Analyses of potential markers of chemo-response in breast cancer

Stacey Jessica Jones MPharm, MBChB, MRCS (Ed)

Submitted in accordance with the requirements for the degree of Doctor of Medicine

The University of Leeds

School of Medicine and Health

December 2021

The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated within the thesis. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

ACKNOWLEDGEMENTS

Throughout my period of research, I have received ample support and guidance from my supervisors Dr Thomas Hughes, Mr Raj Achuthan and Mr Brian Hogan for which I would like to convey my heartfelt gratitude and thanks.

In addition I would like to recognise the following colleagues who have given ongoing advice and support during the research project:

Mrs Sarah Perry, Dr Waleed Al-Amri and Dr Arindam Pramanik who have all provided invaluable support in the laboratory.

Professor Andrew Hanby, Dr Eldo Verghese and Dr Rebecca Millican-Slater who have all provided teaching and guidance in analysis of immunohistochemistry.

I would also like to thank every member of the Leeds Breast Unit who have not only motivated me to pursue this period of research and a career in breast surgery but also provided a happy distraction outside of research during my clinical commitments.

However, not forgetting that this period of study may not have been possible without the funding from the Breast Cancer Action group for which I am grateful.

Last but by no means least I would like to dedicate this work to both my wonderful husband Gunshiam Beekhun for his unconditional help and support and my heavenly father Anthony Jones who was always by my side providing great encouragement during my studies and achievements.

ABSTRACT

Breast cancer is the commonest cancer to affect women worldwide and despite developments in breast cancer management and treatment, around a third of women with early breast cancer progress to metastatic breast cancer, which is generally thought to be an incurable condition. Disease progression is related to therapy resistance, therefore mechanisms and markers of resistance are a research focus. Chemotherapy is a component of breast cancer treatment in around one third of early breast cancer cases. Benefits to chemotherapy have been shown in treating cancers considered to have poorer prognoses including larger and higher grade cancers as well as those with metastatic spread to axillary nodes. Chemotherapy also presents an important treatment in the management of cancers with distant metastatic disease. My aim was to investigate potential markers of chemotherapy resistance.

A single-centre retrospective cohort of 305 women diagnosed with primary breast cancer and treated with adjuvant chemotherapy between 2006 and 2010 was constructed, with a follow-up period of between 11.0 to 166.4 months. Clinical and pathological data were collected, and tumour tissue from each case was assembled into tissue micro-arrays. Four genes were chosen as candidate markers of chemoresistance in breast cancer from previous unpublished work by my research group and from a review of the literature: IFNβ1, MX1, ITGA7 and NR4A1. The expression levels of these proteins across my cohort was determined using immunohistochemistry, and analyses were performed to determine any correlations with clinico-pathological features, with a particular focus on survival after chemotherapy.

Analysis revealed that IFNβ1 expression in fibroblasts was significantly, weakly positively associated with expression of MX1, a down-stream marker of active IFN signalling, in the tumour cells (Spearman's correlation r=0.119; p=0.05) implying that signalling between the cell types was present in some cases. However, expression of IFNβ1 in fibroblasts, or MX1 in tumour cells was not significantly associated with disease-free survival across the whole cohort. Therefore, a second cohort of only triple negative breast cancers (n=109) was obtained, and the analyses repeated. IFNβ1 in fibroblasts was significantly, positively associated with MX1 expression in the tumour cells (Spearman's correlation r=0.210; p=0.028). However, in this case, high expression of IFNβ1 in fibroblasts, and MX1 in tumour cells were each significantly associated with poorer disease-free survival (p=0.01). Dividing the cohort into claudin-low and claudin-high subtypes resulted in a stronger correlation between fibroblast IFNβ1 and tumour cell MX1 in the claudin-low group (r=0.375; p=0.008), whereas it was lost in the claudin-high group (r=0.113; p=0.389). Likewise, correlations

between survival and expression of each of IFNβ1 in fibroblasts and MX1 in tumour cells were maintained in claudin-low cases ($p<0.05$) but lost in claudin-highs.

ITGA7 protein proved to be expressed in both nuclear and cytoplasmic compartments and these were examined separately. High nuclear ITGA7 expression, but not cytoplasmic, was associated with longer disease-free survival, by a mean of 647 days ($p=0.036$) in the whole cohort. A sub-analysis of ER-positive breast cancers also showed that high ITGA7 nuclear protein expression was associated with both a longer disease-free and disease-specific survival; 682 days ($p=0.05$) and 604 days ($p=0.005$) respectively. Kaplan-Meier survival analysis performed separately on the patients who received anthracyclines with or without taxanes revealed a significant increase in disease-free survival of 806 days ($p=0.004$) in primary breast cancers that had high ITGA7 nuclear protein expression and that received anthracycline-based adjuvant chemotherapy.

NR4A1 protein also demonstrated by cytoplasmic and nuclear locations, which were again analysed separately. High NR4A1 nuclear protein expression was associated with a poorer disease-free survival and disease-specific survival, by means of 487 days ($p=0.005$) and 621 days ($p=0.001$) respectively in the whole cohort. A sub-analysis of triple negative and ER-negative breast cancers also showed that high NR4A1 nuclear protein expression was associated with both a shorter disease-free survival (by 1452 days, $p=0.002$ and 1141 days, $p=0.006$ respectively) and disease-specific survival (by 1603 days, $p=0.001$ and 1187 days, $p=0.001$ respectively). In contrast, analyses of the molecular taxonomy of breast cancer international consortium dataset demonstrated no significant influence of NR4A1 mRNA expression on disease-free survival ($p=0.978$) or disease-specific survival ($p=0.288$) in the whole cohort, or in the different molecular subgroups, or depending on use of chemotherapy.

In conclusion, I have demonstrated that each of the four biomarkers IFNβ1, MX1, ITGA7 and NR4A1 are potential clinical markers of chemo-response. However, further work is required to convert these findings into better patient outcomes through validation in additional clinical cohorts and development of stratification strategies or therapeutic targeting based on these proteins.

LIST OF PUBLICATIONS

Gwili N, Jones SJ, Amri WA, Carr IM, Harris S, Hogan BV, Hughes WE, Kim B, Langlands FE, Millican-Slater RA, Pramanik A, Thorne JL, Verghese ET, Wells G, Hamza M, Younis L, El Deeb NMF, Hughes TA. Transcriptome profiles of stem-like cells from primary breast cancers allow identification of ITGA7 as a predictive marker of chemotherapy response. *Br J Cancer*. 2021 Sep;125(7):983-993

Broad RV, Jones SJ, Teske MC, Wastall LM, Hanby AM, Thorne JL, Hughes TA. Inhibition of interferon-signalling halts cancer-associated fibroblast-dependent protection of breast cancer cells from chemotherapy. *Br J Cancer*. 2021 Mar;124(6):1110-1120

Al Amri WS, Allinson LM, Baxter DE, Bell SM, Hanby AM, Jones SJ, Shaaban AM, Stead LF, Verghese ET, Hughes TA. Genomic and Expression Analyses Define MUC17 and PCNX1 as Predictors of Chemotherapy Response in Breast Cancer. *Mol Cancer Ther*. 2020 Mar;19(3):945-955

LIST OF ABBREVIATIONS

ABC ATP- Binding Cassette

ABCB1 ATP- Binding Cassette Sub-Family B Member 1

ABCC1 ATP-Binding Cassette Sub-Family C Member 1

ABCG2 ATP-Binding Cassette Super-Family G Member 2

ACOSOG American College of Surgeons Oncology Group

AIs Aromatase Inhibitors

ANC Axillary Nodal Clearance

APC Adenomatous Polyposis Coli

ATAC Arimidex, Tamoxifen Alone or in Combination

ATP Adenosine Triphosphate

BCRP Breast Cancer Resistance Protein

BCS Breast Conserving Surgery

BCSCs Breast Cancer Stem Cells

Bcl-XL B-Cell Lymphoma Extra Large

Bcl-w Bcl-Like Protein 2

Bcl-2 B-Cell Lymphoma 2

BRCA 1 Breast Cancer 1

BRCA 2 Breast Cancer 2

CAFs Cancer Associated Fibroblasts

CCLE Cancer Cell Line Encyclopaedia

CDKs Cyclin-Dependent Kinases

CD24 Cluster of Differentiation 24

cDNA Complementary Deoxyribonucleic Acid

cfDNA Circulating Free DNA

CI Confidence Interval

CK18 Cytokeratin 18

CK8 Cytokeratin 8

CRC Colorectal Cancer

CSC Cancer Stem Cells

CTLs Cytotoxic T Cells

CTNNB1 Catenin Beta 1

Cyclin D-CDK4/6-Rb Cyclin D-CDK4/6-Retinoblastoma

CYPs Cytochrome P450

DBD DNA Binding Domain

DCIS Ductal Carcinoma in Situ

DFS Disease-Free Survival

DIM Diindoylmethane Derivatives

DNA Deoxyribonucleic Acid

DNA-PK Deoxyribonucleic Acid -Dependent Protein Kinase

DSS Disease-Specific Survival

EBCTCG Early Breast Cancer Trialists' Collaborative Group

ECM Extra Cellular Membrane

EGF Epidermal Growth Factor

EGFR Epidermal Growth Factor Receptor

EMR Electronic Medical Records

EMT Epithelial to Mesenchymal Transition

EMT-TF Epithelial to Mesenchymal Transcription Factor

ER Oestrogen Receptor

ERCC1 Excision Repair Cross Complementation Group 1

FAK Focal Adhesion Kinase

FBXW7 F-Box and WD Repeat Domain Containing 7

FDA Food and Drug Administration

FdUMP Fluorodeoxyuridine Monophosphate

FFPE Formalin-Fixed Paraffin-Embedded

FGF Fibroblast Growth Factor

FUTP Fluorouridine Triphosphate

5-FU 5-Flurouracil

GNAS Guanine Nucleotide Binding Protein

GST Glutathione-S-Transferase

GTP Guanosine Triphosphate

HAS2 Hyaluronan Synthase 2

HERA Herceptin Adjuvant Trial

HER2 Human Epidermal Growth Factor Receptor 2

HGF Hepatocyte Growth Factor

HR Hazard Ratio

IAPs Inhibitor of Apoptosis Proteins

IDC Invasive Ductal Carcinoma

IDC-NST Invasive Ductal Carcinoma-No Special Type

IDH1 Isocitrate Dehydrogenase 1

IFNs Interferons

IFNβ Interferon Beta

IFNAR1 Type 1 Interferon Receptors

IHC Immuno-Histo-Chemistry

IL Interleukin

ILC Invasive Lobular Carcinoma

IRFs Interferon Regulatory Factors

ISGs Interferon Stimulated Genes

ITGA7 Integrin Subunit Alpha 7

JAK Janus Kinase

KM Kaplan Meier

LBD Ligand Binding Domain

LCIS Lobular Carcinoma in-Situ

LTHT Leeds Teaching Hospital Trust

MAC Mitochondrial Apoptosis-Induced Channel

MAPK Mitogen-Activated Protein Kinase

MBC Metastatic Breast Cancer

MCF-7 Michigan Cancer Foundation-7

MDR Multidrug Resistance

MDSCs Myeloid Derived Suppressor Cells

METABRIC Molecular Taxonomy of Breast Cancer International Consortium

MGMT O6-Methylguanine DNA Methyltransferase

MHC Major Histocompatibility Complex

MMPs Matrix Metalloproteinases

MMR Mismatch Repair

MRI Magnetic Resonance Imaging

mRNA messenger Ribonucleic Acid

miRNA micro Ribonucleic Acid

MRP Multidrug Resistance-associated Protein

MX1 Myxovirus Resistance 1

NACT Neoadjuvant Chemotherapy

NAET Neoadjuvant Endocrine Therapy

NBDs Nucleotide-Binding Domains

NER Nucleotide Excision Repair

NF-KB Nuclear Factor Kappa B

NHEJ Non-Homologous End Joining

NHSBSP National Health Service Breast Screening Programme

NICE National Institute for Health and Care Excellence

NPI Nottingham Prognostic Index

NR4A1 Nuclear Receptor 4A1

NSABP National Surgical Adjuvant Breast and Bowel Project

NST No Special Type

OCM Oncostatin M

ORR Objective Response Rate

OS Overall Survival

OSCC Oesophageal Squamous Cell Carcinoma

PAK-1 P21 Activated Kinase 1

PARP Poly (ADP-ribose) Polymerase

pCR Complete Pathological Response

PD-1 Programmed Death Protein 1

PD-L1 Programmed Death Ligand 1

PFS Progression Free Survival

PGE2 Prostaglandin E2

P-gp P-Glycoprotein

PI3K Phosphatidylinositol 3-Kinase

PIK3CA Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PKA Protein Kinase

PPARg Peroxisome Proliferator- Activated Receptor Gamma

PPM Patient Pathway Manager

PR Progesterone Receptor

PTEN Phosphatase and Tensin Homolog

Rb Retinoblastoma

RCTs Randomised Controlled Trials

RNA Ribonucleic Acid

ROC Receiver Operating Characteristic

SDF-1 Stromal Cell-Derived Factor 1

SiRNA Small/Short Interference-Ribonucleic Acid

SLN Sentinel Lymph Nodes

SLNB Sentinel Lymph Node Biopsy

SMAD4 Mothers Against Decapentaplegic Homolog 4

SNV Single Nucleotide Variants

SSPS Statistical Package for the Social Sciences

TAD N-Terminal Transactivation Domain

TAMs Tumour Associated Macrophages

TANs Tumour Associated Neutrophils

TBCRC Translational Breast Cancer Research Consortium

TBS Tris-Buffered Saline

TBS-T Tris-Buffered Saline-TWEEN20

TCGA The Cancer Genome Atlas

TDLUs Terminal Ductal Lobular Units

TGFβ Transforming Growth Factor Beta

TIMP3 Tissue Inhibitor of Metalloproteinase 3

TKI Tyrosine Kinase Inhibitor

TM Tumour Microenvironment

TMA Tissue MicroArray

TMDs Transmembrane Domains

TNBC Triple Negative Breast Cancer

TNF α Tumour Necrosis Factor- α

TNM Tumour Node Metastasis

TP53 Tumour Protein 53

Treg Regulatory T Cells

TRP Transient Receptor Potential

UK United Kingdom

USA United States of America

USS Ultrasound Scan

VEGF Vascular Endothelial Growth Factor

WES Whole Exome Sequencing

WLE Wide Local Excision

TABLE OF CONTENTS

Acknowledgements ii

Abstract iii

List of Publications *v*

List of Abbreviations *vi*

Table of Contents *xii*

List of Tables *xviii*

List of Figures *xxi*

1. INTRODUCTION **1**

1.1 Breast cancer incidence, mortality and survival **1**

1.2 Clinical presentation and diagnosis of breast cancer **1**

1.3 Classification of breast cancer **1**

1.3.1 Histopathological classification 2

1.3.1.1 Carcinoma in-situ 2

1.3.1.2 Invasive breast cancer 3

1.3.2 Histological grade and tumour node metastasis classification 3

1.3.3 Molecular classification 5

1.3.3.1 Further molecular classifications 7

1.3.3.1.1 Claudin low 7

1.4 Overview of breast cancer treatment **11**

1.4.1 Surgical management 12

1.4.1.1 Breast 12

1.4.1.2 Axilla 12

1.4.2 Radiotherapy 13

1.5 Systemic therapies **13**

1.5.1 Adjuvant cytotoxic chemotherapy 13

1.5.1.1 Anthracyclines 14

1.5.1.2 Taxanes 15

1.5.1.3 Alkylating agents 15

1.5.1.4 Anti-metabolites 16

1.5.1.5 Platinum 16

1.5.1.6 Vinca alkaloids 17

1.5.2 Human epidermal growth factor receptor 2 therapy 17

1.5.3 Tyrosine kinase inhibitor 18
1.5.4 Endocrine therapy 18
1.5.5 Neoadjuvant therapies 19
1.5.6 Recent treatment advances 20
1.5.6.1 PARP inhibitors 20
1.5.6.2 PD-L1 inhibitors 21
1.5.6.3 CDK4/6 inhibitors 21
1.6 Mechanisms of cytotoxic chemotherapy resistance in breast cancer 23
1.6.1 Increased efflux of chemotherapeutic agents 25
1.6.2 Modification of drug target 26
1.6.3 Increased repair of DNA damage 26
1.6.4 Impaired apoptosis 27
1.6.5 Decreased drug activation and increased drug inactivation 28
1.6.6 Epigenetic modifications 29
1.6.7 MicroRNAs 29
1.6.8 Epithelial to mesenchymal transition 30
1.6.9 Tumour heterogeneity 31
1.6.9.1 Cancer stem cells are chemo-resistant 31
1.6.9.2 Clonal evolution model: chemotherapy can select for resistance 31
1.7 Tumour microenvironment and its role in chemoresponse 32
1.7.1 Extracellular matrix 34
1.7.2 Tumour associated macrophages 34
1.7.3 Tumour associated neutrophils 34
1.7.4 Myeloid derived suppressor cells 35
1.7.5 Cancer associated fibroblasts 35
1.8 Preliminary work forming the basis of this project 36
1.8.1 Identification of candidate mediators of chemoresistance through therapy-driven selection of somatic variants 36
1.8.2 Transcriptome profiling of stem-like cells from primary breast cancers 39
1.8.3 CAF-induced chemoprotection of breast cancer cells 40
1.9 Selection of candidates for further study in this thesis 41
1.9.1 IFNβ1 and MX1 41
1.9.2 ITGA7 42
1.9.3 NR4A1 43
1.10 Hypothesis and aims 45
2. *MATERIALS AND METHODS* 46
2.1 Ethical approval 46

2.2 Selection of cohort cases **46**
2.3 Tissue MicroArrays **48**
2.4 Selection of appropriate antibodies **49**
2.5 Optimisation of antibodies **50**
2.6 ImmunoHistoChemistry **50**
2.7 Scoring protocol **51**
2.8 cBioPortal for cancer genomics **53**
2.9 Statistical analysis **56**
3*. CONSTRUCTION OF A SINGLE-CENTRE RETROSPECTIVE COHORT OF BREAST CANCER PATIENTS TREATED WITH ADJUVANT CYTOTOXIC CHEMOTHERAPY* **57**
3.1 Abstract **57**
3.2 Introduction **59**
3.3 Results **60**
3.3.1 Construction of a cohort of patients with primary breast cancer treated with adjuvant chemotherapy 60
3.3.2 Validation of tumour grade, lymph node status and molecular subtype as prognostic markers in a retrospective cohort of patients diagnosed with breast cancer and treated with adjuvant chemotherapy 62
3.3.3 Comparison of patient demographics and tumour characteristics of triple negative and the other molecular subtypes of breast cancer 65
3.3.4 Breast cancers treated with adjuvant anthracyclines and taxanes have poorer survival compared to those without taxanes 66
3.3.5 Construction of Tissue MicroArrays representative of 305 patients diagnosed with breast cancer and treated with adjuvant chemotherapy 67
3.4 Discussion **68**
3.5 Conclusion **70**
4. *PARACRINE IFN SIGNALLING BETWEEN CANCER ASSOCIATED FIBROBLASTS* AND CANCER CELLS CORRELATES WITH POOR SURVIVAL AFTER *CHEMOTHERAPY IN TRIPLE NEGATIVE BREAST CANCER PATIENTS* **71**
4.1 Abstract **71**
4.2 Introduction **73**
4.3 Results **74**
4.3.1 Optimisation and analysis of IFNβ1 and MX1 expression in breast cancer 74
4.3.2 Analysis of expression of IFNβ1 and MX1 in breast cancer patients of all 77

molecular subtypes treated with adjuvant chemotherapy (Cohort 1)
4.3.2.1 IFNβ1 and MX1 proteins are expressed at a range of levels across invasive breast cancers 78
4.3.2.2 Expression of IFNβ1 in fibroblasts correlates with MX1 expression in cohort containing all molecular subtypes of breast cancer 79
4.3.2.3 Protein expression of MX1 correlated with grade of tumour in the whole cohort and all breast cancer subtypes, ER-positive, ER-negative, HER2-positive and triple negative 79
4.3.2.4 Kaplan-Meier survival analysis revealed no significant differences in survival relating to high or low IFNβ1 or MX1 protein expression in the whole cohort (cohort 1) 81
4.3.3 Analysis of the relevance of IFNβ1 and MX1 expression in a triple negative cohort treated with adjuvant chemotherapy 83
4.3.3.1 IFNβ1 and MX1 proteins are expressed at a wide range of levels across triple negative invasive breast cancers 85
4.3.3.2 High expression of IFNβ1 in fibroblasts was weakly, but significantly, positively associated with high MX1 expression in the tumour cells in the triple negative cohort 86
4.3.3.3 High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer disease-free survival 86
4.3.3.4 Correlation between fibroblast IFNβ1 and tumour cell MX1 was strengthened in the claudin-low group 88
4.3.3.5 IFNβ1 fibroblast expression and lymph node status were statistically significant independent predictors of disease-free survival in the claudin-low group 88
4.3.3.6 High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer disease-free survival in the claudin-low subgroup of triple negative breast cancers 89
4.4 Discussion **94**
4.4.1 IFNβ1 and MX1 as prognostic markers in Claudin-3 low subtype of triple negative breast cancers 94
4.5 Conclusion **97**
5. *EXPRESSION OF ITGA7 PREDICTS SURVIVAL FROM BREAST CANCER FOLLOWING ADJUVANT CHEMOTHERAPY* **98**
5.1 Abstract **98**
5.2 Introduction **100**
5.3 Results **102**

