalytix) for further ICORG study cohorts.
- The conduct of clinical trials is a laborious and lengthy process that lacks interval outputs. This produces significant challenges for researchers participating in higher degrees of defined periods. For future trials, it is recommended that a dedicated laboratory technician be assigned the duty of sample analysis, rather than researchers pursuing higher degrees.

By analyzing the trend of miRNA expression during neoadjuvant chemotherapy, this ongoing clinical trial seeks to identify patterns that may indicate which patients are responding and which patients are resistant to chemotherapy. Further to this, by assessing association with patient clinicopathologic parameters, the prognostic potential of circulating miRNAs in the neoadjuvant chemotherapeutic setting will be assessed. With an expected end-date of Summer 2017, the final endpoints of this study are eagerly anticipated.

3.6 Conclusion

This clinical trial is the first study to assess the expression of circulating miRNA in the sequential samples of patients undergoing neoadjuvant chemotherapy for breast cancer, in an attempt to determine if expression patterns can be utilized as minimally invasive biomarkers of chemotherapeutic response and disease outcome. During the course of this study lessons were learned regarding the conduct of and participation in clinical trials, and while for the purposes of this thesis final study endpoints cannot be assessed, the trends of miRNA expression observed thus far are promising. The identification of effective prognostic markers with the ability to determine tumour response or resistance to chemotherapy could enable us to tailor therapy to individual patient requirements and potentially, allow us to develop targeted treatments to further improve patient response rates and outcomes from breast cancer.

Chapter 4

ASSESSING THE POTENTIAL ROLE OF ARGONAUTE-2 AS A BREAST CANCER BIOMARKER

4.1 Introduction

In recent years, the management of breast cancer has transformed dramatically, from the use of mastectomy to breast conserving surgery, chemotherapy in the neoadjuvant setting and targeted tumour-specific therapies (Figure 4.1). This transformation is largely due to an increased understanding of the heterogeneity of this disease. Molecular profiling has transformed our management of breast cancer, from the use of Tamoxifen in oestrogen receptor positive disease, to Trastuzumab in HER2 overexpressing. While clinically four main breast cancer subtypes predominate, up to ten subtypes are currently being reported in the literature [26]. The continual advancement of our understanding of this disease is bringing us ever closer to the development of personalised medicine. To achieve this, further molecular characterisation of breast cancer is required so that we may develop targeted treatment strategies that are more effective and efficient, offering fewer patient side effects and better outcomes from disease.

While miRNA have a known regulatory role in various forms of cancer, dysregulation of the upstream miRNA machinery can also play a crucial role in cancer initiation and progression [87, 259]. Argonaute-2 (Ago2) is a protein that is ubiquitously expressed in the body and critical for the conduct of the gene silencing phenomenon RNA interference (RNAi) [157, 158]. One of four mammalian Ago proteins, Ago2 is the only member of the family to play an essential catalytic component of the RNA-induced silencing complex (RISC) (Section 1.9.4) [165]. Within this complex, Ago2 directly binds small RNA molecules, producing mRNA cleavage or translational repression of complementary mRNA to exact gene silencing via RNAi [79]. With such an essential role in the regulation of molecules with known involvement in the initiation and progression of breast cancer, Ago2 represents a very interesting candidate for investigation in this setting.

To date, a high incidence of alteration in the Ago2 gene (both up and down-regulation) has been identified across multiple cancer types, including breast, colorectal, urothelial and prostate [87]. With regards to breast cancer

specifically, increased expression of Ago-2 has been associated with more aggressive breast cancer subtypes, while single-nucleotide polymorphisms of Ago2 have been associated with disease free and overall breast cancer survival [171, 172]. Furthermore, it is reported that Ago2 binds to the tumour metastasis factor focal adhesion kinase promoter to trigger its transcription, suggesting a role in tumour progression [87]. In the clinical setting however, minimal investigation of Ago2 in breast cancer has been conducted to date. With such promising pre-clinical findings, the potential for Ago2 to further characterize the molecular subtypes of breast cancer, and potentially be employed as a novel prognostic or predictive biomarker, or future target for personalized medicine is an exciting prospect that requires further evaluation.

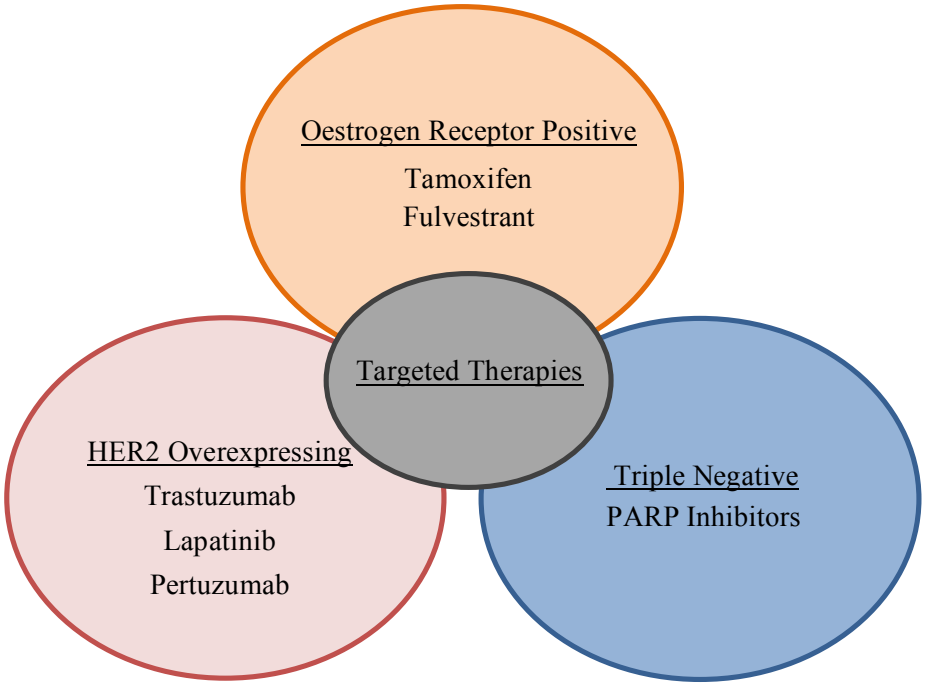


Figure 4.1 Current targeted therapies for breast cancer

4.2 Aim

The aim of this study was to assess the potential of Ago2 levels to stratify breast cancer, to investigate its potential as a further prognostic or predictive biomarker. To achieve this, the ability of Ago-2 to classify breast cancer cell lines was analysed, followed with validation in clinical patient tissue samples.

4.3 Materials and Methods

4.3.1 Cell Culture

All culture conducted for this thesis was via adherent technique and involved the use of immortal breast cancer cell lines as a model for breast cancer. Cell lines for culture were initially purchased from the American Type Culture Collection (ATCC) and were selected based on the molecular subtypes of breast cancer. All cell lines were maintained in cell culture flasks, in cell-line specific media (Table 4.1), in a designated cell line Stericycler CO_2 incubator – HEPA class 100 at 37^{o}C and 5% CO_2. All cell media was supplemented with 10% foetal bovine serum (FBS) and 100 IU/mL Penicillin/ 100µg/mL Streptomycin. To ensure cell health and monitor confluence, light microscopy was regularly employed.

Table 4.1 Outline of breast cancer cell lines utilised

Cell Line	Epithelial Subtype	Receptor Status	Medium
MCF-10A	Normal-like	n/a	DMEM/Hams F-12 + 5% Horse Serum + 20ng/ml EGF + 100ng/ml Cholera Toxin + 0.01 mg/ml Insulin + 500ng/ml Hydrocortisone + Pen/Strep
T47D	Luminal A	ER+, PR+, HER2 -	RPMI 1640 w/L-glutamine + 10% FBS + Pen/Strep
BT-474	Luminal B	ER+, PR+, HER2 +	RPMI 1640 w/L-glutamine + 10% FBS + Pen/Strep
SK-BR-3	Her2 Over-expressing	ER-, PR-, HER2 +	McCoys 5A w/L-glutamine + 10% FBS + Pen/Strep
BT-20	Triple Negative	ER-, PR-, HER2-	EMEM w/L-glutamine + 10% FBS + Pen/Strep

All cell culture activities were conducted in the laboratory's cell culture facility, using strict aseptic technique so as to minimise the likelihood of contamination or cross-infection. Laminar air flow (LAF) hoods were utilised when manipulating cultured cells and personal protective equipment (PPE), including designated cell-culture laboratory coats (red collared) and gloves, were donned before any cell culture paraphernalia was handled. Prior to use, LAF hoods were exposed to 10 minutes of UV before being cleaned with 70% Industrial Methylated Spirits (IMS) solution. All materials exchanged between the water baths, incubators and LAF hoods were also sprayed in IMS solution. In conjunction with ongoing maintenance, each week the cell culture facility was thoroughly cleaned (hoods, incubators, water baths) using autoclaved water and 70% IMS solution. To ensure minimization of contamination and cross-infection strict facility maintenance protocols and laboratory etiquette were adhered to.

4.3.2 Cell Propagation

Every 2-3 days, cell media was replenished, with spent cell media disposed of and fresh media applied. To perform this task, cell-line specific media was placed in a water bath to heat to 37^{o}C. While the media was heating, the LAF hood was exposed to UV light for 10 minutes prior to use, then sprayed down with 70% IMS solution. Prior to changing media, each cell flask was inspected using the light microscope to assess for level of confluence, cell health and any evidence of contamination. Spent media was decanted from the culture flask and replaced with fresh media containing essential nutrients and pen/strep to maintain the cells in an uncontaminated environment. Varying amounts of media were applied according to flask size utilised e.g. T75 flasks 15 ml, T175 flasks 25 ml. Once the media was changed, culture flasks were returned to the incubator at 37^{o}C and 5% CO_2 and media bottles returned to the fridge until next required.

4.3.3 Subculture

Once a cell line achieved 80-90% monolayer confluence, sub-culture was performed to prevent cell senescence. Cell line-specific media and 5 ml (per flask) 0.25% Trypsin/EDTA (T/E) was heated in a water bath and the cell-culture hood prepared for use with UV and 70% IMS. Spent media was decanted from cells before washing with phosphate buffered saline (PBS) to remove any remaining media and ready the cells for trypsinisation. 5ml of T/E was then added and the flask and returned to the incubator for 5 minutes to allow trypsinisation. The light microscope was employed to assess for cell detachment, and if necessary, the side of the flask was tapped gently to encourage detachment. Once detachment was confirmed, the cell T/E suspension was pipetted up and down to disperse cell clumps. Following this, using the cell counter if required (Section 4.3.6), cells were seeded at appropriate densities in fresh cell culture flasks prepared with pre-warmed media, and returned to the incubator until required.

4.3.4 Cryopreservation of cells

To ensure sufficient stocks for on-going personal and future laboratory research, individual cell line suspensions were frozen down. Once a flask of cells had been trypsinised, as outlined in cell line subculture, the cell suspension was transferred to a 15 ml tube containing 5ml of cell-line specific media to deactivate the trypsin, and the tube centrifuged at 1500 rpm for 5 minutes to pellet the cells. The supernatant was removed and the cell pellet reconstituted in 10% Dimethylsulphoxide (DMSO) diluted in FBS. As DMSO is highly toxic to cells at 37^{o}C it was used chilled. Once the cell pellet was reconstituted the cell suspension was quickly transferred to a cryotube (Sarstedt) and placed in an isopropanol bath (Nalgene) before being stored in the -80^{o}C freezer for at least four hours (often overnight). The isopropanol bath allows for controlled freezing of the cells at a rate of -1^{o}C per minute when placed in a -80^{o}C freezer. Cryotubes were then transferred to the liquid nitrogen bio-freezer for long-term storage at approximately -190^{o}C.

4.3.5 Recovering cells from liquid nitrogen storage

Cell-line specific media was warmed to 37^{o}C in the water bath while the LAF hood was readied with UV light and 70% IMS. Once the media was warmed, 15 ml was placed in a T75 flask (or 25 ml in a T175 flask) and this was placed in the incubator to warm. The required cryotube was retrieved from the bio-freezer, and once excess liquid nitrogen had evaporated, placed briefly into the water bath to defrost. Once the vial contents defrosted, the vial was sprayed down with 70% IMS and a Pasteur pipette used to transfer its contents into the warmed flask. The flask was then returned to the incubator, at 37^{o}C, 5% CO_2, to let the cells adhere. Within 24 hours the cells were visually inspected for viability and the media changed to remove any trace of cryopreservative.

4.3.6 Cell Counting

4.3.6.1 Haemocytometer Method

A haemocytometer refers to a modified thick glass microscope slide, used with a cover slip and light microscope, to estimate the concentration of cells in a given volume. The glass slide has a rectangular indentation that creates a chamber of known depth (0.1 mm) and has been engraved with a grid of perpendicular lines (grid area of 1mm). Counting the cells within this given volume enables calculation of the concentration of cells in the overall sample volume i.e. the number of cells per 0.1 mm^2 gives the number of cells per 0.1 µl. Multiplying this by 10 gives the number of cells per µl, and by 10,000 the number of cells per ml.

Prior to use, the shoulders of the haemocytometer were moistened and a cover slip affixed. The cell suspension (10 µl) was applied to the edge of the coverslip and was seen to be drawn under the cover slip by capillary action, filling the chamber. Focusing on one set of 16 corner squares, the number of cells within this area was counted (Figure 4.2). Next, the number of cells in each of the other three 16 corner squares was counted. The average number

of cells in one 16 corner square is equal to the number of cells x 10^4. To calculate viable cell count, an equal volume of trypan blue can be mixed with the cell suspension before placement on the haemocytometer slide. Trypan blue selectively penetrates the cell membranes of dead cells, colouring them blue. The total cell count, less the dead cell count provides the viable cell count.

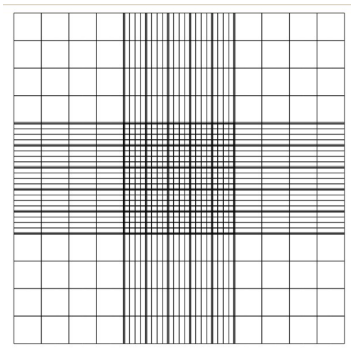


Figure 4.2 Haemocytometer Grid

4.3.6.2 Nucleocounter® Method

The automated Nucleocounter® NC-100™ uses fluorescence from propidium iodide (PI) to determine total and viable cell counts. PI is immobilised within the Nucleocounter's NucleoCassette and has the ability to cross the permeable plasma membrane of dead cells, staining their nuclei and providing a dead cell count. To obtain total cell count, the plasma membranes of all cells must be disrupted with the use of lysis buffer (Reagent A100) and stabilising agent (Reagent B). Subtracting dead from total cell count provides viable cell count.

To use the NucleoCounter®, 100 µl of cell suspension was transferred to two labelled eppendorfs; one eppendorf to calculate total cell count, the other to calculate dead cell count. The cell suspension in eppendorf A was treated with 100 µl of lysis buffer (Reagent A100), vortexed for 15 seconds, then 100 µl of stabilising buffer (Reagent B) was added. These reagents lyse and stabilise all cells for staining and enable calculation of total cell count. The NucleoCasette is loaded with the suspension and placed in the NucleoCounter for analysis. The result provided is multiplied by three to account for the 1:3

dilution of cell suspension, Reagent A100 and Reagent B. Eppendorf B is loaded directly into a fresh NucleoCassette (without the addition of any reagents). PI will in this case stain only cells with permeable membranes, providing a dead cell count.

4.3.7 Freezing down of cell pellets for mRNA analysis

To analyse the expression of a panel of miRNA in cell lines representing the molecular subtypes of breast cancer and a non-cancer control, cell pellets from MCF-10A, T47D, BT-474, SK-BR-3 and BT-20 cell lines were collected. Once a flask of cells was trypsinised, it was transferred to a 15 ml tube containing 5 ml of cell-line specific media and centrifuged at 1500 rpm for 5 minutes to pellet cells. Once the supernatant was discarded and the cell pellet washed in 1ml of PBS, the reconstituted cell pellet was again centrifuged at 1500 rpm for 5 minutes. The supernatant PBS was discarded and 1 ml of Trizol thoroughly mixed through the cell pellet. The eppendorf containing the cell suspension was then stored in the -80^{o}C freezer until required.

4.3.8 Immunohistochemical Staining of Cell Lines

Cell lines for immunohistochemical staining were seeded at a concentration of 1 x 10^4 onto cover slips within the wells of 6 well plates. Each well was washed with PBS before cells were fixed to cover slips with Methanol that had been stored at -20^{o}C. Once chilled methanol was applied, each 6-well plate was incubated at -20^{o}C for 15 minutes before rinsing and storage in PBS at 4^{o}C until required.

The Anti-Mouse Ig ImmPRESS™ *Excel* Staining Kit (Vector Laboratories) in conjunction with a commercially available Argonaute-2 specific antibody (*ChIP Grade, ab57113, Abcam, UK)* was utilised to stain our cell lines of

interest (Appendix 3)[11]. One cover slip for each cell line to be analysed was stained. Firstly, cover slips were incubated in 2.5% Normal Horse Serum for 20 minutes before being incubated in 1:2000 Ago-2 primary antibody for one hour. Following this, PBS-tween (50ml PBS + 50µl Tween 20) was used to wash each cover slip for 5 minutes. Amplifier Antibody was next applied for 15 minutes before a second wash step, using PBS-tween again for 5 minutes. ImmPRESS™ *Excel* reagent was next applied and incubated for 30 minutes before two 5 minutes wash steps, each lasting 5 minutes. To develop the stain, equal volumes of ImmPACT™ DAB EqV Reagent 1 and 2 were mixed before being applied to each coverslip to a controlled standardised length of time (1 minute each). Following the 1 minute exposure to ImmPACT™ DAB EqV Reagent 1 and 2, water was used to wash each cover slip for 5 minutes. As a counterstain, each cover slip was dipped in Haematoxylin for 7 seconds before being washed in water for 10 minutes. DPX was used to fix cover slips to slides and each was left to stand overnight before staining was assessed.

In terms of controls for this staining, two wells from each 6-well plate contained a control. On each plate, one well contained the non-cancer control cell line (MCF-10A), which is known to express little to no Ago-2, and this was stained as per the immunohistochemical protocol outlined above. One further well on each plate was stained as per the delineated protocol, with the exception of the Ago-2 specific primary antibody incubation step, which was omitted. It would be expected that each of these two wells in each 6-well plate therefore would be devoid of staining.

4.3.9 Vector Propagation

EGFP-hAgo2 (Plasmid #21981) was purchased from addgene[12] and propagated via bacterial culture. Luria broth (LB) and LB with agar were prepared by mixing 10g Tryptone, 5g Yeast and 10g NaCl in 1L of ddH_2O,

[11] http://www.abcam.com/argonaute-2-antibody-2e12-1c9-chip-grade-ab57113.html
[12] https://www.addgene.org/21981/

with agar being added to one jar at a concentration of 5g /L**.** Both were autoclaved, and upon cooling, Kanamycin was added to each jar, at a concentration of 50µg/ml, and agar plates were poured. Using sterile loops, Kanamycin –resistant bacteria containing Ago2 plasmid were streaked onto the plates and grown in an incubator at 37^{o}C overnight. The following morning, a single colony was selected from the agar growth using a sterile loop and added to LB broth (200ml containing 200µl Kanamycin). This was then placed in a bacterial shaker at 175 rpm at 37^{o}C overnight to allow the bacteria to multiply. Growth was identified by cloudiness of the media.

4.3.10 Vector Isolation

To isolate the desired vector DNA from bacterial cells the Zyppy™ Plasmid Maxiprep Kit (Zymo Research, The Epigenetics Company) was used as per manufacturers' instruction protocol.

150ml of bacterial culture was centrifuged a 3,400 x g for 10 minutes to pellet bacterial cells. Following this, the supernatant discarded and 15ml of P1 Buffer (Red) was used to re-suspend the pellet by mixing thoroughly and vortexing. 15ml of P2 Buffer (Green) was then added and immediately mixed by inverting 4-6 times. The mixture was then left to stand for one minute at room temperature to allow cell lysis. P3 Buffer (Yellow) was removed from the fridge and 20ml was mixed gently but thoroughly through the solution. This was left to incubate on ice for 5 minutes. During this time the sample was seen to turn yellow with a precipitate becoming visible. The entire mixture was then transferred to a 50ml conical tube and centrifuged at 3,400 x g for 10 minutes to pellet bacterial cell debris. While this mixture was spinning, the kit's *Zymo-Spin™ VI* column was placed into a new 50ml conical tube. 10 ml of the supernatant produced was added to the *Zymo-Spin™ VI* column, the lid replaced and spun at 3,400 x g for 2 minutes. The flow through was discarded and this step repeated until all of the supernatant had been filtered through the column. 10ml of Endo-Wash Buffer was added to the column and centrifuged at 3,400 x g for 30 seconds before the flow through was discarded. 10ml of Zyppy™ Wash Buffer was next added to the

column and centrifuged at 3,400 x g for 1 minute before discarding the flow through. The *Zymo-Spin™ VI* column was transferred into a clean 50ml tube and 2-3 ml of Zyppy™ Elution Buffer added. This was left to stand at room temperature for 1 minute before being centrifuged at 3,400 x g for one minute to elute the plasmid DNA. Finally, the flow through was transferred to a storage tube and placed at -80^{o}C until required.

4.3.11 Vector verification by Restriction Enzyme Digest

Prior to transfecting cell lines, the sequence of the transcript required verification. Argonaute-2 transcript was verified by restriction enzyme digest using the restriction enzymes BamH1-HF (CutSmart #R3136S) and EcoRV-HF (CutSmart #R3195S).

This process commenced with the identification of the 3' and 5' cloning sites of the Ago-2 plasmid (addgene plasmid #21981). BamHI and EcoRI respectively were identified as the restriction sites. EcoRI however was not available in our laboratory. The site addgene plasmid repository was searched and it was identified that EcoRV, which was available in our laboratory, is also located at a single location on our transcript, proximal to EcoRI, without appearing in the construct. As such it was chosen to assess the plasmid using BamHI and EcoRV as our restriction enzymes.

To assess each restriction enzyme individually and in combination, three separate reactions were performed and incubated at room temperature for at least 1 hour. Reaction A assessed both Bam H1 and EcoRV, Reaction B only Bam H1 and Reaction C only EcoRV as outlined in Table 4.2.

Table 4.2 Reagents utilised for transcript verification

Reagents	Reaction (µl)		
	A	B	C
Bam H1-HF	1	1	-
EcoRV-HF	1	-	1
CutSmart Buffer	5	5	5
Plasmid (1µg)	15	15	15
NFW	28	29	29

A 1.5% agarose gel was prepared by mixing 0.75 g of MetaPhor® Agarose, 10 µl Sybergreen and 50ml of 1x Tris Acetate-EDTA (TAE) buffer (Sigma #T9650-4L). This mixture was heated until dissolved and gelatinous. The agarose mixture was poured into the gel template and allowed to solidify for approximately 20 minutes. While the gel was setting, 10 µl of each reaction above (A, B and C) was added to 3 µl loading buffer and mixed. Once the gel hardened, the comb was removed from the wells and the gel was placed in the chamber (orientated with the wells to the black end). 1 x TAE buffer was poured over the gel to fill the chamber. A standard ladder and samples were loaded into the wells before connecting the chamber to a power pack and running at 100 V for 20 minutes. A transilluminator (ultraviolet light box) was then used to visualise the gel and images captured for analysis.

4.3.12 Cell-line Transfection

The *Amaxa® Cell Line Nucleofector® Kit V* (Lonza) was used to transfect selected cell lines.

Cell lines for transfection were cultured as per protocol. Prior to transfection, media was removed from the desired cell lines and each T175 flask was rinsed with 10 ml PBS. For harvesting, cells were incubated in 5 ml of 0.25% trypsin/EDTA for 5 minutes, before trypsin neutralization in 5 ml of cell line-specific culture medium. Cell-counting was next performed to calculate desired volumes for transfection. The required number of cells (1×10^6) per

cell line was centrifuged at 90 x g for 10 minutes at room temperature to pellet the cells and the supernatant removed completely. 100 µl of Nucleofector® Solution was used to re-suspend each cell pellet. In a cuvette, 2 µg of DNA or 2 µg of control vector was mixed with the 100 µl of cells suspended in Nucleofector® Solution. The desired programme was selected (p-020) and each cuvette inserted in turn into the Nucleofector® Cuvette Holder and the program run by pressing the X-button. Once the programme had finished, a pipette was used to transfer the transfected cells into the pre-warmed, antibiotic infused media in the 6-well plates and they were returned to the incubator, humidified to 37°C in 5% CO_2.

Table 4.3 Contents for each Transfection Reaction

Each reaction contains	
Cells	1 x 10^6
Plasmid DNA	2 µg
Cell Line Nucleofector® Solution V	100 µl

For each reaction, 82 µl of Nucleofector® Solution was mixed with 18 µl of supplement to make 100 µl of total reaction volume.

Cell transfection was confirmed using fluorescence microscopy with the EVOS® FL Cell Imaging System (ThermoFisher Scientific) and transfected cell mRNA analysis.

4.3.13 Preparation of DNAse/Buffer RDD

A solution of DNAse in Buffer RDD was required for the extraction of RNA from cell pellets.

Using the RNase Free DNase Set (Qiagen #79254), the DNase was firstly reconstituted using 550 µl of NFW. The enzyme bottle swirled before being left for 5-10 minutes to allow the powdered enzyme to reconstitute. The enzyme solution was aliquoted into labelled tubes before being stored in the

-20°C freezer until required. Prior to use, a solution of DNase in Buffer RDD was prepared using a 1:8 dilution.

4.3.14 RNA extraction from cell pellets

The Qiagen RNeasy® Mini Kit was used for extraction of RNA from cell pellets.

Cell pellets frozen in Trizol® were removed from the -80°C freezer and defrosted on ice. Once thawed, 200µl of chloroform per ml of Trizol previously added to the homogenate was added. Samples were shaken vigorously/vortexed for 15 seconds before being left to stand at room temperature for 5-10 minutes. The homogenate was then centrifuged at 12,000 x g for 15 minutes at 4°C before the upper aqueous phase, containing the RNA, was transferred to a new tube (approximately 600 µl). 3.5 volumes of 100% ethanol was added to the aqueous phase and mixed thoroughly by vortexing. 700 µl of this mixture was then pipetted onto the RNeasy® column and centrifuged at maximum speed at 4°C for 21 seconds. This process was repeated until all of the mixture was passed through the column. Once filtered, 350 µl of buffer RW1 was added to the RNeasy® column before centrifuging at maximum speed at 4°C for 21 seconds. Next, 80 µl of DNase/Buffer RDD mix was added to the filter of each column before incubating at room temperature for 15 minutes. 350 µl of buffer RW1 was again pipetted onto the RNeasy column before centrifuging at maximum speed at 4°C for 21 seconds. 500 µl buffer RPE was then added to the column before centrifugation at maximum speed at 4°C for 21 seconds. The flow through was discarded before another 500 µl of buffer RPE was centrifuged through the column at maximum speed at 4°C, this time for 2 minutes. The RNeasy® column was finally transferred to a 1.5 ml eppendorf before 50 µl of chilled NFW was pipetted directly onto the membrane and centrifuged at maximum speed for 1 minute at 4°C. The final filtrate was transferred to a storage tube, RNA purity and concentration assessed using the Nano spectrophotometer and the sample placed in the -80°C freezer until required.

4.3.15 Reverse Transcription of mRNA

MRNA (from RNA extracted from cell pellets, section 4.3.14) was reversed transcribed to complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). Using spectroscopy results (method outlined in section 3.3.7), 1 µg of RNA from each sample to be analysed was added to the following reagents:

Reagents	Amount
1 µg total RNA	n µl
50 ng/µl random hexamers	1
10 mM dNTP mix	1
DEPC-treated water	Up to 10 µl

Each sample was incubated at 65°C before being placed on ice for 1 minute. The following cDNA synthesis mix was next prepared by adding each component in the indicated order:

Reagents	Volume per Reaction (µl)
10 X RT Buffer	2
25mM $MgCl_2$	4
0.1 M DTT	2
RNase OUT™ (40 U/µl)	1
SuperScript® III RT (200 U/µl)	1

10 µl of this cDNA synthesis mix was added to each sample before brief centrifugation. Samples were then incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes, with a final 5 minutes at 85°C before cooling on ice.

Finally, 1 µl of RNAse H was added to each tube before incubation at 37°C for 20 minutes. cDNA was then stored at -20°C until required.

4.3.16 Real-Time Polymerase Chain Reaction (RQ-PCR)

RQ-PCR was used for the relative quantification of our target gene Ago-2 within our cell lines. Two endogenous control genes, Mitochondrial Ribosomal Protein L19 (MRPL19) and Peptidylprolyl Isomerase A (PPIA) were used for normalisation of our results. TaqMan assays were obtained (pre-formulated forward/reverse primer and MGB probe, Life Sciences) for our target of interest Ago-2, and our endogenous controls PPIA and MRPL-19. For each sample to be analysed the following reagents and volumes were required:

ABI miRNA Kit:	
TaqMan Fast Mastermix	5 µl
NFW	3.5 µl
TaqMan Assay (PDAR)	0.5 µl
cDNA	1 µl
Total Volume	**10 µl**

Each reaction was run on a 96-well plate, with each well containing 10 µl of solution (as above). Each sample being analysed (cell lines) was run in triplicate to determine intra-assay variation. Each plate also contained a 'No Target Control' (NTC) and an RTC (as described in Section 3.3.10) to ensure the purity of the RQ-PCR and reverse transcription processes. These wells contained target-specific premix and 1 µl of NFW or target-specific RTC respectively. Further to this, each plate contained an inter-assay control (IAC) to account for inter-assay variability (threshold standard deviation for inter-assay replicate variability was 0.3). A MicroAmp optical adhesive film was used to seal each plate before being spun in a large centrifuge at 2000 rpm for 1 minute. The plate was then loaded into the AB7900HT instrument and standard fast thermal cycling conditions were used: heating to 95°C for

15seconds x 40 cycles to open the DNA double helix, followed by 60^{o}C for 60sec to allow annealing to take place.