5.3.1 Optimisation and analysis of ITGA7 expression in breast cancer 102
5.3.2 Analysis of ITGA7 expression in breast cancer patients of all tumour subtypes treated with adjuvant chemotherapy 104
5.3.2.1 ITGA7 proteins are expressed at a wide range of levels across invasive breast cancers 105
5.3.2.2 Protein expression of nuclear ITGA7 weakly correlated with tumour grade in breast cancer cases treated with adjuvant chemotherapy 106
5.3.2.3 Kaplan-Meier survival analysis revealed high ITGA7 protein expression is associated with improved survival after chemotherapy in breast cancer 106
5.3.2.4 High ITGA7 nuclear protein expression is associated with longer disease-free survival and disease-specific survival in ER-positive breast cancers 109
5.4 Kaplan-Meier survival analysis revealed a significant increase in disease-free survival in primary breast cancers that had high ITGA7 nuclear protein expression and that received anthracycline-based adjuvant chemotherapy 112
5.5 Discussion 115
5.5.1 ITGA7 as a prognostic marker in breast cancer following adjuvant chemotherapy 115
5.6 Conclusion 118
6. *HIGH NUCLEAR NR4A1 EXPRESSION IS ASSOCIATED WITH POOR PROGNOSIS IN BREAST CANCER PATIENTS* 119
6.1 Abstract 119
6.2 Introduction 121
6.3 Results 122
6.3.1 Optimisation and analysis of NR4A1 expression in breast cancer 122
6.3.2 Analysis of expression of NR4A1 in breast cancer patients treated with adjuvant chemotherapy 124
6.3.2.1 NR4A1 protein is expressed at a range of levels across invasive breast cancers 125
6.3.2.2 Protein expression of NR4A1 did not correlate with any clinical prognostic markers in breast cancer cases that received adjuvant chemotherapy 126
6.3.2.3 High NR4A1 nuclear protein expression is associated with poorer survival after chemotherapy in breast cancer 126
6.3.2.4 High NR4A1 nuclear protein expression is associated with poorer 129

disease-free survival and disease-specific survival in triple negative and ER-negative breast cancer subtypes
6.3.3 NR4A1 mRNA levels correlate very weakly with prognostic factors, and not with outcomes in the METABRIC dataset 132
6.3.3.1 NR4A1 mRNA levels do not correlate with outcomes in the METABRIC datasets in individual subgroup analyses 135
6.3.3.2 NR4A1 mRNA levels do not correlate with outcomes after chemotherapy in the METABRIC dataset 136
6.4 Discussion 140
6.4.1 NR4A1 as a marker of prognosis in breast cancer following adjuvant cytotoxic chemotherapy 140
6.4.2 Is NR4A1 a potential therapeutic target? 142
6.5 Conclusion 145
7. *DISCUSSION* 146
7.1 Prognostic and predictive markers in breast cancer 146
7.2 Potential drug targets 149
7.3 Impact of COVID19 151
7.4 Conclusion 151
8. *REFERENCES* 152
9. *APPENDICES* 166

LIST OF TABLES

Table 1.1: TNM staging for breast cancer 4

Table 1.2: Molecular subtype classification of breast cancers 5

Table 1.3: Candidate genes 37

Table 1.4: The most up - or down-regulated genes in breast cancer stem cells compared to matched non-breast cancer stem cells within the tumour 40

Table 2.1: Summary of the clinico-pathological and pharmacological features for a cohort of adjuvant chemotherapy treated breast cancers 47

Table 2.2: Summary of the clinico-pathological and pharmacological features for a triple negative cohort of breast cancers 48

Table 2.3: Antibodies selected for immunohistochemistry 50

Table 3.1: Comparison of mean disease-free survival and disease-specific survival between tumour grades in breast cancer 62

Table 3.2: Comparison of mean disease-free survival and disease-specific survival between negative and positive lymph node status in breast cancer 63

Table 3.3: Comparison of mean disease-free survival and disease-specific survival between N0, N1, N2 and N3 lymph node status in breast cancer 64

Table 3.4: Comparison of mean disease-free survival and disease-specific survival between ER-positive, Triple negative and HER2-positive molecular subtypes of breast cancer 64

Table 3.5: Comparison of mean disease-free survival and disease-specific survival of breast cancers that received anthracycline without taxane based adjuvant chemotherapy compared with anthracycline with taxane based adjuvant chemotherapy 66

Table 4.1: Core to core correlation of primary breast cancer cores for IFNβ1 and MX1 using Spearman's rho correlation 77

Table 4.2: Correlation of IFNβ1 in fibroblasts with MX1 expression in cancer cells 79

Table 4.3: MX1 cancer cell cytoplasm expression correlates with tumour grade in whole cohort and each breast cancer subtype 80

Table 4.4: Comparison of mean disease-free survival and disease-specific survival between low and high protein expression of IFNβ1 and MX1 81

Table 4.5: Comparison of mean disease-free survival and disease-specific survival between low and high expression of IFNβ1 in fibroblasts and MX1 cancer cell cytoplasm in all subtypes of breast cancer 83

Table 4.6: Comparison of mean disease-free survival and disease-specific survival between low and high expression of IFNβ1 and MX1 in triple negative breast cancers 86

Table 4.7: Multivariate analysis to determine whether IFNβ1 in fibroblasts and MX1 in tumour cells offered prognostic information that was independent of tumour grade and lymph node status 89

Table 4.8: Comparison of mean disease-free survival and disease-specific survival between low and high expression of IFNβ1 and MX1 in claudin low breast cancers 90

Table 4.9: Comparison of mean disease-free survival and disease-specific survival between low and high expression of IFNβ1 fibroblasts and MX1 tumour cell in triple negative claudin high breast cancers 92

Table 5.1: Core to core correlation of primary breast cancer cores for ITGA7 using Spearman's rho correlation 104

Table 5.2: ITGA7 nuclear expression correlates with tumour grade in whole cohort 106

Table 5.3: Comparison of mean disease-free survival and disease-specific survival between low and high expression of ITGA7 107

Table 5.4: Comparison of mean disease-free survival and disease-specific survival between low and high expression of ITGA7 in ER-positive breast cancers 109

Table 5.5: Comparison of mean disease-free survival and disease-specific survival between low and high expression of ITGA7 in HER2-positive, triple negative and ER-negative breast cancers 111

Table 5.6: Comparison of mean disease-free survival and disease-specific survival between low and high expression of ITGA7 in whole cohort of breast cancers that received anthracycline based adjuvant chemotherapy 112

Table 5.7: Comparison of mean disease-free survival and disease-specific survival between low and high expression of ITGA7 in whole cohort of breast cancers that received taxane based adjuvant chemotherapy 114

Table 6.1: Core to core correlation of primary breast cancer cores for NR4A1 using Spearman's rho correlation 124

Table 6.2: NR4A1 expression did not correlate with any clinical prognostic markers 126

Table 6.3: Comparison of mean disease-free survival and disease-specific survival between low and high protein expression of NR4A1 127

Table 6.4: Comparison of mean disease-free survival and disease-specific survival between low and high expression of NR4A1 in triple negative, ER-negative, ER-positive and HER2-positive breast cancers 130

Table 6.5: Summary of the clinico-pathological and pharmacological features for breast cancers within the METABRIC dataset 133

Table 6.6: NR4A1 expression correlated with the clinical prognostic markers; lymph node status, oestrogen expression and triple negative subgroup 134

Table 6.7: Comparison of mean disease-free survival and disease-specific survival between low and high protein expression of NR4A1 134

Table 6.8: Comparison of mean disease-free survival and disease-specific survival between low and high expression of NR4A1 in triple negative, ER-negative, ER-positive and HER2-positive breast cancers 136

Table 6.9: Comparison of mean disease-free survival and disease-specific survival between low and high expression of NR4A1 in breast cancers within the METABRIC dataset that received and did not receive chemotherapy 137

Table 6.10: Comparison of mean disease-free survival and disease-specific survival between low and high expression of NR4A1 in triple negative, ER-negative, ER-positive and HER2-positive subtypes of breast cancers within the METABRIC dataset that received chemotherapy 139

LIST OF FIGURES

Figure 1.1: Three proposed models of claudin-low as a breast cancer phenotype 9

Figure 1.2: A diagram to show the mechanisms of chemotherapy resistance in breast cancer 24

Figure 1.3: Constituents of the tumour microenvironment that affect chemotherapy resistance in breast cancer 33

Figure 2.1: Representative image of the web page from cbioportal 53

Figure 2.2: Representative image of dataset selected 53

Figure 2.3: Representative image of how to customise the layout and selection of the parameters of interest within the dataset 54

Figure 2.4: Representative image of how to select gene of interest 55

Figure 2.5: Representative image of how to download the clinical data 56

Figure 3.1: A flow diagram outlining case selection 61

Figure 3.2: Kaplan-Meier survival analysis of tumour grade in patients with breast cancer treated with adjuvant chemotherapy 63

Figure 3.3: Kaplan-Meier survival analysis of lymph node status in patients with breast cancer treated with adjuvant chemotherapy 63

Figure 3.4: Kaplan-Meier survival analysis of N0, N1, N2 and N3 lymph node status in patients with breast cancer treated with adjuvant chemotherapy 64

Figure 3.5: Kaplan-Meier survival analysis of ER-positive, Triple negative and HER2-positive molecular subtypes of breast cancer treated with adjuvant chemotherapy 65

Figure 3.6: Kaplan-Meier survival analysis of breast cancers that received anthracycline without taxane based adjuvant chemotherapy compared with anthracycline with taxane based adjuvant chemotherapy 66

Figure 3.7: Representative image of a tissue microarray 67

Figure 4.1: Representative images of immunohistochemical staining for IFNβ1 in breast cancers, demonstrating the scoring protocol 75

Figure 4.2: Representative images of immunohistochemical staining for MX1 in breast cancers, demonstrating the scoring protocol 76

Figure 4.3: Score distributions for IFNβ1 in breast cancer patients treated with adjuvant chemotherapy 78

Figure 4.4: Score distributions for MX1 in breast cancer patients treated with adjuvant chemotherapy 78

Figure 4.5: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression 82

Figure 4.6: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression 82

Figure 4.7: Representative images of immunohistochemical staining for Claudin-3 in triple negative breast cancers, demonstrating the scoring protocol 84

Figure 4.8: Score distributions for IFNβ1 in triple negative breast cancer patients treated with adjuvant chemotherapy 85

Figure 4.9: Score distributions for MX1 in triple negative breast cancer patients treated with adjuvant chemotherapy 85

Figure 4.10: Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with triple negative breast cancer 87

Figure 4.11: Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with triple negative breast cancer 87

Figure 4.12: Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with triple negative breast cancer 91

Figure 4.13: Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with triple negative breast cancer 91

Figure 4.14: Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with triple negative breast cancer 93

Figure 4.15: Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with triple negative breast cancer 93

Figure 5.1: Representative images of immunohistochemical staining for ITGA7 in breast cancers, demonstrating the scoring protocol 103

Figure 5.2: Score distributions for ITGA7 in breast cancer patients treated with adjuvant chemotherapy 105

Figure 5.3: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression 108

Figure 5.4: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear ITGA7 expression 108

Figure 5.5: Kaplan-Meier survival analysis for ER-positive breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression 110

Figure 5.6: Kaplan-Meier survival analysis for ER-positive breast cancer outcomes in groups with high versus low nuclear ITGA7 expression 110

Figure 5.7: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression in patients that received anthracycline adjuvant chemotherapy 113

Figure 5.8: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear ITGA7 expression in patients that received anthracycline adjuvant chemotherapy 113

Figure 5.9 A and B: The ITGA7 antibody used in this study is specific for ITGA7 116

Figure 6.1: Representative images of immunohistochemical staining for NR4A1 in breast cancers, demonstrating the scoring protocol 123

Figure 6.2: Score distributions for NR4A1 in breast cancer patients treated with adjuvant chemotherapy 125

Figure 6.3: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic NR4A1 expression 128

Figure 6.4: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear NR4A1 expression 128

Figure 6.5: Kaplan-Meier survival analysis for triple negative breast cancer outcomes in groups with high versus low nuclear NR4A1 expression 131

Figure 6.6: Kaplan-Meier survival analysis for ER-negative breast cancer outcomes in groups with high versus low nuclear NR4A1 expression 131

Figure 6.7: Kaplan-Meier survival analysis for ER-positive breast cancer outcomes in groups with high versus low nuclear NR4A1 expression 132

Figure 6.8: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset 135

Figure 6.9: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset 138

Figure 6.10: Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression in patients that did not receive chemotherapy within the METABRIC dataset 138

Figure 6.11: NR4A1 receptors play a key role in many cellular functions that lead to breast cancer 142

1. INTRODUCTION

1.1. Breast cancer incidence, mortality and survival

Breast cancer is the commonest cancer to affect women in the United Kingdom (UK) [1] and worldwide [2], with approximately 54,700 women diagnosed annually in the UK [1]. With around 11,400 deaths from breast cancer in 2017, the overall survival (OS) of women with breast cancer in the UK has continued to increase. This can be demonstrated by both 5- and 10-year survival rates. The 5-year survival rates have increased from 80% in 2000-2001 to 87% in 2010-2011, whereas 10-year survival rates have increased from 71% to 78% respectively in England and Wales [1]. The overall decrease in mortality from breast cancer is likely due to a combination of factors, such as improvements in surgery and advancements in treatment, such as the use of taxanes as cytotoxic agents in systemic therapy and the development of hormonal and biological therapies [3]. Regardless of advancement in breast cancer management and treatment, around a third of women with early breast cancer will ultimately develop resistance to treatment and will progress to metastatic breast cancer, for which there are many treatment choices but, long term success is limited [4].

1.2. Clinical presentation and diagnosis of breast cancer

Symptomatic breast cancer classically presents as a painless, palpable lump detected by patients or their clinicians. However, 1 in 6 women present with a spectrum of symptoms such as nipple, breast skin and contour abnormalities. Women with asymptomatic early breast carcinomas tend to be identified via the national health service breast screening programme (NHSBSP) [5]. The final diagnosis of breast cancer is confirmed by a pathological assessment of core biopsies obtained from the breast lump [6]. The breast cancer histology, particularly the molecular markers such as ER, PR and HER2 help guide treatments and determine prognosis.

1.3. Classification of breast cancer

The classification of breast cancer is by histopathological or molecular subtyping, and the staging by the Tumour Node Metastasis (TNM) classification system.

1.3.1. Histopathological classification

There are two main histopathological types of breast carcinoma: In-situ cancer and invasive cancer [7].

1.3.1.1. Carcinoma in-situ

Ductal carcinoma in-situ (DCIS) comprises up to 25% of all breast cancers [8] and is a neoplastic proliferation of epithelial cells with atypical cellular and nuclear features [7] that are limited to the basement membrane of the ducts within the breast but, have malignant potential if they were to invade the breast stroma [9]. With DCIS being deemed a precursor for the development of invasive breast cancer, there has been great emphasis on the detection of these lesions [8] of which there are 5 main subtypes: comedo, solid, cribriform, papillary and micropapillary [7] and 3 grades low, intermediate and high via NHSBSP. Women with DCIS have an excellent prognosis [10], with the current standard treatment in the UK being surgery in the form of a mastectomy or breast conserving surgery (BCS) followed by breast radiotherapy in certain cases [11]. There is concern for over treatment in these women and this can be due to the difficulty in deciphering non-invasive DCIS from potentially invasive lesions. Many DCIS lesions may be indolent and low grade DCIS is thought unlikely to progress to invasive disease [8]. The LORIS trial, which is a multi-centre, randomised controlled study whereby patients with low risk DCIS are randomised to active monitoring alone with annual mammography for 10 years or surgery will hopefully address whether low risk DCIS is currently being overtreated [12].

Lobular carcinoma in-situ (LCIS) is the abnormal proliferation of cells within the terminal ductal lobular units (TDLUs) that demonstrate a loss of E-cadherin and is frequently an incidental finding in breast tissue specimens and signifies a risk factor but not necessarily a precursor for the development of invasive breast cancer [13]. A study by King et al of 1032 women with LCIS who received annual surveillance in the form of clinical examination and mammography +/- chemoprevention with a median follow-up of 81 months found the breast cancer incidence rate to be 2% per year. Of the newly diagnosed breast cancers, 65% were invasive and 35% were DCIS [14]. Therefore, the current guidelines on the management of classic LCIS in the UK state that surgical excision is not mandated, and that annual mammographic screening for 5 years will suffice [11]. However, more recently morphologic variants of LCIS known as pleomorphic LCIS have been identified with hostile histopathologic features which are more likely to progress to invasive disease and therefore surgical excision is recommended [13].

1.3.1.2. Invasive breast cancer

Invasive ductal carcinoma (IDC) is the commonest type, accounting for approximately 80% of all invasive breast carcinomas. IDC is classified into several histological subtypes that include, tubular, cribriform, mucinous, medullary, papillary, micropapillary, apocrine and neuroendocrine carcinoma. Approximately 75% of IDCs do not display adequate morphological characteristics to be classified as a specific histological subtype and are therefore, grouped into “no special type” (NST) [7].

The second most common histopathological type is invasive lobular carcinoma (ILC), which comprises 5%–15% of invasive breast carcinomas and has five histological subtypes: classic type, pleomorphic lobular, histiocytoid, signet ring, and tubule-lobular carcinoma [7]. These morphology-based histological subtypes are clinically useful classifications since tumour behaviour and survival varies between them. A study by Liao et al who investigated the prognostic implications of 19,900 women with breast cancer based upon their histological subtype found that medullary and apocrine carcinomas have an excellent 3-year disease-specific survival (DSS) of 96.6% and 97.7% whereas, women with metaplastic or mixed lobular-ductal carcinoma have a worse DSS of 81.9% and 77.1% [15]. However, since a large proportion of breast cancers are classified as invasive ductal cancer of no special type (IDC-NST), the histological classification alone is inadequate to stratify the majority of breast cancers into subtypes with different clinical behaviours [16].

1.3.2. Histological grade and tumour node metastasis classification

As well as histopathological classification, breast cancer can be subdivided based upon grade of tumour, tumour size, lymph node status and presence of metastasis, which all effect breast cancer prognosis [17].

Tumour grade is assessed morphologically to determine degrees of differentiation of tubule formation, mitotic index and nuclear pleomorphism. Tumours are sub-classified into 3 grades, according to the modified Bloom-Richardson grading system; grade 1 (well-differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated), with higher grade tumours having a graver prognosis [18].

Histological grade, together with tumour size and lymph node status, forms part of the conventional prognostication tool, the Nottingham Prognostic Index (NPI), which aids in stratifying individuals for appropriate therapy [19], with higher NPIs indicative of more

aggressive treatment regimens that may include cytotoxic chemotherapy in addition to other therapies.

Breast cancers are also staged according to the TNM classification system, which categories breast cancers into stage 0-IV depending on the size of the primary tumour, nodal status (the presence of tumour cells in lymph nodes that drain the breast) and presence of metastatic disease (Table 1.1). Stage 0 is non-invasive cancer, such as ductal and lobular carcinoma in situ. Stage I-III is considered potentially curable, while stage IV is considered incurable [20].

T Stage (Tumour)			
	Tis	In-situ disease	
	T1	Tumour size 0-2cm, no skin fixation	
	T2	Tumour size >2-5cm, skin distortion	
	T3	Tumour size >5-10cm, skin ulceration over lump	
	T4	Tumour size >10cm, skin involved beyond lump	
N Stage (Nodes)			
	N0	Axillary lymph nodes negative for metastases	
	N1	1 - 3 metastatic axillary lymph nodes	
	N2	4 - 9 metastatic axillary lymph nodes	
	N3	≥10 metastatic axillary lymph nodes	
M Stage (Metastases)			
	M0	No distant metastatic disease	
	M1	Distant metastatic disease	
Overall Stage	**T**	**N**	**M**
Stage 0	Tis	N0	M0
Stage I	T1 T0	N0 N1	M0 M0
Stage IIA	T1 T2	N1 N0	M0 M0
Stage IIB	T2 T3	N1 N0	M0 M0
Stage IIIA	T0 T1 T2 T3	N2 N2 N2 N1/N2	M0 M0 M0 M0
Stage IIIB	T4	Any N	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1.1: TNM staging for breast cancer

Although TNM staging has traditionally been the main classification upon which decisions regarding the most suitable therapy have been made; over the last two decades as a consequence of inadequate prognostic power of the existing classifications, approaches to develop the molecular classification have been sought [16].

1.3.3. Molecular classification

Enhanced knowledge of breast cancer heterogeneity at the molecular level, allowed for the development of a molecular classification, clinically implemented by assessing expression levels of receptors for oestrogen (ER), progesterone (PR), and of the human epidermal growth factor receptor 2 (HER2) and proliferative marker Ki67 [7].

A study by Perou et al that assessed gene expression patterns of 39 breast tumours (36 IDC, 2 ILC and 1 DCIS) was one of the earlier studies to contribute to the molecular classification, as they identified 4 apparent subtypes that they referred to as basal-like, luminal-like/ER-positive, HER-2 overexpressing and normal-like [21]. They later published a further study that assessed the differences in gene expression profiles from 85 complementary deoxyribonucleic acid (cDNA) microarray experiments and correlated this with the characteristics and prognosis of 78 breast tumours (71 IDC, 5 ILC and 2 DCIS). The aforementioned subtypes were once again verified by hierarchical clustering. However, the previous luminal-like/ER-positive subtype was refined and split into Luminal A and Luminal B with their varying expression profiles [22].

Similar to Sorlie et al [22] The Cancer Genome Atlas (TCGA) Network also verified the four breast cancer subtypes i.e., luminal A, luminal B, basal-like and HER2-positive using a larger sample size of 348 primary breast tumours that were analysed by a range of array experiments [23].

These intrinsic molecular subtypes of breast cancer differ greatly in incidence, treatment sensitivity and OS [24], and are identified in the routine clinical setting by testing expression of four markers as shown in Table 1.2. Thus, ER, PR, HER2 and ki67 status together with tumour histopathology and grade are routinely used in clinical practice to envisage responses to newer targeted therapies, customise systemic therapies and aids in prediction of cancer progression and OS [16].

Molecular Subtype	**Receptor Status**			
	ER	**PR**	**HER2**	**Ki67**
Luminal A	Positive	Positive	Negative	Low
Luminal B	Positive	Positive	Positive/Negative	High
Triple Negative	Negative	Negative	Negative	N/A
HER2 +	Negative	Negative	Positive	N/A

Table 1.2: Molecular subtype classification of breast cancers [16]

Luminal A subtype make up 50% of invasive breast cancers and generally have an excellent prognosis. A study by Herr et al that analysed the response of endocrine therapy +/- chemotherapy on 1376 luminal A, breast cancer patients with axillary metastatic disease concluded a 5-year disease-free survival (DFS) of 91% and disease-specific survival (DSS) of 96.5% [25]. Luminal A subtype was also demonstrated to have a better prognosis than all other subtypes in a study by Metzger-Filho et al that assessed DFS and DSS according to subtype of 1,951 patients with node negative breast cancer with a median follow-up of 12.5 years. The 10-year DFS was longer amongst patients with Luminal A subtype (86%), followed by luminal B (76%), then HER2-positive (73%) and lastly the triple negative subtype (71%); $p<0.001$) of breast cancer. Similarly, the 10-year DSS was greater in the Luminal A subtype (89%), followed by Luminal B (83%), HER2-positive (77%) and lastly triple negative subtype (75%); $p<0.001$) of breast cancer [26].

The luminal B subtype comprises approximately 25% [27] of breast cancers. The HER2-positive subtype of breast cancers accounts for 15% of all invasive breast cancers and are often of high grade with lymph node metastasis. Therefore, they tend to be linked with poorer survival, [28] but are sensitive to HER2 targeted agents, such as trastuzumab and pertuzumab [29]. Triple-negative breast cancers (TNBCs) comprise up to 10% of all breast malignancies [30] and tend to be more aggressive tumours with women commonly presenting with large tumours of high grade with metastatic spread to axillary nodes [31] and therefore, have the shortest survival among the subtypes as they are non-responders to endocrine therapy or HER2 targeted therapy [32].

In 2009 the St Gallen Consensus incorporated the measurement of Ki67 a marker of tumour proliferation as a criterion to advise upon the addition of chemotherapy to endocrine treatment for individuals with ER-positive and HER2-negative breast cancer [33]. Later in 2013, they recommended the use of multigene assays namely Oncotype Dx for use in ER-positive, HER2-negative and node negative breast cancers and MammaPrint for use in both ER-positive and negative and node positive breast cancers; to determine the likelihood of an individual developing metastatic disease. Consequently, they can also be used as a tool in aiding treatment decisions in terms of risk versus benefit of chemotherapy; thereby sparing individuals of low risk of metastatic spread from the unpleasant side effects of chemotherapy [34].This is shown in both the TAILORx [35] and RxPONDER [36] trials. TAILORx is a prospective trial of approximately 10,000 women diagnosed with ER-positive, HER2-negative and node negative breast cancer with an Oncotype Dx recurrence score of 11-25. These women were randomly allocated to receive either endocrine therapy alone or in combination with chemotherapy. The findings were that endocrine therapy alone was non-inferior to endocrine therapy in addition to chemotherapy in terms of DFS, (HR 1.08; 95% CI, 0.94 to

1.24; P=0.26) [35]. Whereas, the RxPONDER, a prospective trial of 5083 women diagnosed with ER-positive, HER2-negative, node positive (1 to 3 lymph nodes) breast cancer with a Oncotype Dx recurrence score of 0 to 25 found no benefit in DFS (HR 1.02; 95% CI 0.82 to 1.26; P=0.89) upon the addition of chemotherapy to endocrine therapy in postmenopausal women. While pre-menopausal women significantly benefited from the addition of chemotherapy in terms of DFS at 5 years (89.0% vs 93.9%, HR 0.60; 95% CI 0.43 TO 0.83; P=0.002) [36].

1.3.3.1 Further molecular classifications

There is great importance on further sub-molecular classification of breast tumours in order to identify molecular targets to develop new and effective treatments [37].

1.3.3.1.1 Claudin low

The claudin-low molecular subtype of breast cancer is not currently fully established and there remains some controversy of its presence as a subtype. However, there are now an increasing number of studies highlighting both its molecular and clinical significance which will be discussed.

The breast cancer subtype claudin-low was first established in 2007 by gene expression profiling that compared 232 human breast tissue samples with 108 mammary tumours in mice [38]. They comprise up to 14% of all breast cancers and are found to be mainly invasive ductal, triple negative breast tumours associated with a poor DFS and DSS, although, only a small percentage of TNBCs are claudin-low [39]. Claudin-lows are distinguished from other molecular subtypes based upon their decreased expression of genes concerned with tight junctions, cell-cell adhesion, E-cadherin production and luminal epithelial differentiation such as cluster of differentiation 24 (CD24), cytokeratin 8 (CK8) and cytokeratin 18 (CK18). Conversely, they overexpress epithelial mesenchymal transition (EMT) genes and display characteristics of immune cell, cancer stem cells (CSCs) and stromal cell infiltration [39].

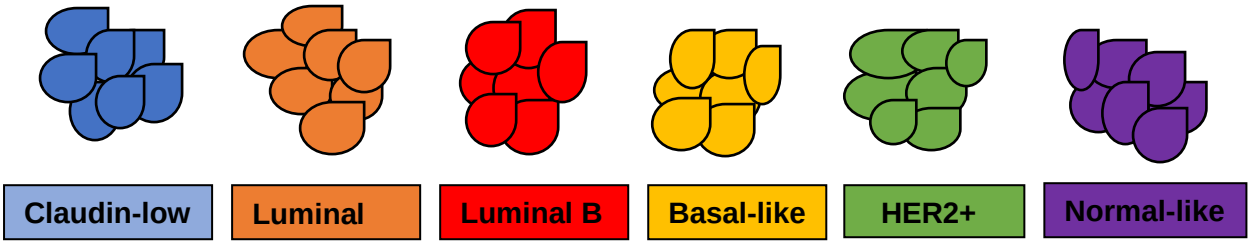
A study by Dias et al that performed immunohistochemistry (IHC) on Tissue MicrroArrays (TMAs) comprising of 942 breast tumours with a claudin-low incidence of 8.4%, concluded that both claudin-low and basal-like subtypes had the worse OS compared with the other molecular subtypes, albeit that this difference was not statistically significant (p=0.4) [39]. A further study by Sabatier et al that analysed expression in a larger data set of breast cancers using whole-genome DNA microarrays found that claudin-low tumours also had a decreased

expression of P53, signifying inhibition of apoptosis as well as displaying an increased activity of the epidermal growth factor receptor (EGFR), SRC and transforming growth factor beta (TGFβ) pathways. With regards to prognosis and response to chemotherapy, the claudin-low subtype is similar to both basal and HER2-positive breast cancers with a 5-year DFS of 67% [40]. Whereas, Prat et al found that claudin-low breast tumours were associated with mesenchymal and mammary stem cell-like characteristics and a shorter survival in comparison to the other intrinsic molecular breast cancer subtypes [41].

In 2011 Lehmann et al examined the gene expression profiles of 587 TNBCs and identified six subtypes: basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and a luminal androgen receptor. Of the six subtypes, the mesenchymal stem-like was found to express low levels of claudin-3, 4, and 7 in keeping with claudin-low tumours. Thereby, implying that this subtype is partly made up of the claudin-low tumours [37].

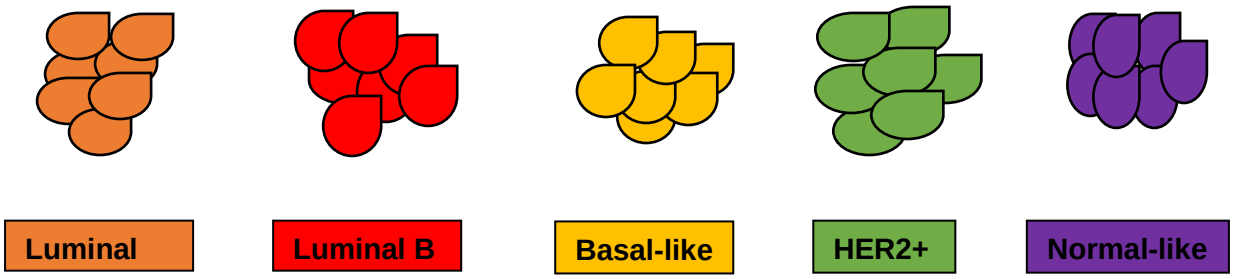
In comparison Fougner et al believed claudin-low to be an additional phenotype which may infiltrate the breast cancers belonging to each intrinsic molecular subtype. An alternative theory is that breast tumours exhibit a range of claudin-lowness from (1) non-claudin low, (2) moderately claudin-low, (3) extensively claudin-low or (4) purely claudin-low subtype (Figure 1.1) [42].

A. Established Model

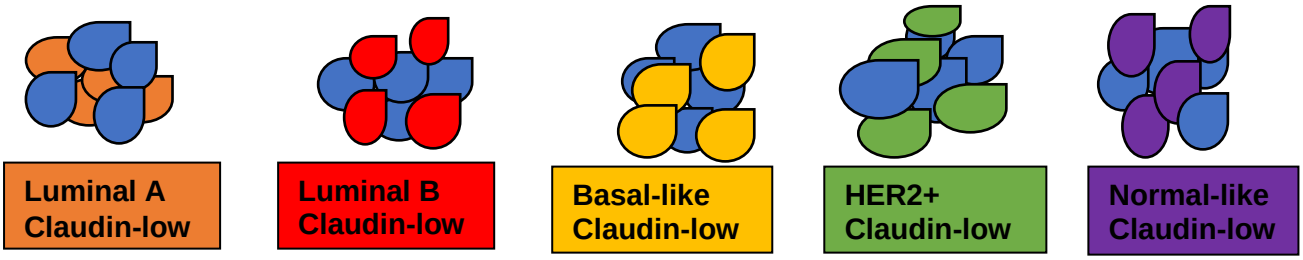


B. Binary Model

1. Non-claudin-low



2. Claudin-low



C. Continuous Model

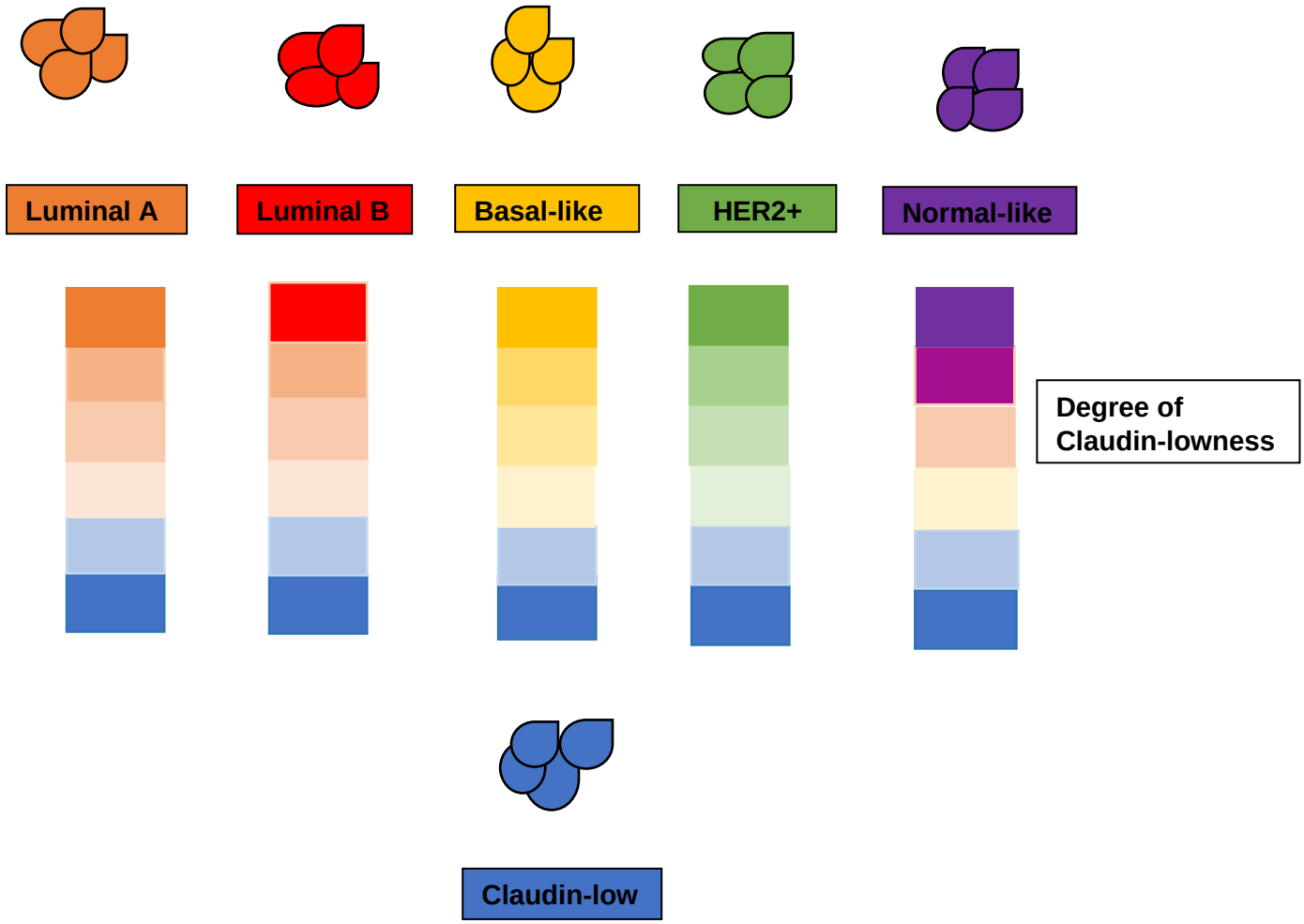


Figure 1.1. Three proposed models of claudin-low as a breast cancer phenotype

A. Established model: Claudin-low tumours are of their own unique subtype

B. Binary model: Breast tumours can possess both a claudin-low phenotype and their intrinsic subtype. Therefore, breast tumours are classified as claudin-low or non-claudin low

C. Continuous model: Claudin-low may be a continuous feature within the intrinsic subtype of breast tumours, with tumours displaying varying degrees of claudin-lowness

Adapted from [42]

At present there are a minimum of 24 known claudins with their own tissue specific expression which is commonly altered in cancer. The claudins 1, 3, 4, 7 and 8 are commonly expressed in invasive breast cancer and display a membranous staining pattern. As claudins have a high specificity of expression in cancer they may act as a valuable biomarker for potential therapies in overcoming chemoresistance [43].

Creighton et al demonstrated the association between breast cancer cells with low claudin expression and chemoresistance when residual breast tumours following treatment with either letrozole or docetaxel were enriched with a marker of breast cancer stem cells (BCSCs), (CD44+/CD24-/low) and claudin-low signatures. They also reported an elevated expression of vimentin, a mesenchymal marker. Therefore, the residual tumour may comprise of a subpopulation of cells with both CSC-like properties and mesenchymal characteristics. This therefore suggested that reduced claudins contribute to chemoresistance in cancer cells via EMT or CSCs [44].

Claudins comprise of 4 transmembrane domains and 2 extracellular loops which makes them potential targets for antibody-based therapies, with anti-claudin 3 and 4 antibodies currently in use and have shown to have encouraging anti-tumor properties both in vitro and in vivo [45,46, 47].

The aforementioned breast cancer classifications are highly effective in distinguishing individuals who will have a graver outcome.

1.4. Overview of breast cancer treatment

Treatment choices for primary breast cancer comprise surgery, radiotherapy, cytotoxic chemotherapy, endocrine therapy, and biological therapy. Patients generally undergo surgery to remove the cancer, followed by a combination of the aforementioned treatments depending upon the molecular subtype and stage of cancer. When these treatments are given following surgery they are known as adjuvant treatments. In some cases chemotherapy may be suggested before surgery to try to downstage the cancer and this is called neo-adjuvant chemotherapy (NACT). Both adjuvant and neo-adjuvant chemotherapy have been proven to decrease mortality from breast cancer by lowering the probability of recurrence by treating small deposits of metastatic disease that may not have been apparent at the time of surgery [48].

1.4.1. Surgical management

Surgical management of breast cancer is offered to women who do not have distant metastatic disease at presentation.

1.4.1.1 Breast

Treatment of the breast comprises of breast conserving surgery (BCS) in the form of a wide local excision (WLE) or a mastectomy. The intention of BCS is to remove the tumour with a 1mm margin of normal breast tissue [49] to reduce the chance of local recurrence. Generally, women are given the choice of BCS if the tumour volume to breast volume ratio is adequate in achieving clear margins and an acceptable cosmetic outcome [48].

1.4.1.2 Axilla

The management of the axilla has seen many changes over the years with a greater emphasis now being placed on more of a conservative surgical approach to the axilla. Prior to the sentinel lymph node biopsy (SLNB) in the 1990's [50], staging (detect any tumour cells in) the axillary nodes was by an axillary node clearance (ANC). With the SLNB becoming the gold standard procedure for staging the axilla in clinically and radiologically node-negative patients there has been a significant reduction in the associated arm and shoulder morbidity such as lymphoedema, pain and paraesthesia associated with an ANC [51]. Advancements in pre-operative axillary ultrasonography with guided fine needle aspiration cytology or core biopsy has also led to the stratification of individuals with axillary disease to proceed directly to ANC [52].

A randomised phase 3 trial (National Surgical Adjuvant Breast and Bowel Project (NSABP B-32)) which compared the survival outcomes of 5611 women with clinically node-negative breast cancers found that those randomised to a SLNB then an ANC if malignant nodes were found had an 8-year DFS estimate of 81.5% compared with 82.4% (p=0.54) in those that proceeded directly to an ANC. Estimated 8-year OS was also similar between groups; 90.3% and 91.8% respectively (p=0.12) [53].