4.3.17 Relative Quantification

As detailed in section 3.3.13, RNA expression levels require normalization to those of endogenous controls. For mRNA analysis, the endogenous controls utilized were PPIA and MRPL-19. Raw fluorescence data from RQ-PCR was exported into the software package qBase plus and relative quantification assessed.

4.3.18 Proliferation Assay

The *CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay* (MTS, Promega) was utilised to assess cellular proliferation at 24, 48 and 72 hours post gene transfection. This assay provides a colorimetric method for determining the number of viable cells in proliferation at a given timepoint. Transfected cell lines were cultured as per protocol. Once sufficiently confluent, cells were seeded at a density of 1 x 10^4 in a 96 well plate. Three 96 well plates were constructed as per Figure 4.3, one to be assessed for each of the three timepoints of interest. At the 24 hour, 48 hour and 72 hour timepoints, as per manufacturer protocol, a mixture of tetrazolium compound (MTS) and phenazine methosulphate (PMS) were added to each well containing cells. The 96 well plate was then returned to incubation at 37°C and 5% CO2 for 3 hours before reading absorbance on a luminometer (Multiskan RC, Thermo, Fisher Scientific) at 490 nm.

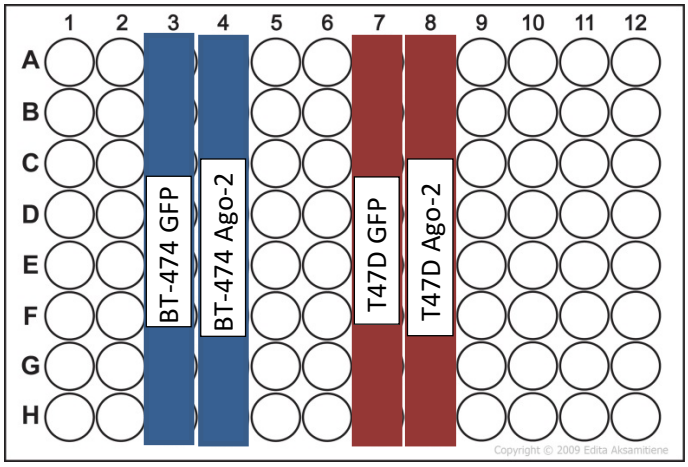


Figure 4.3 Loading of 96-well plate for proliferation assay

4.3.19 Tissue Microarray (TMA)

In collaboration with the department of pathology, a tissue microarray of approximately 700 breast cancer specimens was constructed and stained for Ago2 (Figure 4.4). A specified protocol for Ago2 staining, via ImmPACT/ImmPRESS DAB Immunohistochemistry, was optimised by collaborators in the department of pathology. To achieve this a number of known Ago-2 positive specimens (e.g. colorectal specimens) were stained and included in the array for reference. Clinical breast tissue samples comprised core biopsies, wide local excisions and mastectomy specimens received by the pathology department during the period 1996 to 2007. Patient clinicopathological data corresponding to each individual specimen was collected and collated.

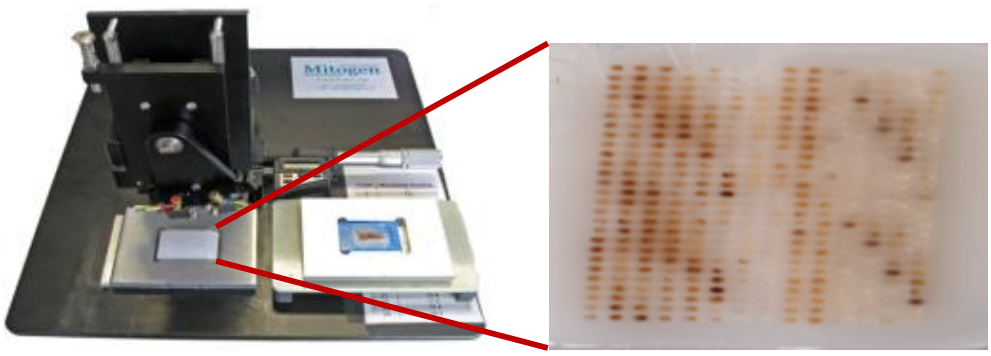


Figure 4.4 Tissue Microarray Construct (0.6µm cores 0.8µm apart)

4.3.20 TMA Scoring

In collaboration with clinical pathologists a scoring system was developed based on the observed pattern of Ago2 staining in control and breast cancer specimens (see Section 4.4.4.2/3). This scoring system was employed by two study researchers, who scored the TMA images in a blinded manner, independent of one another. Independent TMA score databases were then sent to study biostatisticians to investigate clinicopathological correlation.

4.3.21 Statistical Analysis

4.3.21.1 Laboratory Statistical Analysis

Gene expression values obtained from RQ-PCR were exported from qBase to Minitab (v17) statistical software for analysis. As the mRNA expression data was not normally distributed, all results were log transformed for analysis. One-way ANOVA was used to test for significance between cell line mRNA expression values. Significance was reached if $p \leq 0.05$.

4.3.21.2 Biostatistician Analysis

Study biostatisticians performed the statistical analysis of the tissue microarray data. A range of association tests were employed. The Pearson X^2 test was used to test for general association between two nominal categorical variables. Cochran-Mantel-Haentzel was employed when taking ordinal variables into account. ANOVA and Kruskal-Wallis H tests were used to test for differences in distributions of a continuous variable for different populations. To calculate the effect size of significant differences a series of Mann-Whitney tests were used with Bonferroni correction to control for inflation of Type I error. Significance was reached if $p \leq 0.05$.

4.4 Results

4.4.1 Endogenous Ago2 mRNA in Breast Cancer Cell Lines

Baseline Ago2 mRNA expression was assessed in cell lines representing the four molecular subtypes of breast cancer and a control cell line. Ago2 mRNA was successfully detected in each of four breast cancer cell lines (T47D, BT-474, SKBR3, BT-20), and one non-cancer control cell line (MCF-10A). Expression was assessed in triplicate from each of these cell lines with results outlined in Figure 4.5. The T47D cell line, which represents Luminal A breast cancer, expressed Ago2 mRNA at significantly higher levels than either the SKBR3 (HER2 breast cancer) and BT-20 (triple negative breast cancer) cell lines (Anova p= 0.005).

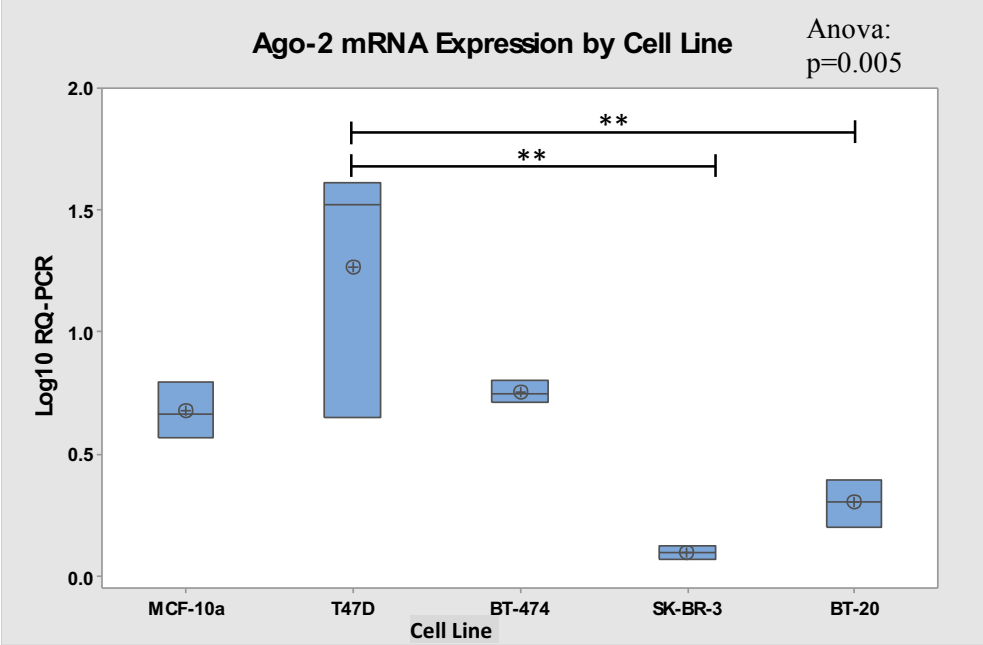


Figure 4.5 Ago2 mRNA expression by cell line
(Expression was assessed in triplicate from each cell line)

4.4.2 Ago2 Staining Pattern & Intensity in Breast Cancer Cell Lines

Further to assessing Ago2 mRNA in cell lines, Ago2 protein content was also investigated. Immunohistochemistry, with staining for Ago2 protein, was conducted on four breast cancer cell lines (T47D, BT-474, SKBR3, BT-20) and one non-cancerous control cell line (MCF-10A), to assess Ago2 staining pattern and intensity *in-vitro*. The control cell line and the negative control (no primary antibody used) displayed a complete lack of staining (predominantly grey in colour, Figure 4.6A). Ago2 protein was detected in each breast cancer cell line, represented by the orange/brown colouration (Figure 4.6B). Ago2 staining pattern and intensity varied by breast cancer cell line, as outlined in Figure 4.6. Luminal cell lines (T47D and BT-474) displayed predominantly cytoplasmic Ago2 staining, of weak to moderate intensity. HER2 (SKBR3) and the Triple Negative (BT-20) cell lines displayed stronger Ago2 staining that was present both within the cytoplasm and, particularly in the HER2 cell line, within the nucleus.

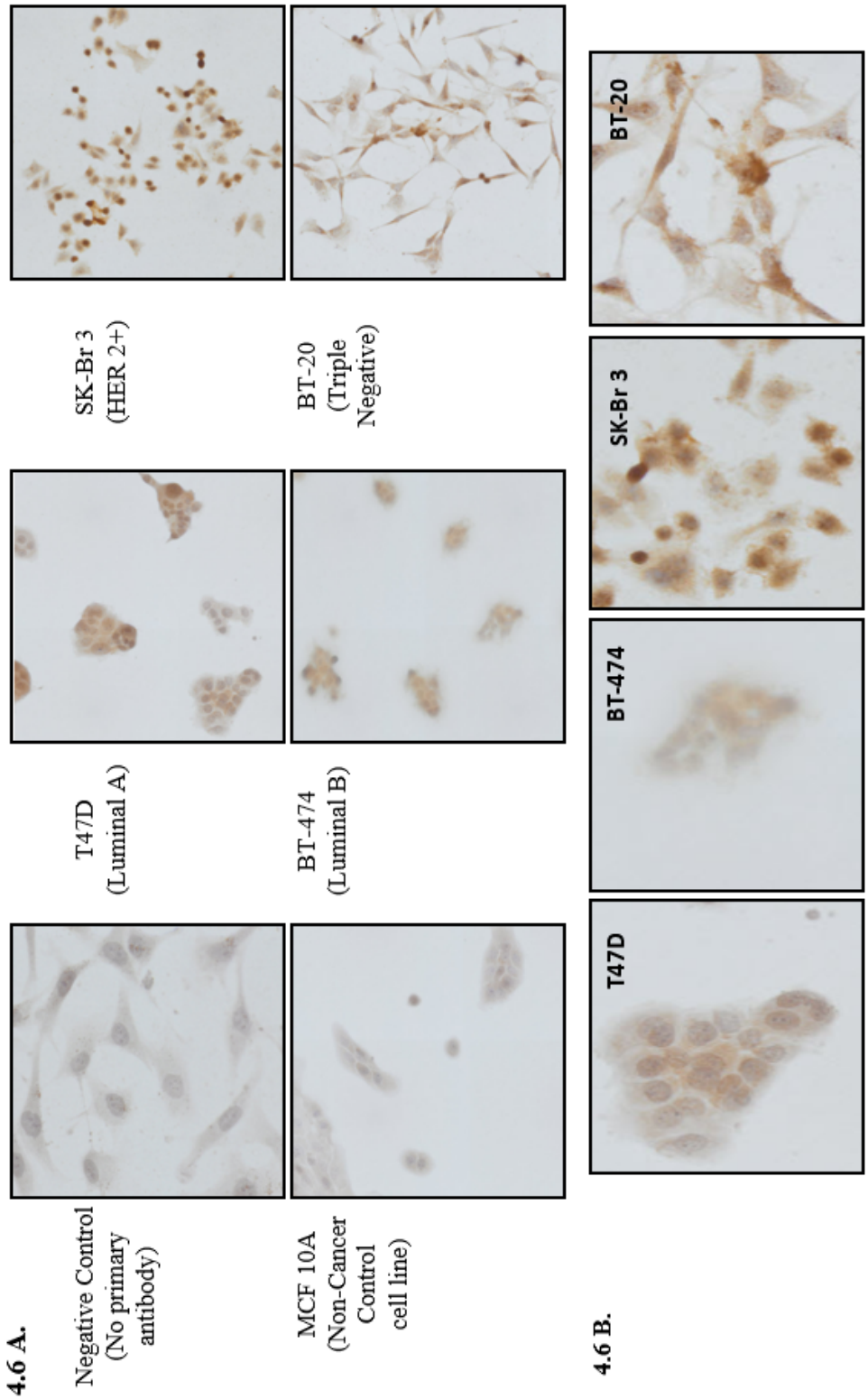


Figure 4.6 A Breast Cancer Cell Line IHC Images (20X)
B. Higher Magnification of each Breast Cancer Cell Line (60X)

4.4.3 Ago2 Transfection

To assess the functional impact of Ago2 expression, Ago2 was transfected into selected cell lines and a proliferation assay conducted.

4.4.3.1 Transcript Verification

Prior to transfection of cell lines, the size of the Ago2 construct was verified by restriction enzyme digest using the restriction enzymes BamH1-HF (CutSmart #R3136S) and EcoRV-HF (CutSmart #R3195S), as outlined in Figure 4.7. Vector backbone size was 4.7 kb and gene insert size of 2.6 kb. In independent digest, BamH1 and EcoRV each resulted in a single band of the full size of the vector, ~7.6 kb (red boxes), and combined digest matched the backbone size of 4.7 kb and the insert size of 2.6 kb (blue box). As the results on this gel match the predicted sizes from the plasmid information, the Ago2 gene sequence was confirmed.

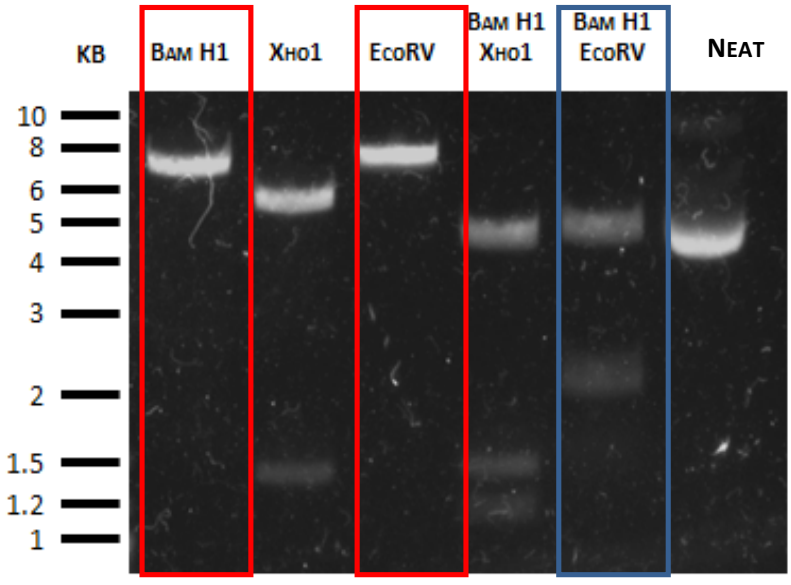


Figure 4.7 Restriction enzyme digest confirming Ago-2 vector size

4.4.3.2 Cell Line Selection

Based upon the immunohistochemical cell line analysis, Ago2 protein levels appeared lowest in luminal cell lines T47D and BT-474. Ago2 mRNA analysis revealed the T47D cell line to have significant levels of Ago2

mRNA, with BT-474 cells having intermediate amounts. As such, BT-474, the Luminal B cell line, was chosen for transfection, so that the effect of Ago2 over-expression could be assessed.

4.4.3.3 Confirmation of Transfection

The *Amaxa® Cell Line Nucleofector® Kit V* was used to transfect the BT-474 cell line, as outlined in Section 4.3.13. To confirm effective transfection, fluorescence microscopy was employed. The EVOS® FL Cell Imaging System (ThermoFisher Scientific) was used to visualise cells transfected with Ago2-GFP or the GFP alone control. Transfected cells survived culture by vector-mediated antibiotic-resistance.

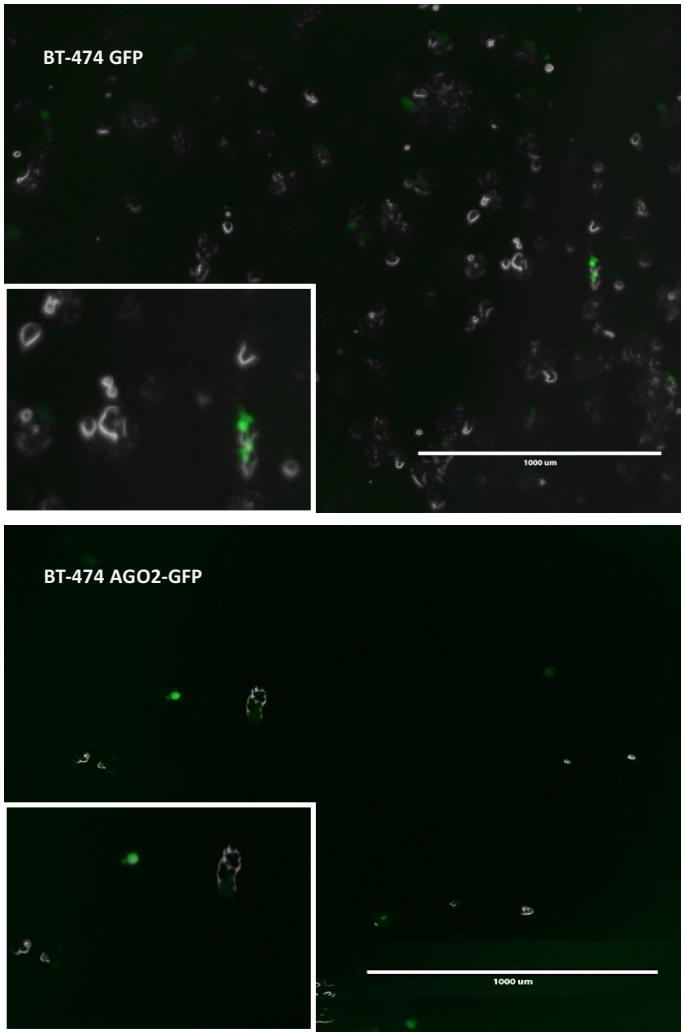


Figure 4.8 Images of cell line BT-474 successfully transfected with A. GFP alone control and B. Ago2-GFP.

4.4.3.4 Ago2 mRNA Analysis in Transfected Cells

To further confirm the expression of Ago2 mRNA in the transfected BT-474 cells, the level of Ago2 mRNA within the Ago-GFP transfected cells and the GFP-alone transfected cells (control) was assessed by RQ-PCR as outlined in Section 4.3.16. The expression of Ago2 was not up-regulated in the transfected cells compared to control (Figure 4.9).

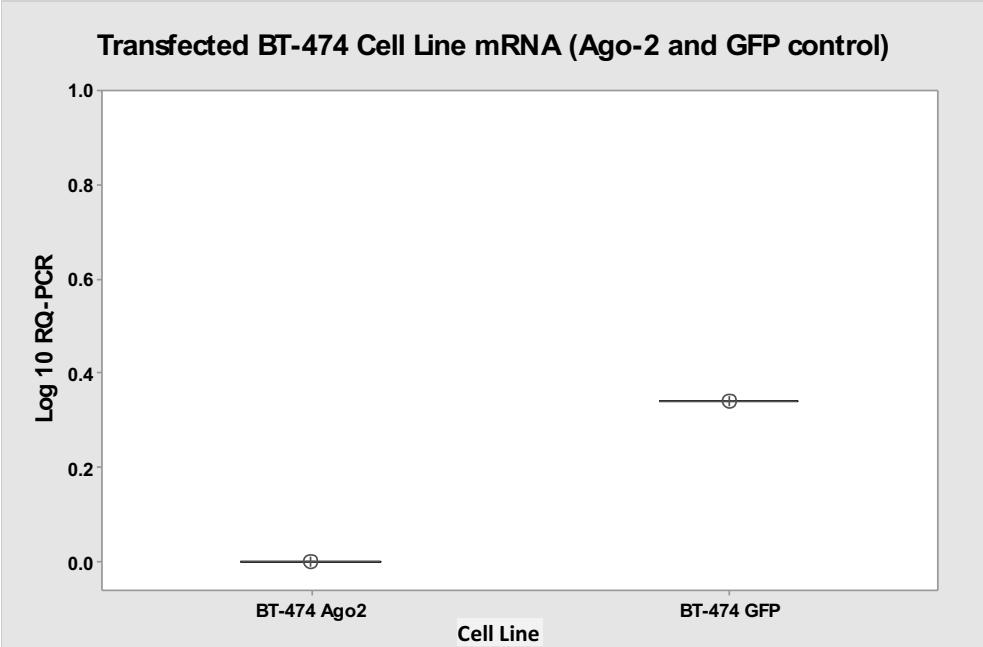


Figure 4.9 Ago2 mRNA levels in transfected BT-474 cells

4.4.3.5 Proliferation Assay of Transfected Cells

To assess the functional effects of Ago2 transfection into BT-474 cells, a proliferation assay was conducted in duplicate as outlined in Section 4.3.18. The Ago2 transfected cells were seen to proliferate at a mildly increased rate, particularly at the 48 hour timepoint.

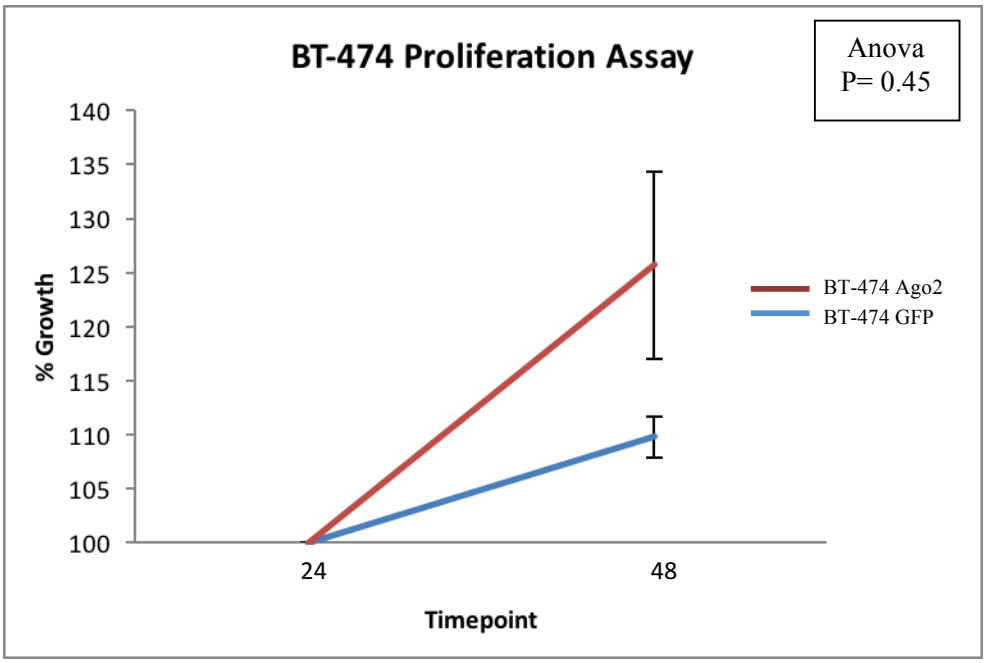


Figure 4.10 Proliferation assay revealing a mild increase of proliferation in cells transfected with Ago2.

4.4.4 Tissue Microarray

Having analysed Ago2 in breast cancer *in-vitro* and detecting differences in expression and localisation between breast cancer subtypes, investigation of Ago2 in clinical patient samples was undertaken to assess the clinical relevance and potential application and translation of Ago2 analysis.

Of the initial 700 clinical breast specimens included in the array, 391 samples were effectively transferred upon visual analysis, with the remaining 309 specimen sections exhausted. The following results represent the findings from these 391 samples.

4.4.4.1 Array Specimen Clinicopathological Parameters

Regarding patient demographics, median patient age was 58 years (range 29-93 years). The remaining sample data outlined is in Table 4.4. The majority of samples were from post-menopausal patients, with invasive ductal carcinoma, of Luminal A subtype. Most tumours were stage 2, grade 3, without lymph node involvement or metastasis. Mastectomy specimens predominated the array. As specimens were collected between 1996-2007, 126 specimens were not subtyped, as this predated HER2 testing.

Table 4.4 TMA Clinicopathological Data

Clinicopathological Data of Array Specimens			
Menopausal Status		**Procedure**	
Pre	91	Mastectomy	142
Peri	10	Biopsy	137
Post	246	Wide Local Excision	94
N/A	44	Segment/Quadrantectomy	18
Pathology		**Subtype**	
Invasive	383	Luminal A	133
Non-Invasive	6	Liminal B	35
N/A	2	HER2	21
Ductal	270	TNBC	76
Lobular	59	N/A	126
Other	62		
Tumour (T Score)		**Nodes (N Score)**	
Is	6	0	172
1	129	1	100
2	174	2	52
3	29	3	30
4	32	X	28
X	6		
N/a	15		
Grade		**Metastasis (M Score)**	
0	39	0	343
1	36	1	9
2	149	X	29
3	159	N/A	10
N/A	8		

4.4.4.2 Ago2 Stain Intensity and Pattern

The intensity of Ago2 staining was seen to vary from negative staining, where no stain was visible, to strong staining, where a vivid orange/brown colouring was visible in cell cytoplasm, as outlined in Figure 4.11. Ago2 protein staining was primarily confined to cell cytoplasm in all specimens, with minimal nuclear staining observed. This staining pattern and the stain intensity was in keeping with that observed in the *in-vitro* analysis conducted (Section 4.4.2) A predominance of epithelial staining occurred, with stromal cell preservation from stain (Figure 4.12).

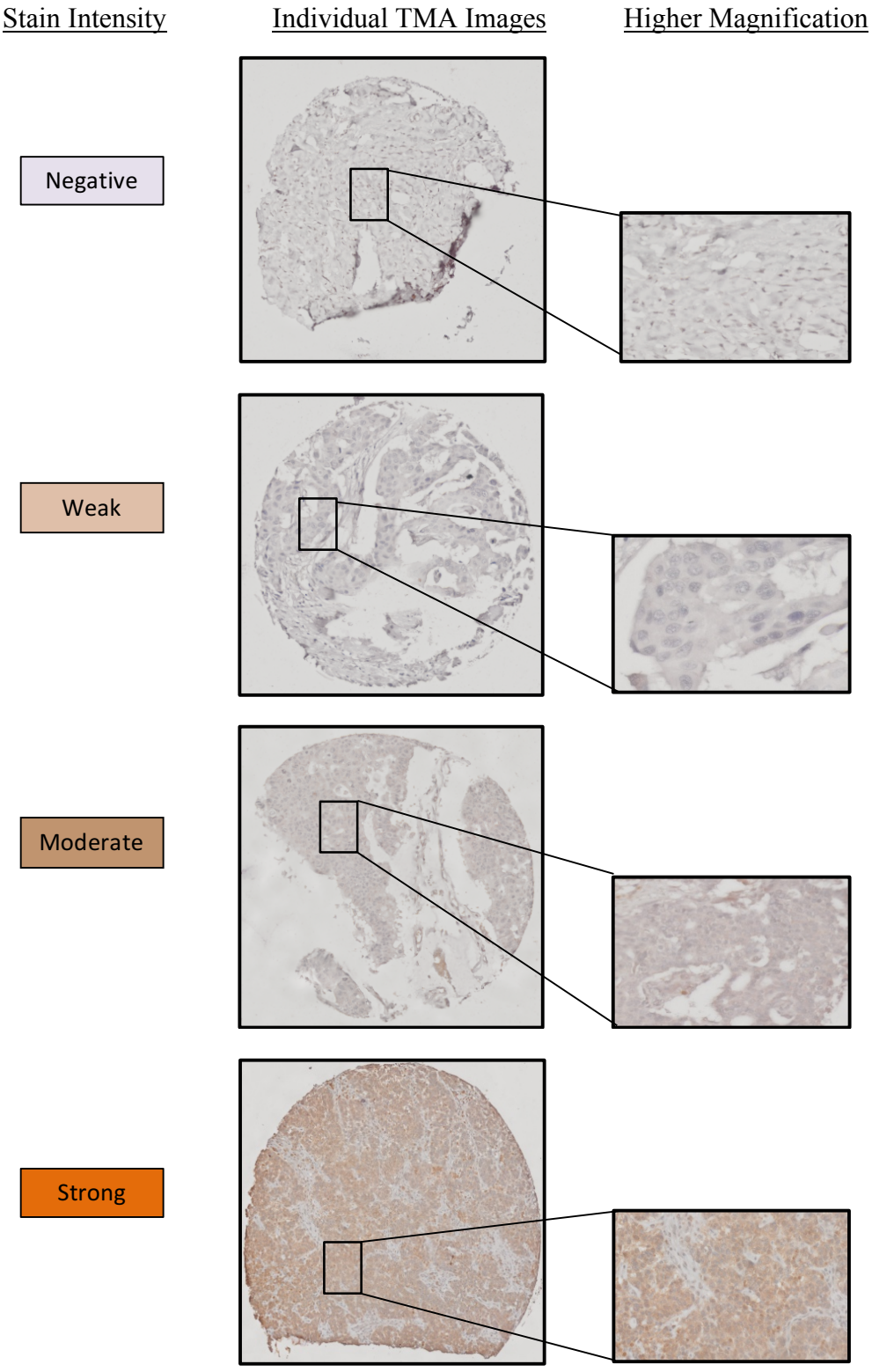


Figure 4.11 Examples of individual TMA images with stain intensity

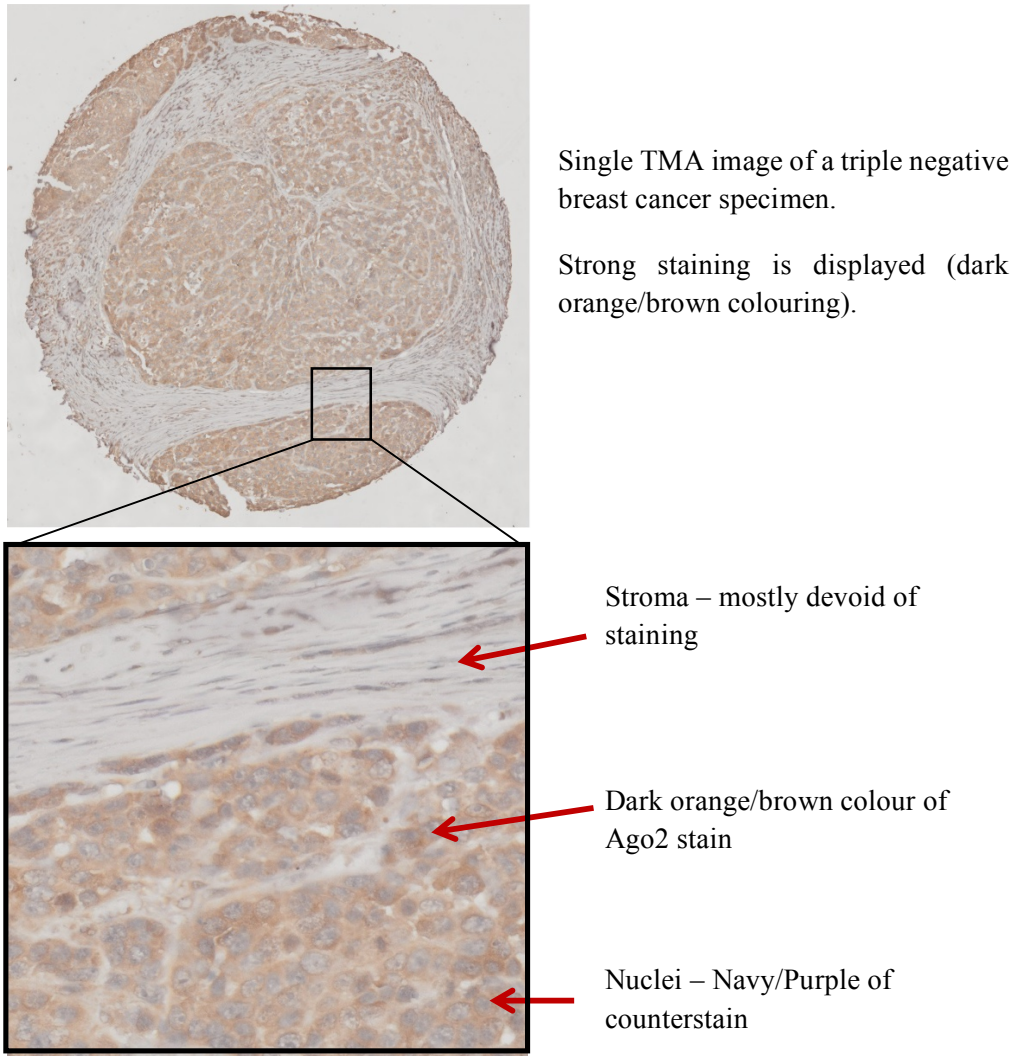


Figure 4.12. Example of Ago2 staining pattern

4.4.4.3 Custom Array Scoring Sheet

In collaboration with clinical pathologists, a custom scoring system was developed for this Ago2 tissue microarray (figure 4.13). Two independent researchers who were blinded to sample/patient clinicopathological details analysed the array images using this customised scoring system.

Hospital Numbe	Path ID	TMA specimen number	TMA coordinate (Row, Column)	TMA identifer	Ago 2 Cytoplasm staining
			R10C1_01_PAIV14	1107 PA IV 14 t/m 17	
			R10C2_01_PAIV14	1107 PA IV 14 t/m 17	
			R10C3_01_PAIV14	1107 PA IV 14 t/m 17	
			R10C4_01_PAIV14	1107 PA IV 14 t/m 17	

Figure 4.13. Brief excerpt from the customised Ago2 scoring system that was devised in collaboration with clinical pathologists. The final column is blank and was populated by two independent researchers who were blinded to sample details.

Of the 391 specimens effectively arrayed and stained, negative and weak staining predominated, with a lesser number of moderate and strongly stained samples (Table 4.5).

Table 4.5 Summary of Ago2 categories by stain intensity

Intensity	No. Samples	(%)
Negative	121	(31)
Weak	121	(31)
Moderate	77	(20)
Strong	66	(18)
Total	385	

4.4.4.4 Ago2 staining intensity association analysis

Ago2 staining intensity results were analysed for association with patient demographics and clinicopathological parameters, as outlined in the following sections.

4.4.4.4.1 Patient Demographics

No significant difference in age distribution was identified between the Ago2 stain intensity categories (Table 4.6, Figure 4.14). Further to this, a similar pattern of menopausal status was identified within each category, without evidence of association with Ago2 staining (Figure 4.15).

Table 4.6 Age distribution of patient samples

Ago 2	Mean Age	Median Age
Negative	58.6	57
Weak	57.8	57
Moderate	58.4	56
Strong	58.3	56

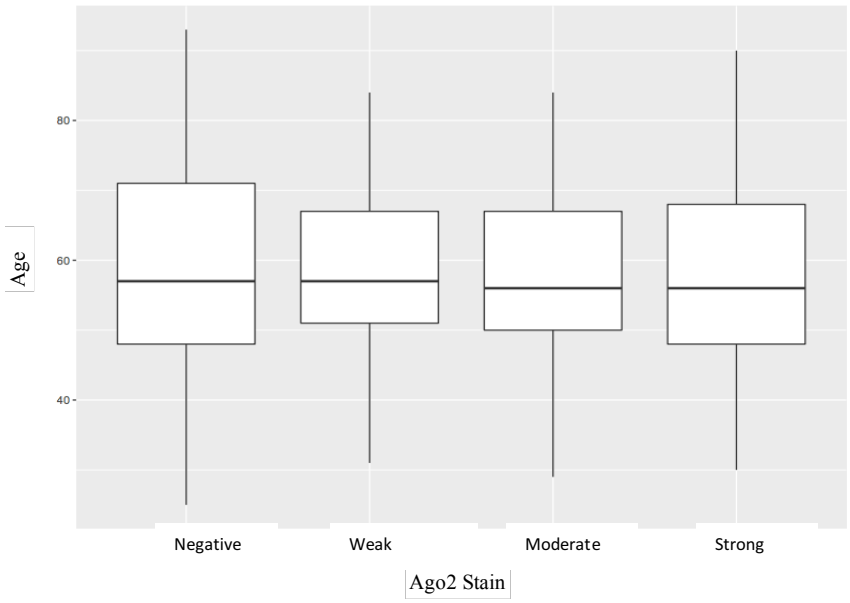


Figure 4.14 Age distribution within each Ago2 staining category

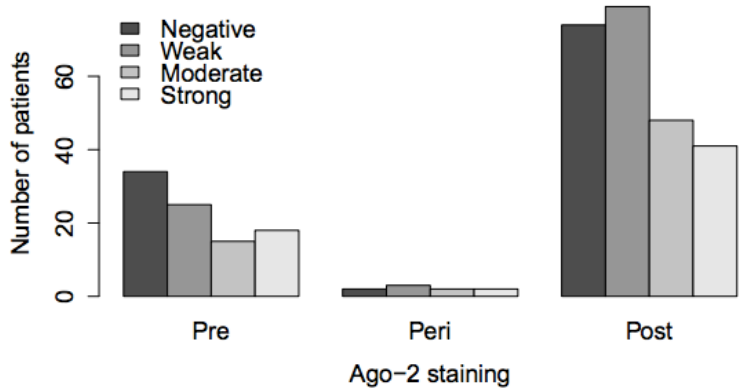


Figure 4.15 Ago2 staining intensity by menopausal status

4.4.4.4.2 Breast Cancer Subtype

Of the 391 effectively stained specimens, 263 samples had a known definitive subtype, and were assessed for association with Ago2 stain intensity. A significant association was identified (p=0.0001), whereby Luminal A specimens stained predominantly negative to weak, with basal specimens predominantly strongly stained (Figure 4.16).

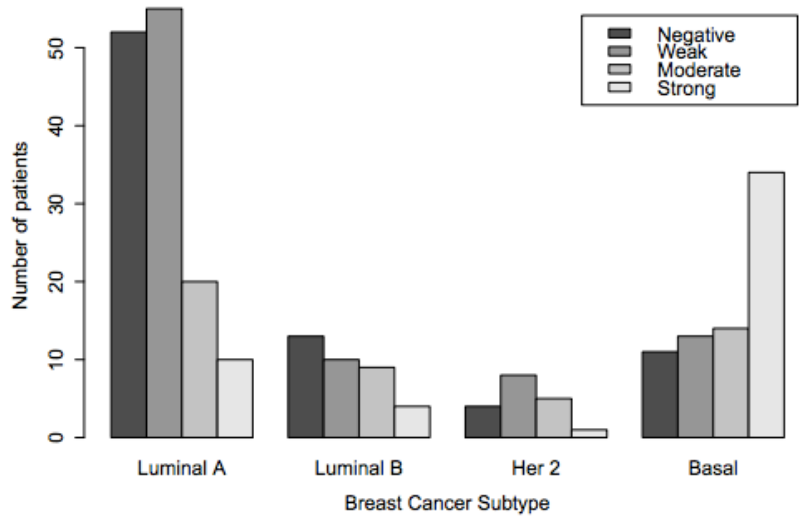


Figure 4.16 Ago2 staining by breast cancer subtype. Luminal A stained predominantly negative-weak and basal primarily strongly.

4.4.4.4.3 Oestrogen Receptor Status

Of specimens effectively stained, 353 had known oestrogen receptor status and were analysed for association with Ago2 stain intensity. A significant inverse relationship was identified between the presence of the oestrogen receptor and stain intensity (p=0.0001). Oestrogen receptor positive specimens were predominated by negative stain intensity, with numbers of oestrogen positive samples reducing as stain intensity increases. Strong stain intensity was predominated by oestrogen negative samples (Figure 4.17).

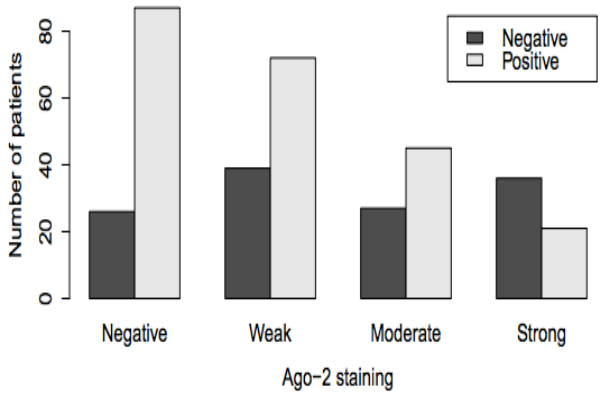


Figure 4.17 Ago2 staining intensity by oestrogen receptor status

4.4.4.4.4 Progesterone Receptor Status

For the analysis of progesterone receptor status, 349 specimens were included. A statistically significant inverse relationship was identified, whereby stain intensity decreased with progesterone receptor positivity. Negatively stained samples were predominantly progesterone receptor positive, with strongly stained samples progesterone receptor negative.

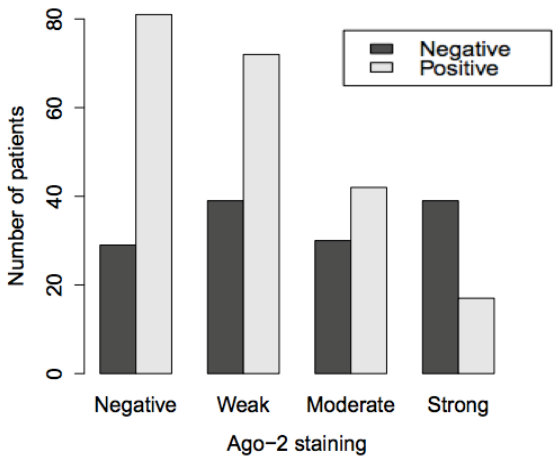


Figure 4.18 Ago2 staining intensity by Progesterone receptor status

4.4.4.4.5 HER2 Status

A total of 265 patient samples had known HER2 status and were assessed for association with Ago2. No association was identified between HER2 negativity or positivity and stain intensity, as outlined in Figure 4.19.

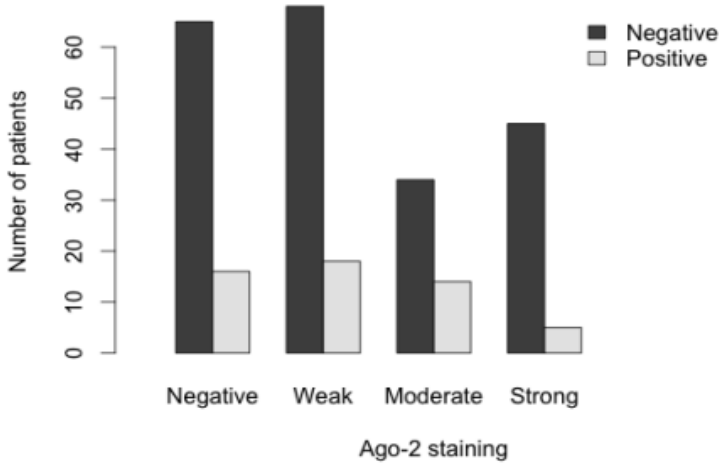


Figure 4.19 Ago2 staining intensity by HER2 status

4.4.4.4.6 Tumour Size

A significant difference in Ago2 staining was identified in association with tumour size, with larger tumours displaying less Ago2 staining. Using a series Mann-Whitney test with Bonferroni correction, a significant difference between negative and weak Ago2 staining was identified ($p<0.01$) with estimated effect size of 0.23 mm in favour of negative staining. A further significant difference was identified between negative and moderate Ago2 staining ($p=0.02$) with estimated effect size of 0.21 mm, again in favour of negative staining.

Table 4.7 Tumour size distribution by Ago2 stain intensity

	Ago2	Tumour.Size.mean	Tumour.Size.median
1	Negative	34.14	28.00
2	Weak	24.41	23.00
3	Moderate	26.51	21.00
4	Strong	29.97	25.00

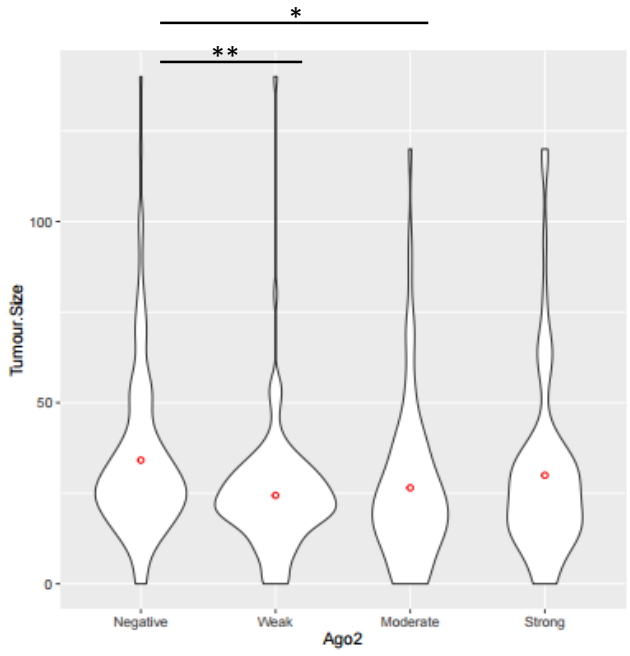


Figure 4.20 Ago2 staining by tumour size. Negatively stained specimens were significantly larger than weak or moderately stained specimens

4.4.4.4.7 Tumour Stage (T Score)

As outlined in the introduction Section 1.3, tumour stage is represented by the T score, which increases from T1 to T4 based on increasing tumour size. The majority of specimens were T2 and T1. A statistically significant difference was identified between Ago2 staining and tumour stage, whereby T3 specimens were predominantly stained negatively compared to the other stain intensities, and negative stain intensity has a lower proportion of T1 specimens than the remaining staining categories (Figure 4.21). This is in keeping with the findings of tumour size analysis.

Table 4.8 Tumour stage distribution of specimens

T Score	No Samples	(%)
T1	121	(33)
T2	178	(49)
T3	32	(9)
T4	33	(9)
Total	364	

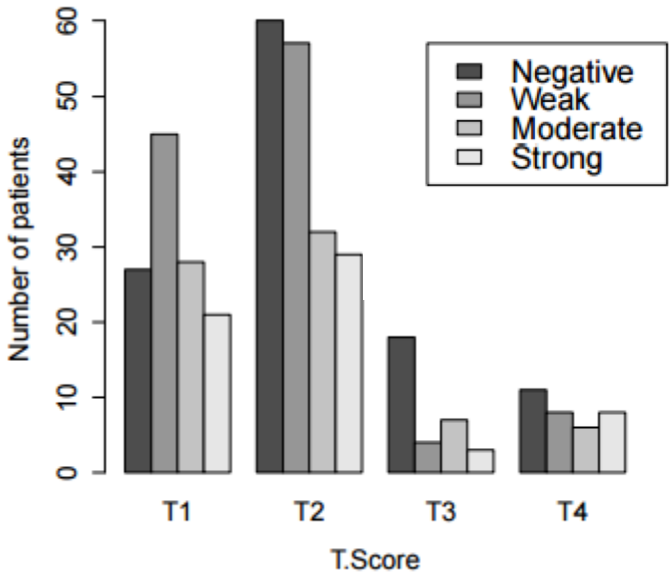


Figure 4.21 Ago2 staining intensity by T score. T3 specimens are predominantly negatively stained with a lower proportion of negatively stained T1 samples compared to other T scores

4.4.4.4.8 Nodal Status (N Score)

The majority of specimens were not associated with nodal disease (Table 4.9). Upon assessing association between Ago2 stain intensity and specimen nodal status (N0, N1, N2 and N3, outlined in Section 1.3) a similar staining pattern was observed across N scores, with no significant difference identified (figure 4.22).

Table 4.9 Distribution of specimen Nodal Status

N Score	No Samples (%)
N0	168 (48)
N1	99 (28)
N2	54 (16)
N3	28 (8)
Total	349

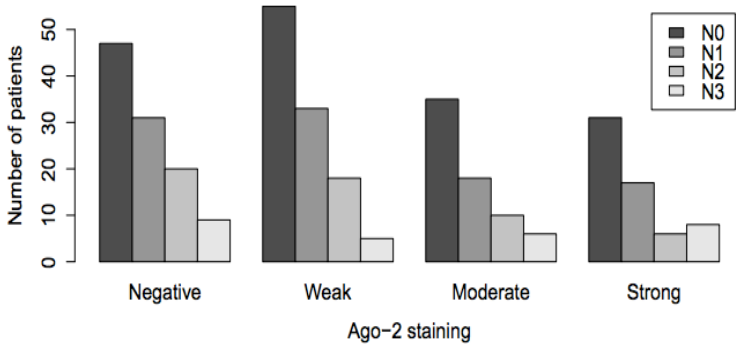


Figure 4.22 Ago2 staining intensity by N score. No significant difference identified

4.4.4.4.9 Metastasis (M Score)

An 'M' or metastasis score (outlined in Section 1.3) was available on 355 patients (92%), with the majority of patients being metastasis free, or M0 as outlined in Table 4.10. Although only a minority of patients had metastatic disease, a significantly stronger Ago2 stain was identified in association with metastasis.

Table 4.10 Distribution of specimen M Score

M Score	No Patients (%)
M0	338 (95)
M1	17 (5)
Total	355

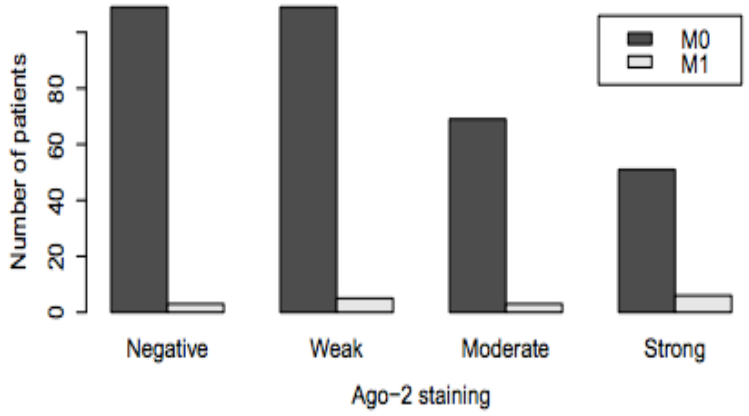


Figure 4.23 Ago2 staining intensity by M score. M1 specimens were predominantly strongly stained

4.4.4.4.10 Tumour Grade

Grade refers to the differentiation of cancer cells within the tumour upon microscopic analysis. Increasing score denotes less differentiated thus more aggressive tumours. The majority of specimens in this array were Grade 2 or Grade 3, indicating aggressive disease (Table 4.11). No evidence of association was identified between Ago2 staining intensity and breast cancer grade.

Table 4.11 Distribution of specimen Tumour Grade

Grade	No Patients (%)
0	40 (10)
1	35 (9)
2	152 (40)
3	158 (41)
Total	385

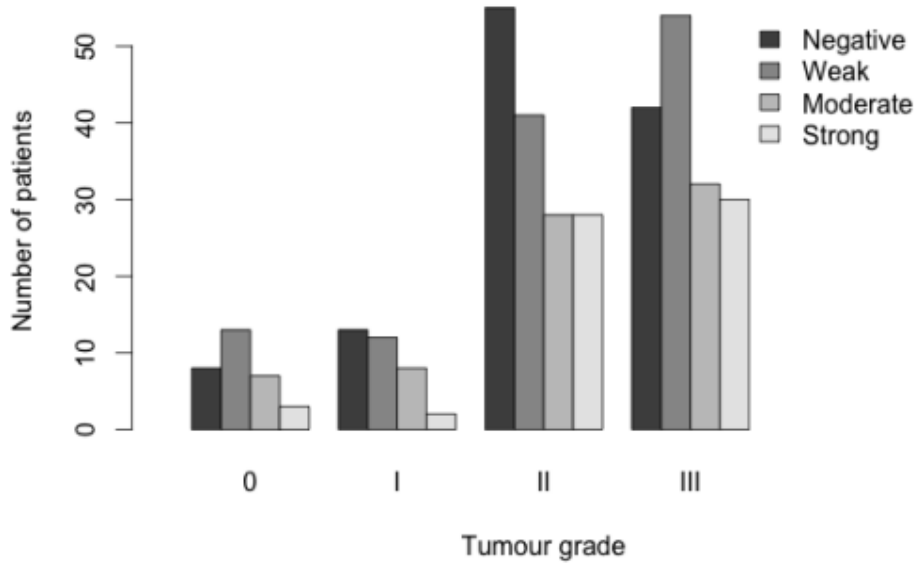


Figure 4.24 Ago2 staining intensity by Tumour Grade. No significant difference was identified

4.4.4.4.11 Procedure

A significant difference was identified between Ago2 staining and surgical intervention performed (p=0.001). The more aggressive surgical intervention of mastectomy was significantly associated with negative staining, while the majority of patients undergoing wide local excision were weakly stained.

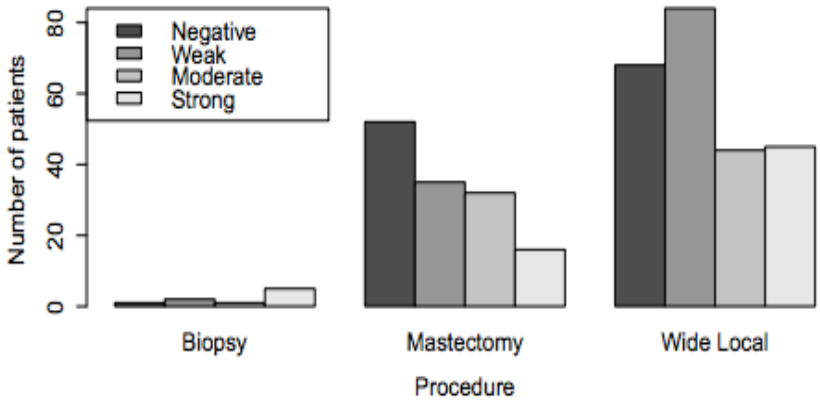


Figure 4.25 Ago2 staining intensity by procedure performed

4.4.4.4.12 Ductal Carcinoma in Situ (DCIS)

Of specimens analysed, 363 samples were free of DCIS (94%), with only 22 samples DCIS positive (6%). No significant evidence of association was identified between Ago2 stain intensity and the presence or absence of DCIS.

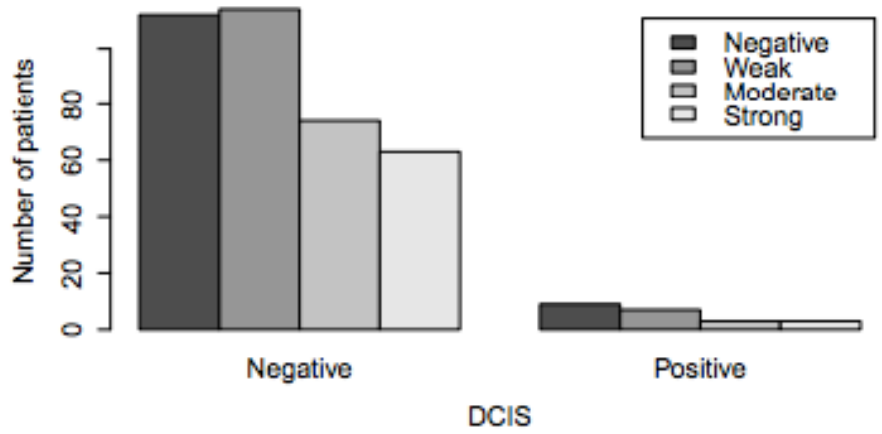


Figure 4.25 Ago2 staining intensity by presence or absence of DCIS

4.4.4.4.13 Nottingham Prognostic Index

The Nottingham Prognostic Index (NPI) is a formula used to determine patient prognosis in breast cancer. It is calculated using three pathological criteria: tumour grade, tumour size and the number of lymph nodes involved. This index was calculated for each patient specimen and association with Ago2 staining was investigated.

Table 4.5 outlines the NPI score and its associated prognosis. For the purpose of this analysis, scores were simplified into index 1-4 as outlined. No significant evidence of association between Ago2 staining and NPI was identified.

Table 4.12 NPI Scores with Prognosis and corresponding score used for analysis

NPI	Prognosis	Analysis Score
<2.4	Excellent	Index 1
>2.4<3.4	Good	Index 2
3.4-5.4	Moderate	Index 3
>/= 5.5	Poor	Index 4

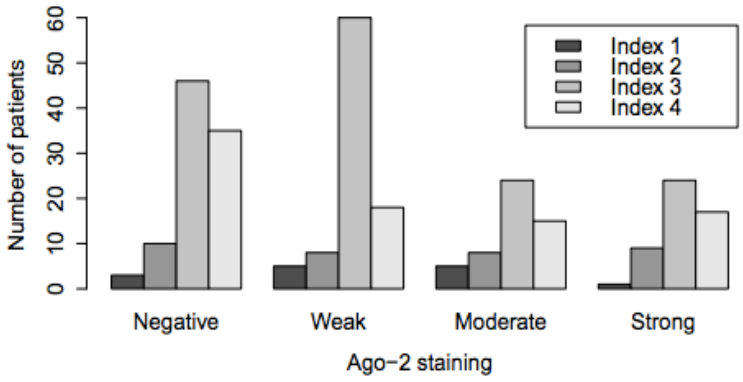


Figure 4.26 Ago2 staining intensity by Nottingham Prognostic Index. No significant difference

4.5 Discussion

In this current era of personalised medicine and individualised cancer management, the identification of prognostic markers and therapeutic targets that can better tailor management and stratify patient outcome proves essential. As the first study of Ago2 protein in clinical breast specimens, this analysis outlines the significant association between Ago2 protein and distinct pathological parameters of breast cancer, thus highlighting Ago2s potential future application in this setting.

In-vitro analysis highlighted variable Ago2 levels in cell lines representing the molecular subtypes of breast cancer, with the more aggressive subtypes being associated with a higher Ago2 protein content. Cell line Ago2 mRNA analysis however, showed the inverse of this, with HER2 overexpressing and triple negative cell lines containing reduced Ago2 mRNA compared to the less aggressive luminal breast cancer subtypes and the non-cancer control. One potential explanation for this could be the consumption of Ago2 mRNA in the more aggressive cell lines to produce the higher volumes of Ago2 protein product they contain. While contrary to the findings of Adams, Claffey [172], who reported higher expression of Ago2 mRNA in association with oestrogen receptor negative cell lines, a finding of reduced Ago2 mRNA expression in breast cancer was also identified by Kwon, Lee [260]. With conflicting reports in the literature, further analysis is undoubtedly warranted to fully elucidate the explanation for this altered Ago2 mRNA expression in breast cancer.