The American College of Surgeons Oncology Group (ACOSOG) Z0011 trial [54] contested the apparent benefit of proceeding to an ANC on the finding of macro-metastasis on SLNB in women with clinically node-negative, T1-2 breast tumours. The trial involved 891 women who were found to have one or two metastatic nodes on SLNB, of which 446 women were randomised to no further axillary treatment and the remaining 445 women proceeded to an ANC. The study concluded that women with T1-2 breast tumours that are clinically node-

negative, but later found to have low axillary burden on SLNB could be adequately treated by avoiding an ANC as there is no statistically significant difference in DFS at 5-years in the SLNB only group (83.8%) compared with SLNB proceeding to ANC group (82.2%) (p=0.13) and 5-year OS of 92.5% and 91.9% respectively (p=0.24) [55].

1.4.2. Radiotherapy

Following BCS the majority of women are likely to receive radiotherapy to the breast to eliminate any residual tumour cells. A randomised prospective study spanning 8-years by the National Cancer Institute on 237 women with either Stage I or Stage II breast cancer with a follow-up of approximately 18-years, concluded that there was no detectable difference with regards to an estimated 20-year DFS of women managed with mastectomies (67%) in comparison to BCS and radiotherapy (63%) (p=0.64), with a DSS of 58% versus 54% (p=0.67) [56].

A phase III, multi-centre trial known as PRIMETIME is currently active and seeking recruitment of women aged 60 years and over with T1, grade 1 or 2, ER-positive, HER2-negative and node negative breast cancers suitable for BCS; to determine if radiotherapy can be safely omitted in these women with a potential low risk of local recurrence thereby, avoiding the associated risks [57] such as skin irritation, pulmonary fibrosis and angiosarcoma [58].

Adjuvant post mastectomy radiotherapy is offered to individuals who are believed to be at increased risk of loco-regional recurrence including patients with larger tumours and where there is a heavy burden of disease in the axillary nodes [59].

1.5. Systemic therapies

1.5.1. Adjuvant cytotoxic chemotherapy

Adjuvant cytotoxic chemotherapy is offered to patients with Luminal subtypes of breast cancer with an advanced stage due to large tumour size and metastatic axillary lymph nodes [60]. Individuals with HER2 over-expressing tumours will be offered chemotherapy in combination with HER2 targeted therapies such as trastuzumab [61]. Lastly, chemotherapy is usually recommended to individuals with triple negative tumours, as there is no response to endocrine or HER2-target therapy [62].

There are numerous chemotherapeutic agents for breast cancer in clinical use and patients are normally administered a combination of drugs, depending on their overall health and ability to tolerate the associated side effects. Common chemotherapy agents include anthracyclines, alkylating agents, taxanes, anti-metabolites and vinca alkaloids [63]. However, not all individuals diagnosed with breast cancer are given adjuvant cytotoxic chemotherapy, as a proportion of women will have excellent prognoses following surgery combined with radiotherapy and endocrine therapy and the addition of chemotherapy provides no added benefit [60]. Oncotype Dx and MammaPrint multiple gene assays as well as the online prognostic and treatment benefit tool known as PREDICT are useful in guiding these treatments [64].

1.5.1.1. Anthracyclines

Anthracyclines were first established as chemotherapeutic agents in the 1970s and epirubicin and doxorubicin are still routinely used as breast cancer chemotherapy [65]. Anthracyclines have multiple mechanism of actions to limit cancer growth. They can intercalate between deoxyribonucleic acid (DNA) base pairs leading to nucleic acid damage and ultimately interfering with the synthesis of DNA and ribonucleic acid (RNA) in highly proliferating cells thereby, inhibiting transcription and replication. They also inhibit topoisomerase II enzyme activity, which is normally responsible for making temporary double strand DNA breaks to allow for relaxation of supercoiled DNA to enable DNA synthesis. Anthracyclines form a DNA-topoisomerase II complex thereby, preventing the repair of double-strand DNA breaks which leads to growth arrest and programmed cell death. Lastly, they form free radicals destroying cell membranes [66].

A meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), which comprised of more than 100,000 individuals enrolled in clinical trials, evaluating the role of anthracycline as adjuvant chemotherapy in breast cancer concluded that anthracycline based chemotherapy in comparison to no chemotherapy lowered the risk of breast cancer recurrence over the next 10-years by 8% (47.4% vs. 39.4%), confidence interval (CI) 0.68-0.79 and lowered the overall mortality over the same time period by 5% (39.6% vs. 34.6%), CI 0.78-0.91 [67].

However, the main concerns associated with use of anthracyclines are cardiac toxicity and haematological malignancies [66]. Nonetheless, despite these concerns, to date no adjuvant chemotherapy regimen for treatment of breast cancer has been more effective than an anthracycline-taxane regimen as the Early Breast Cancer Trialists' Collaborative Group

(EBCTCG) meta-analysis also concluded that adding a taxane resulted in a further reduction in recurrence over the next 8-years of 4.6% (34.8% vs. 30.2%) and mortality rate of 3.2% (26.7 vs. 23.5%) [67]. Therefore, anthracyclines are commonly administered together with an alkylating agent and/or taxanes.

1.5.1.2. Taxanes

Taxanes, such as docetaxel and paclitaxel, are well known chemotherapeutic agents for treating breast cancer and interfere with microtubule stability [68]. Microtubules consist of αβ-tubulin and are one of the main constituents of the cytoskeleton that play a significant role in cell division and are therefore, important in the multiplication of cancer cells. Microtubules exhibit dynamic instability as they fluctuate between phases of lengthening and shortening by the addition or subtracting of subunits of tubulin, which are required during mitosis for successful cell division. The taxanes bind to the β-tubulin of the microtubule and suppress microtubules dynamic instability during the mitotic stage of the cell cycle, which leads to mitotic arrest and induces apoptosis in cells during division [69].

Peripheral neuropathy is the most common side effect associated with taxanes which can occur in up to 83% of patients taking paclitaxel and is the main reason for its discontinuation amongst patients [70]. This is surprising since neurones are non-dividing cells however, the mechanisms responsible for taxane induced neuropathy are multifactorial. These include impaired axonal transport due to microtubule disturbance and modification of the ion channels; Na+, K+ and transient receptor potential (TRP) resulting in overexcitability of peripheral neurons. Mitochondrial damage induced by taxanes also leads to the overproduction of reactive oxygen species which results in damage to proteins and lipids and interference of calcium homeostasis within neurons which results in the demyelination of peripheral nerves [71].

1.5.1.3. Alkylating agents

Cyclophosphamide is the most common alkylating agent used in the treatment of breast cancer. Its mechanism of action involves the introduction of an alkyl group at N7 position of guanine in DNA forming a strong covalent bond. This disturbs cross linkage of DNA strands and inhibits DNA replication as well as causing abnormal base pairing; with alkylated guanine

pairing with thymine rather than cytosine which gives rise to a defective protein resulting in apoptosis [72].

Administration of a cyclophosphamide together with an anthracycline and a taxane is favoured in the treatment of women who are at a high risk of recurrence. A randomised controlled trial (RCT) by Mackey et al compared sequential and concurrent combinations of doxorubicin and cyclophosphamide with docetaxel chemotherapy in 3298 women with metastatic axillary node breast cancer. Of the women that received sequential treatment in the form of four cycles of doxorubicin and cyclophosphamide followed by four cycles of docetaxel the 10-year DFS was 66.5% compared with 66.3% (p=0.749) in women who received doxorubicin, cyclophosphamide and docetaxel concurrently for six cycles. OS was 79.9% and 78.9% (p=0.506) respectively [73].

Although, the efficacy did not differ between the regimens, the side effects and toxicity profile of the sequential regimen was associated with fluid retention, neuropathy and myalgia whereby, the concurrent regimen has a shorter duration of treatment with a better side effects profile [73].

1.5.1.4. Anti-metabolites

5-flurouracil (5-FU) and capecitabine are antimetabolites routinely used in the treatment of breast cancer, with the later primarily administered in the case of metastasis. Capecitabine is a pro-drug of 5-FU [74] whereas, 5-FU is broken down intracellularly into two metabolites namely fluorodeoxyuridine monophosphate (FdUMP) and fluorouridine triphosphate (FUTP) which block the effects of thymidylate synthetase; thereby preventing DNA synthesis [75].

A meta-analysis of RCTs by Natori et al found that the addition of capecitabine to standard chemotherapy regimen (epirubicin/cyclophosphamide/docetaxel or epirubicin/docetaxel or doxorubicin/cyclophosphamide/docetaxel or 5-FU/epirubicin/cyclophosphamide/docetaxel) in women with TNBC increased both DFS and OS, albeit resulting in early cessation of treatment due to intolerable side effects such as severe diarrhoea and hand-foot syndrome [76].

1.5.1.5. Platinum

Carboplatin is the most widely used platinum chemotherapeutic agent in treating breast cancer but is not considered a first line therapy. Its mechanism of action comprises of forming intra

and inter strand cross-links with DNA, thereby preventing replication and transcription leading to apoptosis [77].

A randomised phase III trial comparing the effects of carboplatin and docetaxel in terms of objective response rate (ORR) and progression free survival (PFS) in patients with advanced TNBC found that individuals with a breast cancer 1 (BRCA 1) or breast cancer 2 (BRCA 2) mutation had a greater response to carboplatin as compared to docetaxel in terms of ORR of 68% vs. 33.3% (p=0.03), and an increased PFS of 2.4 months (6.8 vs. 4.4 months; p=0.002), but no difference in OS [78].

1.5.1.6. Vinca alkaloids

Vinca alkaloids are a class of chemotherapeutic agents that attach to tubulin in the M-phase of the cell cycle, thereby preventing the formation of microtubules and ultimately cell division. Vinblastine is one of two vinca alkaloids used in the treatment of metastatic breast cancer and as well as inhibiting cell division it prevents angiogenesis. Vinorelbine has a similar mechanism of action as vinblastine [79].

1.5.2. Human epidermal growth factor receptor 2 therapy

HER2-overexpressing breast cancers have a relatively unfavourable prognosis among the molecular subtypes [80] (Section 1.3.3). The availability of the therapeutic monoclonal antibody trastuzumab over the last two decades has considerably improved patients' outcomes and highlighted the importance of targeted therapy in breast cancer management [81]. Overexpression of HER2 triggers multiple downstream growth-related pathways required for proliferation. The most established mechanism of action of trastuzumab is that it attaches to the HER2 receptor and hinders its activation and successive signalling to downstream proliferation pathways such as MAPK and PI3K, which results in inhibition of proliferation and growth and ultimately cell death [80].

The Herceptin adjuvant trial (HERA), which randomly allocated women with HER2-positive breast cancer following primary therapy to have trastuzumab for 1 year, 2 years or observation only, concluded that after a follow-up of approximately 10 years, those women that received trastuzumab for 1 year significantly reduced their risk of a recurrence (Hazard ratio (HR) 0.76, 95% CI 0.68–0.86) and risk of death (HR 0.74, 0.64–0.86) in comparison to the observation

only group. Additionally, in those individuals that received adjuvant trastuzumab for 2 years did not yield further benefit in terms of DFS outcomes when compared to those women who received trastuzumab for 1 year (HR 1.02, 95% CI 0.89–1.17) [69]. However, if trastuzumab was ineffective and disease progression was to occur, National Institute for Health and Care Excellence (NICE) recommends the use of lapatinib in the setting of a clinical trial for individuals with metastatic disease [82].

Dual anti-HER2 therapy in the form of trastuzumab and pertuzumab is now routinely used in the UK in women with HER2-positive and node positive breast cancer following the outcome of the APHINITY trial, which concluded an increase in the 3-year DFS in the pertuzumab group compared with placebo (92.0% vs 90.2%; HR 0.77; 95% CI 0.62 to 0.96; P=0.02) [83].

1.5.3. Tyrosine kinase inhibitors

Lapatinib is a tyrosine kinase inhibitor (TKI) that has been granted use together with capecitabine in the treatment of HER2-positive metastatic breast cancer that has advanced despite the use of standard chemotherapy that included anthracycline, taxanes and trastuzumab [84]. This was based upon the results of a phase III RCT involving 324 individuals with HER2-positive breast cancer that had progressed on standard chemotherapy and compared capecitabine as monotherapy versus in combination with lapatinib. 37% of individuals within the study had a disease progression event, 30% in the combination therapy group and 45% who received monotherapy ($p<0.001$), and time to progression was 8.4 and 4.4 months respectively [85].

It's mechanism of action involves inhibition of HER1 and HER2 thereby, preventing activation of MAPK and PI3K signalling and ultimately leading to apoptosis of cancer cells [84]. Brain metastases occur in around a third of women with HER2-positive breast cancer and unlike trastuzumab, lapatinib has the potential to treat metastatic brain disease due to its potential to invade the blood brain barrier [86].

1.5.4. Endocrine therapy

Endocrine therapy is routinely offered to women with ER-positive tumours (defined as an Allred score ≥3), as an adjuvant therapy, or as the only form of treatment for elderly women with ER-positive breast cancers who are not suitable for surgery due to co-morbidities or

unable to tolerate aggressive chemotherapy regimens. The therapies aim to inhibit the up-regulated oestrogen signalling pathway, thereby interfering with oestrogen-dependent proliferation and survival of the cancer. This is achieved by either targeting hormone synthesis or by blocking receptor function. Only individuals with luminal A or luminal B breast cancers are appropriate for endocrine therapy as only they express ER-alpha receptors [87].

Tamoxifen is a selective ER modulator which is given to pre-menopausal women and binds to ER and antagonises oestrogen function [88]. Several studies have been carried out over the years by the National Surgical Adjuvant Breast and Bowel Project (NSABP) evaluating the effect of adjuvant endocrine therapy in the management of breast cancer. The NSABP B-14 study that involved randomizing 2,644 women with oestrogen positive and node negative breast cancer to adjuvant treatment with tamoxifen or placebo for 5-years, concluded a significant increase in DFS in those individuals that received tamoxifen. Further follow-up identified continual DFS advantage at 10-years in women that received tamoxifen, 69% vs 57%, respectively together with an OS increase of 4%, 80% vs. 76% [89].

The Arimidex, Tamoxifen Alone or in Combination (ATAC) trial which comprised of 9,366 post-menopausal women with node negative breast cancer found that anastrozole was superior to tamoxifen in decreasing time to recurrence (402 *vs* 498 events, p=0·0005), and decreased the possibility of distant metastatic disease (324 *vs* 375 events, p=0·04) [90].

In post-menopausal women, Aromatase Inhibitors (AIs) such as anastrozole and letrozole are given that block aromatase, which plays a role in converting androgen to oestrogen and thereby ultimately leading to reduced oestrogen levels [91]. However, AIs are not suitable for pre-menopausal women as they only decrease the production of oestrogen at extra gonadal sites, most commonly adipose tissue. Thereby, oestrogen produced by the ovaries is not inhibited and contributes to the growth of the breast tumour [92].

1.5.5. Neoadjuvant therapies

Both chemotherapy and endocrine therapy can be given before surgery in the neoadjuvant setting. Chemotherapy is more commonly used in the neoadjuvant approach though for both therapies the adjuvant setting remains the most common [93, 94]. Women that receive neoadjuvant chemotherapy (NACT) have comparable outcomes in terms of DFS and OS in comparison to women that receive adjuvant chemotherapy [95].

However, neoadjuvant treatments are advantageous when the aim is to reduce tumour size before surgery, which might facilitate BCS. This aids recovery of patients post operation both physically and psychologically as it allows for less distressing surgery [96].

Secondly, NACT permits the modification or alteration of chemotherapy regimens based on responses of primary tumours, which can be monitored by magnetic resonance imaging (MRI) or ultrasound scan (USS) and clinically by palpation, with an ultimate aim of improving response [97]. A study by Samiei at al demonstrated that complete pathological response (pCR) in primary tumours (i.e., the absence of invasive cancer cells remaining in the breast tissue as assessed by histopathology) strongly correlated with absence of axillary metastasis. Of women with a clinically node negative axilla, approximately 98% with breast pCR had no nodal metastatic disease on final histology in comparison to 72% of women who did not achieve breast pCR ($p<0.001$). Women with a clinically node positive axilla, 45.0% with breast pCR were node negative on final histology compared with 9.4% who did not achieve breast pCR ($p<0.001$) [98]. It is important to note that monitoring of response to therapy is not possible in the adjuvant setting, as tumours have been excised and any occult metastatic disease cannot be monitored [99]. Similarly, tailoring of downstream adjuvant therapies such as the addition of capecitabine to women with HER2-negative breast cancer that have residual invasive carcinoma following NACT (CREATE-X) [100] or the use of Trastuzumab Emtansine for residual invasive HER2-positive breast cancer (KATHERINE) [101] is not possible.

1.5.6. Recent treatment advances

1.5.6.1. PARP inhibitors

A poly (ADP-ribose) polymerase (PARP) inhibitor, Olaparib has been shown to be effective in treating ovarian malignancy in women who carry BRCA gene mutations, by inhibiting PARP enzyme activity and preventing DNA damage repair, and it was approved for use in metastatic breast cancer by the United States of America (USA) food and drug administration (FDA) in January 2018 [102] following the OlympiAD trial. The phase III global, multicentre, randomised trial concluded a PFS benefit but no OS benefit in individuals with germline BRCA 1 or BRCA 2 mutations, HER2-negative metastatic breast cancer. PARP inhibitors are the latest class of agents to demonstrate benefit in treatment of BRCA 1 or BRCA 2 mutations, HER2-negative metastatic breast cancer and may be beneficial in combating chemotherapy resistance in breast cancer [103].

1.5.6.2. PD-L1 inhibitors

The growth and progression of breast tumours are highly influenced by the immune system in a process known as immunoediting and consists of three stages, elimination, equilibrium and escape [104,105]. Current developments in breast cancer immunotherapy include the identification of immune checkpoint antagonists for PD-1/PD-L1 [106].

In 2019 the USA FDA approved the use of atezolizumab, a programmed death ligand 1 (PD-L1) inhibitor, together with paclitaxel in the management of women with either locally advanced or metastatic TNBC that are identified to have the PD-L1 protein [107].

Both programmed death protein 1 (PD-1) and PD-L1 play a key role in T-cell mediated immune responses [108] and PD-L1 is more likely expressed in triple negative and HER2-positive breast tumours [106]. Over expression of PD-L1 on tumours is thought to result in a decrease in the activation of cytotoxic T-cells that are responsible for identifying and eliminating tumour cells. Thus, by atezolizumab inhibiting the PD-1/PD-L1 interaction, T-cell activation and anti-tumor properties are restored [108]. However, a phase 3 trial by Schmid et al found that 15.9% of patients prescribed atezolizumab in combination with albumin bound paclitaxel in women with metastatic TNBC had to discontinue treatment due to the associated serious side effects such as colitis, hepatitis and pneumonitis in comparison to 8.2% of those administered only albumin bound paclitaxel [109].

1.5.6.3. CDK4/6 inhibitors

Cyclin-dependent kinases (CDKs) belong to the protein kinase family and play an active role in both cell cycle and transcriptional regulation [110]. Both CDK4 and CDK6 are principally responsible for regulating the cell cycle and therefore, can influence breast tumour growth and development. The cyclin D-CDK4/6-retinoblastoma (cyclin D-CDK4/6-Rb) signalling pathway is key to controlling the G1 to S transition phase of the cell cycle [111]. Cyclin D attaches to CDK4/6 which results in phosphorylation of retinoblastoma (Rb) thereby, detaching Rb from the Rb-E2F complex, allowing cells to enter S phase of the cell cycle and begin DNA replication. Therefore, any atypical activation of the cyclin D-CDK4/6-Rb pathway due to cell cycle gene mutations or anomalies in the regulators of cyclin D and CDK4/6 can lead to tumourigenesis [112]. Therefore, CDK4/6 is a key target in the development of new therapeutic agents for malignant tumours [113].

CDK4/6 inhibitors impede the progression from G1 to S phase of the cell cycle by preventing phosphorylation of Rb and ultimately the release of the transcription factor E2F thereby, leading to cell cycle arrest at G1 phase, which inhibits growth and development of tumour cells. Also, as tumourigenesis requires the interaction of numerous signalling pathways such as PI3K, RAF, Wnt, Janus kinase (JAK) 2, NOTCH and Myc and that CDK4/6 related signalling molecules are downstream of these pathways, alludes that inhibition of CDK4/6 may also inhibit the aforementioned signalling pathways which reveals their vast anti-tumour effect [112].