In keeping with the *in-vitro* findings, a variable Ago2 protein stain intensity was identified in the tissue microarray, ranging from no stain (negative) to a bright orange/brown colour (strong), which indicated a higher concentration of Ago2 protein. The stain was predominantly epithelial and cytoplasmic and was seen to significantly associate with current molecular markers of breast cancer. Consistent with the *in-vitro* analysis, Ago2 protein was significantly less abundant in the less aggressive Luminal A breast cancer subtype compared to the more aggressive triple negative breast cancer. In conjunction

with this, Ago2 protein was significantly associated with the absence of the oestrogen and progesterone receptor. While only a minority of patient samples were associated with metastatic disease, a significant association was identified between the presence of Ago2 protein (strong staining) and metastasis. These findings support a potential oncogenic role for Ago2, which is in keeping with current literature. Upon analysing the effect of hypoxia-induced phosphorylation of Ago2 in breast cancer, an association between elevated phosphorylated Ago2 levels and increased cell invasion and reduced survival in breast cancer was reported [261]. Similarly, in renal cell carcinoma elevated Ago2 protein was associated with poorer overall survival [262]. While incomplete at the time of writing this report, an analysis of association between Ago2 protein levels and disease-free and overall survival in breast cancer is currently being conducted on this dataset.

Interestingly, Ago2 protein was significantly less abundant in larger tumours compared to smaller tumours. This finding was maintained upon analysing T score, whereby larger T2-T4 tumours showed more negative staining results than smaller T1 tumours (Figure 4.21). This could potentially explain the finding that less abundant Ago2 (negative staining) was associated with the more aggressive surgical intervention of mastectomy, as larger tumours mandate a more invasive procedure.

The ability of Ago2 to classify breast tumours by pathologic variables currently used to guide management and prognosticate for patients with breast cancer highlights the potential for Ago2 to serve as a novel biomarker for breast cancer. The use of Ago2 in this setting however remains in its infancy, with further investigation required to fully elucidate mechanisms ofAgo2 regulation and the cancer-specific functions of the Ago2 gene and its resultant protein.

4.6 Conclusion

This analysis highlights for the first time the association between tissue Ago2 levels and distinct molecular and biological parameters of breast cancer. Such an association highlights the potential for Ago2 to serve as a novel prognostic or predictive marker, with a potential to guide patient management strategies and facilitate treatment optimization and individualisation.

Chapter 5

PHOTOACOUSTIC IMAGING OF THE BREAST

5.1 Introduction

While the management of breast cancer has transformed dramatically in recent years, inherent deficiencies in breast imaging remain. The challenge of early breast cancer diagnosis continues, while the ability to accurately detect *in vivo* tumour response to systemic therapy remains elusive. Conventional imaging techniques each have recognised limitations, hindering their applicability, including low specificity, the requirement of intravenous contrast agents and high cost [11, 15, 20]. The requirement of a novel imaging modality which is both highly sensitive and specific for breast cancer, capable of detecting tumour response to neoadjuvant chemotherapy is therefore warranted.

Photoacoustic imaging (PAI) represents an exciting and novel approach for detailed visualisation of breast tissue. Based on light excitation and ultrasound detection, high-resolution micro-ultrasound and PA signals are simultaneously captured, enabling extensive structural and functional tissue analysis not obtainable with conventional imaging techniques such as mammography, ultrasonography, magnetic resonance imaging or positron emission tomography [210]. Using haemoglobin as an endogenous contrast agent, this non-ionising technology permits visualisation of tissue vasculature and assessment of tissue oxygenation [211]. This is a very exciting prospect, as tissue hypoxia is known to correlate with tumour progression and treatment resistance, while an increase in tumour oxygenation is reported to correlate with tumour response to therapy [233]. The ability to monitor the vascular dynamics within the tumour microenvironment could potentially enable monitoring of tumour progression, regression and treatment response.

The clinical applicability of various light-based, or optoacoustic, imaging systems has been assessed on multiple tissues and organ systems [218-224]. In the breast setting, the ability of optoacoustics to differentiate malignant tumour from benign tissue has been demonstrated [225, 226, 228]. Further to this, recent studies have highlighted the exclusive ability of optical imaging to detect tumour signals indicative of therapeutic response. Specifically, increased tumour oxyhaemoglobin was seen to significantly associate with

tumour partial or complete pathological response to chemotherapy, perfectly differentiating treatment responders from non-responders [229, 233]. These studies and systems have recognised limitations however, including small subject numbers, shallow penetration depth and lack of confirmatory measures of study findings.

The VisualSonics Vevo® LAZR PAI system has multiple characteristics making it suitable for clinical application. As co-registered high-resolution ultrasound is displayed with PA signals via a hand-held probe, this system could be easily incorporated into clinical practice by clinicians familiar with ultrasonography. It is non-invasive, non-ionising and suitable for bedside use. Pre-clinical studies of the VisualSonics Vevo® LAZR PAI system in functional models and animal studies have confirmed its ability to detect tumour distinct from healthy tissue and tissue vasculature disruption in response to neoadjuvant chemotherapy [263, 264]. However, this system has not yet been investigated in the clinical breast setting.

The identification of an imaging system capable of expediting breast cancer diagnosis and monitoring *in vivo* tumour response to systemic therapy could potentially enable tailored, individualised breast cancer management. The ability to detect a partial response could potentially enable alteration of a patient's chemotherapeutic regimen e.g. extended treatment, alternative agents. The identification of tumours unresponsive to chemotherapy could enable expedited surgical intervention for the patient, obviating the deleterious effects of systemic therapy. This could enable more effective, tailored patient-care programmes that offer fewer patient side effects and better outcomes from disease.

5.2 Aims

The primary aim of this study was to assess the ability of the VisualSonics Vevo® LAZR Photoacoustic Imaging system to image real-time patient breast tissue in the clinical setting. Further to this, optimisation of the system for breast imaging was performed so that *in-vivo* tumour response to neoadjuvant chemotherapy could be investigated.

5.3 Methods

5.3.1 Ethical Approval

Ethical approval was sought to utilise this novel system for patient imaging in the clinical setting. As the system is non-invasive and non-ionising, ethical approval was granted by the Clinical Research and Ethics Committee, Galway University Hospital (Appendix 4).

5.3.2 System Placement

The Symptomatic Breast Unit (SBU), Galway University Hospital, is a referral clinic whereby patients with specific breast complaints e.g. lump, discharge, pain, are referred by their General Practitioner for review by a breast surgeon. Each patient is reviewed and examined by a breast surgeon, before conventional imaging (mammography or ultrasonography as appropriate) is performed and reviewed by a consultant radiologist. Where necessary, breast biopsy is also performed within the department. Each week 145 new patients present to the SBU for review [265]. Of these, approximately 7 patients per week are newly diagnosed with breast cancer, with the majority of patients diagnosed with non-malignant pathologies. To facilitate patient access and imaging, the PAI system was set-up in one of the SBU's clinic rooms (Figure 5.1).

Figure 5.1 The PAI System in one of the Symptomatic Breast Unit clinical rooms

5.3.3 Patient Recruitment

The initial application of the system required the imaging of a variety of patient pathology and healthy breast tissue, so that baseline characteristics and tissue parameters could be established and the system could be optimised. As such, any patient presenting to the SBU who satisfied the following inclusion criteria were invited to participate in this study:

1. Aged 18 years or over.
2. Capable of providing written informed consent.

Written informed consent was obtained from each patient prior to enrolment and imaging and a patient information proforma (Appendix 5 & 6) completed. Patient clinicopathological data was collected from patient notes and Galway University Hospital imaging and laboratory systems (e.g. imaging and histopathology reports). At the Galway University Hospital weekly breast cancer multidisciplinary meeting (MDM), any patient who is undergoing investigation for a breast abnormality is discussed by consultant breast surgeons, oncologists, radiologists and radiotherapists, so that a definitive diagnosis and treatment plan is decided upon. Once the imaging system was

optimised for breast tissue imaging, attendance at the MDM enabled the identification of patients newly diagnosed with breast cancer, scheduled to undergo neoadjuvant chemotherapy for the purposes of assessing the ability of the system to detect tumour response to neoadjuvant chemotherapy.

5.3.4 Patient Demographics and Clinicopathological Data

A customised patient demographic and clinicopathological data pro forma was developed for this study so that an in-depth analysis of patient images could be undertaken (Appendix 6). Patient records and hospital laboratory and radiology systems were studied to collect all required data.

5.3.5 Equipment detail

The VisualSonics Vevo® LAZR PAI system comprises a tuneable photoacoustic excitation laser system, a multi-element linear-array transducer, an amplifier and digitizer.

5.3.5.1 Laser System

The lasing medium utilised in this system is the crystal neodymium-doped yttrium aluminium garnet or Nd:YAG. Light is produced from the photoacoustic laser system via an optical parametric oscillator, pumped by frequency-doubled Nd:YAG at 680-970nm, with a 20 Hz repetition rate and 5 ns pulse width. This light is coupled to two fiber bundles mounted on each side of the imaging surface of the transducer probe, delivering two laser beams to the tissues. PA waves generated propagated back to the transducer probe, coupled through ultrasound gel, and were acquired by the transducer array. From here, the information was transferred to the system computer via the amplifier and digitizer.

5.3.5.2 Multi-element Liner-array Transducer Probes

During this study, three individual multi-element linear-array transducer probes were utilised, with central frequencies of 40, 21 and 15 MHz (Table 5.1). Each transducer probe consisted of 256 elements, divided into four quadrants, each having 64 elements. With each laser pulse, PA signals produced were captured by a single quadrant of the transducer array. As such, four laser pulses were required for a single image (frame rate one-fourth the laser repetition rate, i.e. 5 Hz).

Table 5.1 Linear-array Transducer Probe characteristics

Transducer Probe (Frequency)	Broadband Frequency (MHz)	Axial Resolution (μm)	Imaging Depth (mm)	Imaging Width (mm)
40	32 - 55	40	15	14.1
21	13 - 24	75	20	23
15	9 – 18	100	36	32

5.3.6 Imaging Procedure

Once each patient provided informed consent, they were asked to undress to the waist and to lie on the examination bed in the SBU clinic room so that PAI could be performed. PAI was conducted in the same manner as ultrasonography, whereby a hand-held probe was applied to the contour of the breast, using ultrasound gel (Figure 5.2). Due to the use of laser sight, safety goggles were worn by the imaging technician and the patient. Single breast measurement took approximately 10-15 minutes and where possible, bilateral breast imaging was performed. All images captured were stored on the system computer for subsequent analysis.

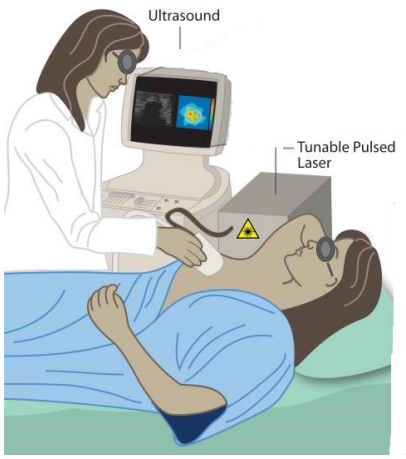


Figure 5.2 PA Imaging Procedure. A handheld probe is applied to the contour of the breast. Safety goggles are worn by the patient and imaging technician (adapted from [213]).

5.3.7 Image Parameters & Analysis

For each tissue area analysed, a selection of images was obtained by adjusting various system settings, as outlined in the following sections. Within each setting, a continuous stream of image frames was recorded in real-time from a fixed field of view over a particular region of interest (ROI).

5.3.7.1 PA Images

The 800 nm wavelength was chosen for PA imaging because of the low light absorbance in tissue components, such as water and oxy/de-oxyhaemoglobin, in the near-infrared (NIR) wavelength range (600-800 nm). However, to optimise the system and ensure the superiority of the 800nm wavelength, Nanostepper and Spectro images were also recorded (Section 5.3.7.4). PA images were assessed for gross structural and anatomical details, and overlap with B-mode ultrasound images. Further to this, depth penetration was analysed and image gain (Section 1.13.1.2) optimised.

5.3.7.2 B-mode Ultrasound

B-mode ultrasound images were automatically co-registered with PA images, enabling accurate localisation of tissue pathology so that targeted PA signals could be analysed.

5.3.7.3 Oxy-Haemoglobin Image

To determine the average concentration of oxygen (sO_2) and average total haemoglobin (Hb_T) within a given image region of interest (ROI), dedicated imaging software was utilised. This software was based upon an equation developed by Wang, Xie [266] that calculates sO_2 and Hb_T using a two-wavelength approach as follows:

$$\mu_a^{\lambda 1}(r) = \frac{P^{\lambda 1}(r)}{\Gamma \cdot F^{\lambda 1}(r)}$$

$$\mu_a^{\lambda 2}(r) = \frac{P^{\lambda 2}(r)}{\Gamma \cdot F^{\lambda 2}(r)}$$

$$HbT(r) = \frac{a_1\mu_a^{\lambda 1}(r) - a_2\mu_a^{\lambda 2}(r)}{a^3}$$

$$StO_2(r) = \frac{a_4\mu_a^{\lambda 2}(r) - a_5\mu_a^{\lambda 1}(r)}{a_6\,\mu_a^{\lambda 1}(r) - a_7\mu_a^{\lambda 2}(r)}$$

P(r), the detected pressure at point r; Γ, the Gruneisen parameter (0.1 – 0.2 in soft tissue); F(r), estimated fluence of the excitation light; μ_a, the calculated absorption coefficient; λ_1, first wavelength of light; λ_2, second wavelength of light.

5.3.7.4 Nanostepper and Spectro Images

By acquiring images at multiple wavelengths, quantitative spectroscopic measurements of blood oxygenation and haemoglobin concentration can be achieved [263]. 'Nanostepper' and 'Spectro' refer to system settings that captured PA images at variable wavelengths across the biological window. The *Nanostepper* setting recorded PA images at five distinct wavelengths at increments of 50 nm, beginning at 700 nm and increasing to 900 nm (Figure 5.3). The *Spectro* setting operated in a similar fashion, capturing PA images every 10 nm across the biological window of the spectrum, 680 – 970 nm.

The purpose of employing these system parameters was twofold. The currently employed two-wavelength technique for calculating sO_2 and Hb_T is not ideal, as only two wavelengths are taken into account. Utilising multiple wavelengths when imaging patients will enable adjustment of the two-wavelength technique so that more accurate sO_2 and Hb_T can be calculated. Further to this, analysis of PA images from a range of wavelengths enables the identification of the optimal wavelengths for clinical breast imaging.

5.3.7.5 Doppler Image

Doppler ultrasound of the ROI was recorded to accurately determine the location of vessels within the tissue. Once tissue vasculature was correctly mapped, the PA and oxy-haemo images could be more accurately analysed.

5.3.7.6 Statistical Analysis

As outlined in Section 5.3.7.3, the VisualSonics Vevo® LAZR PAI system has dedicated software that calculates tissue oxygen and haemoglobin concentration. Further calculations were conducted on Microsoft Excel® (2016).

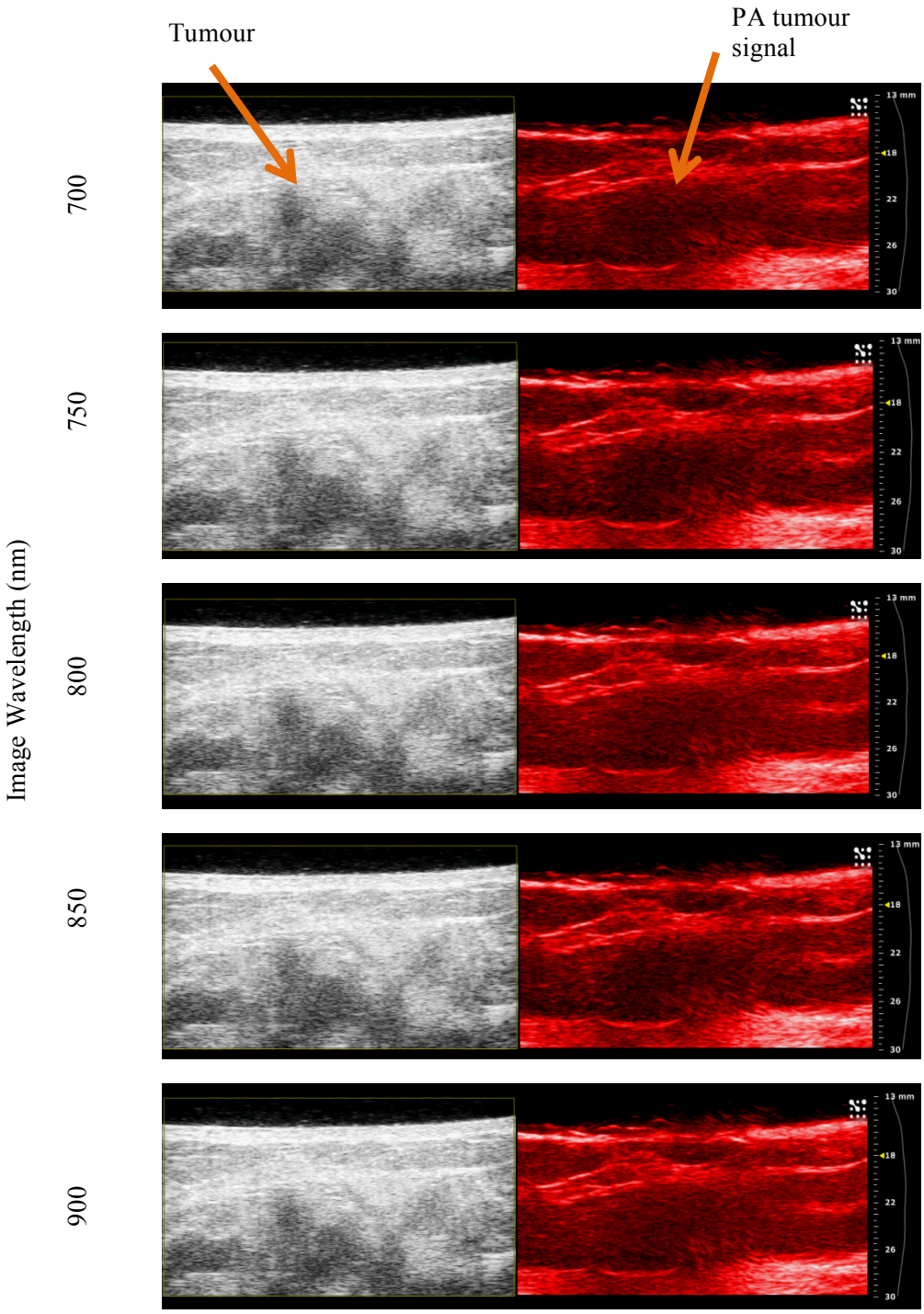


Figure 5.3 Images of a breast tumour captured using the *Nanostepper* system function. As images were being captured the PAI system cycled through five distinct wavelengths at increments of 50 nm. The variation in light saturation is appreciable (darker initially at 700 nm with more light saturation at 900 nm)

5.4 Results

5.4.1 Clinical Application of PAI

Throughout the course of this study, PAI was successfully employed in a total of 36 patients. A range of patient pathology was visualised across a spectrum of demographic and clinicopathologic parameters, as outlined in Table 5.2.

Table 5.2 Patient Clinicopathological data

Menopausal Status	
Pre	12
Post	24
Age	
Mean	46
Range	19 – 77
Pathology	
Normal Control	14
Cyst	6
Fibroadenoma	8
Tumour	11 (>1 in 2 patients)
Cancer Subtypes	
Luminal A	8
Luminal B	0
HER2	2
Triple Negative	1
Tumour Size (cm)	
Mean	2.74
Range	2 - 5.1

During the optimisation of the system (further outlined in subsequent sections) three transducer probes were employed for clinical breast imaging, visible in Figure 5.3.

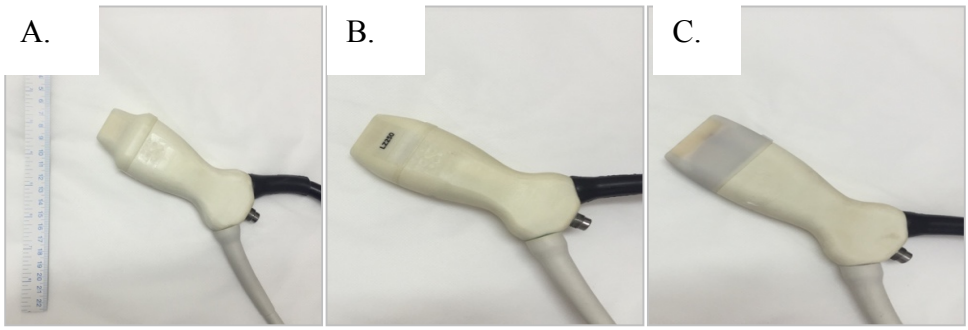


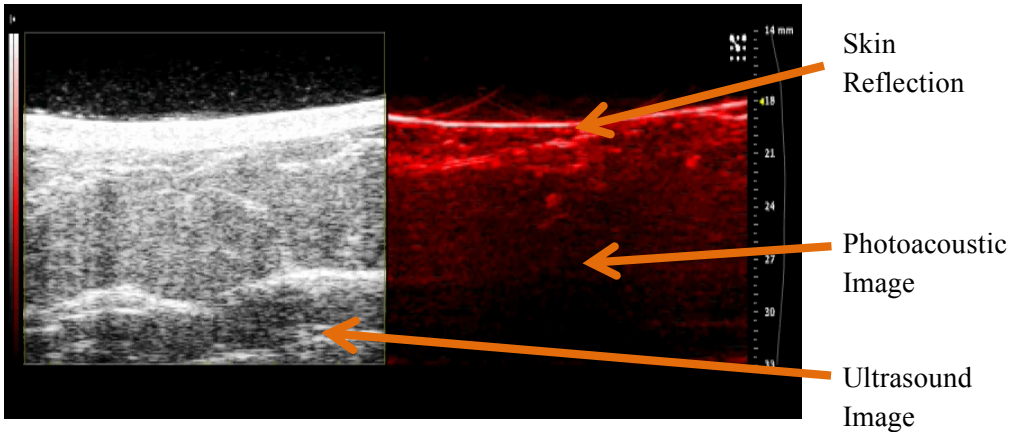
Figure 5.3 Transducer probes for PAI. A. 40 MHz probe B. 21 MHz probe C. 15 MHz probe

5.4.2 Images Captured

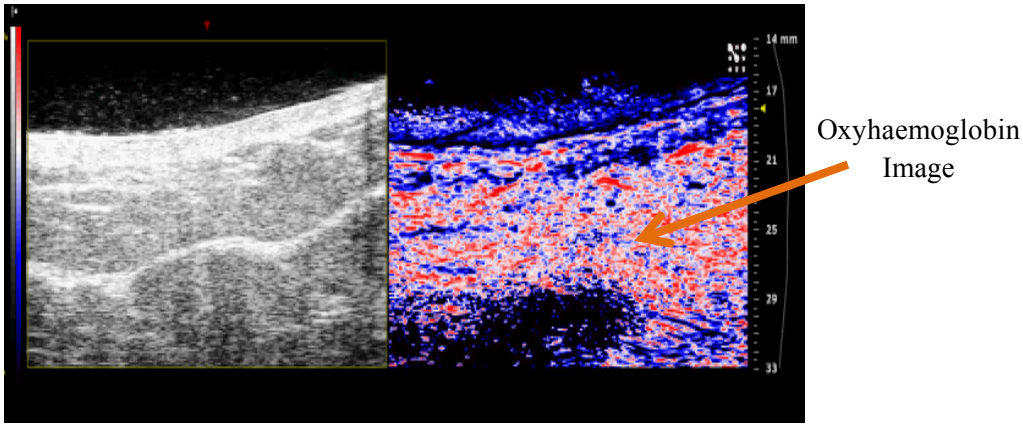
As outlined in Section 5.3.7, a range of images were captured upon each patient imaging session. Figure 5.4 A. depicts a standard co-registered image with conventional ultrasound visible to the left of the image and photoacoustic image visible to the right. The ultrasound image is utilised to locate the region of interest and enables focused acquisition of the PA image. As normal breast tissue was imaged here no distinct signals (other than the skin) are appreciable.

A co-registered oxyhaemoglobin image is shown in Figure 5.4 B. Using the captured imaged and known light absorption coefficients of haemoglobin, dedicated system software enabled calculation of oxygenated and deoxygenated haemoglobin and average total haemoglobin concentration within specific regions of interest within the tissue (Section 5.3.7.3). Figure 5.4 C. outlines a doppler imaged, used to localise blood vessels. Examples of *Nanostepper* and *Spectro* images are displayed in Section 5.3.7.4.

A.



B.



C.

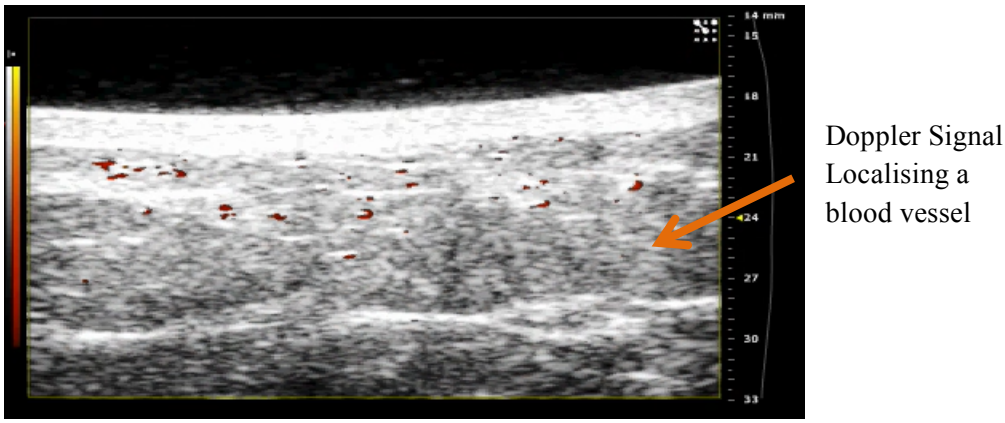


Figure 5.4 Examples of PA images captured with each distinct system setting.
A. Baseline photoacoustic image. Used to assess structural information
B. Oxyhaemoglobin image, enabling determination of tissue oxy and deoxyhaemoglobin C. Doppler Image, utilized to accurately map tissue vasculature

5.4.3 System Optimisation

As a novel imaging system, the VisualSonics Vevo® LAZR PAI system had not previously been applied clinically. As such, system optimisation and customisation for use in the clinical setting for breast imaging had to be undertaken, as outlined in the following sections. While each section is discussed separately, all aspects of the system were continually and concurrently being optimised.

5.4.3.1 Depth Penetration

Initial imaging was performed with a 40 MHz probe. Upon analysis of the images captured of one normal control breast and one breast cancer, poor depth penetration was recognised, as displayed in Figure 5.5.

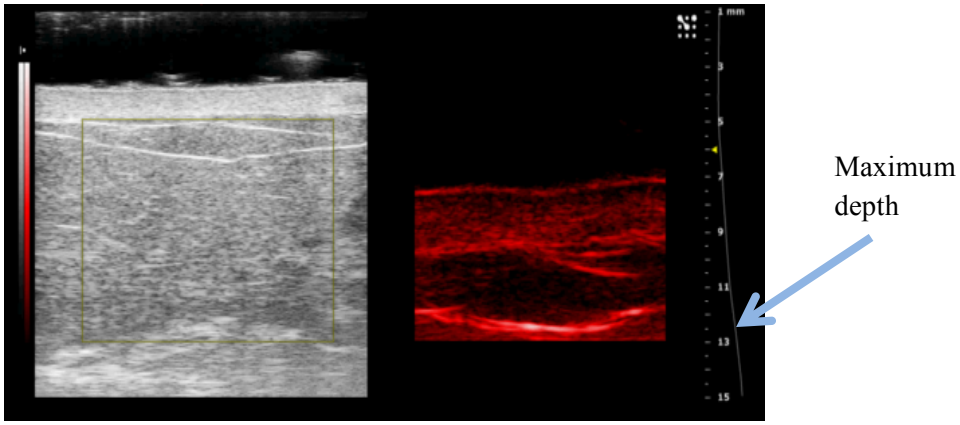


Figure 5.5 Image of normal breast tissue captured on 40 MHz probe. In this image maximum depth can be seen to reach 13 mm (blue arrow)

It was recognised that a lower frequency probe would be required to better visualise deeper into breast tissue, as such a 21 MHz probe was employed. A total of 6 patients were imaged with the 21 MHz probe, three normal controls, one fibroadenoma, one cyst and one breast cancer (Figure 5.6).

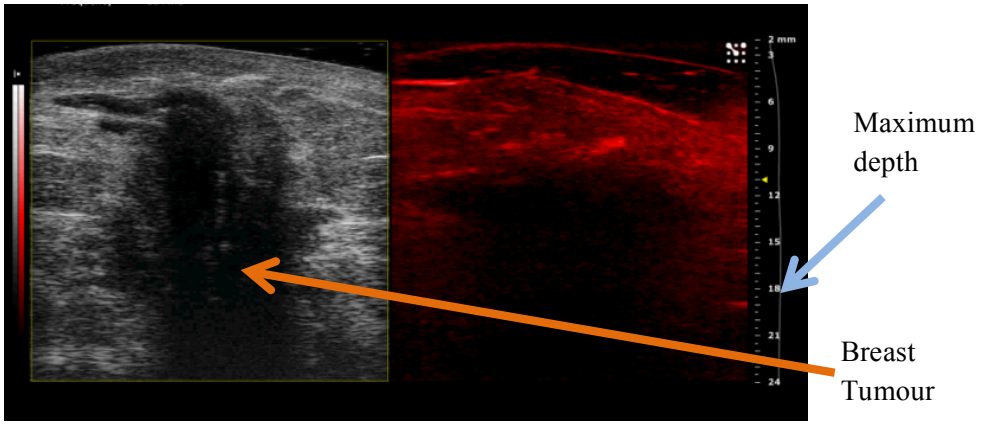


Figure 5.6 Image of breast tumour captured using the 21 MHz probe. In this image maximum depth can be seen to reach 18 mm (blue arrow)

Images captured were analysed in collaboration with biomedical physicists who were developing the VisualSonics Vevo® LAZR PAI system for clinical use. Once more it was recognised that the depth penetration of the photoacoustic signal was not optimal for clinical breast imaging. Based on these initial images, a customised 15 MHz probe was manufactured specifically for clinical breast imaging. With this probe, optimal depth penetration for clinical breast imaging was finally achieved (Figure 5.7). During the course of this study a total of 22 patients were imaged with the 15 MHz probe.

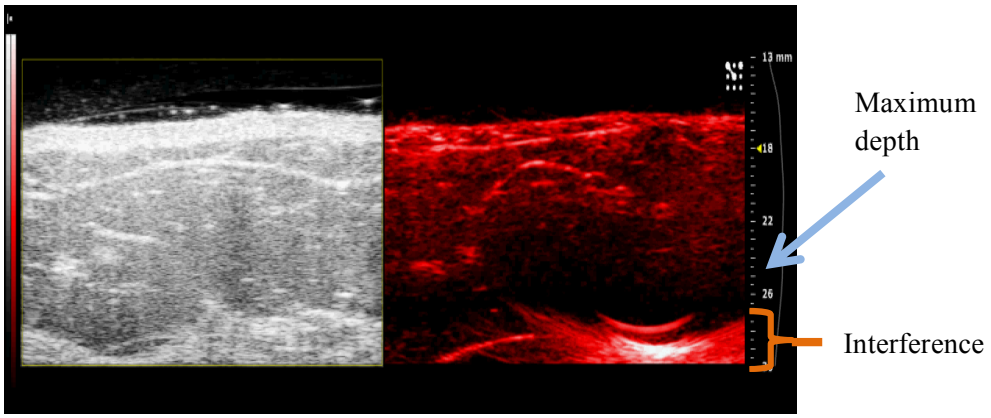


Figure 5.7 Image of normal control captured using the 15 MHz probe. In this image maximum depth can be seen to reach 25 mm (blue arrow)

5.4.3.2 Reflection Artefact and Image Interference

Analysis of initial images captured revealed significant interference with the pulsed laser light, manifested as reflection artefact within the image (Figure 5.8). This reflection was due to bubbles within the ultrasound gel. A range of ultrasound gels were trialled before the most suitable formation was identified, *Aquasonic ® 100 Ultrasound Transmission Gel* (Parker Laboratories, USA).

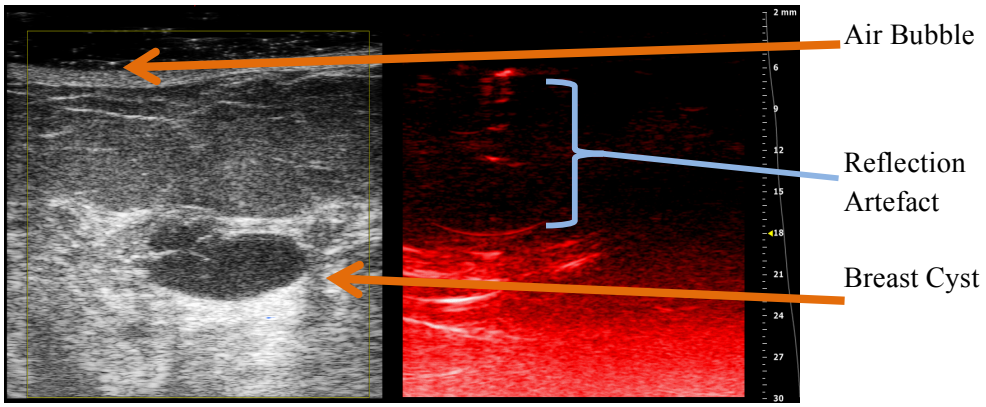


Figure 5.8 Image of a breast cyst. Reflection artefact is visible within the PA image (blue arrow) caused by the presence of an air bubble within the ultrasound gel on the probe.

Further to the gel utilised, another challenge encountered was the reflection of the acoustic wave off the aperture of the ultrasound transducer. Once reflected the acoustic waves would travel back into the tissues, producing signal interference that was visible within the PA image, as outlined in Figure 5.9.

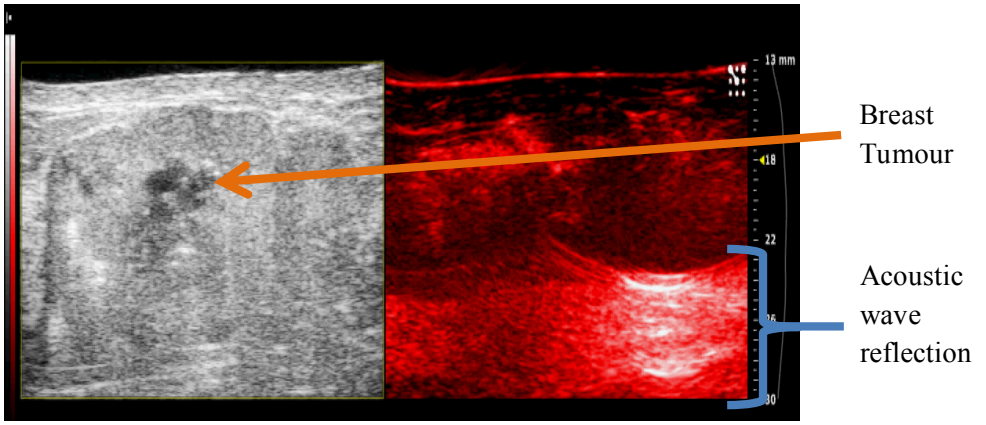


Figure 5.9 Image of a breast tumour. Acoustic wave reflection. produced when sound waves reflect from the surface of the probe back into the tissues causing interference and producing signal artefact.

To overcome this, study physicists developed the concept of applying a gel pad (*Aquaflex® Ultrasoud Gel Pad,* Parker Laboratories, USA) to the aperture of the ultrasound transducer (Figure 5.10). Composed of ultrasound compatible gel, this pad allowed free passage of pulsed laser light into tissues while increasing the distance between the probe aperture and the skin, preventing acoustic waves from reflecting back into tissues. This development enabled improved resolution at greater depth and obviated the acoustic wave reflection.

[The gel pads were cut to size each day prior to imaging, with the overlying cling replaced between patients to maintain clinical hygiene.]

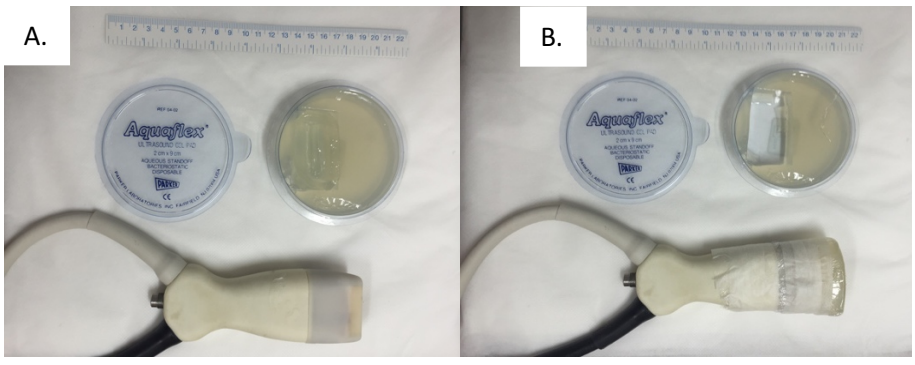


Fig 5.10 The 15MHz probe with Gel Pad modification A. The probe with the gel pad cut to size. B. Gel pad has been applied to the probe aperture, covered in cling film and taped in place.

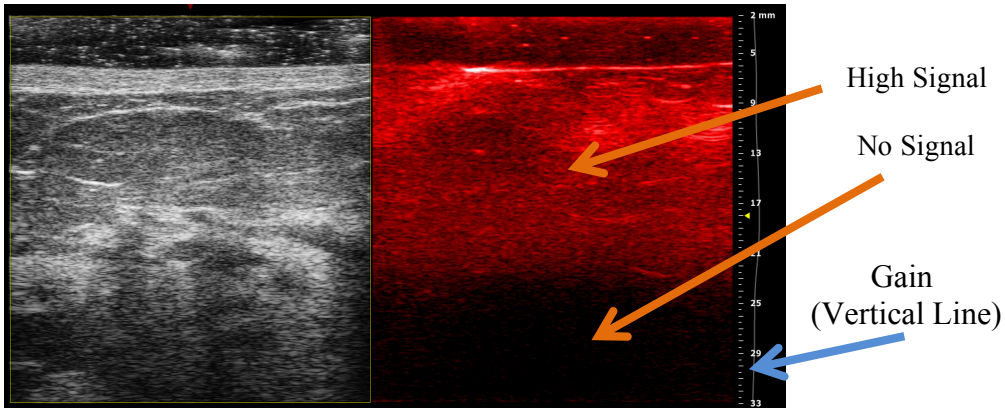
In addition to abolition of the acoustic wave reflection, application of the gel pad also resolved the challenge of the concave transducer aperture. The concavity of the structure permitted the introduction of multiple air bubbles in a given imaging session. The presence of the gel pad however produced a more convex probe surface, decreasing the occurrence of bubbles within the gel which resulted in reduction of image artefact.

5.4.3.3 Gain Optimisation

As light and sound travel through the tissues being imaged, its signal becomes attenuated as depth penetration increases (Section 1.13.1.2). Without correction, signal attenuation produces low intensity images that appear lighter in superficial layers and darker in deeper layers. To compensate for this attenuation, the signal intensity of the returning sound waves can be amplified. The degree to which the signal is amplified is called the gain. However, different tissues display different attenuation coefficient. As this system had not previously been applied to clinical breast imaging, breast tissue attenuation had to be investigated, with consequent gain optimisation.

An example of a healthy normal control breast captured prior to gain optimization is seen in Figure 5.11 A. Higher PA signal is readily apparent in superficial tissue (bright red, saturated) whereas the deeper tissue appears dark (no PA signal being detected). The scale to the right of the PA image shows the gain setting, and can be seen to be standard from the skin to 33 mm depth (straight vertical line). This gain has to be optimised to achieve an optimal PA image from the region of interest. 5.11 B. is another example where gain adjustment can be appreciated at the depths of the image (the gain line is no longer vertical), however excessive amplification is displayed with saturation of the signal producing a bright red image.

A.



B.

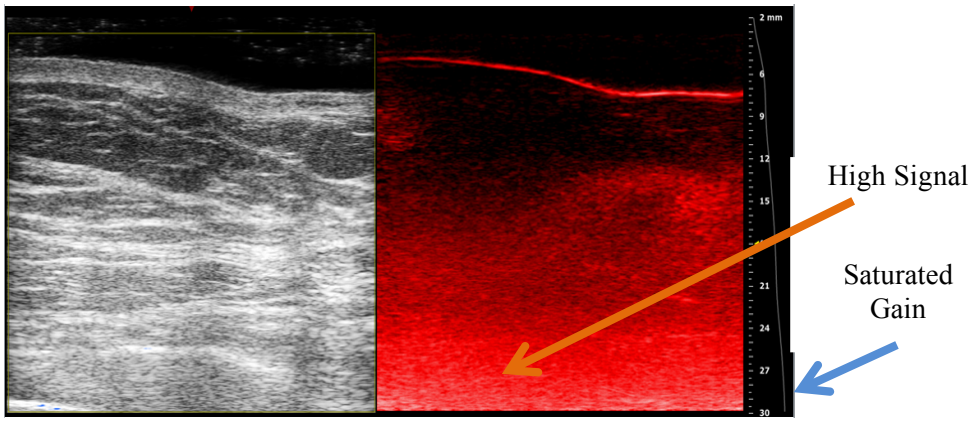


Figure 5.11 A. Image of normal control tissue. The gain line (blue arrow) is vertical, indicating that no gain adjustment has taken place. B. Image of normal control tissue. The gain line (blue arrow) has been adjusted but excessively so

With regular use of the system, imaging technique improved and the analysis of a variety of images enabled appreciation of the optimal gain to acquire effective standardised visualisation of breast tissue. Study physicists then programmed the system gain settings so that prospectively captured images would have optimal standardised gain, as depicted in Figure 5.12.

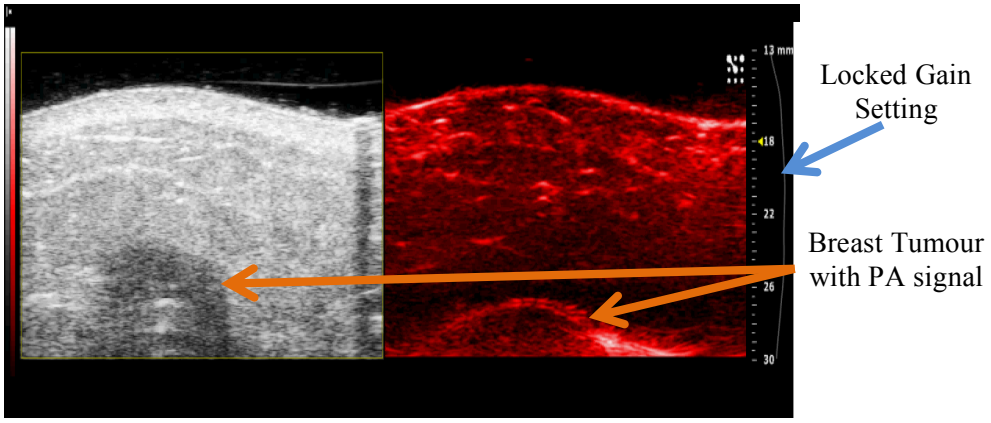


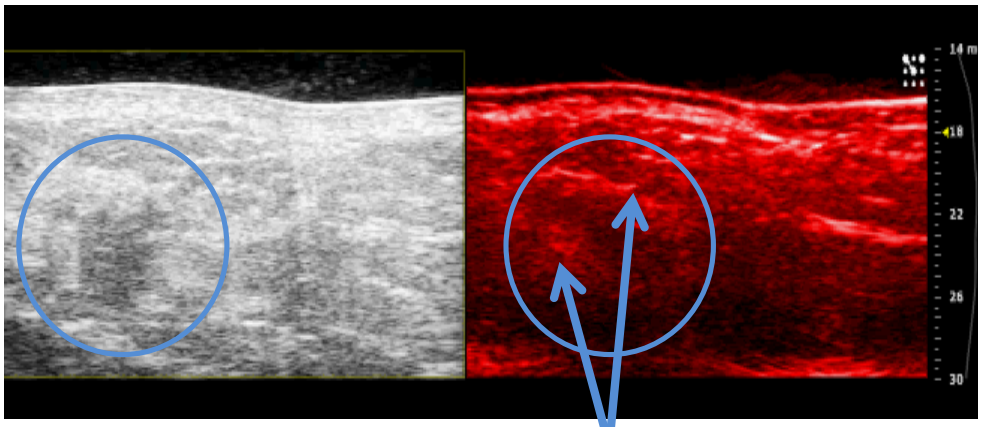
Figure 5.12 Optimised image of a breast tumour with gain line pre-programmed and standardised (blue arrow)

5.4.4 Image Analysis

5.4.4.1 Photoacoustic Signal

With the successful optimization of the system, effective clinical imaging of patient pathology was permitted. Figure 5.10 A depicts a 2cm Luminal A tumour in the right breast of a 35 year old female. While the tumour was localised on B-mode ultrasound imaging distinct photoacoustic signals corresponding to the tumours location are appreciable (blue circle). Figure 5.13 B depicts the photoacoustic signal produced upon imaging a 2.5cm fibroadenoma in the breast of a 19 year old girl.

A.



B.

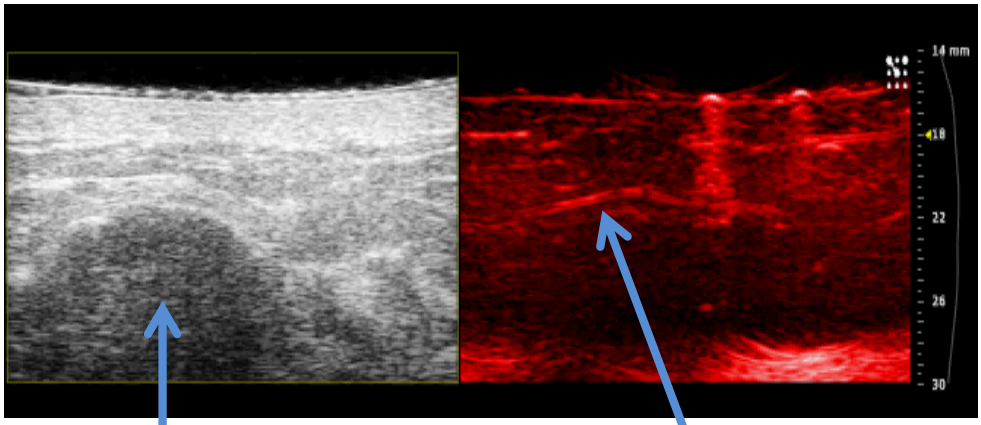


Figure 5.13 Examples of two breast pathologies effectively imaged following system optimization. A. Image of breast tumour highlighting PA signals (one signal from the tumour surface and one centrally) B. Image of breast fibroadenoma highlighting a single PA signal from the surface of the fibroadenoma

5.4.4.2 Oxyhaemoglobin Concentration

As a goal of this study was to employ PAI to detect tumour response to neoadjuvant chemotherapy via tissue oxyhaemoglobin (sO_2) changes, once system optimisation was achieved, assessment of baseline normal-control sO_2 was established. Analysing the sO_2 of the 14 normal-control patients imaged identified an average breast sO_2 of 68%, ranging from 66 – 72% (Table 5.3). Further to baseline normal measurements, intra-tumoral sO_2 was also calculated. Analysing the results of the 11 tumours imaged to date identified an average tumour sO_2 of 64.2%, which is more hypoxic than the baseline normal identified (Table 5.3). In conjunction with this, analysis of the 8 fibroadenomas imaged revealed an average sO_2 of 65.6%.

Table 5.3 Average tissue sO_2 and range

Tissue Imaged	Average sO_2	Range
Normal-Control (n=14)	68%	66 – 72%
Tumour (n=11)	64.2%	57.2 – 69%
Fibroadenoma (n=8)	65.6%	59.3 – 70%

5.4.4.3 Neoadjuvant Chemotherapeutic Response Detection

With the system optimised for effective clinical application, the potential for the system to detect *in-vivo* tumour response to neoadjuvant chemotherapy (NAC) had to be assessed. This required the development of a prospective study. To inform study structure and protocol, a laboratory visit was organised to the Beckman Laser Institute and Medical Clinic Laser Microbeam and Medical Program, University of California, Irvine. This laboratory group, led by Professor Bruce Tromberg, has recently published their findings of a statistically significant increase in tissue oxyhaemoglobin concentration in tumours responding to NAC, as detected with a light-based imaging system [233]. Upon meeting with the group and partaking in their ongoing study, it was recognised that following the administration of NAC, the optimal time at which to image patients was within the first 24 hours. Subsequent to this, a further image at a one week interval was recommended, with a final image performed pre-operatively. This information enabled the development of a more effective study protocol that is now being implemented by my successor, who is pursuing the NAC arm of this study.

At the time of writing, three patients have provided informed consent to partake in this study (two are due to commence NAC in the coming weeks with one patient due to commence palliative chemotherapy) and baseline images have been captured. Once definitive chemotherapy schedules have been organised for these patients further PAI sessions will be organised accordingly.

5.5 Discussion

Photoacoustic Imaging (PAI) is a novel non-ionising imaging modality that is suited to clinical bedside imaging. It does not require the administration of contrast agents and enables extensive structural and functional tissue analysis not obtainable with conventional imaging techniques. Using haemoglobin as an endogenous contrast agent, PAI can visualise tissue microvasculature and quantify tissue oxygenation. The ability to monitor vascular dynamics within the tumour microenvironment highlights the exciting prospect of neoadjuvant chemotherapeutic (NAC) response monitoring. It is recognised that current imaging modalities employed for disease response monitoring are limited, with more recent studies advocating for the implementation of functional imaging techniques to more effectively detect tumour response to therapy [204].

As outlined in Section 1.13.2, several light-based imaging systems have been investigated in both pre-clinical and clinical imaging studies to date with very promising results. However, each system displayed inherent deficiencies including technical difficulties, such as inadequate depth penetration or lack of reproducibility, to poor sensitivity [226-228, 267]. This study presents the optimisation of the VisualSonics Vevo® LAZR photoacoustic imaging system and its first ever application in the clinical setting for patient breast tissue visualisation. This is the only light-based system studied to date that enables co-registered conventional ultrasound imaging, which permits more accurate localisation of pathology within tissues, thus targeted photoacoustic analysis.

Initial challenges with depth penetration were overcome with the manufacture of a customised 15 MHz transducer. With this probe, effective imaging to depths of 3.5 cm was achievable. This is impressive given the reported maximum depths of current light-based imaging systems; *Twente Photoacoustic Mammoscope* ~2cm [267], and *Diffuse Optical Spectroscopy* 1.5 cm [229]. While increasing depth penetration negatively impacted on image resolution, experience garnered from repeated imaging enabled the identification of ideal gain settings for optimal photoacoustic signal detection

and image procurement. In addition to this, modifications such as the application of a gel pad further improved quality.

Once the system was optimised, photoacoustic signals from distinct tissue pathologies could be appreciated and tissue oxyhaemoglobin concentrations (sO_2) calculated. It is interesting to note that the average tissue oxyhaemoglobin level in tumours and fibroadenomas was less than that of normal-control tissue (Table 5.3). Fibroadenomas are a benign disease, although they do have the potential to progress into carcinoma. A potential explanation for this hypoxia could be due a high turnover within the benign mass or alternatively less vasculature within the benign disease process. The sO_2 values identified in this study and the finding of lower sO_2 in breast tumour tissue (mean sO_2 64.2 %) compared to normal breast tissue (mean sO_2 of 68 %) were in keeping with current literature, whereby a mean normal breast tissue sO_2 of 70.7 % is reported and tumour tissue of 68.4% [229]. This is an interesting finding, as a hypoxic tumour environment is known to correlate with tumour progression and resistance to systemic therapy [233]. It has been reported that patients whose tumours are responding to NAC, experience an increase in tumour oxygenation [229, 233]. The ability to non-invasively monitor tumour oxygen levels could potentially enable the detection of patients' individual responses to therapy, permitting personalised adjustment of treatment strategies to optimise patient outcomes from disease. The ongoing aspect of this study, being conducted with the optimised imaging protocol by my successor, is addressing this very question. To date, two patients about to commence NAC for breast cancer have been enrolled onto a prospective study and baseline images captured. One further patient who will receive palliative chemotherapy for metastatic breast cancer will also be studied for response monitoring.

The ability of PAI to detect *in-vivo* tumour response, or resistance to NAC could potentially support the individualisation of breast cancer management by enabling the customization of each patient's care plan based on their unique tumour response to treatment. The development of tailored patient management plans could enable fewer patient side effects from treatment and better outcomes from disease.

5.5.1 Technical Challenges of this Study

Significant challenges were encountered in the setting up and running of this study. The primary issue with this study was that this was a new technology that had never before been applied on humans, in the clinical setting. To introduce a new technology into the hospital and use it in patient imaging is not a straightforward process. Ethical approval had to be sought from the hospital committee, with rigorous testing of the system conducted by colleagues in the discipline of physics to confirm laser safety, which was ensured with the acquisition of customised eye protection goggles. Following several months, the application was deemed successful and the process of obtaining the system begun.

The VisualSonics Vevo® LAZR photoacoustic imaging system was sourced from Fujifilm, Toronto. Once acquired, the next challenge faced was where to locate and apply the system. In any public setting, particularly in the hospital, space is a premium. As expected, monopolising a clinical suite with this large, cumbersome, alien system, was initially not permitted. The system was therefore positioned in an unused room in the upstairs of the symptomatic breast unit, Galway University Hospital. Significant challenges were encountered with regards to patient recruitment as patient flow was being interrupted by removing patients from the clinical area. Following ongoing discussion, it was finally agreed that the system could be moved down to one of the clinical rooms on a trial basis. While the physical presence of the system, and the heat it generated in the confines of the clinic room, were not welcomed by the unit's permanent staff, my continual presence and persistence were required to ensure it was not banished to the unused room upstairs.

With the system in place in the clinical area, patient recruitment and imaging could now be conducted. However, as this system had never previously been applied in the clinical setting on humans, and neither hospital radiology staff, nor myself, had prior experience in the use of the system, it really was quite uncertain as to what to expect. The learning curve therefore, was substantial. Imaging was initially conducted by consultant radiologist colleagues, however due to the demands of the public health system, their regular

participation was not sustainable. These colleagues kindly trained me in the use of ultrasonography and it was my role, in conjunction with patient identification and recruitment, to perform the photoacoustic imaging. It was incumbent upon me to promptly and effectively learn the technique of ultrasonography (in conjunction with photoacoustic imaging). This included the use and application of the probes and system parameters and the recognition of ultrasonographic breast tissue features, both normal and pathological. Following this, the technique and application of photoacoustic imaging could be undertaken.

To develop effective technique, appreciate imaging parameters and optimise findings, regular meetings and discussions were held between breast clinicians, study physicists, radiologists and researchers, where I would present the images I had captured and we would attempt to define optimised parameters and to recognise features that required adjustment and refinement. As the system was sourced from Fujifilm, Toronto, physical meetings with system developers and technicians were infrequent, and upgrades to the system's technology, probes and lasers required significant organisation and incurred temporal restraints. Further to this, each time a parameter was adjusted (e.g. gain altered, new probe developed, new gel lubricant applied, new imaging feature developed (e.g. oxyhaemoglobin setting)), further images had to be acquired and assessed.

The results section of this chapter outlines in detail the optimization that was required for this system to be potentially valid. My active participation was required in all areas of this study, with significant problem-solving required: from system acquisition, placement and application, to patient recruitment, imaging and collection of clinicopathological data, in identifying and recognising breast tissue features, analysing and optimizing images and interacting with the variety of parties involved to ensure study survival and optimization.

5.6 Conclusion

In conjunction to the requirement of systemic disease response biomarkers, further non-invasive mechanisms of tumour detection and *in-vivo* therapeutic response monitoring are necessary. Current breast imaging techniques are inherently deficient at detecting tumour response to neoadjuvant chemotherapy. The validation of a novel non-invasive imaging technique in this setting could revolutionise the monitoring of treatment response in breast cancer to guide and individualise the administration of chemotherapy.

Chapter 6

DISCUSSION & CONCLUDING REMARKS

6.1 Thesis Rationale: The pursuit of personalised medicine

Breast cancer is the most frequently diagnosed female malignancy worldwide, and is responsible for the majority of cancer-related deaths amongst women [1]. While the management of breast cancer has transformed dramatically in recent years, personalised breast cancer management has not yet been achieved. Currently employed disease biomarkers require invasive tissue sampling, while imaging techniques utilised to detect *in-vivo* tumour response to therapy are less than ideal (Section 1.11). To enable the optimal treatment of patients in an individualised manner there exists a requirement for further, less invasive prognostic and predictive markers, additional therapeutic targets and an effective modality to detect early *in-vivo* tumour response to neoadjuvant chemotherapy (NAC). Such factors could be used to better stratify patient outcomes and to develop targeted treatment strategies and personalised breast cancer management plans that are more effective and efficient, offering fewer patient side effects and better outcomes from breast cancer.

6.2 MicroRNAs as novel biomarkers of NAC response

MicroRNAs (miRNA) are relatively novel gene expression regulators whose potential role as disease biomarkers has garnered much attention of late. In 2005, genome-wide miRNA expression analysis enabled identification of miRNAs that were differentially expressed in breast cancer tissue compared to healthy control tissue [103]. The pattern of miRNA expression correlated with distinct tumour phenotypes, ER and PR expression and tumour stage. Since this time, these findings have been corroborated by numerous further authors. The ability of miRNA expression profiles to classify breast tumours by pathologic variables currently utilized to determine responsiveness to neoadjuvant chemotherapy highlights the potential of miRNA signatures as novel predictive and prognostic biomarkers. Further to this, these RNA molecules are readily detected and quantified from patient blood samples that can be obtained via minimally-invasive means.

In Chapter 2, a bibliometric analysis of miRNA research produced from point of discovery to present revealed the maturity of this field of research. The ubiquitous applicability and popularity of miRNA research confirmed its acceptance as a valid research avenue worldwide. However, this study highlighted the requirement of a novel research direction. This led to the recognition of a research question that warranted further investigation; miRNAs as novel biomarkers of neoadjuvant chemotherapeutic (NAC) response in breast cancer. Whilst the role of miRNA as disease response markers had recently been investigated across a variety of pathologies, including colorectal and gastric carcinoma, at the time of writing, no such investigation had been conducted in the breast cancer setting. The ICORG 10-11 National Clinical Trial was pursued to answer this very question (Chapter 3).

The interim analysis of the expression of a panel of miRNA from the circulation of breast cancer patients undergoing NAC is outlined in Chapter 3. MiRNA were readily extractable and quantifiable from the circulation of all patients analysed, highlighting their suitability as minimally-invasive biomarker candidates. Expression patterns were consistent with current literature i.e. lower expression of miR-145 and miR-10b in cancer compared to Let-7a, miR-21, miR-155 and miR-195, which have known increased expression in association with breast cancer. Although overall study endpoints could not be achieved during the scope of this thesis, interim findings were promising, with miRNA readily detectable and quantifiable from blood samples. The definitive study findings are eagerly anticipated as they have the potential to guide and individualise the administration of chemotherapy.

Further lessons were learned from this study with regards to the conduct of a clinical trial. It is a lengthy and laborious process that mandates the allocation of a dedicated laboratory technician and analyst, to guide study centres, ensure the timely analysis of samples and the effective collation of all study data. On a technical level, it was recognised that blood samples for miRNA analysis should be collected in Paxgene tubes so that RNA can be stabilised

to ensure the validity, reproducibility and optimisation of study findings and results.

6.3 Assessing Argonaute-2 as a prognostic and predictive biomarker

While the definitive utility of miRNA in NAC response monitoring was underway in the ICORG 10-11 trial, a further avenue of investigation up-stream of miRNAs was pursued for its potential application as a further prognostic or predictive breast cancer biomarker. Argonaute-2 (Ago2) is a protein that is expressed ubiquitously throughout the body, playing an essential role in the regulation of gene expression via small non-coding RNAs, including siRNAs, PIWI-interacting RNAs (piRNAs) and miRNAs [155-158]. A high incidence of alteration in the Ago2 gene is reported across multiple cancer types, including breast, colorectal, urothelial and prostate, in conjunction with an association with more aggressive breast cancer subtypes and enhanced tumour metastasis [87, 170, 171]. As a result, Ago2 represented a very interesting candidate in the search for a novel prognostic or predictive biomarker, or potential future target for personalized medicine.