Furthermore, Goel et al also demonstrated that CDK4/6 inhibitors also initiate anti-tumor immunity in breast cancer cell lines by downregulation of E2F gene expression and upregulation of major histocompatibility complex (MHC) class I expression**.** They also prevent the proliferation of regulatory T (Treg) cells and suppression of E2F release [114]. Lastly, Deng et al concluded that CDK4/6 inhibitors encourage removal of cancerous cells via enhancing cytotoxic T cells (CTLs) [115]**.**

There are presently three CDK4/6 inhibitors, palbociclib, ribociclib, and abemaciclib, that have been permitted by the FDA for treatment of metastatic breast cancer [116].

Palbociclib was approved for use in post-menopausal women in combination with an AI as first-line treatment of ER/PR-positive and HER2-negative metastatic breast cancer [117] based upon the results of a randomised phase II study PALOMA-1/TRIO-18 [118]. Palbociclib was later approved for use with fulvestrant as second line therapy in postmenopausal women with metastatic ER/PR-positive and HER2-negative breast cancer who did not achieve adequate response from endocrine therapy [119] based on the results of the phase III study PALOMA-3 [120].

The results of the phase III MONALEESA-2 study [121] led to the approval of Ribociclib as first-line therapy of ER/PR-positive and HER2-negative metastatic breast cancer in postmenopausal women in 2017 [122].

Abemaciclib was the last CDK4/6 inhibitor to be approved, initially as second line therapy in combination with fulvestrant in individuals with ER/PR-positive and HER2-negative metastatic breast cancer who developed disease progression on endocrine therapy [123] based on the results of the randomised phase III MONARCH-2 study. This study randomised individuals to receive either abemaciclib and fulvestrant or placebo and fulvestrant. The median PFS in the abemaciclib arm of the trial was significantly improved at 16.4 months, compared with 9.3

months respectively ($p<0.001$) [124]. Abemaciclib is also now approved for use in combination with an AI for first-line therapy of ER/PR-positive and HER2-negative metastatic breast cancer in post-menopausal women [123] based on results from a phase III study, MONARCH-3 [125]. Abemaciclib is also the only CDK4/6 inhibitor that has been approved as monotherapy for treatment of ER/PR-positive and HER2-negative metastatic breast cancer following progression of disease despite receiving both chemotherapy and endocrine therapy [123]. Unlike, palbociclib and ribociclib, abemaciclib also results in less adverse neutropenia and leukopenia but otherwise has a similar side effect profile in terms of gastrointestinal effects [126].

Women within the cohort I have constructed for my research did not receive any of the aforementioned recent treatments (PARP inhibitors, PD-L1 inhibitors or CDK4/6 inhibitors) as the cohort comprised only of women diagnosed with primary breast cancer and not metastatic breast cancer. Additionally, these drugs were not in clinical use during the timeframe of the study, 2006-2010.

1.6. Mechanisms of cytotoxic chemotherapy resistance in breast cancer

Chemotherapy resistance is a substantial obstacle in successful treatment of breast cancer and around a third of individuals newly diagnosed will progress to a local recurrence or metastasis due to treatment failure [127]. There are two forms of chemotherapy resistance: innate resistance, which develops prior to treatment, and acquired resistance, which develops following exposure. In some women, continued exposure to chemotherapy drugs may result in resistance to structurally dissimilar chemotherapeutic drugs resulting in multidrug resistance (MDR). MDR can also arise in innate resistance where there is no preceding exposure to chemotherapy.

Chemotherapeutic drug resistance can be observed via various mechanisms, such as increased drug efflux pumps, modification of drug target, increased repair of DNA damage, changes in apoptotic signalling pathways, alteration in drug metabolism and epithelial to mesenchymal transition (EMT) (Figure 1.2) [128].

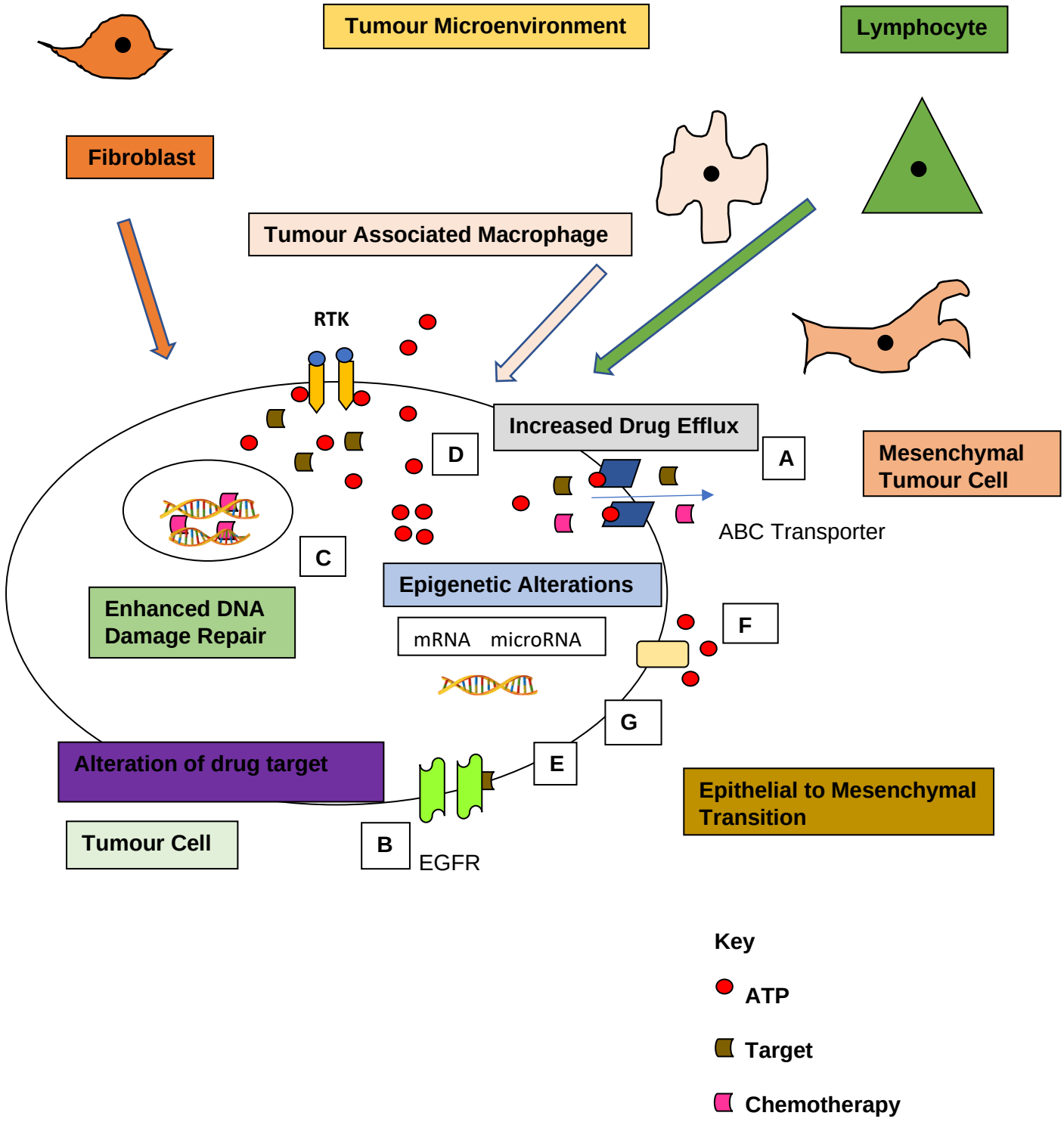


Figure 1.2 A diagram to show the mechanisms of chemotherapy resistance in breast cancer that include increased efflux of chemotherapeutic agents (A), modification of drug target (B), increased repair of DNA damage (C), impaired apoptosis (D), decreased drug activation and increased drug inactivation (E), epigenetic modifications (F) and microRNAs (G), epithelial to mesenchymal transition (H) and tumour microenvironment (I). ABC: ATP binding cassette; RTK: receptor tyrosine kinases; EGFR: epidermal growth factor receptor; TAM: tumour-associated macrophage. Adapted from [128]

1.6.1. Increased efflux of chemotherapeutic agents

One of the main mechanisms of resistance to chemotherapeutic agents is the expression of drug efflux pumps that give tumour cells the abilities to evade chemotherapeutic drugs by reducing intracellular drug accumulation [129].

A well-studied family of proteins with members that perform this function are the ATP-binding cassette (ABC) transporters, of which there are 49 known human ABC genes with 7 subfamilies named ABCA through to ABCG [130]. These transporters are mainly expressed in organs that are involved in the excretion of waste products of metabolism and play a key role in maintaining normal physiology by having the ability to excrete and prevent the accumulation of potentially harmful substances, as well as playing a role in MDR [131].
ABC transporters comprise of two cytoplasmic nucleotide-binding domains (NBDs) and at least two transmembrane domains (TMDs). Following the binding of the substrate to the TMDs, the NBDs attach and hydrolyse adenosine triphosphate (ATP), resulting in release of the phosphate group and a conformational change of the ABC transporter due to the energy generated. This structural change, results in the substrate moving through the protein channel and being released into the extracellular space. Lastly, the protein returns to its original conformation following an additional ATP hydrolysis at its binding site [132].

Although the NBDs amongst all ABC transporters are comparable in both structure and function, this is not the case for TMDs. Thereby, some ABC transporters may have a well-defined substrate specificity, in comparison to others that are able to transport an extensive range of substances which include chemotherapeutic agents [133]. The ABC transporters with an extensive substrate specificity include, ATP-binding cassette sub-family B member 1 (*ABCB1*), ATP-binding cassette sub-family C member 1 (*ABCC1*) and ATP-binding cassette super-family G member 2 (*ABCG2*) and are largely implicated in the resistance of many chemotherapeutic agents [128].

Nemcova-Furstova et al performed mRNA expression profiling followed by western blot analysis of ABC transporters found that ABCB1 proteins were up-regulated in paclitaxel resistant variants of the breast cancer cell lines, SK-BR-3 and MCF-7. Whereas ABCG2 was only up-regulated in paclitaxel-resistant SK-BR-3 cell lines. Other ABC transporters, ABCB4 and ABCC2 were found to be up-regulated in the paclitaxel-resistant MCF-7 cell line. However, the silencing of ABCB1 transporter protein expression by siRNA almost completely restored the cell to be fully sensitive to the taxane paclitaxel [134].

Therefore, mutations and overexpression of the ABC family of transporters can greatly affect chemotherapy response in breast cancer. Importantly, these can be potentially targeted to reverse resistance.

1.6.2. Modification of drug target

A well-known example of chemotherapy resistance as a consequence of altered drug target is that of tyrosine kinase inhibitor (TKI), which arises due to a modification of the epidermal growth factor receptor (EGFR) as a consequence of a missense mutation. The T790M mutation, results in substitution of methionine instead of threonine [135]. This structural change causes a steric hindrance and prevents the binding of TKI [136] as well as elevating intracellular ATP affinity thereby, reducing the affinity for TKI [137] which then results in activation of MAPK, JAK and PI3K signalling pathways and tumour proliferation, angiogenesis and metastasis [84].

Resistance to endocrine therapy in the form of tamoxifen also arises in part due to a conformational change in the receptor due to phosphorylation of ER-α by activation of molecular pathways such as protein kinase (PKA), MAPK and p21-activated kinase1 (PAK-1) [138].

With respect to traditional cytotoxic chemotherapy agents, taxanes provide a notable example. Microtubules are crucial elements of the cytoskeleton and mitotic apparatus. Microtubule-targeting agents such as taxanes bind to the β-tubulin of the microtubule and suppress microtubules dynamic instability during the mitotic stage of the cell cycle which leads to mitotic arrest, inducing apoptosis in cells during division [69]. Resistance to anti-mitotic agents can arise due to either structural or functional changes of the microtubules. Ranganathan et al reported an overexpression of the β-tubulin isotypes III and IVa resulted in paclitaxel resistance due to less efficient binding of taxanes to these isotypes compared with other isotypes [139].

1.6.3. Increased repair of DNA damage

The repair of DNA damage has a key role in chemotherapeutic resistance. Platinum agents such as cisplatin are well known chemotherapeutic agents that cause DNA damage. Cisplatin does this by forming intra and inter-strand crosslinks with purine bases at N7 thereby forming

DNA adducts that prevent transcription and DNA synthesis. The cell cycle is arrested, and cells undergo apoptosis if DNA repair mechanism are unable to repair the damage [140].

Resistance to platinum-based chemotherapeutic agents often occurs due to the up-regulation of DNA repair mechanisms such as, nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination and nonhomologous end joining (NHEJ). The NER pathway is liable for eliminating DNA adducts whereby, the MMR pathway is accountable for rectifying mismatches, insertions and deletions of single-strand DNA that occurred during replication whereas, homologous recombination and NHEJ pathways repair DNA double strand breaks [141].

A study by Li et al analysed the relationship between excision repair cross complementation group 1 (ERCC1) and the repair of DNA adducts induced by platinum agents in vitro and concluded that tumours resistant to cisplatin therapy highly expressed ERCC1, which is a rate limiting protein in the NER pathway responsible for the excision of platinum adducts [142]. Lastly, a study by Chen et al who evaluated the expression levels of a DNA repair enzyme, O6-methylguanine DNA methyltransferase (MGMT) in 83 individuals with nasopharyngeal cancer that received cisplatin therapy concluded that tumour cells with high expression levels of MGMT were increasingly resistant to cisplatin therapy, which correlated with a poor PFS, and OS compared with tumour cells that expressed low levels of MGMT [143].

1.6.4. Impaired apoptosis

Alterations regulating cellular damage from chemotherapy agents is another mechanism that can contribute to chemotherapy resistance [144].

In tumourigenesis there are several methods in which the apoptotic pathways can be altered leading to a decrease in apoptosis or apoptosis resistance. These comprise of the down-regulation of pro-apoptotic proteins and an up-regulation of anti-apoptotic proteins, altered death receptor signalling, decreased function of the caspases as well as alteration in the function of p53 [145].

A study by Qin et al found that microRNA let-7i reduced chemotherapy resistance in MCF-7 breast cell lines via a down-regulation of pro-survival protein B-cell lymphoma 2 (Bcl-2) expression [146]. The Bcl-2 protein family comprises of the following proteins Bcl-2, B-cell lymphoma-extra-large (Bcl-XL), Bcl-like protein 2 (Bcl-w) which have anti-apoptosis effects via the prevention of the mitochondrial apoptosis-induced channel (MAC) configuration, while

Bax, Bak, Bik, Bid have pro-apoptosis effects by encouraging the formation of MAC. Inhibitors of apoptosis proteins (IAPs) are another family that exert these effects via inhibition of caspases 3, 7 and 9 [145].

Zhao et al also demonstrated that cIAP 1 and 2 proteins are also upregulated, likely via the nuclear factor kappa B (NF-κB) signalling pathway, in breast tumours which contributed to tumour growth as well as preventing apoptotic death induced by chemotherapeutic agents [147].

Lastly, mutations in the TP53 tumour suppressor gene have been associated with evasion of apoptosis and therefore chemotherapy resistance in breast cancer [148]. TP53 proteins are normally activated upon DNA damage which are then responsible for the management of downstream genes concerned with DNA repair, cell cycle arrest and apoptosis such as, p21, FAS or BAX/BCL2. However, atypical expression of p53 can downregulate Bax expression which inhibits the configuration of MAC, decreasing the pro-apoptotic effect and upregulate Bcl-2 expression which inhibits the release of cytochrome C from mitochondria inhibiting p53-mediated apoptosis [148].

1.6.5. Decreased drug activation and increased drug inactivation

One of the main enzymes responsible for drug activation and inactivation comprise of the cytochrome P450 (CYPs) and glutathione-*S*-transferase (GST) [149]. CYPs are key in the metabolism of many chemotherapeutic agents such as cyclophosphamide, taxanes, vinca alkaloids, tamoxifen and imatinib [150]. However, modification in CYPs activity can arise due to mutation for example a mutation in *CYP2B6* correlates with a poor outcome in individuals with breast cancer [151] and mutations of *CYP2D6* gives rise to tamoxifen resistance [152]. The GSTs are a group of detoxifying enzymes mainly responsible for metabolism of the platinum based chemotherapeutic agents cisplatin and oxaliplatin as well as cyclophosphamide and increased expression of GST in tumour cells increases the detoxification of the aforementioned agents resulting in less efficient cytotoxic damage of the cells [149].

Resistance to chemotherapeutic agents can also arise via a decreased prodrug activation or increased drug inactivation by phase I and or II enzymes [150]. Resistance to cytarabine can occur in the treatment of acute myeloid leukaemia due to reduced drug activation because of downregulation or mutation of deoxycytidine kinase, which is responsible for the initial

phosphorylation of cytarabine to cytarabine-monophosphate which is then phosphorylated further to its active form cytarabine-triphosphate by nucleotide kinases [153].

1.6.6. Epigenetic modifications

Epigenetic modifications in the form of DNA methylation and histone alterations give rise to chemotherapy resistance as well as participating in other drug resistance mechanisms such as enhanced drug efflux, increased repair of DNA damage and impaired apoptosis. DNA methylation involves the attachment of methyl groups to cytosines within CpG islands, found mainly upstream of gene promoter regions. Whereas, histone modifications change the conformation of chromatin by either histone acetylation or deacetylation, thereby, regulating gene expression [144]. A study by Kantharidis et al concluded that demethylation of the MDR1 promoter gene correlated with a phenotype representing multidrug resistance and the administration of a demethylating agent, 5'- azadeoxycytidine to cells lacking P-glycoprotein (P-gp) changed the methylation sequence of the MDR1 promoter that were representative of that of P-gp positive cells [154].

1.6.7. MicroRNAs

MicroRNAs (miRNAs) perform a key role in chemotherapy resistance in breast cancer and are non-coding RNAs less than 25 nucleotides in length [155]. They are responsible for the regulation of post-transcriptional gene expression of many genes such as those implicated in cell proliferation, cell cycle and cell death signalling pathways, drug metabolism, drug transport and DNA damage repair genes [156].

A study by Wang et al confirmed that miRNA-21 encourages doxorubicin resistance by the downregulation of phosphatase and tensin homolog (PTEN) in MCF7- cells [157]. PTEN is normally responsible for inhibition of the PI3K signalling pathway which ultimately leads to the prevention of tumour proliferation and cell death [156]. Similarly, miRNA-21 induced trastuzumab resistance in the MDA-MB-453 cell line via silencing of PTEN pathway [158]. Gan et al found that downregulation of miRNA-221 and or miRNA-222 increased MDA-MB-468 cell line sensitivity to tamoxifen via the upregulation of tissue inhibitor of metalloproteinase-3 ($TIMP_3$) [159]. Bao et al found that miRNA-298 targeted ABCC1 in breast cancer cell lines

MDA-MB-231 and a decrease in miRNA-298 expression correlated with increased P-gp expression which resulted in cells becoming resistant to doxorubicin [160].

1.6.8. Epithelial to Mesenchymal Transition

Epithelial to Mesenchymal Transition (EMT) is the manner in which epithelial cells lose their characteristics such as lack of motility and cell adhesion by a decrease in E-cadherin and occludin and acquire a mesenchymal phenotype by expressing fibronectin, vimentin and N-cadherin and an enhanced activity of matrix metalloproteinases [128]. This transition is mediated via signalling pathways that include, Wnt-β-catenin, Notch, TGF-β, Hedgehog and receptor tyrosine kinases, which then mediate EMT transcription factors (EMT-TFs) that comprise of Snail family transcriptional repressor 1 and 2 (SNAIL1/2), Twist and Zinc finger E-box-binding homeobox 1 and 2 (ZEB 1/2) [161].

These signalling pathways and transcription factors can give rise to chemotherapy resistance in breast cancer. A study by Xu et al assessed the expression of TGF-β and EMT markers E-cadherin and N-cadherin in triple negative breast cancer cell lines in response to epirubicin. The findings were that an upregulation of TGFβ correlated with a decrease in E-cadherin and an increase in N-cadherin expression and ultimately epirubicin resistance in triple negative cancer cell lines [162]. Similarly, Wu et al demonstrated that trastuzumab resistance in HER2 overexpressing cell lines was due to Wnt3 overexpression which stimulated the Wnt/β-catenin signalling pathway thereby resulting in activation of EGFR which induced EMT leading to increased expression of N-cadherin [163]. Many studies have also shown that EMT-TFs contribute to chemotherapy resistance in breast cancer. A study by Wang et al examined the expression of multidrug resistance associated protein (MRP), P-gp and Twist in response to paclitaxel in breast cancer. MCF-7 cells were exposed to high concentrations of paclitaxel to establish a paclitaxel resistant cell line. These cell lines were then transfected to over-express Twist to determine the correlation between Twist and MRP and P-gp expression. The findings were that Twist increased resistance to paclitaxel by the upregulation of MRP and P-gp as the overexpression of ABC transporters in tumour cells can increase the removal of chemotherapeutic agents and result in resistance [164].