The differential expression of Ago2 protein in association with breast cancer subtype, hormone receptor status, tumour size, T score and M score is outlined in Chapter 4. Both in breast cancer cell lines and clinical tissue specimens, Ago2 protein was significantly less abundant in the less aggressive Luminal A breast cancer subtype compared to the more aggressive tripe negative breast cancer. In conjunction with this, Ago2 protein was significantly associated with the absence of the oestrogen and progesterone receptor. While only a minority of patient samples were associated with metastatic disease, a significant association was identified between the presence of Ago2 protein (strong staining) and metastasis. Upon proliferation assay, cells transfected with the Ago2 gene were seen to have increased proliferation. These findings support a potential oncogenic role for Ago2, which is in keeping with current literature. Upon analysing the effect of hypoxia-induced phosphorylation of Ago2 in breast cancer, an association between elevated phosphorylated Ago2 levels and increased cell invasion and reduced survival in breast cancer was reported [261]. Similarly, in renal cell

carcinoma elevated Ago2 protein was associated with poorer overall survival [262]. While incomplete at the time of writing this report, an analysis is being conducted between breast tissue Ago2 protein levels and patient disease-free and overall survival from breast cancer.

The ability of Ago2 to classify breast tumours by pathologic variables currently used to guide therapeutic decisions and prognosticate for patients with breast cancer highlights the potential for Ago2 to serve as a novel biomarker for breast cancer. The use of Ago2 in this setting however remains in its infancy, with further investigation required to fully elucidate mechanisms of Ago2 regulation and the cancer-specific functions of the Ago2 gene and its resultant protein.

6.4 Photoacoustic Imaging in disease-response monitoring

In conjunction with circulatory and tissue biomarkers, a further non-invasive mechanism for disease response-detection, disease monitoring and patient prognostication was being developed for application in the clinical setting. Chapter 5 outlines the first application, optimization and baseline measurements of the VisualSonics Vevo® LAZR photoacoustic imaging system in the clinical setting on patient breast tissue. Based on light excitation and ultrasound detection, this novel imaging system was successfully optimised specifically for the visualisation and evaluation of breast tissue and breast pathology, with effective imaging of breast tissue, comprising healthy tissue, fibroadenoma and tumours. Imaging depth was optimised to 3.5cm, which is superior to other light-based imaging systems currently reported in the literature [229, 267]. Tissue oxyhaemoglobin concentrations (sO_2) were effectively calculated, and a lower sO_2 was identified in tumours compared to healthy control tissue. These results were in keeping with current reports in the literature [229]. With the system effectively and reliably visualising breast tumours and calculating sO_2 values, an optimised study protocol was developed so that the system's ability to detect NAC response could be evaluated, and this study is currently underway by my successor. Patient enrolment onto the NAC arm of this study is ongoing with baseline images

being effectively captured. Further images will be captured during NAC and pre-operatively, and pathological analysis of breast specimen will be examined. Images and sO2 measurements will be assessed and investigated for association with definitive patient response. The ability of PAI to effectively detect and monitor *in-vivo* tumour response to NAC would enable individualised patient management, ceasing chemotherapy where no response is identified, or potentially altering regimen, or even obviating the surgical intervention when reliable pCR is identified.

6.5 Future Directions

Molecular profiling has increased our understanding of the heterogeneity of breast cancer by enabling the identification of distinct breast cancer subtypes that have known clinical behaviours and responses to therapy. This discovery enabled the development of targeted therapies, such as Tamoxifen in oestrogen receptor positive disease, and Trastuzumab in HER2 overexpressing. Further molecular characterisation of breast cancer is undoubtedly warranted to identify further prognostic markers and therapeutic targets. In conjunction with this, the development of novel imaging modalities is required to enable effective and reliable detection and monitoring of disease response to systemic therapy. This thesis provides an analysis of the role of miRNAs in breast cancer, outlines the investigation of two novel prognostic and predictive markers, miRNAs and Ago2, and their potential roles in targeted therapeutics and chemotherapeutic administration, and details the optimisation and development of the novel PAI in disease response monitoring. The content of this thesis contributes to breast cancer research locally, nationally and internationally. Study findings have already informed several currently ongoing studies in this laboratory and hospital group, improved sample collection and storage techniques, and greatly contributed to a national clinical trial that is currently near completion. On a global scale, chapters have been published in peer-reviewed international journals, with further manuscripts currently in preparation, and multiple presentations have been delivered both nationally and internationally. By further informing

mechanisms to tailor breast cancer management on an individualised basis, work presented here contributes to the development of personalised or precision medicine, pursuing tailored chemotherapeutic administration and superior patient outcomes from this disease.

6.6 Conclusion

The goal of translational research is to develop personalised medicine. To enable the optimal treatment of patients in an individualised manner, there exists a requirement for further, less invasive prognostic and predictive breast cancer biomarkers, additional therapeutic targets and an effective imaging modality to detect early *in-vivo* tumour response to neoadjuvant chemotherapy. The development of tailored breast cancer care plans would enable the administration of more effective and efficient treatments with fewer patient side effects and better outcomes from disease.

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Appendices

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: _____

	Please Initial box.
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: Anti-Argonaute-2 Antibody Product Datasheet

abcam

Product datasheet

Anti-Argonaute-2 antibody [2E12-1C9] - ChIP Grade ab57113

9 Abreviews 39 References 4 Images

Overview

Product name	Anti-Argonaute-2 antibody [2E12-1C9] - ChIP Grade
Description	Mouse monoclonal [2E12-1C9] to Argonaute-2 - ChIP Grade
Tested applications	**Suitable for:** CHIPseq, WB, ICC/IF, IHC-P, IP, Flow Cyt, ChIP, ICC
Species reactivity	**Reacts with:** Chicken, Human, Zebrafish **Does not react with:** Xenopus laevis
Immunogen	Recombinant fragment corresponding to Human Argonaute-2 aa 483-859.

Properties

Form	Liquid
Storage instructions	Shipped at 4°C. Store at +4°C short term (1-2 weeks). Upon delivery aliquot. Store at -20°C or -80°C. Avoid freeze / thaw cycle.
Storage buffer	pH: 7.20 Constituent: PBS
Purity	Protein G purified
Clonality	Monoclonal
Clone number	2E12-1C9
Isotype	IgG1
Light chain type	kappa

Applications

Our Abpromise guarantee covers the use of **ab57113** in the following tested applications.

The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

Application	Abreviews	Notes
CHIPseq		Use at an assay dependent concentration. PubMed: 23592263
WB		Use a concentration of 1 - 5 µg/ml. Predicted molecular weight: 97 kDa.
ICC/IF		Use a concentration of 10 µg/ml.

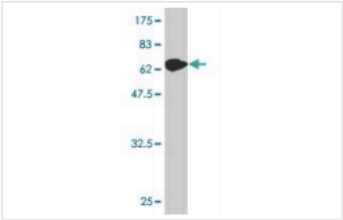
1

IHC-P	★★★★☆	Use a concentration of 3 µg/ml.
IP	★★★★★	Use at an assay dependent concentration. PubMed: 19574298
Flow Cyt		Use 1µg for 10^6 cells. ab170190-Mouse monoclonal IgG1, is suitable for use as an isotype control with this antibody.
ChIP		Use at an assay dependent concentration. PubMed: 20010811
ICC	★★★★☆	Use at an assay dependent concentration.

Target

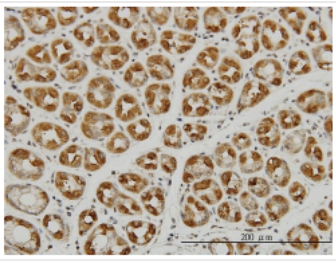
Function	Required for RNA-mediated gene silencing (RNAi) by the RNA-induced silencing complex (RISC). The 'minimal RISC' appears to include EIF2C2/AGO2 bound to a short guide RNA such as a microRNA (miRNA) or short interfering RNA (siRNA). These guide RNAs direct RISC to complementary mRNAs that are targets for RISC-mediated gene silencing. The precise mechanism of gene silencing depends on the degree of complementarity between the miRNA or siRNA and its target. Binding of RISC to a perfectly complementary mRNA generally results in silencing due to endonucleolytic cleavage of the mRNA specifically by EIF2C2/AGO2. Binding of RISC to a partially complementary mRNA results in silencing through inhibition of translation, and this is independent of endonuclease activity. May inhibit translation initiation by binding to the 7-methylguanosine cap, thereby preventing the recruitment of the translation initiation factor eIF4-E. May also inhibit translation initiation via interaction with EIF6, which itself binds to the 60S ribosomal subunit and prevents its association with the 40S ribosomal subunit. The inhibition of translational initiation leads to the accumulation of the affected mRNA in cytoplasmic processing bodies (P-bodies), where mRNA degradation may subsequently occur. In some cases RISC-mediated translational repression is also observed for miRNAs that perfectly match the 3' untranslated region (3'-UTR). Can also upregulate the translation of specific mRNAs under certain growth conditions. Binds to the AU element of the 3'-UTR of the TNF (TNF-alpha) mRNA and upregulates translation under conditions of serum starvation. Also required for transcriptional gene silencing (TGS), in which short RNAs known as antigene RNAs or agRNAs direct the transcriptional repression of complementary promoter regions.
Sequence similarities	Belongs to the argonaute family. Ago subfamily. Contains 1 PAZ domain. Contains 1 Piwi domain.
Domain	The Piwi domain may perform RNA cleavage by a mechanism similar to that of RNase H. However while RNase H utilizes a triad of Asp-Asp-Glu (DDE) for metal ion coordination, this protein appears to utilize a triad of Asp-Asp-His (DDH).
Post-translational modifications	Hydroxylated. 4-hydroxylation appears to enhance protein stability but is not required for miRNA-binding or endonuclease activity.
Cellular localization	Cytoplasm > P-body. Nucleus. Translational repression of mRNAs results in their recruitment to P-bodies. Translocation to the nucleus requires IMP8.

Anti-Argonaute-2 antibody [2E12-1C9] - ChIP Grade images



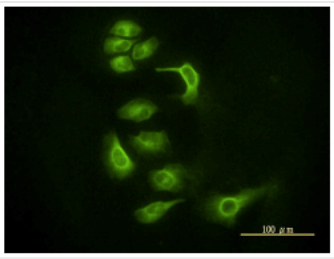
Western blot - Anti-Argonaute-2 antibody - ChIP Grade (ab57113)

Predicted band size : 97 kDa
Western Blot detection against the recombinant fragment imunogen (68.4 KDa).



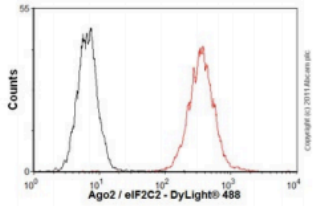
Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections) - Anti-Argonaute-2 antibody - ChIP Grade (ab57113)

Ago2 / eIF2C2 antibody (ab57113) used in immunohistochemistry at 3ug/ml on formalin fixed and paraffin embedded human stomach.



Immunocytochemistry/ Immunofluorescence - Anti-Argonaute-2 antibody - ChIP Grade (ab57113)

Ago2 / eIF2C2 antibody (ab57113) used in immunofluorescence at 10ug/ml on HeLa cells.



Flow Cytometry - Anti-Argonaute-2 antibody - ChIP Grade (ab57113)

Overlay histogram showing HeLa cells stained with ab57113 (red line). The cells were fixed with 80% methanol (5 min) and then permeabilized with 0.1% PBS-Tween for 20 min. The cells were then incubated in 1x PBS / 10% normal goat serum / 0.3M glycine to block non-specific protein-protein interactions followed by the antibody (ab57113, 1µg/1x10^6 cells) for 30 min at 22°C. The secondary antibody used was DyLight® 488 goat anti-mouse IgG (H+L) (ab96879) at 1/500 dilution for 30 min at 22°C. Isotype control antibody (black line) was mouse IgG1 [ICIGG1] (ab91353, 2µg/1x10^6 cells) used under the same conditions. Acquisition of >5,000 events was performed. This antibody gave a positive signal in HeLa cells fixed with 4% paraformaldehyde/permeabilized in 0.1% PBS-Tween used under the same conditions.

Appendix 4: Ethical Approval Form for Photoacoustic Study

Feidhmeannacht na Seirbhíse Sláinte
Health Service Executive

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

Clinical Research Ethics Committee
Main Administration Building
Merlin Park Hospital
Galway.

16th January, 2014.

Professor Michael J. Kerin
Professor of Surgery
National Breast Cancer Research Institute
Discipline of Surgery
Clinical Science Institute
NUI
Galway.

Ref: C.A. 1004 – Potential Role of Photocoustic Tomography in Breast Cancer Imaging

Dear Professor Kerin,

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

Yours sincerely,

p.p. Colette Collins

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

Appendix 5: Photoacoustic Study Patient Information and Consent Form

GALWAY UNIVERSITY HOSPITALS – PHOTOACOUSTIC TOMOGRAPHY

PATIENT INFORMATION and CONSENT FORM

INTRODUCTION

We would like to invite you to participate in a clinical research initiative at Galway University Hospitals to investigate a new imaging modality for use in the breast setting. Although major advances have been made in the management of cancer, many aspects of the disease are not fully understood. It is hoped that our understanding of the disease will be improved through research.

The purpose of the study is to identify whether photoacoustic tomography, a novel form of ultrasound imaging, could improve imaging of the breast.

Although this study may have no direct benefit to you, it is hoped that the results may benefit patients like you in the future. You are under no obligation to take part and if, having read the information below, you would prefer not to participate, we will accept your decision without question.

YOUR INVOLVEMENT

We are inviting patients who have been reviewed at the symptomatic breast unit to participate in this study. If you volunteer to participate in our study, you give us consent to perform photoacoustic investigation(s).

Procedure

Photoacoustic tomography is performed like an ultrasound exam. You will be brought into an examination room and asked to lie on the examination table. Both you and the doctor performing your examination will be required to wear a pair of safety goggles to protect your eyes from the laser contained within the photoacoustic probe. The exam consists of a probe, covered in jelly, being pressed against and scanned over the contour of your breast. You will not feel any pain or discomfort during the procedure, and it should take no more than thirty minutes.

Clinical Information

By participating, you give us consent to store information relating to your investigation on a database. We will not disclose the result of the investigation with anyone outside of the study group, and it will not affect your diagnosis or treatment in any way. Your identity will remain confidential. Your name will not be published or disclosed to anyone outside the study group. All research is covered by standard institutional indemnity insurance and is approved by a Research Ethics Committee that ensures the ethical nature of the research. Nothing in this document restricts or curtails your rights. You may withdraw your consent at any time. If you decide not to participate, or if you withdraw your consent, your standard of treatment will not be affected in any way.

Further Information

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

If you would like further information about research projects that may be conducted, please contact your Consultant.
Thank you in anticipation of your assistance. Please read and sign the Consent section.
I have read the attached information sheet on the above project, dated

_____

Please Initial Box ☐

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

(Name of sponsor): ……..…….

PARTICIPANT'S NAME: ……..……

CONTACT DETAILS: ……

PARTICIPANT'S SIGNATURE: ……

DATE: ……

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

NAME OF RESEARCH NURSE OR INVESTIGATOR:……

DATE: ……..……..……

CONSULTANT: ……..……..…….

Appendix 6: Pro Forma for Patient Clinicopathological Data Collection PAI

Patient details: -Name -DOB -Board No.	Addressograph:
History:	Presenting Complaint:
Examination:	
Imaging: -Other imaging (CT/MRI)	Mammogram Report: Ultrasound Report:
Histology:	
Family History	
Other Relevant History: -Previous breast disease -Past surgical/medical history -Medication	

Publications

PeerJ

Evolution of a research field—a micro (RNA) example

Máire-Caitlín Casey, Michael J. Kerin, James A. Brown and Karl J. Sweeney

Discipline of Surgery, School of Medicine, National University of Ireland, Galway, Ireland

ABSTRACT

Background. Every new scientific field can be traced back to a single, seminal publication. Therefore, a bibliometric analysis can yield significant insights into the history and potential future of a research field. This year marks 21 years since that first ground-breaking microRNA (miRNA) publication. Here, we make the case that the miRNA field is mature, utilising bibliometrics.

Methods. Utilising the Web of Science™ (WoS) database publication and citation information, we charted the history of miRNA-related publications, describing and dissecting contributions by publication type (plus category, pay-per-view or open access), journal (highlighting dominant journals), by country, citations and languages.

Results. We found that the United States of America (USA) publishes the most miRNA papers, followed by China and Germany. Significantly, publications attributed to the USA also receive the most citations per publication, followed by a close grouping of England, Germany and France. We also describe the relevance and acceptance of the miRNA field to different research areas, through its uptake in areas from oncology to plant sciences. Exploring the recent momentous change in publishing, we find that although pay-per view articles vastly out-number open-access articles, the citation rate of pay-per-view articles is currently less than double that of open-access.

Conclusions. We believe the trends described here represent the typical evolution of a research field. By analysing publications, citations and distribution patterns, key moments in the evolution of this research area are recognised, indicating the maturation of the miRNA field and providing guidance for future research endeavours.

Subjects Bioinformatics, Cell Biology, Molecular Biology, Science and Medical Education
Keywords miRNA, microRNA, *Lin-4*, Bibliometric, *Let-7*

Submitted 13 January 2015
Accepted 18 February 2015
Published 17 March 2015

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Additional Information and Declarations can be found on page 15

DOI **10.7717/peerj.829**

INTRODUCTION

With expanding scientific production and unprecedented access to information, a means of independently assessing and analysing research output becomes apparent and essential. Consequently, various data analysis tools and internet-based search engines have been devised to enable the processing and organisation of this scientific output into a manageable form. Bibliometric parameters are one such tool utilised for this assessment. First described by Paul Otlet in 1934 (*Rousseau, 2014*), bibliometrics refers to the quantitative statistical analysis of publications to enable activity and dynamics within research fields to be mapped.

The onset of the digital age revolutionised the manner in which scientific knowledge is produced and distributed. *Gibbons et al. (1994)* provide an account of this fundamental change in their concept of '*Mode 2*' knowledge production. This refers to the development of a highly interactive and transdisciplinary research system that is socially distributed. *Nowotny, Scott & Gibbons (2001)* elaborated this concept still further, highlighting novel and contemporary scientific practices with an increasing range of '*knowledge producers*.' Various theories of science evolution have been proposed, with some authors analogising the progression of research to the evolution of living organisms: the introduction of new concepts, development of novel research directions and the emergence and loss of hypotheses (*Chavalarias & Cointet, 2013*).

The field of microRNA research presents an exceptional opportunity to observe the progression of a novel area of scientific investigation from point of discovery to rapidly maturing field, using bibliometrics. The term microRNA (miRNA) was coined by *Ruvkun (2001)*, to refer to a naturally-occurring class of short, non-coding RNA molecule between 19 and 21 nucleotides long. *Lee, Feinbaum & Ambros (1993)* discovered the first miRNA in 1993, isolating *Lin-4* from the nematode *Caenorhabditis elegans*. It took seven years before a second miRNA, *Let-7*, was discovered by *Pasquinelli et al. (2000)*. The revelation that *Let-7* sequence, expression and function were conserved across animal phylogeny (*Pasquinelli et al., 2000*), from nematodes to humans, resulted in a research revolution. Subsequently, thousands of miRNAs have been identified in eukaryotes, including plants, fish, fungi and mammals. In humans alone approximately 2,555 unique mature miRNAs have been identified (http://www.mirbase.org/). While the exact function of all recognized miRNAs remains to be fully elucidated, they are known to regulate gene expression via binding target messenger RNA (mRNA), inhibiting translation or triggering mRNA degradation. Importantly, it has been demonstrated that in addition to their inhibitory role miRNAs can function to induce or activate transcript levels (*Vasudevan, Tong & Steitz, 2007*; *Place et al., 2008*). Through these mechanisms miRNAs fulfil a regulatory role in various cellular processes, including cell development, differentiation, proliferation and apoptosis (*Place et al., 2008*; *Filipowicz, Bhattacharyya & Sonenberg, 2008*; *Bartel, 2004*).

Deregulated miRNA expression patterns have been noted across all organisms, encompassing a wide spectrum of pathological processes, from immunological defects in fish, altered developmental phase transition and flowering time in plants, and neurodegeneration, cardiovascular disease and cancer in humans (*Calin et al., 2002*; *van Rooij et al., 2006*). Discovery of deregulated miRNA expression led to the hypothesis that miRNAs could potentially be used as diagnostic or prognostic markers of disease. Furthermore, miRNA are attractive therapeutic targets for the treatment of various conditions, including cancer. The novel role of miRNA and their importance to many different processes has led to an explosion into the scientific enquiry of miRNA function. The aim of our study is to utilise one data analysis tool, Web of Science^{TM}, in conjunction with current theories of research evolution, to quantitatively analyse a novel field of investigation from its point of discovery, to outline its progression and potentially predict its future course.

MATERIALS AND METHODS

Database

Citation data was retrieved from the *Web of Science*™ (WoS) database (http://apps.webofknowledge.com/), produced by Thomson Reuters (see results section, "database"). Search results from WoS encompassed entries from the *WoS Core Collection*, comprising *Science Citation Index Expanded* (SCI-EXPANDED), *Social Sciences Citation Index* (SSCI), *Arts & Humanities Citation Index* (A&HCI), *Book Citation Index* (BKCI), *Conference Proceedings Citation Index* (CPCI), *Current Chemical Reactions* (CCR Expanded) and *Index Chemicus* (IC) (http://images.webofknowledge.com/WOKRS513R8.1/help/WOS/hp_database.html).

Search terms and methods

The WoS database was searched utilising the terms "miRNA" and "microRNA" with the Boolean operator "OR". Upon analysis of initial search findings, conflicting results were identified, namely publications containing the following: *mirna estuary, mirna bay, mirna river, mirna equation, Mirna A (author)* and *mirna SC*. As such, the Boolean operator "NOT" was utilised to exclude these findings and refine our search results. Further to this, the research category of "*Agriculture*" was excluded from our search, as several inaccurate search results were identified within this classification.

Although recognised as the first miRNAs discovered, the initial publications pertaining *Lin-4* and *Let-7* did not utilise the terms microRNA or miRNA (coined in *Ruvkun, 2001*), as such these papers did not feature in the search results. To account for this, the *Web of Science Core Collection* database was searched for *Lin-4* and *Let-7* in isolation, utilising the Boolean Operator "NOT" to exclude miRNA and microRNA, so as to prevent overlap with original search results.

The publication timeframe analysed encompassed January 1993 to December 2013. Publications of all languages were accepted, comprising all peer-reviewed articles, including reviews, letters to the editor and editorials.

Data analysis

WoS data tools were utilised to perform certain elements of result analysis e.g. generating *Journal Citation Reports*. Additional data analysis was performed using *Microsoft Excel 2010*© and *Minitab* version 16 ®.

N.B. The results returned from WoS upon searching study criteria were found to increase with the passage of time. This is thought to be due to delayed indexing of journals among other factors. As a result, some figures containing quantitative numbers may differ slightly among sections of this manuscript, when totalled.

RESULTS

Database

Several research platforms currently exist for examining bibliometrics, including the Web of Science™, Highwire© and PubMed™. Prior to commencing this study, the suitability

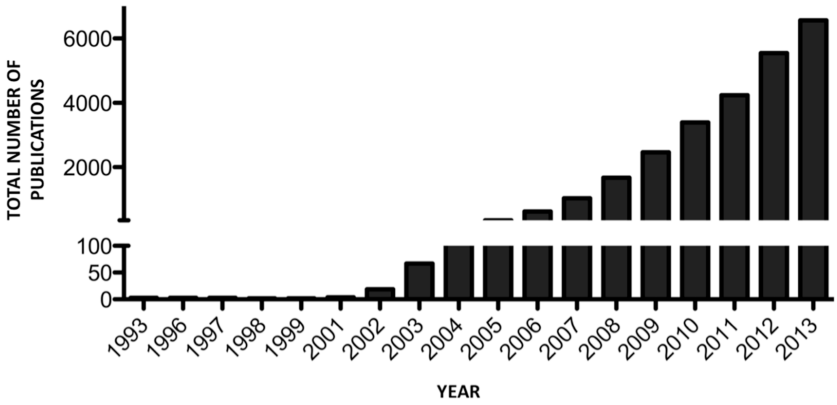

Figure 1 **Number of miRNA publications per year.**

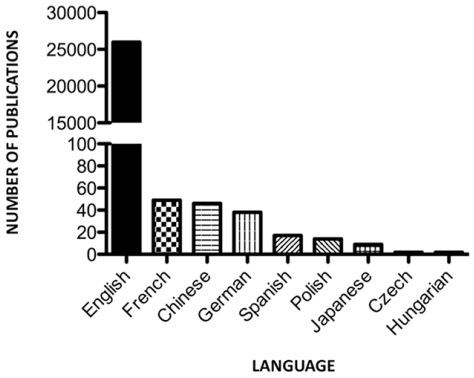

Figure 2 **Language of publication.**

of these databases was individually analysed to allow selection of the optimal data resource. Ultimately, WoS was chosen for the purpose of this study due to superior journal coverage, approximately 9,300 compared to HighWire (1,700) and PubMed (5,669). An additional significant factor was the return of a substantially higher proportion of results using the defined study search criteria (see section 'Search terms and methods').

Publication distribution

The number of published items identified pertaining to miRNAs, as catalogued in the Web of Science™ Core Collection database (1993–2013), totals 26,177 publications. The first publication occurred in 1993, with minimal additional publications (35) until 2003 (Fig. 1). Sixty-two percent of all miRNA literature (16,348 publications) was published within the period 2011–2013 inclusive. Currently, the highest output occurred in 2013,

Table 1 Rank of 25 countries publishing miRNA material most prolifically with cumulative number of publications per country.

Rank	Country	No. publications
1	United States	11,056
2	People's Republic of China	5,584
3	Germany	2,083
4	United Kingdom	1,527
5	Japan	1,474
6	Italy	1,455
7	Canada	904
8	France	848
9	Australia	650
10	South Korea	645
11	Netherlands	625
12	Spain	621
13	Switzerland	492
14	Denmark	420
15	Taiwan	411
16	India	371
17	Israel	348
18	Sweden	335
19	Belgium	292
20	Ireland	254
21	Singapore	239
22	Austria	203
23	Brazil	201
24	Poland	184
25	Greece	158

with 25% of total miRNA publications (6,560). Of total publications identified, 99.2% of items were published in English (25,980 publications), with the remainder of articles in French (49 publications), Chinese (46 publications), German (38 publications), Spanish (17 publications), Polish (14 publications), Japanese (9 publications), Czech (2 publications) and Hungarian (2 publications) (Fig. 2).

Publications by country

To further analyse miRNA-related literature, the distribution of publications by country was determined. In total, 84 countries contributed to the miRNA literature, as outlined in Table 1 (Top 25). The top 5 most prolific countries account for 83% of total publications (21,652). The USA is responsible for 42% of all miRNA literature (11,056 publications) followed by the Peoples' Republic of China 21% (5,584 publications), Germany 8% (2,083 publications), the United Kingdom (1,527 publications) and Japan 6% (1,474 publications). Further nations featuring within the top 10 most prolific countries include Italy 6% (1,455 publications), Canada (904 publications), France (848 publications), Australia (650 publications) and South Korea (645 publications) (Fig. 3). Interestingly,

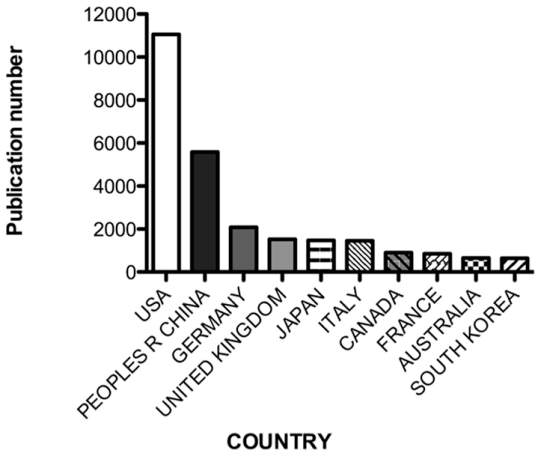

Figure 3 **Number of publications per country.**

separating the United Kingdom into its constituent countries England alone would rank 6th (1,239 publications) behind the USA, the Peoples' Republic of China, Germany, Japan and Italy (Fig. S1).

Research categories

Analysing the ontological research categories by which miRNA publications are classified in the WoS database, the top 50% of miRNA publications identified can be categorised into medicine and health sciences, which comprises of Biochemistry Molecular biology (23.5%, 6,163 publications), Oncology (15.5%, 4,070 publications), Cell biology (15%, 3,923 publications) and Genetics heredity (10%, 2,554 publications) (Table 2). Further ontological categories of miRNA research listed include horticulture, marine science and entomology.

Peer-reviewed publications

The categories of research, as defined in Table 2, are comprised of miRNA publications that feature in various international peer-reviewed journals. Table 3 outlines the 25 most prolific journals publishing miRNA research (Table S1, 50 most prolific). In the 20 year period analysed, the youngest journal *Public Library Of Science ONE* (*PLoS ONE)* delivered the largest number of miRNA-related publications at 1,589 (6% of total), with *Nucleic Acids Research* responsible for 489 publications (2% of total) and *Proceedings of the National Academy of Science of the USA (PNAS)* 451 publications (2% of total). We found the top 50 journals were responsible for 38% of total miRNA publications, of which the top 10 journals represent 18% of total miRNA publications identified.