1.6.9. Tumour heterogeneity

Breast cancer heterogeneity is a potential explanation for chemotherapy resistance and can be divided into both inter-tumour and intra-tumour heterogeneity. Inter-tumour heterogeneity signifies variation in morphology, genetic make-up and molecular composition between tumours. While, intra-tumour heterogeneity denotes diversity among different cells of a specific tumour as a consequence of epigenetic and genetic differences between the individual tumour cells [165]. There are two proposed models to account for intra-tumoural heterogeneity.

1.6.9.1. Cancer stem cells are chemo-resistant

In the cancer stem cells (CSCs) model, tumours are mainly composed of tumour cells with a restricted ability to replicate and differentiate but also have a small sub-group of cells known as CSCs that have the ability to self-renew and differentiate into the malignant component that drives tumour progression [165].

CSCs play a key role in chemoresistance in breast cancer as they are involved in numerous mechanisms such as the overexpression of ABC transporters, enhanced DNA repair, inhibition of cell apoptosis and the ability to transition between epithelial and mesenchymal behaviours [166]. A study by Britton et al found an overexpression of ABCG2 in breast cancer stem cells (BCSCs) that were resistant to mitoxantrone in comparison to non-CSCs [167]. Whereas, Zhang et al concluded that BCSCs upregulate DNA repair genes in a p53-null murine tumour model [168].

1.6.9.2. Clonal evolution model: chemotherapy can select for resistance

In the clonal evolution model, mutations occur on an individual tumour cell basis which gives rise to selective advantages to the tumour microenvironment and growth, resulting in these variants developing altered sensitivity to chemotherapies based upon Darwinian selection. Thus, original chemotherapies would only eradicate a specific subset of tumour cells, whereas less sensitive tumour cells would survive, proliferate and grow resulting in an altered tumour cell configuration that is resistant to the original chemotherapies [165].

Using next generation sequencing and circulating free DNA (cfDNA) liquid biopsy, Palmieri et al demonstrated in an individual with breast cancer up to 10 clones were present at initial primary tumour biopsy which comprised of varying mutational load within gene including Isocitrate dehydrogenase 1 (IDH1), Catenin Beta 1 (CTNNB1), F-Box and WD repeat domain containing 7 (FBXW7), Adenomatous polyposis coli (APC), EGFR, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase catalytic subunit alpha (PIK3CA), PTEN, Mothers against decapentaplegic homolog 4 (SMAD4), CDKN2A and Guanine nucleotide binding protein (GNAS). However, between initial diagnosis and 180 days post recurrence, mutational load for PIK3CA and CDKN2A doubled from 25-50%. Thereby, having the potential to identify key mutations leading to cancer recurrence could allow for these mutations to be targeted specifically [169]. Balko et al who investigated the targeting sequence of exons of cancer related genes to determine changes in variants of TNBCs that were given NACT, concluded that only a minority of variants were to be found in both pre and post samples [170]. These findings are similar to work performed by Al Amri and colleagues who performed whole exome sequencing (WES) on 6 ER-positive cancers pre and post NACT [171]. While, Balko et al found PI3K pathway genes and cell-cycle regulators to be increased in the post-NACT samples [170], whereas Al-Amri et al found MUC17 and PCNX1 to be regulators of chemoresponse [171]. Similarly, Kim et al who investigated matched pre and post exomes in 20 TNBCs treated with NACT demonstrated that chemotherapy is responsible for encouraging the growth of resistant clones or a decrease in sensitive clones [172].

1.7. Tumour microenvironment and its role in chemoresponse

The tumour microenvironment (TM) consists of the extracellular matrix (ECM), endothelial cells, myeloid cells (tumour associated macrophages (TAMs), tumour associated neutrophils (TANs) and myeloid derived suppressor cells (MDSCs)), cancer associated fibroblasts (CAFs), adipocytes and several signalling molecules which collectively form the tumour stroma. The aforementioned components together with TM acidity and oxygen availability all play a role in contributing to chemoresistance in breast cancer (Figure 1.3) [173].

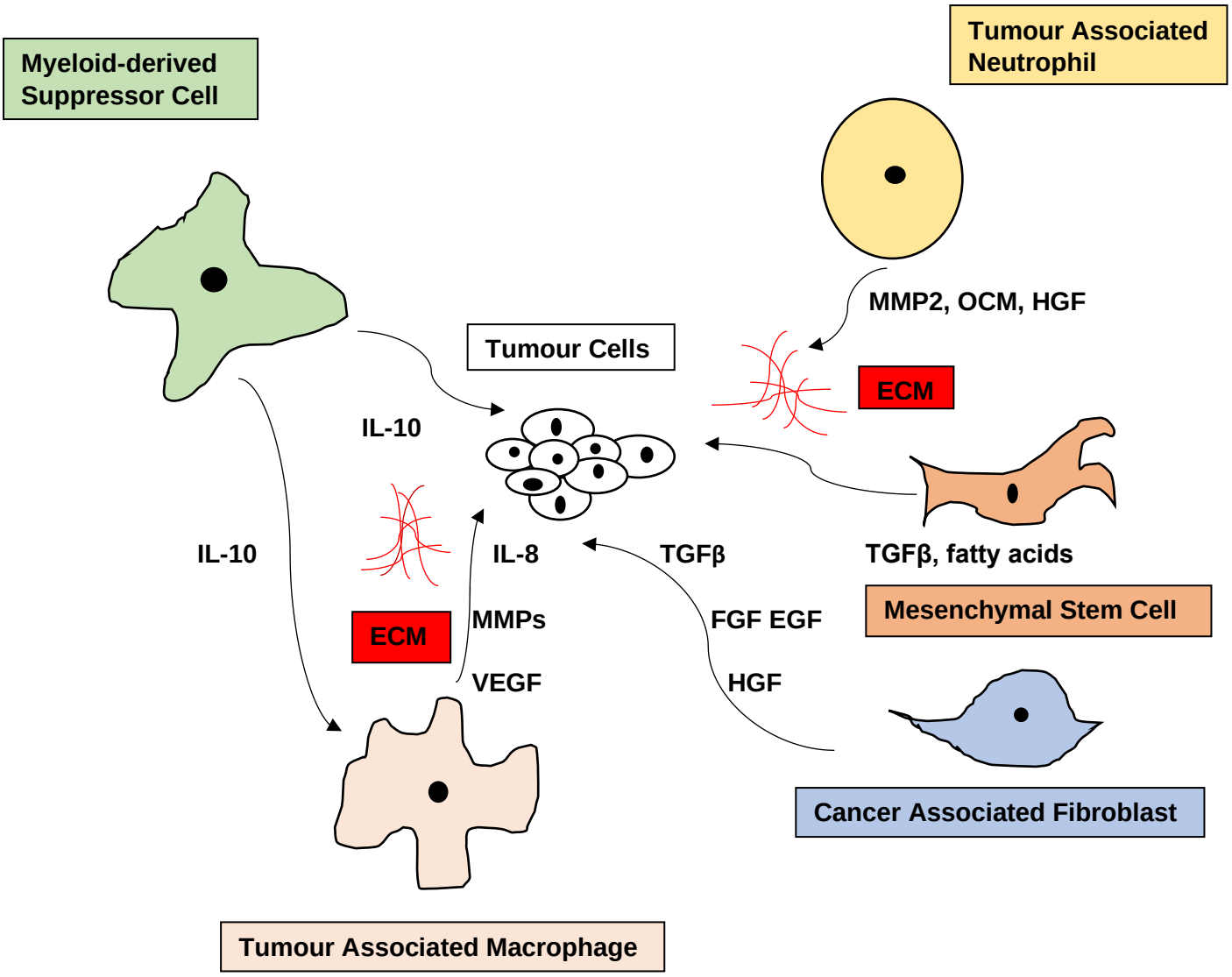


Figure 1.3 Constituents of the tumour microenvironment that affect chemotherapy resistance in breast cancer. Tumour cells are in close proximity with their microenvironment which consists of extracellular matrix, endothelial cells, myeloid cells (tumour associated macrophages, tumour associated neutrophils and myeloid derived suppressor cells, cancer associated fibroblasts, adipocytes and several signalling molecules which collectively form the tumour stroma. Together with tumour microenvironment acidity and oxygen availability all play a role in contributing to chemoresistance in breast cancer. Epidermal growth factor (EGF); Fibroblast growth factor (FGF); Hepatocyte growth factor (HGF); Interleukin-8/10 (IL-8/10); Matrix metalloproteinases (MMPs); Oncostatin M (OCM); Transforming growth factor beta (TGF-β); Vascular endothelial growth factor (VEGF). Adapted from [174]

1.7.1. Extracellular matrix

Chemotherapy resistance arises due to ECM remodelling which results in increased elasticity and stiffness of ECM proteins such as collagen and fibronectin which makes it difficult for chemotherapeutic agents to penetrate the tumour thereby, drug delivery time is lengthened which can lead to drug resistance [175]. A stiffened ECM also results in the induction of miRNAs which decreases the expression of PTEN protein involved in tumour suppression. Thereby, enhancing the activity of the PI3K-Akt signalling pathway involved in cancer growth, invasion and metastasis [173].

1.7.2. Tumour associated macrophages

Tumour associated macrophages (TAMs) can give rise to chemoresistance via many mediators [174]. Firstly, they can secrete both TGF-β and tumour necrosis factor- α (TNF α) that are key inducers of the EMT of tumour cells which leads to tumour invasion and drug resistance [176]. Jinushi et al also concluded that TAMs secrete a range of proteins that aid angiogenesis such as matrix metalloproteins (MMPs), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [177]. TAMs also secrete immunosuppressive mediators such as prostaglandin E2, IL-10 and various chemokine C-C ligands that inhibit Th 1 immune response that give rise to chemoresistance [174]. Lastly, a study by Shree et al concluded that TAMs that express the proteases cathepsins decrease chemotherapeutic response to paclitaxel, etoposide and doxorubicin in breast cancer. Surprisingly, the chemoprotective effect of TAMs incorporates drugs with different mechanisms of action such as stabilization of microtubules, inhibition of topoisomerase activity, or DNA intercalation, which implies that TAMs can aid tumour survival in a cathepsin-dependent manner via a wide range of stimuli [178].

1.7.3. Tumour associated neutrophils

Tumour associated neutrophils (TANs) induce chemoresistance via secretion of hepatocyte growth factor (HGF), oncostatin M (OCM) and MMP2 which are involved in ECM remodelling thereby, leading to increased tumour motility and invasion [179]. Like TAMs they secrete MMPs and VEGF that enhance angiogenesis [180].

1.7.4. Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are associated with chemoresistance via the repression of immunogenic activity mainly a consequence of IL-10 secretion [174]. Beury et al concluded that IL-10 prevented both the stimulation of macrophages as well as the production of immune cytokines such as IL-6 and TNF-α from macrophages [181] as well as decreasing intra-tumoural immunity as a consequence of inadequate dendritic cell maturation [182].

1.7.5. Cancer associated fibroblasts

Cancer associated fibroblasts (CAFs) comprise the majority of cells within the TM and contribute to cancer cell growth, invasion and metastasis as well as chemotherapy resistance via the secretion of tumour-promoting proteins and growth factors, cytokines such as stromal cell-derived factor 1 (SDF-1) and IL-6, exomes and factors that remodel ECM [173].

TGF-β, HGF, FGF and EGF encourage tumourigenesis and chemoresistance via the EMT process [174]. TGF-β activates the Smad 2/3 signalling pathway whereby, the other growth factors mainly activate the PI3-AKT and mitogen-activated protein kinase pathway giving rise to chemoresistance as a consequence of cross activation [174]. Furthermore, FGF signalling has been shown to downregulate the expression of pro-apoptotic and apoptosis-inducing factors which decreases apoptosis in response to chemotherapeutic agents [183]. Whereas, HGF is associated with resistance to lapatinib in women with HER2-overexpressing breast cancers [184], over secretion of IL-6 by breast CAFs has been shown to stimulate resistance to tamoxifen via activation of the JAK/STAT3, or PI3K/AKT signalling pathways [185].

CAFs are also responsible for the activation of the Wnt signalling pathway of tumour cells in a paracrine manner. A direct target of the Wnt/β-catenin signalling pathway is the MDR1 gene which encodes an efflux pump, P-gp, overexpression of which is associated with multi-drug resistance [186].

Exosomes derived from CAFs are also thought to play a role in chemoresistance via the activation of the NOTCH signalling pathway and upregulation of NOTCH target proteins. Also, P-gp in exosomes prolongs tumour survival by efflux of chemotherapeutic agents [187]. Whereas, miRNAs within exomes can enter the cytosol and can inhibit target mRNA

expression. A study by Yeung et al found that miR-21 inhibited the apoptotic protease activating factor 1 in tumours resulting in paclitaxel resistance [188].

Lastly, CAFs overexpress ECM remodelling proteins such as MMPs, fibronectin and ECM remodelling results in epithelial plasticity giving rise to chemotherapy resistance as a consequence of acting as a physical barrier to the diffusion of chemotherapy agents [189].

The aforementioned chemotherapy resistance mechanisms do not act independently but instead there is great interplay between them which poses significant challenges and therefore further development of better and targeted therapies is required.

1.8. Preliminary work forming the basis of this project

Previous investigations in the Hughes laboratory using three separate approaches identified candidate genes that could be involved in chemoresistance in breast cancer. For my work, I examined these datasets and selected genes for further experimental testing. Below, I describe each of these three separate initial experiments and the resulting datasets.

1.8.1. Identification of candidate mediators of chemoresistance through therapy-driven selection of somatic variants

The hypothesis behind the first approach was that somatic mutations that increase in number after chemotherapy may encourage therapy resistance, while somatic variants that are reduced or eliminated may be linked with sensitivity to the therapy [190].

Cancer cells were collected by laser capture microdissection from pre-chemotherapy biopsy samples and post-chemotherapy resection samples from a small cohort of breast cancers showing partial responses to a NACT regimen of epirubicin/cyclophosphamide (n=6). Whole exome sequencing (WES) was carried out on normal cells, pre-NACT cancer cells and post-NACT cancer cells. Data was then analysed pairwise (either pre-NACT or post-NACT cancer cells vs. normal cells) to identify somatic variants in the cancer cells, in particular single nucleotide variants (SNV) and small insertions or deletions (Indels).

A number of different strategies were then implemented for sifting and prioritising somatic variants to produce a list of candidate genes demonstrating evidence for these mutations affecting chemotherapy response of cells. The number of mutated genes amongst the 6

individuals, the group in which the gene was present (i.e., unique to pre-NACT samples, unique to post-NACT samples, or variants shared between pre-NACT and post-NACT and showing evidence of an alteration in prevalence), the degree of alteration in mutant allele frequencies for variants shared between pre-NACT and post-NACT, and the likely effects of the mutations on gene function were taken into consideration [171, 190].

The following candidate genes were identified as potentially suitable for further investigation. Table 1.3 lists these genes, along with some details of the variants identified, and whether these variants were selected against or for by chemotherapy.

Gene	Patient No	Variant Type	Effect	Impact
ABL1	2	SNV	Missense	Moderate
	5	DEL	Frameshift	High
AP3B1	2	SNV	Missense	Moderate
	6	SNV	Missense	Moderate
CCDC88C	2	SNV	Missense	Moderate
	3	DEL	Deletion	Moderate
CENPF	2	SNV	Missense	Moderate
	5	INS	Frameshift	High
CEP350	1	SNV	Missense	Moderate
	4	DEL	Frameshift	High
COL6A3	2	SNV	Missense	Moderate
	3	DEL	Deletion	High
CRIPAK	3	INS	Insertion	Moderate
	5	SNV	Missense	Moderate
DMBT1	2	SNV	Intron	Moderate
	3	DEL	Deletion	Moderate
	5	INS	Intron	Moderate
EFEMP1	2	DEL	Deletion	High
	4	SNV	Missense	Moderate
EGFLAM	2	SNV	Missense	Moderate
	5	INS	Frameshift	High
FRYL	2	DEL	Frameshift	High
	4	INS	Frameshift	High
IGSF-10	2	SNV	Missense	Moderate
	3	SNV	Missense	Moderate

	5	SNV	Missense	Moderate
ITGA7	1	SNV	Missense	Moderate
	2	SNV	Missense	Moderate
MYO10	2	SNV	Missense	Moderate
	3	SNV	Intron	Low
NCOA3	2	SNV	Missense	Moderate
	4	DEL	Frameshift	High
NLRC5	3	DEL	Frameshift	High
	4	DEL	Frameshift	High
NOTCH2	2	SNV	Stop_gained	High
	4	DEL	Frameshift	High
NR4A1	2	SNV	Missense	High
	5	DEL	Deletion	Moderate
PKD2L1	2	SNV	Missense	Moderate
	6	INS	Insertion	Moderate
PTPN14	2	SNV	Missense	Moderate
	5	DEL	Frameshift	High
RPTN	1	SNV	Stop_gained	High
	5	INS	Insertion	Moderate
S100PBP	2	SNV	Missense	Moderate
	4	INS	Frameshift	High
SEZ6	1	SNV	Missense	Moderate
	5	INS	Stop_gained	High
SYNE1	2	SNV	Synonymous	Low
	3	DEL	Frameshift	High
	4	SNV	Stop_gained	High
TENM4	4	DEL	Frameshift	High
	5	INS	Frameshift	High
THADA	2	SNV	Missense	Moderate
	5	SNV	Missense	Moderate
TTN	2	SNV	Missense	Moderate
	4	SNV	Missense	Moderate
ZBTB49	2	SNV	Missense	Moderate
	3	DEL	Frameshift	High
ZFHX4	2	SNV	Missense	Moderate

	5	INS	Insertion	Moderate
SSPO	5	SNV	Missense	Moderate
	3	SNV	Missense	Moderate
XDH	2	SNV	Missense	High
	4	SNV	Missense	Moderate
KIAA1161	2	SNV	Missense	Moderate
	4	SNV	Stop_gained	High

Table 1.3. Candidate genes. Genes are listed with distribution of the variants across the 6 patients and their effect

1.8.2. Transcriptome profiling of stem-like cells from primary breast cancers

The aim of this study was to identify transcripts associated with breast cancer stem cells (BCSCs) from primary breast cancers, which may give an understanding of the difference in function between BCSCs and non-BCSCs within the tumour [191]. This is relevant to understanding differential chemotherapy responses since CSCs are reportedly chemoresistant.

Aldefluor assays were used to fluorescently label BCSCs of 17 primary breast cancers of various histopathological and molecular subtypes. Transcriptomes of BCSCs and matched non-stem cancer cells were then established using RNA-seq (n=6) and analysed to ascertain significantly differentially expressed transcripts amongst BCSCs and the remaining tumour cells, using all 6 paired samples, or only 5 of the pairs since one pair was regarded as an outlier in principle component analyses. The most differentially expressed genes (both up- and down-regulated) within BCSCs are listed in Table 1.4.

	Down Regulated		Up Regulated	
	Gene	**Mean Fold Difference**	**Gene**	**Mean Fold Difference**
All	HBA2	207	LINC01279	5.3
	HBA1	175		
	GJA4	57		
	NDUFA4L2	42		
	BTNL9	39		
	ANGPT2	25		
	ITGA7	24		
5 Pairs	HBB	1053	PDGFRA	8.8
	HBA2	303	DCN	8.8
	HBA1	183	LUM	7.8
	GJA4	83	SFRP2	6.3
	RGS5	66	LINC01279	5.9
	CDH6	48	RARRES2	4.0

Table 1.4. The most up- or down-regulated genes in breast cancer stem cells compared to matched non-breast cancer stem cells within the tumour. Expression in breast cancer stem cells and matched non-breast cancer stem cells within the tumour were compared in all 6 cases (All) or in only 5 cases (5 pairs). The most up- or down-regulated genes are listed, together with mean fold-changes

1.8.3. CAF-induced chemoprotection of breast cancer cells

Two further candidate genes, Interferon-β1 (IFNβ1) and Myxovirus Resistance 1 (MX1), were identified from a different data set within the Hughes group as genes of interest in chemotherapy resistance [192].