Document type

MiRNA publications are comprised of various document types including original articles, review articles, news items, editorial material, corrections, reprints, database

Table 2 **Rank of 25 research categories featuring miRNA publications most frequently, with number of publications per research category, and percentage of overall publication.**

Rank	Category	No. works	% Total works
1	Biochemistry Molecular Biology	6,163	23.53
2	Oncology	4,070	15.54
3	Cell Biology	3,923	14.98
4	Genetics Heredity	2,554	9.75
5	Science Technology Other Topics	2,474	9.45
6	Biotechnology Applied Microbiology	1,634	6.24
7	Research Experimental Medicine	1,623	6.19
8	Haematology	1,131	4.32
9	Cardiovascular System Cardiology	1,068	4.077
10	Neurosciences Neurology	1,057	4.04
11	Pharmacology Pharmacy	935	3.57
12	Gastroenterology Hepatology	906	3.46
13	Pathology	895	3.42
14	Biophysics	893	3.40
15	Plant Sciences	745	2.84
16	Immunology	701	2.68
17	Developmental Biology	696	2.66
18	Chemistry	575	2.19
19	Endocrinology Metabolism	573	2.19
20	Life Sciences Biomedicine Other Topics	516	1.97
21	Virology	482	1.84
22	Mathematical Computational Biology	411	1.57
23	General Internal Medicine	344	1.31
24	Surgery	333	1.27
25	Physiology	308	1.18

reviews, proceedings papers, letters and meeting abstracts. Of the 26,177 publications identified, 69% of all documents were original research articles (18,111 publications). In addition to original articles, 14% of published items comprised meeting abstracts (3,659 publications), 13% review articles (3,314 publications) and 3% were editorial material (647 publications). The remaining publications comprised of a minimal number of article corrections, news items and reprints. Table S2 shows an analysis of published document types for the top 10 journals which published miRNA-related documents. Constant with the overall trend, articles featured most prominently, comprising 88% of publications for these top 10 journals (4,789 publications), followed by meetings abstracts 8% (393 publications), reviews 1.5% (73 publications), editorial material 0.6% (28 publications) and corrections 0.4% (20 publications). Proceedings papers, letters and database reviews comprise the remainder, providing minimal input.

Publication citations

For all miRNA related publications, 837,898 citations were found by querying the *WoS Core Collection* Database. In concordance with publication numbers, citations per year

Table 3 **Rank of 25 journals publishing miRNA material most frequently, with cumulative number of publications per journal.**

Rank	Journal	No. publications
1	PLoS ONE	1,589
2	Nucleic Acids Research	489
3	PNAS	451
4	BLOOD	432
5	Journal of Biological Chemistry	346
6	Biochem Biophys Res Communications	329
7	RNA-a Publication of the RNA Society	303
8	Cancer Research	302
9	BMC Genomics	296
10	Hepatology	252
11	Circulation	246
12	Oncogene	211
13	Faseb Journal	210
14	Cell Cycle	200
15	Febs Letter	180
16	Modern Pathology	179
17	Gastroenterology	176
18	Laboratory Investigation	171
19	Genes Development	167
20	Journal of Virology	162
21	Cell	159
22	RNA Biology	146
23	International Journal of Cancer	136
24	Nature	136
25	Oncology Reports	136

increased exponentially peaking in 2008, at which point citation rate decreased (Fig. 4A). Ranking the total number of citations by country, publications originating from the USA received the highest total number of citations ($n = 475{,}300$) followed by China ($n = 72{,}265$), Germany ($n = 71{,}051$), Italy ($n = 47{,}084$) and England ($n = 42{,}970$) (Fig. 4B). Analysing the average citation per item for the top 10 countries publishing miRNA material, the USA retained the first position (44.3 citations per item). However, the remaining positions changed with the second position now held by England (35.9 citations per item) followed by Germany (35.4 citations per item), France (34.4 citations per item) and Italy (34.2 citations per item). Interestingly, of the top ten countries China now displays the least number of citations per item ($n = 14.5$) (Fig. 4C). Examining the citations per item from the top 20 countries (as described in Table 1), the rankings change considerably with Switzerland (13th) now displaying the highest number of citations per item at 55.1, followed by USA (44.3 citations per item), then the Netherlands (39.6 citations per item), England (35.9 citations per item) and Sweden (35.8 citations per item) (Table S3).

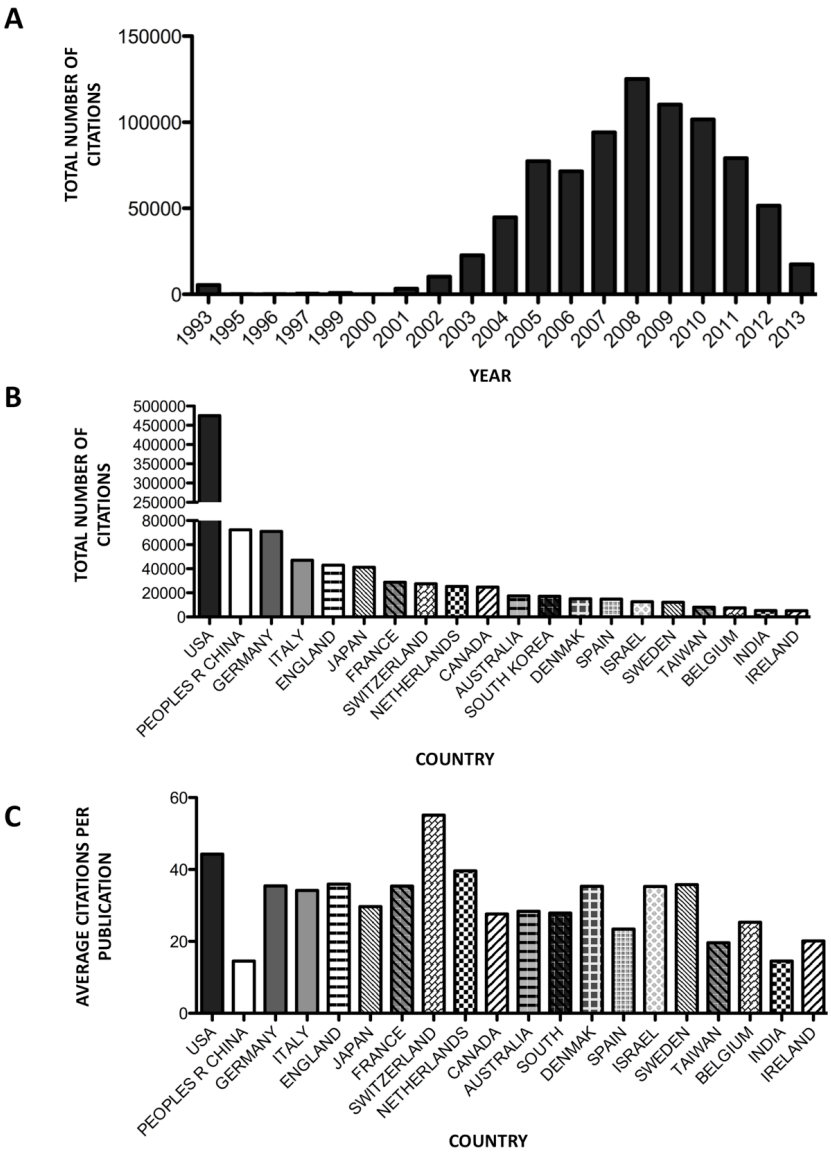

Figure 4 Publication citations. (A) Number of citations per yearly publication. (B) Number of cumulative citations per country. (C) Average citation per publication per country.

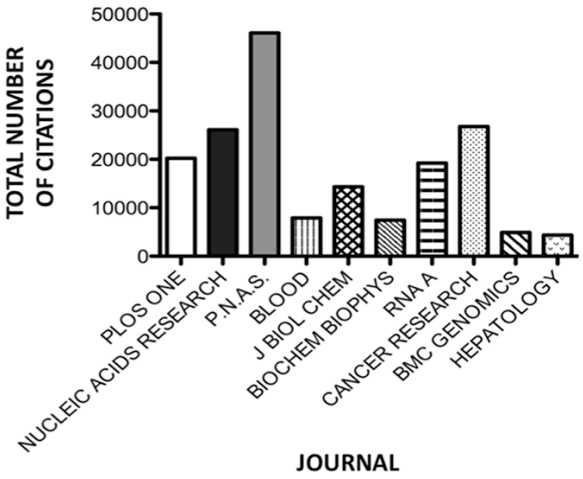

Figure 5 Number of citations per journal for the 10 journals publishing miRNA material most prolifically.

To further analyse the citation pattern, we investigated citations of the top 10 journals publishing miRNA material (Fig. 5). *Proceedings of the National Academy of Science of the USA (PNAS)* was the most frequently cited with 6% of the total citations (46,112 citations), followed by *Cancer Research, Nucleic Acids Research* and *PLoS ONE* each with 3% of total citations (26 763, 26 118 and 20 242 citations respectively). The final entry in this list is *RNA A Publication of the RNA Society* with 2% of the total citations (19, 217 citations).

Considering citations per publication, Table S4 outlines the 10 most cited miRNA publications since the discovery of this research field. The top 3 publications cited featured in the journal *Cell*, with an accumulative total of 11,581 citations (1.4% total citations). 6 of the top 10 publications cited featured in *Nature, Nature Reviews Cancer or Nature Methods*, with one publications featuring in *PNAS*. The first, seminal, miRNA publication by Lee et al. ranks third (3,671 citations) and the second key miRNA publication by *Pasquinelli et al. (2000)* features 28th (931 citations). The top 10 cited papers feature over a 16 year period, with three publications in 2005, three in 2006 and one publication in each of the years 1993, 2004, 2008 and 2009. Of this top 10, four items are review articles. To further examine significant miRNA publications, the top 10 most cited primary research miRNA publications were identified (Table 4). *Cell* was still seen to contribute 3 of the top 10, with *Nature, Nature Genetics* and *Nature Methods* contributing 5 publications and *PNAS* one publication, with the addition of one publication by *Science*.

Open access versus pay-per-view

Of the miRNA related publications identified, 17% of publications were open access ($n = 4,560$), with 83% of publications pay-per-view access ($n = 22,788$) (Fig. 6A). Analysing the citations of these two categories of publication, open access items were cited

Table 4 Top 10 cited primary research miRNA publications.

Rank	Citations	Title	Author	Journal	Year
1	4,167	Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets	Lewis BP, et al.	Cell	2005
2	3,671	The C-Elegans heterochronic gene Lin-4 encodes small RNAs with antisense complementarity to Lin-14	Lee RC, et al.	Cell	1993
3	3,512	MicroRNA expression profiles classify human cancers	Lu J, et al.	Nature	2005
4	2,337	Mapping and quantifying mammalian transcriptomes by RNA-Seq	Mortazavi A, et al.	Nature Methods	2008
5	2,291	A microRNA expression signature of human solid tumors defines cancer gene targets	Volinia S, et al.	PNAS	2006
6	2,168	Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs	Lim LP, et al.	Nature	2005
7	2,151	Prediction of mammalian microRNA targets	Lewis BP et al.	Cell	2003
8	2,009	Identification of novel genes coding for small expressed RNAs	Lagos-Quintana M et al.	Science	2001
9	1,954	Combinatorial microRNA target predictions	Krek A et al.	Nature Genetics	2005
10	1,851	The nuclear RNase III drosha initiates microRNA processing	Lee Y et al.	Nature	2003

84,864 times, representing 10% of overall citations. However, pay-per-view publications were cited 731,470 times, which represented 90% of overall citations (Fig. 6B). Average citation per publication reveals 32.1 citations per pay-per-view publication, compared to 18.6 citations per item for open access publications (Fig. 6C).

Hallmarks of miRNA research

Utilising the bibliometric data retrieved, we identified the key discoveries in the field of miRNA research. The seminal miRNA publication, outlining the discovery of these short RNA molecules, by *Lee, Feinbaum & Ambros (1993)* is certainly the first hallmark of miRNA research. Subsequent to this, recognition of conservation of miRNA sequence expression across animal phylogeny from nematodes to humans by *Pasquinelli et al. (2000)*, with the identification of further miRNAs, also represents a significant key advancement in this field. As previously discussed (section 'Publication citations'), these papers feature 3rd and 28th respectively in the most cited miRNA publications, highlighting their visibility and influence.

Following these crucial findings, discovery of the regulatory roles of miRNAs in various cellular processes, from differentiation to apoptosis, should be considered highly significant in furthering our understanding of the functionality of these short RNA molecules (*Place et al., 2008*; *Filipowicz, Bhattacharyya & Sonenberg, 2008*; *Bartel, 2004*). The next, related key event in the miRNA field was the discovery of deregulation of miRNA expression associated with human diseases, such as cancer (*Calin et al., 2002*; *van Rooij*

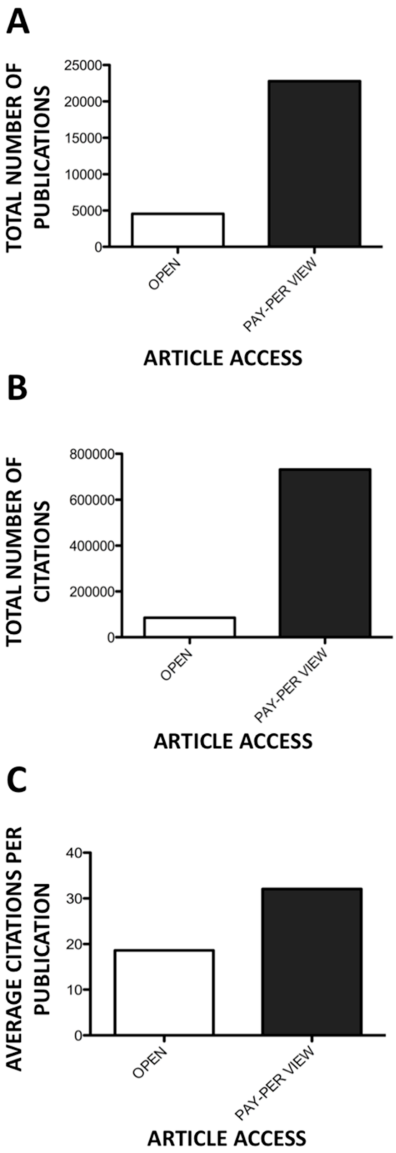

Figure 6 Open access versus pay-per-view publications. (A) Number of open access versus pay-per-view publications. (B) Number of cumulative citations by open versus non-open access publications. (C) Average number of citations per open and pay-per-view publication.

et al., 2006; *Schaefer et al., 2007*). This discovery raises a potential use of miRNA, as both predictive and prognostic biomarkers of disease. At present, multiple clinical trials are currently registered with ClinicalTrials.gov, investigating the ability of miRNA to function as biomarkers of disease and response to current therapies.

Another key event in the evolution of the field has been the discovery that miRNA are capable of extra cellular signalling (*Valadi et al., 2007*). This novel finding significantly added to our knowledge of the mechanisms that can be employed for cell–cell signalling and communication.

The most recent development in the field that should be considered significant is the therapeutic use of miRNAs as targeted therapies to modulate disease (*Kota et al., 2009*). The attainment of personalised disease management via the use of miRNAs is highly appealing, though many obstacles currently remain, including identification of optimal delivery methods, off-target effects and safety.

DISCUSSION

To our knowledge, this work represents the first bibliometric analysis of the miRNA field. Our analysis revealed an exponential increase in research output, with yearly publications more than quadrupling between 2005 ($n = 356$) and 2008 ($n = 1{,}672$), and increasing eighteen-fold by 2013 ($n = 6{,}560$) (Fig. 1). In describing phylomemetic patterns of science evolution, *Chavalarias & Cointet (2013)* outline the importance of '*special events*' in the progression of a research field, with scientific output increasing significantly during the subsequent time period (*Chavalarias & Cointet, 2013*). *Special events* pertaining to the miRNA field can be identified by analysing the most cited of all primary research miRNA publications since the discovery of this research field (Table 4). The timeline covered by the 10 most cited primary research miRNA publications spans from 1993 to 2008, highlighting the continual key discoveries being made in the miRNA field. Of these 10 publications, 3 were published in the journal *Cell*, with 5 published in *Nature* or one of its subsidiary journals, one publication in *PNAS* and one publication in *Science*. The average *journal impact factor* of the 10 most cited primary research miRNA publications is 27.8, highlighting their visibility and influence as a driving force behind the exponential increase in yearly miRNA publications. Currently, one key event in the evolution of the field was identified that was not found by our bibliometric analysis: miRNA involvement in extracellular signalling (*Valadi et al., 2007*). This publication did not make it into our current top 10 (although it has >1,200 citations), possibly due to its relatively recent publication date (2008). However we anticipate in the future that this publication will enter the top 10 most cited papers in the field. The trend of miRNA publications therefore, adheres to Chavalarias and Cointet's association, with output increasing exponentially in concordance with hallmarks of their brief history—discovery (*Lee, Feinbaum & Ambros, 1993*), recognition of deregulation in cancer (*Calin et al., 2002*), cardiovascular disease (*van Rooij et al., 2006*), autoimmune and neurodegenerative disease (*Schaefer et al., 2007*; *Sonkoly et al., 2007*), potential use as disease biomarker (*Cortez & Calin, 2009*), expression in serum/plasma (*Cortez & Calin, 2009*) and use of anti-miR's as targeted therapy (*Kota et al., 2009*).

While 84 countries have contributed to miRNA literature to date, five countries dominate scientific production in this research field (Table 1). The USA, China, Germany, the United Kingdom and Japan are collectively responsible for more than 80% of all current miRNA literature with 96% of total citations. The distribution of citations per country differs considerably when considering average citations per publication. Switzerland exhibits the highest number of citations per item, with the USA featuring 2nd, followed by a grouping of the Netherlands, England, Sweden and Germany (Fig. 4C). While China features second in the top 10 countries publishing miRNA material, it exhibits the lowest number of citations per publication ($n = 14.5$), reflecting a large portion of uncited literature. Further analysing citations by journal access type (open access journals to pay-per-view), it was observed that pay-wall restricted journals, which represented the majority of publications (83%), accounted for 90% of total citations. Interestingly, the average citation per publication was 1.7 times higher for pay-wall restricted journals compared to open access journals (Fig. 6C). While this adheres to currently observed citation patterns in the literature, due to the growing popularity of open access journals and open-access publication requirements from funding agencies, it is proposed that this discrepancy will no longer be apparent in future years, with open-access publications and citation numbers currently increasing (*Bjork & Solomon, 2012*).

Of the miRNA publications identified in the WoS database, it is interesting to note that 69% of all documents were original articles, reflecting the relative youth of this novel field of investigation, with only ∼10 years of sustained multi-group research efforts. In an analysis of the progression of a field of science, *Bonaccorsi (2008)* outlines increasing diversity within research paradigms as an instrumental factor, attributing this to various scientific hypotheses and the investigative techniques applied to examine them (*Bonaccorsi, 2008*). With the discovery of a definitive role for miRNA in the pathogenesis of multiple disease processes, the predominance of medicine and health sciences becomes apparent (Table 2). Categories of published research span the gamut of medical domains, from immunology to oncology, haematology to virology and neuroscience to surgery. While further areas of investigation feature such as entomology and agriculture, their contribution to overall research output is currently negligible.

In an analysis of the dynamic interest in research topics within the biomedical scientific community, *Michon & Tummers (2009)* identified trends that are exemplified by our analysis of miRNAs. When novel research is initially published, it generally appears in high impact journals, followed by a lag in scientific output, prior to subsequent progression of publications. The initial miRNA publication outlining the discovery of lin-4 appeared in 1993 in the journal '*Cell*' which then brandished an impact factor of 37.2. Subsequent to this however, miRNA output did not significantly progress further until 2003. While miRNA publications began to escalate, the average journal impact factor of the top 10 publishing journals was 13.6. Five years later, with miRNA output increasing still further, the equivalent impact factor decreased to 9, with a further drop to 7.4 by 2012. With increasing scientific output, a shift towards lower impact journals is seen, producing a *long-tail distribution* of publishing when viewed by host journal impact factor. Originally

described by Vilfredo Pareto, a social economist, the *long-tail distribution* can refer to a number of observable phenomena. In this context, it is used to describe a publishing pattern whereby high and medium impact factor journals feature in the minority, with the majority of journals having minimal citation impact (*Michon & Tummers, 2009*). Presence of this distribution is recognised as a sign of acceptance of a research topic as valid within the scientific community (*Michon & Tummers, 2009*). Reaching this stage of publication saturation, *Pfeiffer & Hoffmann (2007)* advocate the development of novel research directions within a given field as particularly advantageous, with pioneering work potentiating publication in high impact journals, and thus returning the cycle to the beginning of the *long-tail distribution* once more.

CONCLUSION

When we consider the ongoing remodelling of scientific production, our analysis of publication trends, citations and distribution patterns was very informative. Recognising the developmental stage of a particular research field provides researchers with direction and guidance, both in current and future investigative goals. The current unprecedented access to scientific material and bibliometric information provides an opportunity to analyse the dynamics of scientific landscapes, enabling the production of informed, targeted scientific outputs.

Abbreviations

miRNA	microRNA
WoS	Web of Science
PLoS ONE	Public Library Of Science
PNAS	Proceedings of the National Academy of Science of the USA

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Maire-Caitlin Casey, James A. Brown and Michael J. Kerin are funded by BREST-PREDICT and the National Breast Cancer Research Institute (NBCRI). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
BREST-PREDICT.
National Breast Cancer Research Institute.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Máire-Caitlín Casey conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Michael J. Kerin analyzed the data, reviewed drafts of the paper.
- James A. Brown conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Karl J. Sweeney conceived and designed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.829#supplemental-information.

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IJC
International Journal of Cancer

Exploring circulating micro-RNA in the neoadjuvant treatment of breast cancer

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Mini Review

Breast cancer is the most frequently diagnosed malignancy amongst females worldwide. In recent years the management of this disease has transformed considerably, including the administration of chemotherapy in the neoadjuvant setting. Aside from increasing rates of breast conserving surgery and enabling surgery *via* tumour burden reduction, use of chemotherapy in the neoadjuvant setting allows monitoring of *in vivo* tumour response to chemotherapeutics. Currently, there is no effective means of identifying chemotherapeutic responders from non-responders. Whilst some patients achieve complete pathological response (pCR) to chemotherapy, a good prognostic index, a proportion of patients derive little or no benefit, being exposed to the deleterious effects of systemic treatment without any knowledge of whether they will receive benefit. The identification of predictive and prognostic biomarkers could confer multiple benefits in this setting, specifically the individualization of breast cancer management and more effective administration of chemotherapeutics. In addition, biomarkers could potentially expedite the identification of novel chemotherapeutic agents or increase their efficacy. Micro-RNAs (miRNAs) are small non-coding RNA molecules. With their tissue-specific expression, correlation with clinicopathological prognostic indices and known dysregulation in breast cancer, miRNAs have quickly become an important avenue in the search for novel breast cancer biomarkers. We provide a brief history of breast cancer chemotherapeutics and explore the emerging field of circulating (blood-borne) miRNAs as breast cancer biomarkers for the neoadjuvant treatment of breast cancer. Established molecular markers of breast cancer are outlined, while the potential role of circulating miRNAs as chemotherapeutic response predictors, prognosticators or potential therapeutic targets is discussed.

Breast cancer is the most frequently diagnosed cancer among women worldwide, accounting for 23% of total cancer cases.[1] In 2012, worldwide 1.7 million women were diagnosed with breast cancer, representing a >20% increase in incidence since 2008. Concurrently, worldwide more than 520,000 women died from breast cancer, representing a 14% increase in annual breast cancer related mortality and confirming breast cancer as the most common cause of cancer-related deaths amongst women.[1] Within developed countries the incidence of breast cancer continues to rise. This is likely due to the implementation of screening programs and improved imaging techniques, leading to many breast cancers being diagnosed at an earlier stage. Furthermore, our improved molecular understanding of breast cancer and the use of increasingly effective chemotherapeutics has resulted in improved patient outcomes, with mortality decreasing by 2 to 3% per year in developed countries.[2]

Historically, neoadjuvant chemotherapy (NAC) was reserved for locally advanced breast carcinoma, converting technically inoperable tumours into candidates for mastectomy. With the increasing trend toward breast conserving surgery, however, the use of primary systemic therapy was extended to include patients with invasive, early-stage operable tumours. The adoption of NAC has led to increasing rates of breast conserving surgery and provides an opportunity to assess *in vivo* tumour responsiveness to chemotherapeutics.[3] Although previous studies failed to identify any improvement in disease free and overall survival between neoadjuvant and adjuvant therapies, it has been established that patients achieving *complete pathological response* (pCR) to NAC therapy experience improved outcomes, while unresponsive patients or patients with progressive disease during NAC experience worse outcomes.[4–7] Supporting this, it has been shown that the early response to neoadjuvant treatment can predict pCR and therefore may serve as a predictor of long-term outcome.[8,9]

Key words: miRNA, micro-RNA, neoadjuvant chemotherapy, breast cancer, circulating

Abbreviations: ER: oestrogen receptor; Her2: Erbb2 receptor; miRNA: micro-RNA; NAC: neo-adjuvant chemotherapy; NICE: National Institute for Health and Care Excellence; pCR: complete pathological response; PR: progesterone receptor

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This article was published online on 30 January 2016. An error was subsequently identified. This notice is included in the online and print versions to indicate that both have been corrected on 4 February 2016.

Grant sponsor: Breast Cancer Research (BCR)

DOI: 10.1002/ijc.29985

History: Received 11 June 2015; Accepted 22 Dec 2015; Online 12 Jan 2016

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Unfortunately at present there is no reliable, clinically validated, method for predicting chemotherapeutic responders from non-responders. While the likelihood of achieving pCR varies greatly by breast cancer subtype (from 7.5% in luminal cancers to 45% in HER2/Triple negative cancers[10,11]), many patients are exposed to the potential morbidity and mortality associated with chemotherapy, without any certainty of benefit from treatment. This has resulted in global efforts to discover breast cancer biomarkers that can predict and detect response to neoadjuvant therapy. Such biomarkers could confer multiple benefits, including tailored patient-care programs, reduced chemotherapy-induced morbidity or mortality and potentially expedite the identification of effective new therapies for the treatment of breast carcinoma. At present, circulating micro-RNAs (miRNAs) represent an important avenue in the search for a non-invasive biomarker for *Breast Cancer Res*ponse prediction and monitoring for neoadjuvant chemotherapy. The evidence for this is discussed further in the following sections.

Micro-RNAs

Micro-RNAs are a naturally-occurring class of short, non-coding RNA molecules ~19 to 25 nucleotides in length. miRNAs have been demonstrated to regulate gene expression at the post-transcriptional level, *via* binding primarily to 3' or 5' untranslated regions of target messenger RNAs (mRNA), leading to inhibition of translation or mRNA degradation.[12] Interestingly in addition to their inhibitory role, miRNAs have recently been demonstrated to facilitate increases in transcript levels, under certain conditions.[13,14]

Since their discovery in 1993, knowledge of the role of miRNAs in regulating gene expression across a spectrum of pathological processes has grown exponentially.[15] It is now recognized that certain miRNAs are highly specific for tissue and developmental stages, exerting a regulatory effect on a myriad of cellular processes including cell development, differentiation, proliferation and apoptosis.[12,14,16] Many miRNA are expressed in tissue- and disease-specific patterns and are known to correlate with clinicopathological features and prognostic indices across a spectrum of pathologies.[17–22] However, to date few studies have investigated the effect of neoadjuvant chemotherapy on miRNA expression patterns in breast cancer.

miRNA biosynthesis and mechanisms of action

miRNA generation is a complex process that commences in the nucleus. miRNA genes are transcribed by RNA polymerase II/III as primary miRNAs (pri-miRNAs). These pri-miRNAs are processed by the Drosha-DGCR8 complex, becoming pre-miRNAs. Pre-miRNAs are transported into the cell cytoplasm by the nuclear export protein Exportin 5, where they are cleaved by the RNase III enzyme Dicer, with either TRBP (Trans-activator RNA-binding protein) or PACT (protein activator of PKR), into a double stranded miRNA duplex. One strand of the duplex represents a mature miRNA and is incorporated into the RNA-induced silencing complex (RISC), while the other strand is degraded. The miRNA:RISC complex (miRISC) then targets mRNA containing complementary sequences to the mature miRNA, inhibiting translation or inducing mRNA degradation (Fig. 1).[12,16,23]

Further to their intracellular function, it has been recognized that miRNAs function at an intercellular level, transmitting information from one cell population to another and inducing changes through this novel extracellular signaling mechanism.[24,25] Although small RNAs were detected in the circulation as early as 2004,[26] it was in 2008 that the presence of miRNAs in the circulation was confirmed, with significant differences in expression patterns detectable between patients with cancer compared to controls.[27,28] These circulating miRNAs were found to be present with remarkable stability, indicating that they must be protected from the digestive action of circulatory RNases. Recent studies have confirmed that miRNAs are transported by a variety of mechanisms that shield them from this RNase degradation, including packaged into membrane-derived vesicles, such as exosomes, bound to lipoproteins and as part of ribonucleoprotein complexes.[24,25,29–31] While some mechanisms regulating the packaging and export of membrane-bound miRNA are understood,[32–34] the process of non-membrane-bound miRNA export from cells remains unclear.[35,36] The exact sources of all circulating miRNA remains unconfirmed. It appears there are two, complimentary, sources of circulating miRNA: (*i*) miRNA released passively into the bloodstream following tissue injury and cell death and (*ii*) miRNA actively exported from cells into the bloodstream. However, in both cases miRNA could be either protein bound "un-encapsulated" miRNA or miRNA protected inside membrane coated vesicles (such as exosomes). Of note, it has been demonstrated that an estimated ≥90% of circulating miRNAs are bound to argonaute-2 (Ago2) containing complexes, with only a minority being transported packaged in vesicles.[30,37] However, controversy regarding the proportions of free and membrane-bound circulating miRNA remains, due to a lack of standardization of sample processing techniques, preventing data from individual studies from being directly compared.