Breast CAFs were found to protect TNBC cell lines MDA-MB-231 and MDA-MB-157 from the anthracycline chemotherapeutic epirubicin *in vitro*. CAF induced protection was found to be due to CAF-dependent activation of the interferon signalling pathway in the cancer cells, as CAFs secreted IFNβ1 in the presence of cancer cells, blocking of IFNβ receptors by specific antibodies resulted in decreased protection of the cancer cells by CAFs, and addition of exogenous IFN was sufficient to induce protection. Thus, expression of IFNβ1 in breast CAFs, and of MX1, a marker of active IFN-signalling, in the cancer cells were potential markers of chemoresistance.

1.9. Selection of candidates for further study in this thesis

The aforementioned candidate genes were further examined by literature review in an effort to identify published literature to strengthen the case for specific candidates having roles in cancer biology and/or chemoresistance. Finally, four candidate genes were chosen: both IFNβ1 and MX1 as markers of active IFN signalling as identified in the CAF-cancer cell cross-talk study. Integrin subunit alpha 7 (ITGA7) and Nuclear receptor 4A1 (NR4A1) from the genomics screen, the former because it was also identified in the CSC transcriptomics experiment.

I will now discuss each of these proteins in detail.

1.9.1. IFNβ1 and MX1

These markers were selected with the expectation that one, IFNβ1, would be most relevant in the CAF compartment, while the other, MX1, would be relevant in the tumour cells.

Interferons (IFNs) are members of the cytokine family with IFNβ1 falling within the type I IFNs, which activate transcription of many genes such as those involved in apoptosis, angiogenesis as well as immunoregulatory effects [193]. Hosein et al found that IFN signalling can be activated by breast CAFs that secrete IFNβ thereby, encouraging growth of the MCF-7 cell lines [194]. Franci et al found an upregulation of JAK/STAT and NOTCH pathways as well as epigenetic modifiers in murine breast tumours following chemotherapy with docetaxel, doxorubicin and cyclophosphamide [195] and a further study using MRC5 lung fibroblasts was shown to protect both MDA-MB-231 and MDA-MB-157 cell lines from chemotherapeutic agents by stimulation of NOTCH3 and STAT1, a key IFN-signalling intermediate, which was linked with the up-regulation of IFN target genes OAS1 and MX1 [196].

Although there is substantial evidence within the literature that suggests that CAFs mediate resistance to many chemotherapeutic drugs in the management of breast cancer, via their secretion of growth factors and cytokines such as TGF-β [197], and SDF-1 [198,199] which activate the downstream signalling pathways such as P13K/AKT and MAPK/ERK1/2 [200]. There is paucity of the literature in terms of the CAFs and IFNβ signalling in breast cancer chemoresistance.

MX1 protein is part of the dynamin family and is an interferon induced GTPase encoded by Myxovirus resistance 1 (MX1) gene. However, its function in breast cancer is unidentified, but is likely to be stimulated by the interferons, IFN α and β [196].

A study by Aljohani et al who assessed the expression level of MX1 by TMA-IHC comprising 845 breast cancer cases found that overexpression of MX1 correlated with increased tumour size ($p=0.01$), histological grade, NPI and ER/PR negativity (all $p<0.0001$) as well as associated with a worse prognostic outcome in terms of breast cancer specific survival ($p=0.028$) [201]. Croner et al concluded that high expression of MX1 was associated with metastasis in colorectal cancer (CRC) and knockdown of MX1 prevented progression and invasion of cancer cells [202]. MX1 expression has not specifically been implicated previously as a mediator of breast cancer chemoresponse.

1.9.2. ITGA7

ITGA7 encodes the α-7 integrin protein which belongs to the integrin family [203] and forms a heterodimer with integrin β1 in the plasma membrane which then acts as a receptor for the attachment of laminin 1 and 2 [204]. Numerous studies have demonstrated ITGA7's involvement in cell proliferation, migration and invasion in a range of cancers. However, with respect to ITGA7 specifically, the literature on its function in breast cancer is conflicting. Bai et al showed that a high expression of ITGA7 in breast cancer, correlated with increased tumour size ($p=0.004$), grade of tumour ($p=0.017$) and TNM stage ($p=0.038$) in addition to a poor OS ($p<0.001$). Whereas, knockdown of ITGA7 in MCF7 cell lines resulted in increased apoptosis and inhibition of cell growth and migration of breast cancer cells [205].

Other studies have confirmed the association of upregulation of ITGA7 with adverse outcome in oesophageal cancer [206]. Whereas, in vitro experiments have demonstrated the oncogenic properties of ITGA7 in pancreatic cancer [207]. Knockdown of ITGA7 in hepatocellular cancer prevented cell invasion [208] and ITGA7 knockdown in glioblastoma cells blocked cell proliferation [209]. Proposed mechanisms for ITGA7 oncogenic effects include regulation of FAK/Akt and P13K/Akt [207].

Conversely, Bhandari et al concluded that ITGA7 functions as a tumour suppressor in breast cancer as ITGA7 expression was low in breast cancer tissue in comparison to normal mammary tissue. Performing RT-qPCR and knocking down ITGA7 in MDA-MB-231 and BT-549 cell lines using siRNA was thought to encourage tumour invasion by enhancing c-met and vimentin activity which enhances the EMT process and ultimately breast cancer progression [203]. ITGA7 as a tumour suppressor gene is supported by a study by Guan et al who concluded that downregulation of ITGA7 in papillary thyroid cancer resulted in increased growth, motility and invasion of tumour cells and ITGA7 knockdown prompted invasion of

tumour cells via epithelial to mesenchymal transition (EMT) as a consequence of elevated vimentin and N-cadherin and low E-cadherin expression [210]. Whereas Tan et al concluded that ITGA7 behaved as a tumour suppressor in prostate cancer by interacting with TIMP3 and causing arrest of cell growth as a consequence of low levels of cyclin D1 [211].

ITGA7 has also been associated with chemotherapy resistance however, not in the context of breast cancer. Ming et al concluded that ITGA7 gives rise to chemoresistance in oesophageal squamous cell carcinoma (OSCC). OSCC cells within the cisplatin resistance group demonstrated the capability to form spheroids in comparison to the tumour cells within the control group as well as an increased expression of CSC associated genes [206].

1.9.3. NR4A1

The orphan nuclear receptor 4A1 (NR4A1) belongs to the nuclear receptor family of which there are 48 known human receptors. NR4A1 acts as a transcription factor that regulates downstream gene expression and plays a key role in a wide range of actions such as cell growth and differentiation, migration, DNA repair and apoptosis. NR4A receptors comprise of three domains, an N-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) and are classified as orphan as their endogenous ligands have not yet been identified, which may make it more difficult to consider NR4A1 as a target for targeted therapy approaches [212].

Within the current literature NR4A1's involvement in carcinogenesis is inconsistently reported. Numerous studies have described NR4A1 as a tumour suppressor. Wu et al performed immunohistochemical quantification of NR4A1 expression on TMAs comprising of 148 TNBC cases and 60 normal breast tissue cases and concluded that a downregulation of NR4A1 expression in TNBC cases correlated with a higher tumour stage, metastatic axillary lymph nodes and shorter DFS. Whereas, restored NR4A1 expression in the TNBC cell line MDA-MB-231 resulted in a significantly lower rate of proliferation, survival and invasion of these cells in tissue culture, which was considered to be most likely a consequence of the reduced action of the JNK-AP-1-cyclin D1 pathway [213]. Alexopoulou et al also concluded a decrease in NR4A1 expression corresponded with an increase in tumour grade and metastasis of both lobular and invasive ductal breast cancers, and it was hypothesised that NR4A1 plays a role in adhesion and migration of tumour cells. Ectopic expression of NR4A1 in two cell lines, PMC42 and ZR-75-1 could prevent cell migration while propagation and apoptosis of cells was not affected [214]. Also, Mullican et al performed knockout of NR4A1 in mice, resulting in the development of acute myeloid leukaemia [215].

In contrast, a study by Zhou et al found that NR4A1 expression encouraged tumour invasion and disease progression in breast cancer by initiating TGF-β/SMAD signalling. TGF-β functions to contain tumour growth and restore homeostasis in both normal and pre-cancerous cells via regulating the arrest of cells and apoptosis. However, progression to invasive cancer occurs when there is deregulation of TGF-β signalling and consequently tumour cells evade the tumour suppressive activity of TGF-β. NR4A1 has also been shown to encourage cell motility, tumour invasion and progression via TGF-β-induced epithelial to mesenchymal transition [216]. Similarly, Hedrick and Safe support these findings but also report that TGF-β induces MAPK14 which is crucial in EMT in TNBC, [217]. Further studies in ER-positive MCF-7, ER-negative MDA-MB-231 and SKBR3 breast cancer cell lines have shown that NR4A1 has a role in regulation of β1-integrin expression, as an increase in expression is associated with a poor DFS and OS [218]. NR4A1 overexpression has also been reported in ovarian, pancreatic and colon tumours. Similarly, NR4A1 has also been shown to regulate β1-Integrin expression in both colon and pancreatic tumours and is associated with a poor prognostic outcome due to its involvement in cell migration, adhesion and invasion [219]. Delgado et al found NR4A1 to be overexpressed in serous ovarian tumours of high grade which was associated with a poor PFS [220].

Again, there is paucity in the literature with regards to the role of NR4A1 in chemotherapy resistance in breast cancer. However, a study by Wilson et al reported that a low nuclear expression of NR4A1 correlated with cisplatin resistance in ovarian cancer due to abnormal nuclear sequestration, which can be partly suppressed by activation of NR4A1 phosphorylation and export from nucleus to cytoplasm [221].

1.10. HYPOTHESIS AND AIMS

My hypothesis is that levels of expression of specific proteins in breast cancers control chemotherapy response and could potentially be used as predictive markers to guide selection of appropriate treatment regimens.

Specific aims:

1. To identify a large cohort of patients diagnosed with primary breast cancer and treated with adjuvant chemotherapy and collect cancer tissue and extensive clinico-pathological data, including survival follow-up.

2. To examine expression of candidate proteins IFNβ1, MX1, ITGA7 and NR4A1 using immunohistochemistry and assess correlations with chemotherapy response as measured by disease-free survival and disease-specific survival.

3. To investigate whether NR4A1 is a predictive or prognostic marker using the molecular taxonomy of breast cancer international consortium (METABRIC) data set for breast cancer.

2. MATERIALS AND METHODS

2.1. Ethical approval

Ethical approval was granted from Leeds (East) Research Ethics Committee reference 06/Q1206/180 (Appendix 9.1) to enable retrieval of breast cancer tissue from the pathology tissue block archive at St James's University Hospital (Leeds Teaching Hospital NHS Trust [LTHT]) and clinical data from patient notes or hospital databases.

2.2. Selection of cohort cases

Cohort 1 – Adjuvant chemotherapy

360 patients diagnosed with primary invasive breast cancer between 2006 to 2010 who underwent surgical resection and were treated with adjuvant chemotherapy at LTHT were identified from hardcopy diaries kept by the Breast Oncology Department. Comprehensive data including patient demographics (age at diagnosis, date of diagnosis, breast laterality), pathological data (tumour histological subtype, tumour grade, hormone and HER2 receptor status as well as nodal status), surgical data (date of surgery and surgical procedure performed), oncological data (chemotherapy regimen, radiotherapy, endocrine treatment) outcome data (progression of disease to, local and/or metastatic recurrence with dates) were collected from pathology reports, breast multi-disciplinary team meeting records, and breast surgery and oncology clinical letters using the LTHT computer database, Patient Pathway Manager (PPM). Exclusion criteria were; individuals who received NACT or who had not received adjuvant chemotherapy, individuals with metastatic breast cancer, individuals with a recurrence in breast cancer with primary breast cancer being diagnosed prior to 2006 and males with breast cancer. Disease-free survival (DFS) was defined as the time between the diagnosis date and either the date of occurrence of either locoregional or distant metastasis, or for patients without an event, the most recent disease-free follow-up evidence. Whereas, disease-specific survival (DSS) was defined as the time between the diagnosis date and either the date of death from breast cancer or for patients without an event, the most recent follow-up evidence. Following retrieval of tumour slides and corresponding Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks, 305 cases were suitable for Tissue MicroArray (TMA) construction. The clinical and pathological data for these patients are summarised in Table 2.1.

Characteristics	n=305 (100%)
Age: Median 52 (range 25-74) years	
Follow-up: Median 120.4 (range 11-166.4) months	
Tumour Histopathology	
Ductal NST	222 (72.8)
Lobular	23 (7.5)
Metaplastic	6 (2.0)
Other	6 (2.0)
Mixed	48 (15.7)
Tumour Grade	
1	18 (5.9)
2	124 (40.7)
3	163 (53.4)
Lymph Node Status	
N0	117 (38.4)
N1	120 (39.3)
N2	42 (13.8)
N3	26 (8.5)
Molecular Subtype	
Luminal A	91 (29.8)
Luminal B (HER2+/ER+, HER2-/ER+)	116 (38.0)
Triple Negative	68 (22.3)
HER2 Positive enriched	30 (9.8)
Chemotherapy Regimens	
Endocrine Therapy	190 (62.3)
Anti-HER2 Therapy	59 (19.3)
Anthracycline based	
- without Taxanes	149 (48.9)
- with Taxanes	116 (38.0)
- with others	40 (13.1)

Table 2.1 Summary of the clinico-pathological and pharmacological features for a cohort of adjuvant chemotherapy treated breast cancers

Cohort 2 – Triple negative cases

A cohort of 109 patients with triple negative primary breast cancer diagnosed between 2007 and 2013 at LTHT who underwent surgical resection and received chemotherapy was provided through collaboration with Dr Laura Wastall (Consultant Histopathologist, LTHT). 22 of these patients also appear in cohort 1 due to the dates overlapping. Patients were identified from PPM the LTHT computer database. Inclusion criteria were defined as triple negative molecular classification, absence of neoadjuvant therapies, adequate archival tumour tissue availability and at least 2 months of follow up data at the point of data collection. Exclusion criteria were; individuals who presented with metastatic breast cancer, individuals with a recurrence in breast cancer with primary breast cancer being diagnosed prior to 2007 and

males with breast cancer. The clinical and pathological data for these patients are summarised in Table 2.2.

Characteristics	n=109 (100%)
Age: Median 57.7 (33-90) years	
Follow-up: Median 65.6 (2.1-123.2) months	
Tumour Histopathology	
Ductal NST	96 (88.1)
Lobular	1 (0.9)
Mixed	3 (2.8)
Metaplastic	2 (1.8)
Adenoid cystic	2 (1.8)
Neuroendocrine	1 (0.9)
Clear cell	1 (0.9)
Medullary	2 (1.8)
Inflammatory	1 (0.9)
Tumour Grade	
1	1 (0.9)
2	14 (12.8)
3	91 (83.5)
Unknown	3 (2.8)
Lymph Node Status	
N0	69 (63.3)
N1	25 (22.9)
N2	9 (8.3)
N3	6 (5.5)
Chemotherapy Regimens	
Anthracycline-based	
- without taxanes	56 (51)
- with taxanes	42 (39)
- with others	5 (5)
Non anthracycline-based	6 (6)

Table 2.2. Summary of the clinico-pathological and pharmacological features for a Triple Negative cohort of breast cancers treated with adjuvant chemotherapy

2.3. Tissue MicroArrays (TMAs)

For cohort 1, haematoxylin/eosin stained tumour slides of 305 patients with primary breast cancer that were treated with adjuvant chemotherapy were retrieved from the pathology files, reviewed and marked microscopically to select areas of tumour representative of the overall tumour histology and avoiding areas of necrosis and representing central and peripheral regions where possible, by the author, Dr Eldo Verghese and Dr Rebecca Millican-Slater (Consultant Breast histopathologists, LTHT). The corresponding paraffin embedded blocks of resected breast tumours were identified and collected. TMAs were then constructed by the author, using a manual tissue microarrayer (MTA1; Beecher Instruments, USA). Three 0.6mm tissue cores of tumour were obtained from the marked representative tumour regions (central

and peripheral where feasible) within each paraffin embedded block and placed into a TMA donor block, with 1.0mm intervals between cores. The entire cohort was spread across five separate TMA blocks. Recipient blocks also contained control tissues of placenta, liver, appendix, spleen, pancreas, testes, tonsil, brain and lung, arranged in such as pattern so as to aid identification of each of the 5 TMAs. The tumour cores were also surrounded by a 'wall' of non-tumour cores on all sides of the grid. TMA blocks 1 to 4 each comprised of 70 cases (210 cores) of breast tumour. TMA 5 comprised of 25 cases (75 cores) of breast tumour. From each TMA block, 5µm sections were cut using a microtome (RM2255; Leica Wetzlar, Germany) and placed onto Superfrost Plus slides (ThermoFisher Scientific; Massachusetts, USA) for IHC.

For cohort 2, the TMAs of the 109 triple negative primary breast cancer cases that received chemotherapy were obtained through collaboration with Dr Laura Wastall (Histopathologist, LTHT). These TMAs had been constructed previously by her, as described above.

2.4. Selection of appropriate antibodies

Antibodies were selected based upon the evidence of specificity stated in the manufacturers' data published on their website (Thermofisher and Cell Signaling Technology) and/or in published papers.

There are several polyclonal antibodies available for NR4A1. However, PA5-32949 was chosen to detect NR4A1 due to its validated use in IHC [222]. Similarly, PA5-20390 a polyclonal antibody was chosen to detect IFNβ1 as it has been validated for IHC [223] and by the presence of a single protein band of 17kDa in western blot experiments [224]. Similarly, the polyclonal antibody # 37849S was chosen to detect MX1 as it has been widely used for immunohistochemistry in breast cancer tissue and again by the presence of a single protein band of 76kDa in western blot experiments [225]. Finally, Claudin 3 polyclonal antibody PA5-16867 was chosen as this antibody underwent enhanced verification of its specificity by siRNA facilitated knockdown of the target protein. MCF-7 cell lines were transfected with Claudin 3 siRNA, and loss of the single protein band of 18kDa was confirmed using Western Blot analysis. IHC analysis of PA5-16867 also showed the expected membrane/cytoplasmic staining, compared with a negative control without primary antibody [226].

All 5 antibodies used are shown in Table 2.3.

Antibody	Type	Code and Manufacturer	Optimised Primary Antibody Concentration
NR4A1	Rabbit polyclonal	PA5-32949, ThermoFisher Scientific (Massachusetts, USA)	1:75
ITGA7	Rabbit polyclonal	ThermoFisher Scientific (Massachusetts, USA)	1:100
IFNβ1	Rabbit polyclonal	PA5-20390, ThermoFisher Scientific (Massachusetts, USA)	1:800
MX1	Rabbit polyclonal	# 37849S, Cell Signaling Technology (Leiden, Netherlands)	1:50
Claudin 3	Rabbit polyclonal	PA5-16867, Invitrogen, ThermoFisher Scientific (Massachusetts, USA)	1:500

Table 2.3. Antibodies selected for immunohistochemistry

2.5. Optimisation of antibodies

Antibodies for use in IHC were optimised by the author using off-cuts of the TMAs that were assembled to represent the primary breast tumours that received adjuvant chemotherapy (Cohort 1). Negative controls omitted the primary antibody; these produced no staining. A range of antibody concentrations were used to obtain appropriate tissue location staining and low background staining. Optimisation slides were reviewed by breast histopathologists Dr Eldo Verghese and Professor Andrew Hanby (Consultant Breast Histopathologists, LTHT) and the desired concentration of antibodies for optimal staining are shown in Table 2.3.