Breast Cancer and Neoadjuvant Chemotherapy

The first successful chemotherapeutic regimen for operable breast cancer was described in 1976 by Bonadonna *et al.*[38] The combination adjuvant therapy with *cyclophosphamide, methotrexate* and *fluorouracil* (CMF) was shown to significantly reduce post-operative recurrence rates. By the early 1990s, *anthracycline*-containing regimens were introduced and are now recognized as superior to treatment with CMF alone.[39] Importantly, it was during this time that administration of chemotherapy in the preoperative or neoadjuvant period began. The *National Surgical Adjuvant Breast and Bowel Project B-27* and the *Aberdeen trial* recognized the addition of a *taxane* to an *anthracycline*-based chemotherapy further reduced the risk of recurrence, and in the neoadjuvant setting improved the rates of complete pathological remission and therefore overall outcome (Fig. 2).[4,40,41]

In recent years, it is recognized that breast cancer is a heterogeneous disease characterised by discrete breast cancer subtypes.[42] While the exact number of subtypes remains to be elucidated, in a landmark paper, Sorlie *et al.*[43] described

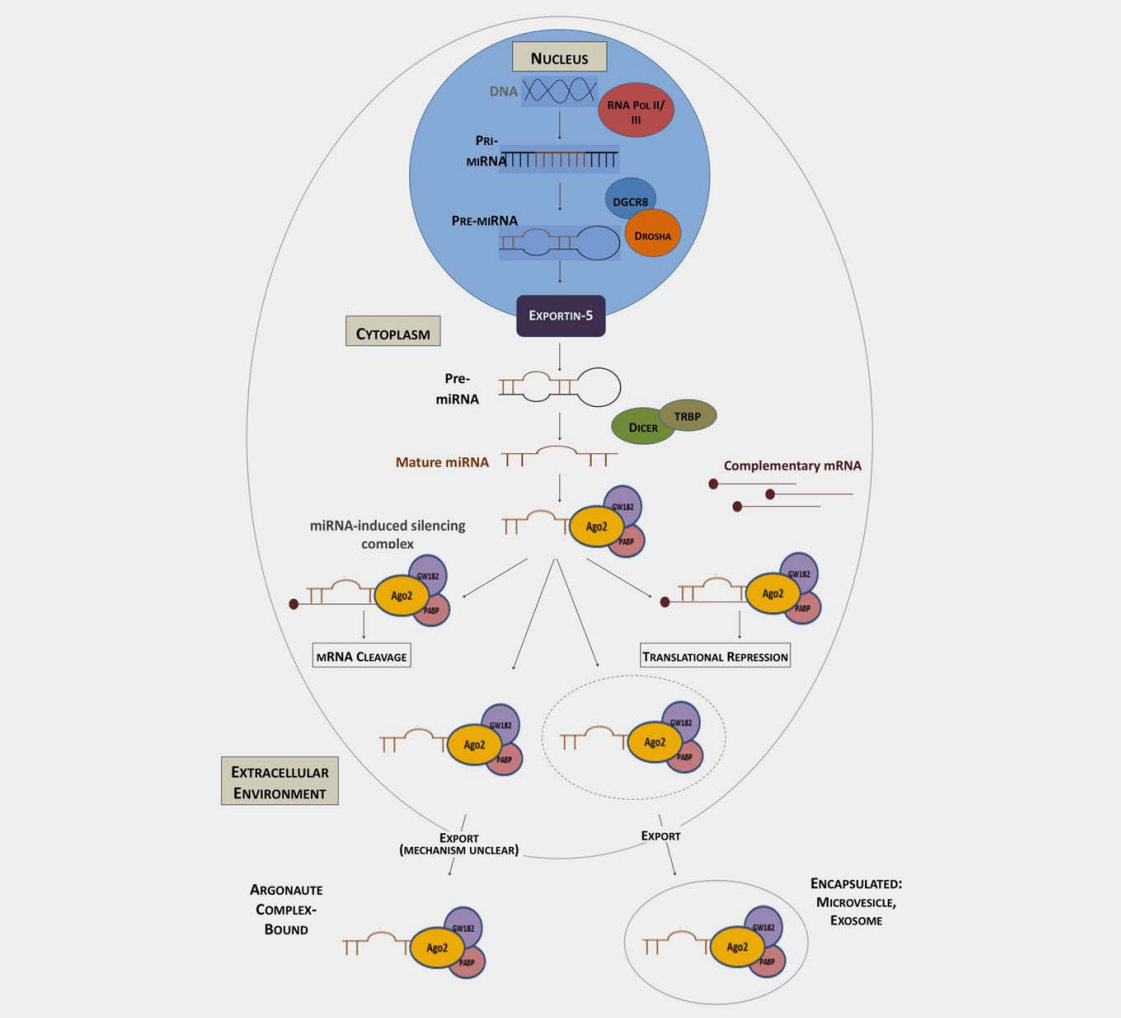

Figure 1. Model of miRNA biogenesis and cellular export.

Luminal A, *Luminal B*, *Basal* (also known as "*triple negative*" tumours) and *HER2* over-expressing (Table 1). More recently, 10 distinct breast cancer subtypes have been proposed, although this stratification is not yet applied clinically.[4]5 Currently utilized breast cancer subtypes have known distinct clinical behaviors and responses to therapy and are stratified according to presence or absence of the *oestrogen receptor* (ER), *progesterone receptor* (PR) and the *human epidermal growth factor receptor 2* (HER2) (discussed further in Current and Potential Molecular Markers Used to Guide the Administration of Chemotherapy section). While no gold-standard chemotherapeutic regimen currently exists for breast cancer, it is generally accepted that an *anthracycline*-based regimen be utilized, with the addition of a *taxane*.[46,47] For patients with breast cancer overexpressing the HER2 receptor, targeted therapy with the humanized monoclonal antibody *Trastuzumab* is recommended by NICE clinical guidelines[48] (Fig. 2; Table 2).

Current and Potential Molecular Markers Used to Guide the Administration of Chemotherapy

The current gold standard molecular markers for *Breast Cancer Res*ponse prediction are the ER, PR and HER2 receptors and the proliferation marker Ki67. The *American Society of Clinical Oncology* guidelines mandate that the immunohistochemical markers ER, PR and HER2 be assessed in all cases of invasive breast carcinoma to guide management decisions, including choice of chemotherapeutic regimen.[53] Further markers such as Claudin and specific miRNAs have been proposed and are presently undergoing further validation (Fig. 3).[54]

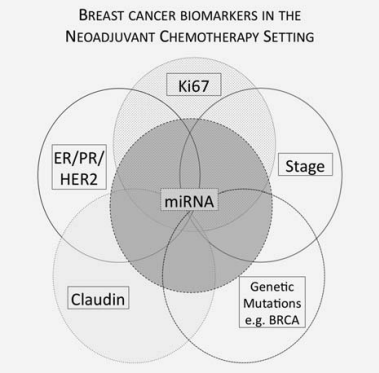

Figure 2. Breast cancer biomarkers in the neoadjuvant chemotherapy setting. Currently used clinical biomarkers (solid lines); new potentially clinically relevant biomarkers (dotted lines).

ER/PR receptor

ER/PR expression, as identified by immunohistochemistry, provides an index for sensitivity to endocrine treatment and acts as a marker of chemosensitivity. Approximately two thirds of tumours display ER-positivity, correlating with improved responsiveness to endocrine therapy and improved patient outcomes. PR expression is strongly dependent on ER expression, with <1% of breast cancers displaying sole PR-positivity. In this instance, limited benefits from endocrine therapy have been described.[55] Associating chemotherapeutic treatments and ER/PR status, ER positivity correlates with poor tumour response.[56] However, ER-negative tumours are more likely to achieve complete pathological remission and thus experience improved outcomes with chemotherapy.[57]

HER2 (ERBB2)

HER2 is a membrane tyrosine kinase receptor that upon activation affects cell proliferation and survival.[58] It is located on chromosome 17q12 and is an oncogene, amplified in ~15 to 20% of breast cancer cases. Initially identified as a prognostic marker, HER2 overexpression is associated with increased relapse rates, increased incidence of metastases and worse overall survival.[59,60] However, the development of therapies specifically targeting HER2 has resulted in significant improvements in outcomes for patients with HER2-positive breast cancer.[61] Risk of relapse and death are reduced by approximately 50% and 30% respectively, improving disease-free and overall survival.[62] In 2013 the addition of Trastuzumab to neoadjuvant chemotherapy in patients with HER2-positive tumours was found to double complete pathological remission rates (compared to chemotherapy alone) and was associated with a longer event free survival.[63] Most recently, use of neoadjuvant followed by adjuvant HER2 has demonstrated sustained benefit in event-free survival and a strong association with complete pathological remission.[64]

Basal/triple negative breast cancers

"Basal" and "triple negative (TNBC)" breast cancer subtypes overlap greatly in terms of their immunophenotype (ER, PR & HER2 negative), aggressive clinical behavior and increased prevalence in younger, African-American patients. However, they are not synonymous, as not all basal cancers determined by gene expression analysis lack ER, PR and HER2 and not all triple-negative cancers show a basal phenotype by expression array analysis.[65] Due to a more aggressive clinical pathology, both of these subtypes are associated with a higher risk of mortality. Both subtypes lack all known effective biomarkers and therefore targeted therapies. However, both subtypes are highly sensitive to neoadjuvant chemotherapy.[66–68] The highest rates of complete pathological remission have been achieved in TNBC tumours utilising a neoadjuvant regimen of docetaxel, doxorubicin and cyclophosphamide, while the addition of bevacizumab is expected to increase this rate further.[63]

Ki67

Ki67 is a nuclear non-histone protein utilized as a marker of proliferation, as it is absent in quiescent cells, yet universally expressed among proliferating cells. Immunohistochemical staining of, Ki67 expression levels is associated with the percentage of tumour cell nuclei positively stained and are used to determine a Ki67 score. In early and advanced breast cancer the Ki67 score can predict the response to chemotherapy.[69] A high pre-treatment score is associated with a good chance of complete pathological remission to therapy and therefore improved long-term outcome.[70] Lee *et al.*[71] describe a significant decrease in Ki67 index following neoadjuvant chemotherapy, a finding that is recognized as a strong predictor of recurrence-free and overall survival. Of concern regarding the use of Ki67 is the lack of standardization of analytical practice, with laboratories utilising differing cut-off points to differentiate between "high" and "low" Ki67. Further to this, as with all of the above described markers, determination of expression levels requires tumour tissue, mandating invasive sampling techniques, which further emphasizes the need for a non-invasive breast cancer biomarker.

Claudins

More recently, a potential fifth subtype of breast cancer has been described, classified as "Claudin-low."[72,73] Claudins are a family of proteins that function in tight-junctions and cell-cell adhesion, including Claudin 3, 4, 7 and E-cadherin. A new subtype of breast cancer, "Claudin-low" has recently been described, characterised by lack of expression of the Claudin proteins.[72,74] Claudin-low tumours are typically triple-negative and display high expression of epithelial-to-mesenchymal transition (EMT) markers.[75] The expression of EMT markers has known associations with resistance to therapeutics and higher metastatic potential.[76] Claudin-low cancers display lower complete pathological remission rates following NAC. Overall Claudin-low cancers were found to

Table 1. Breast cancer subtypes with receptor status and prevalence[44]

Breast cancer subtype	ER	PR	HER2	Prevalence (%)
Luminal A	+	±	−	40
Luminal B	+	±	+	20
HER2	−	−	+	15–20
Basal/triple negative	−	−	−	10–15

This table outlines the prevalence and receptor status of current clinically utilized breast cancer subtypes.
Abbreviations: ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

have an intermediate prognosis, worse than basal-like breast cancer, but better than luminal cancer.[72] However, the "Claudin-low" breast cancer subtype remains poorly described.[77] Further definitive characterization is required before it is fully accepted into clinical practice.

miRNA

In 2005, genome-wide miRNA expression analysis enabled identification of miRNAs that were differentially expressed in breast cancer tissue.[78] The panel of 29 miRNAs identified differentiated tumours from normal tissues with an accuracy of 100%. Importantly, miRNA expression correlated with distinct tumour phenotypes, ER and PR expression and tumour stage. More recently, a miRNA expression pattern of 31 miRNAs evaluated in 93 tumour samples was found to predict hormone receptor status, and thus classify tumours by genetic subtype.[79] These findings were independently corroborated by Lowery *et al.*[20] who profiled 453 miRNAs in 29 early-stage breast cancer specimens, identifying a distinct panel of miRNAs corresponding to expression of ER, PR and HER2.

Translating these tissue findings into the circulation, it was found that miR-195 expression was significantly elevated in breast cancer patients ($n = 148$) compared to controls ($n = 44$), and that these levels reduced postoperatively.[80] Furthermore, high levels of circulating miR-21 and miR-10b were found to be associated with ER negativity, thus a poorer prognosis.[80] In a further study, elevated miR-155 expression was associated with PR positivity.[81]

The ability of miRNA expression profiles to classify breast tumours by biopathologic variables currently utilized to determine responsiveness to neoadjuvant chemotherapy highlights the potential of miRNA signatures as novel predictive and prognostic biomarkers that could allow individualization of breast cancer treatment and improved selection of patients for neoadjuvant chemotherapy (Fig. 3).

miRNA as Novel Biomarkers of NAC Response

The role of miRNA in neoadjuvant chemotherapeutic response prediction and monitoring has been investigated across a variety of pathologies. In colorectal carcinoma an association between miRNAs and tumour response to neoadjuvant chemoradiotherapy was proposed.[82] This association was confirmed by identification of a distinct miRNA expression signature that could effectively predict colorectal cancer response to neoadjuvant chemoradiotherapy.[83] In human gastric cancer, decreased let-7i expression was found to have a significant association with a poorer response to chemotherapy and shorter overall survival.[84] In breast cancer the ability of a panel of miRNAs to predict response of triple negative breast carcinoma to neoadjuvant chemotherapy was investigated.[85] Although study numbers were limited (11 patients), results indicated higher miR-200b-3p and miR-190a expression and lower miR-512-5p expression was associated with a better pathologic response to chemotherapy.

While all the above findings involved miRNA analysis from tumour samples, some peri-neoadjuvant studies of circulating miRNA have also been conducted. One study investigated miRNA extracted from the sera of stage II-III locally advanced and inflammatory breast carcinoma patients preneoadjuvant chemotherapy.[86] A two-gene signature of miR-375 and miR-122 was identified with the ability to predict metastatic disease relapse with a sensitivity of 80% and specificity of 100%. Patients relapsing following neoadjuvant chemotherapy were found to have significantly up-regulated expression of miR-122 while patients with higher circulating miR-375 experienced a good clinical outcome. Fluctuation within a panel of miRNA was also found in patients with primary operable or locally advanced breast cancer receiving neoadjuvant chemotherapy.[87] Of a panel of eight miRNAs, miR-221, miR-195 and miR-21 were noted to decrease most significantly with the administration of chemotherapeutics, although correlation with response to systemic therapy was not conducted. A further study recognized expression of two particular miRNAs to be induced by treatment with chemotherapeutics, namely miR-34a and miR-122. Elevated expression of these miRNAs was detected both in tumour tissue and serum, and was particularly associated with anthracycline-based regimens in patients achieving partial response to neoadjuvant chemotherapy.[88]

Some interesting *in vitro* findings that show promise for translation to the clinical setting have been conducted. Investigating targeted therapies for triple negative breast cancers, the overexpression of miR-181a/b was found to associate with more aggressive breast cancer subtypes.[89] Utilizing a range of cell lines (MDA-MB-231, HEK 293GP, MDA-MB-468, SUM159PT, OVCAR, HT29, PANC1 and Sk-Br-3) the overexpression of miR-181a/b was found to dampen the DNA damage response, thus increasing the sensitivity of the triple negative cells in which it was expressed to poly (ADP-Ribose) polymerase-1 (PARP-1) inhibition. It was proposed that profiling miR-181a/b expression in patients with triple negative breast cancer could identify patients for PARP-1 inhibition or platinum-based chemotherapy.

miRNA and chemoresistance

It is recognized that specific miRNA expression signatures are associated with resistance to all forms of breast cancer treatment, including chemotherapy, anti-endocrine therapy and radiotherapy.[90–94] Regarding chemotherapeutic

Table 2. Chemotherapeutics used to treat breast cancer

Drug class	Mechanism of action	Example	Reference
Anthracyclines	Inhibition of DNA and RNA synthesis Disruption of DNA damage response Inhibition of Topoisomerase II	Doxorubicin Epirubicin Mitoxantrone	49
Taxanes	Disruption of microtubule function	Docetaxel Paclitaxel	50
Alkylating Agent: Nitrogen Mustard	Interference with DNA replication	Cyclophosphamide	40
Anti-metabolites	Prevention of folate use for DNA generation	Methotrexate Fluorouracil Capecitabine	51
Anti-HER2/EGFR	Tyrosine kinase inhibition Arrest of cell cycle Suppression of angiogenesis	Trastuzumab Pertuzumab Lapatinib	52

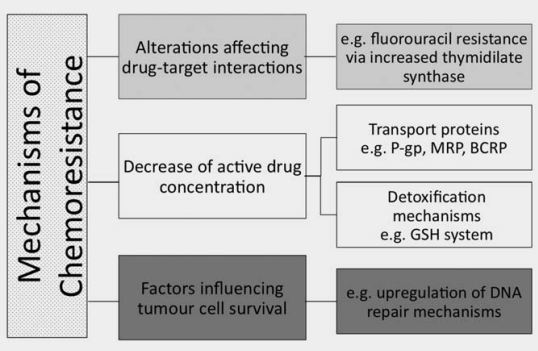

Figure 3. Mechanisms of chemoresistance.

resistance, several recent reports have revealed the key regulatory role of miRNAs affecting drug resistance proteins and targeting proteins involved in apoptosis (Table 3, Fig. 3).

In the circulation, an association between serum miR-125b levels from patients with invasive ductal breast carcinoma receiving NAC and chemoresistance has been described.[105] Increased expression of miR-125b was found to have a significant association ($p = 0.008$) with non-response to chemotherapy. Further to this, forced miR-125b overexpression in breast cancer cells *in vitro* increased chemotherapeutic resistance, with subsequent reduction in miR-125b levels sensitizing the cells to chemotherapy once more. Similar results were observed regarding miR-210, where increased plasma miR-210 levels correlated with a reduced sensitivity of HER2 breast carcinoma to *Trastuzumab* therapy. High pre-treatment circulating mir-210 was found to be associated with lower pCR rates and lymph node metastasis. Recently the radiological and clinical response of breast cancer to either neoadjuvant chemotherapy or hormonal therapy was assessed using a low density miRNA array. Significantly increased Let-7a was found in the plasma of patients achieving a radiological response following neoadjuvant chemotherapy, but not hormonal therapy.[117]

In breast cancer tissue, down-regulation of miR-200c was found in patients who were non-responsive to NAC.[110] Subsequently, up-regulation of miR-200c in human breast cancer cell lines enhanced chemosensitivity and decreased expression of multi-drug resistance (MDR) proteins P-glycoprotein (P-gp) and MDR-associated protein (MRP-1).

Utilizing breast cancer cell lines, differential miRNA expression has been noted to correlate with resistance to chemotherapeutics. Using MCF-7/MX100 cell miR-328 was identified as a negative regulator of breast cancer-resistance protein (BCRP), with higher miR-328 levels facilitating an improved Mitoxantrone response.[118] Up-regulated miR-19 (in MCF-7/TX200, MCF-7/VP-17, MCF-7/MX100 and MCF-7/WT cell lines) correlated with overexpression of three MDR-related transport proteins (MDR-1, MRP-1 and BCRP). Importantly, miR-19 inhibitors decreased the expression of these MDR proteins.[96] MiR-451 was also found to regulate expression of MRP-1, with up-regulation of miR-451 in doxorubicin-resistant MCF-7 cells returning chemotherapeutic sensitivity.[92] Mir-326 exhibited the same effect on MRP-1, inducing sensitivity to doxorubicin in MDR MCF-7 cells.[119] Further miRNAs noted to target MRP-1 include miR-345 and miR-7, which were found to decrease cellular levels of MRP-1.[95]

In BT474, SKBR3, and MDA-MB-453 breast cancer cell lines miR-21 conferred resistance to Trastuzumab, *via* down-regulation of its target PTEN.[97] Subsequently, this pathway was also found to modulate resistance to doxorubicin in doxorubicin-resistant MCR-7 cell lines.[98] In addition, miR-137 was found to be down-regulated in MDR MCF-7 cells.[107]

Cells lines can provide conflicting data however. MCF-7 and MDA-MB-231 cells with acquired docetaxel resistance, showed increased expression of miR-34a, with miR-34a inhibition enhancing chemotherapeutic response in docetaxel-resistant MCF-7 cell lines.[102] However, decreased levels of miR-34a were described in the Adriamycin-resistant MCF-7 cell line,[104] with forced overexpression of miR-34a found to increase chemotherapeutic sensitivity of Adriamycin-resistant MCF-7 cell lines.[103] The exact role of miR-34a in breast cancer chemotherapeutic response is not fully understood and requires

Table 3. miRNAs with a validated involvement in chemotherapeutic resistance in breast cancer

miRNA	Expression	Target(s)	Drug Ass[n]	Source (# patient samples)	Reference
miR-7	Down-regulation	MDR-1	Cisplatin	Cell line	95
miR-19	Up-regulation	MDR-1, MRP-1 and BCRP *via* PTEN	Paclitaxel Mitoxantrone VP-16	Cell Line	96
miR-21	Up-regulation Up-regulation	PTEN PTEN	Doxorubicin Trastuzumab	Cell Line	97, 98
miR-25	Up-regulation	Inhibits autophagic cell death	Epirubicin	Cell Line	99
miR-30c	Down-regulation Down-regulation	YWHAZ TWF1, IL-11	Doxorubicin Paclitaxel Doxorubicin	Cell Line Human breast tissue (n = 51) Cell Lines	100 101
miR-34a	Up-regulation Down-regulation Down-regulation	BCL-2, Cyclin D1 Notch-1 E2F3, PXR	Docetaxel Adriamycin Doxorubicin	Cell Line Cell Line Cell Line	102 103 104
miR-125b	Up-regulation Up-regulation	E2F3 Bak 1	5-Fluorouracil Paclitaxel	Blood Serum Cell Line	105 106
miR-137	Down-regulation	P-glycoprotein, *via* YB-1	Vincristine Doxorubicin Paclitaxel	Cell Line	107
miR-149	Down-regulation	NDST1	Adriamycin	Cell Line	108
miR-155	Up-regulation	FOXO3a	Doxorubicin VP-16 Paclitaxel	Human Breast Tissue (n = 126)	109
miR-200c	Down-regulation Down-regulation	P-glycoprotein MDR mRNA TrkB, Bmi1	Doxorubicin Doxorubicin	Human Breast Tissue (n = 39) Cell Line Cell Line	110 111
miR-210	Up-regulation	Not studied	Trastuzumab	Blood Serum (n = 43) Cell Lines	91
miR-221	Up-regulation	Not studied	Adriamycin	Blood Plasma (n = 125)	112
miR-288	Down-regulation	MDR-1, P-glycoprotein	Doxorubicin	Cell Line	113
miR-320a	Down-regulation	TRPC5, NFATC3	Adriamycin Paclitaxel	Cell Line	114
miR-345	Down-regulation	MDR-1	Cisplatin	Cell Line	95
miR-451	Down-regulation	P-glycoprotein, MDR-1	Doxorubicin	Cell Line	92
miR-489	Down-regulation	Smad3	Adriamycin	Cell Line	115
miR-663	Up-regulation	HSPG2	Adriamycin	Cell Line	116

This table presents an overview of miRNAs that have a validated role in chemotherapeutic resistance, referencing the drug and source examined and the identified miRNA targets.
Abbreviations: HSPG2: heparin sulfate proteoglycan 2; Smad3: mothers against decapentaplegic homolog 3; NDST1: GlcNAc *N*-deacetylase/*N*-sulfotransferase-1; TRPC5: transient receptor potential channel C5; NFATC3: nuclear factor of activated T-cells isoform C3; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta; PXR: pregnane X receptor; BCL-2: B-cell lymphoma 2; YB-1: Y-box binding protein-1; MRP-1: multidrug resistance-associated protein-1; BCRP: breast cancer resistance protein; TWF1: Twinfilin 1; IL-1: interleukin-1; TrkB: tyrosine receptor kinase type 2; Bmi1: B-cell-specific Moloney murine leukemia virus integration site 1; Bak 1: Bcl-2 antagonist killer 1.

further investigation. This seemingly contradictory data highlights the complex nature of miRNAs and their role in chemoresistance, particularly in relation to taxane resistance.[120]

miRNAs as Targeted Therapies in the Neoadjuvant Setting

The use of miRNAs in the treatment of breast cancer includes using miRNAs as therapeutic treatments and manipulating miRNA expression to enhance existing treatments. As miRNAs function as oncomirs and tumour suppressors, two therapeutic potentials exist: overexpression of targeted miRNAs (miRNA replacement therapy) or down-regulation (silencing) of miRNAs.

Oncomir inhibition

Antisense-inhibition of miRNA activity can be achieved by using miRNA antagonist oligonucleotides (anti-miRs),

locked-nucleic acids (LNA), or targeted miRNA silencing (antagomiRs).[121] The efficacy of this targeted therapy has been demonstrated by several studies. MCF-7 cells transfected with anti-miR-21 oligonucleotides were grown *in vitro* and in a xenograft murine model. Anti-miR-21 suppressed both cell growth *in vitro* and tumour growth in the mouse model. In addition, cell growth inhibition was associated with increased apoptosis and decreased cell proliferation.[122]

In the chemotherapeutic setting, down-regulation of mir-21 was seen to increase sensitivity of MCF-7 cells to taxol therapy,[123] with miR-203 knockdown increasing cisplatin sensitivity.[124] Increased sensitivity in response to miRNA knockdown has also been demonstrated, in HS578T cells whereby by miR-155 down-regulation (using antisense-*miR-155* oligonucleotides) increased apoptosis in response to treatment with Paclitaxel, Doxorubicin and VP-16.[109]

miRNA replacement therapy

This strategy involves the reintroduction of function of a tumour suppressing miRNA.

In NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice, SK-3rd cells over-expressing Let-7 or miR-30 displayed significantly reduced tumourigenicity and lung and liver metastasis.[125,126] In a further study in chemoresistant MDA-MB-231 and BT-549 cells, overexpression of miR-200c was found to restore chemosensitivity to microtubule-directed agents.[127]

Although rapid and continual advancements are being made regarding the manipulation of miRNAs for the treatment of breast cancer, this area of research remains in its infancy, with many obstacles to overcome prior to mainstream implementation in breast cancer therapeutics. The majority of studies conducted to date are *in vitro,* examining miRNAs and their effects in various cell lines. To validate these studies, large clinical trials are required to support these preliminary findings. Present obstacles to overcome include the identification of optimal delivery methods and the prevention of off-target effects and safety optimization.

Discussion

The management of breast cancer, in terms of diagnosis, chemotherapeutics and surgical intervention, continues to adapt in line with translational research and evidence. Whilst the use of chemotherapy in the neoadjuvant setting is now acceptable for any patient considered a candidate for adjuvant therapy,[128] there currently exists no clinically validated means of differentiating chemotherapeutic responders from non-responders. While miRNA expression analysis holds significant promise in this setting, further studies investigating miRNAs in the neoadjuvant breast cancer setting are undoubtedly warranted. Presently, as per ClinicalTrials.gov, two clinical trials are currently recruiting breast cancer patients undergoing neoadjuvant chemotherapy for miRNA profiling and analysis.

A further challenge affecting the use of circulating miRNA as breast cancer biomarkers is the lack of accepted standardized protocols for sample collection, handling and processing. As a consequence, many previous studies cannot be easily compared. This is due to intrinsic differences in: Patient cohorts (treatment regimes, timing of sample collection), Biofluid collected and analyzed (whole blood, plasma, serum), Collection methods (EDTA, Paxgene tubes, sample handling), Processing/extraction techniques (diverse extraction kits, timing of extraction), Investigation of miRNA pool (total, free or membrane bound), Use of multiple non-standardized endogenous controls and Detection methods utilized [arrays (which are constantly being updated) or different RQ-PCR platforms].[129–132] The development and adoption of a standardized set of operating and technical protocols would facilitate study comparison, improved reproducibility and the development of improved targeted future studies.

The use of miRNAs as targeted therapies, although in its infancy, holds immense promise, although further evaluation and validation of current findings are required. The identification of a biomarker that could predict or potentially monitor tumour response to neoadjuvant chemotherapy could revolutionize the manner in which chemotherapeutics are administered, bringing us ever closer to personalized breast cancer management.

Future directions

In addition to miRNAs, dysregulation of miRNA machinery can play a crucial role in cancer initiation and progression.[133] miRNA-binding proteins mediate miRNA-dependent cleavage or degradation of target mRNAs, with all miRNAs studied to date assembling into miRNA-silencing complexes. Studies have shown that genes involved in miRNA biogenesis are dysregulated in various breast cancer subtypes.[79] Down-regulation of Drosha and Dicer, two key elements of the miRNA machinery, has been associated with more aggressive breast cancer subtypes.[134,135] Furthermore, increased expression of exportin-5, a pre-miRNA transporting nuclear receptor, has been associated with increased breast cancer susceptibility.[136] In addition, expression of the key miRNA-binding protein Argonaute-2 protein (Ago2), required for miRNA extracellular transport, is elevated in basal-like breast cancer subtypes, with elevated Ago2 levels producing enhanced proliferation, reduced cell-cell adhesion and increased migratory ability,[137] implicating Ago2 in more aggressive breast cancer subtypes. Further to this, single-nucleotide polymorphisms of Ago2 have been associated with disease free and overall survival in breast cancer.[138]

The use of miRNA machinery genes as breast cancer biomarkers is still in its infancy, however, with further investigation required to fully elucidate mechanisms of miRNA maturation, miRNA-machinery gene regulation and the cancer-specific functions of these miRNA machinery genes and their resultant proteins.

Conclusion

Whilst breast cancer management continues to improve the requirement of a breast cancer biomarker that is both predictive and prognostic remains. Investigation of the potential for

miRNAs to fulfill this role holds much promise, although further clinical studies are required, particularly in the neoadjuvant chemotherapeutic setting.

Acknowledgement

We thank all the members of Prof Kerins' group for stimulating discussions.

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