2.6. ImmunoHistoChemistry

Immunohistochemistry was performed by the author according to the following steps. TMA slides were dewaxed with xylene (3 changes, 5min each), and rehydrated with absolute ethanol (3 changes, 1min each). This was followed by a wash in running tap water for 5min. Antigen retrieval was performed by immersing slides in 750ml 10mM citric acid buffer, pH 6.0 (adjusted with 1M NaOH if needed) and heating using a 900W microwave. The citric acid

buffer was pre-warmed in a pyrex dish for 2min at high power and the slides were then placed in the citric acid buffer and heated for 10min at high power. The slides were then left to cool for 20min at room temperature and transferred to running tap water for 5min. Next, slides were placed in 200ml of methanol mixed with 2ml of 30% v/v hydrogen peroxide and incubated for 10min at room temperature, to block endogenous peroxidase activity. The slides were next washed in running tap water for 5min, followed by rinsing with Tris-Buffered Saline (TBS). Next, slides were transferred to a humidified chamber and 100µl of antibody diluent reagent solution (ThermoFisher; Massachusetts, USA) was added for 5min. Antibodies shown in Table 2.3 were separately added to slides at the concentrations listed in the table (diluted in Antibody diluent reagent) to cover the entire tissue section and incubated overnight at 4^{o}C. Antibody diluent reagent only was added for the no primary antibody controls. Following overnight incubation, slides were washed with TBS-T (0.1% Tween 20; Sigma; St Louis, USA) twice for 5min and TBS twice for 5min. Next, 100µl of SignalStain Boost IHC detection Reagent (HRP, Rabbit) (Cell Signalling Technology; Massachusetts, USA) was applied to the slides and left at room temperature for 30min. Slides were then washed with TBS-T and TBS as previously. Next, 100µl of SignalStain DAB substrate working solution (Cell Signalling Technology; Massachusetts, USA) was added at room temperature for 5min and slides washed in running tap water for a further 5min. Mayer's Haematoxylin was used to counterstain the slides for 1min, followed by washing in running tap water for 1min. Slides were then placed in Scott's water for 1min and then again in running tap water for 1min. Slides were then dehydrated through absolute ethanol (3 changes, 1 minute each) and through xylene (3 changes, 1 minute each). Lastly, slides were mounted under coverslips with DPX (Fluka; Gillingham, UK) and left overnight at room temperature. Stained sections were scanned digitally using ScanScopeXT at x20 magnification and were manually scored using Webscope (Aperio; Vista, CA, USA).

2.7. Scoring protocol

Scoring protocols to score expression for every antigen were developed in discussion with Prof Andrew Hanby and Dr Rebecca Millican-Slater (Consultant Breast Pathologists, LTHT). Scoring was performed remotely using scanned slides accessed through the internet. It was found that NR4A1 and ITGA7 located both in the nucleus and cytoplasm of cancer cells. For NR4A1, scoring of epithelial cancer cells was based on cytoplasmic intensity (1-3); 1 being weak, while 3 represented strong staining and the proportion of cells showing nuclear staining (0-4); (0=0%, 1=1-5%, 2=6-25%, 3=26-75%, 4=>75%). For ITGA7, scoring was based on cytoplasmic and nuclear staining, intensity (0-3), 0 being negative, 1 being weak, 2 intermediate and 3 strong and proportion (0-5); (0=0%, 1=<1%, 2=1-10%, 3=11-33%, 4=34-

66% and 5=67-100%). For IFNβ1, staining was present in stromal fibroblasts and sometimes in cancer cells. Intensity of staining of fibroblasts was scored as (1-3); 1 being weak, 2 moderate and 3 strong. For MX1, staining was noted in the cytoplasm of cancer cells. Cytoplasmic intensity in tumour cells was scored as 0-3; 0 being negative, 1 weak, 2 moderate and 3 strong. For both IFNβ and MX1, only intensity was scored as the proportions of cells staining at these intensities were consistently the vast majority of cells therefore, proportion was not informative. For claudin-3, staining of cytoplasm/membrane of tumour cells was scored as negative (0) or positive (1), in order to support a definition of claudin-low (negative) or claudin-high (positive).

For all antibodies except claudin-3, scores of individual cases were averages of the cores scored for that case, and expression was dichotomised into high and low groups using Receiver Operator Characteristic (ROC) analyses [227]. For claudin-3, all cores within each case were consistently positive or negative, therefore dichotomisation was simply positive in all or negative in all.

The whole cohort was scored by the author following a training period by Prof. Andrew Hanby, and 10% of the tissue cores were identified for independent scoring by Prof. Hanby or Dr Millican-Slater to allow statistical analysis of scoring reproducibility.

2.8. cBioPortal for cancer genomics

Firstly, cBioPortal was accessed via https://www.cbioportal.org/ [228].

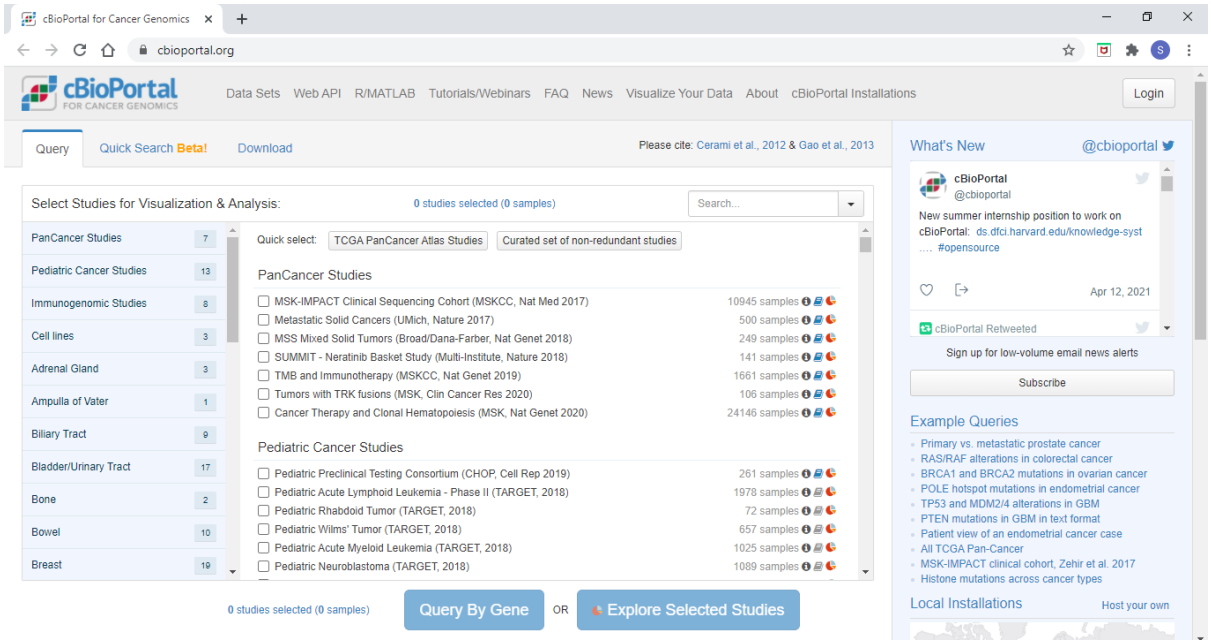


Figure 2.1 Representative image of the Web page from cbioportal

A dataset was then loaded by selecting Breast on the left-hand column and the dataset of interest (Breast cancer-METABRIC).

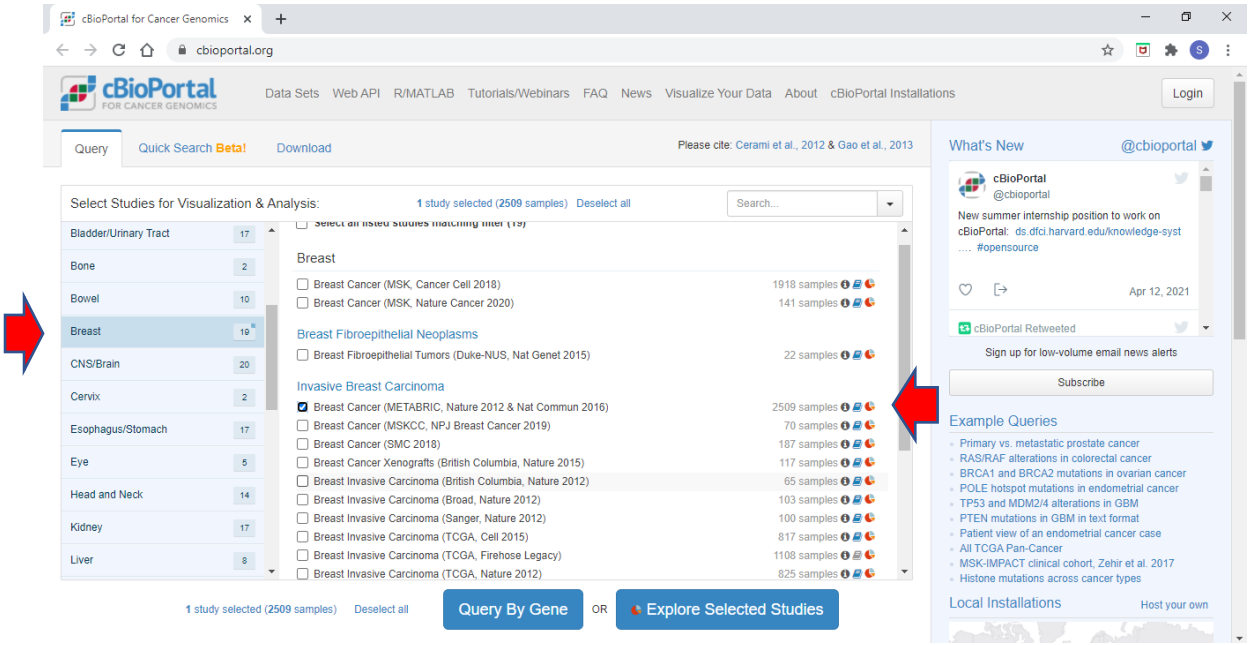


Figure 2.2. Representative image of dataset selected

Next, clicking on the pie chart of the dataset of interest allowed for the customisation of the layout and selection of the parameters of interest by clicking on “CHARTS”.

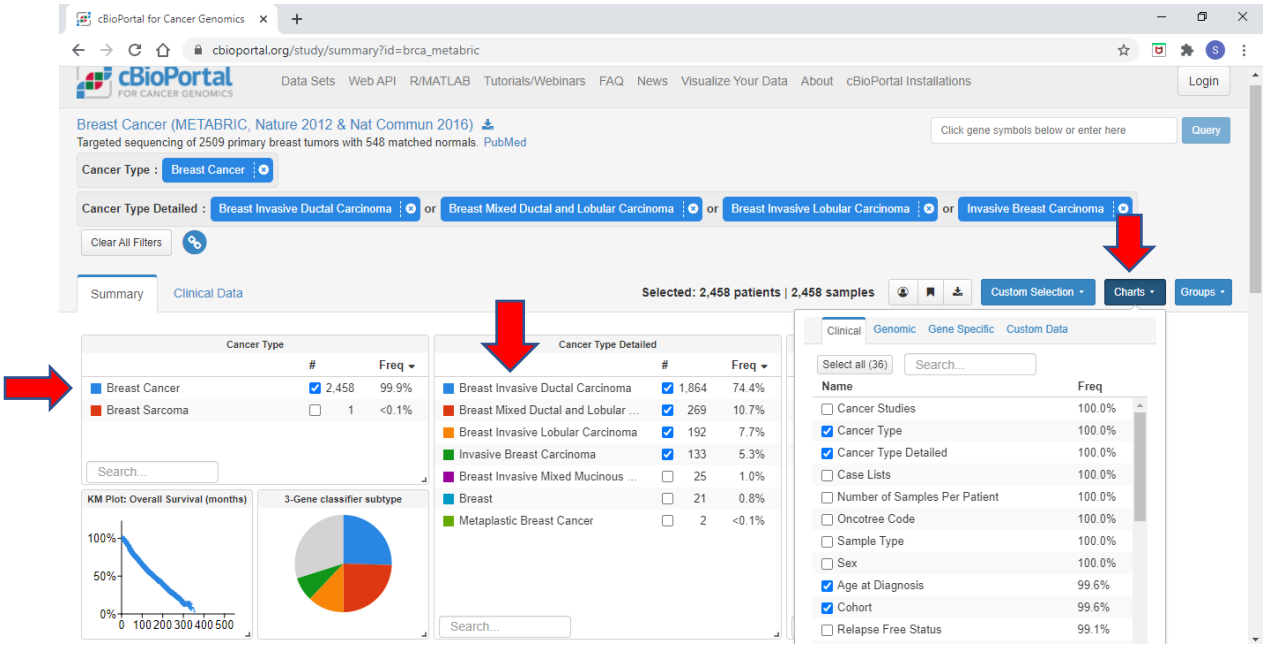


Figure 2.3. Representative image of how to customise the layout and selection of the parameters of interest within the dataset

Once the appropriate filter(s) from “CHARTS” were selected (cancer type, age at diagnosis, ER, PR and HER2 status, tumour grade, lymph node status and chemotherapy and survival) it was possible to visualize pie charts for those filter(s) on the main page.

The genes of interest were inserted into the search bar and ‘query’ clicked.

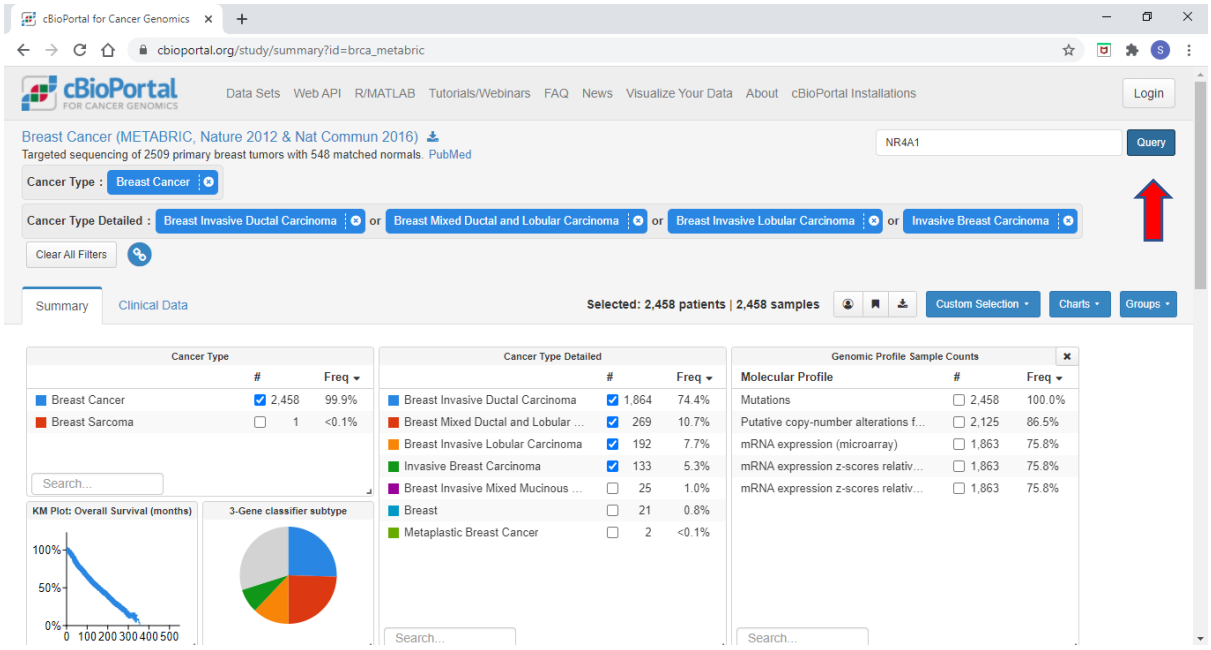
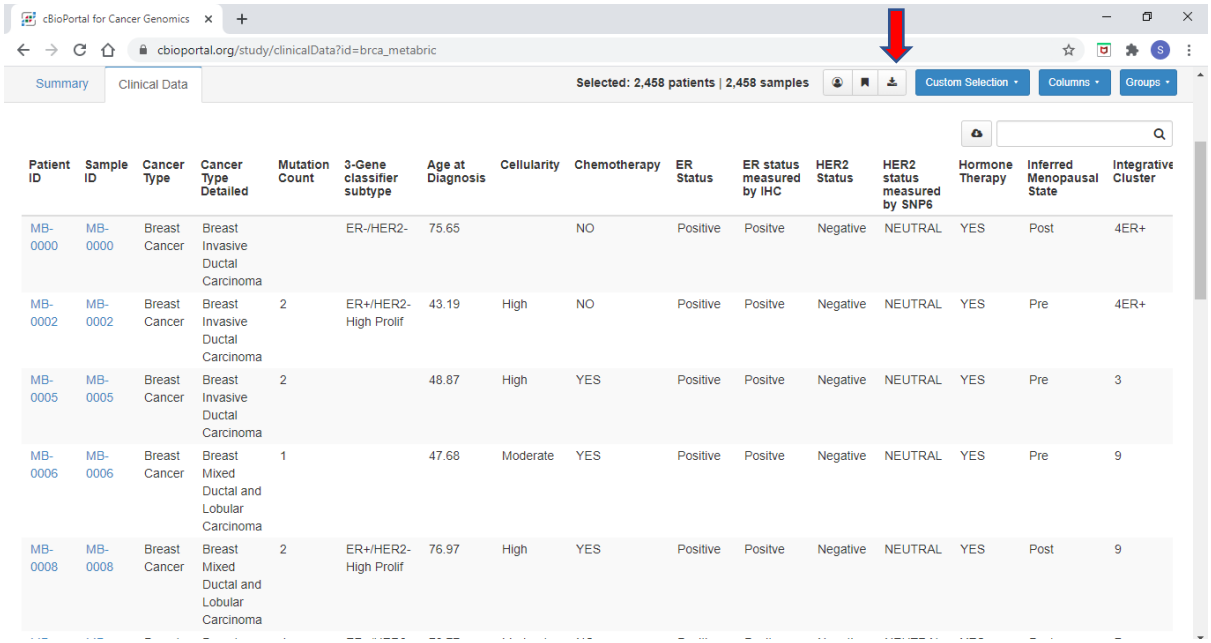


Figure 2.4. Representative image of how to select gene of interest

The Download button was then pressed to download the data regarding the gene of interest. The following list of files then appeared: Copy-number Alterations (OQL is not in effect), Mutations (OQL is not in effect), Altered samples: List of samples with alterations. Unaltered samples: List of samples without any alteration, Sample matrix: List of all samples where 1=altered and 0=unaltered, Capped relative linear copy-number values, mRNA expression (microarray), mRNA expression z-scores relative to diploid samples (microarray), microRNA expression. microRNA expression Z-scores, mRNA/miRNA expression Z-scores (all genes), mRNA expression z-scores relative to all samples (log microarray), Methylation (HM27), Protein expression (RPPA). Based upon the data required the “Tab Delimited Format” button was then clicked.

Next, to download the clinical data required the symbol  on the top right of the main page was clicked.



Patient ID	Sample ID	Cancer Type	Cancer Type Detailed	Mutation Count	3-Gene classifier subtype	Age at Diagnosis	Cellularity	Chemotherapy	ER Status	ER status measured by IHC	HER2 Status	HER2 status measured by SNP6	Hormone Therapy	Inferred Menopausal State	Integrative Cluster
MB-0000	MB-0000	Breast Cancer	Breast Invasive Ductal Carcinoma		ER-/HER2-	75.65		NO	Positive	Positve	Negative	NEUTRAL	YES	Post	4ER+
MB-0002	MB-0002	Breast Cancer	Breast Invasive Ductal Carcinoma	2	ER+/HER2- High Prolif	43.19	High	NO	Positive	Positve	Negative	NEUTRAL	YES	Pre	4ER+
MB-0005	MB-0005	Breast Cancer	Breast Invasive Ductal Carcinoma	2		48.87	High	YES	Positive	Positve	Negative	NEUTRAL	YES	Pre	3
MB-0006	MB-0006	Breast Cancer	Breast Mixed Ductal and Lobular Carcinoma	1		47.68	Moderate	YES	Positive	Positve	Negative	NEUTRAL	YES	Pre	9
MB-0008	MB-0008	Breast Cancer	Breast Mixed Ductal and Lobular Carcinoma	2	ER+/HER2- High Prolif	76.97	High	YES	Positive	Positve	Negative	NEUTRAL	YES	Post	9

Figure 2.5. Representative image of how to download the clinical data

2458 sample data was retrieved but only 1886 were analysed due to insufficient data. Spearmans Rho analysis for correlation and Kaplan-Meier analyses for survival were performed in statistical package for the social sciences (SPSS) (version 26) for the whole cohort, breast cancer subtypes (ER-positive, ER-negative, HER2-positive and triple negative) and those that did and did not receive chemotherapy.

2.9. Statistical analysis

Cohen's Kappa statistics for inter-scorer concordance, ROC curves analysis, Spearman's rank correlation coefficient and Kaplan-Meier (KM) survival analyses were performed using IBM SPSS (version 26). Bar graphs were made with Microsoft Excel. P values less than or equal to 0.05 were deemed to be statistically significant.

3. CONSTRUCTION OF A SINGLE-CENTRE RETROSPECTIVE COHORT OF BREAST CANCER PATIENTS TREATED WITH ADJUVANT CYTOTOXIC CHEMOTHERAPY

3.1. Abstract

Background: Electronic medical records are commonly used when collecting data in clinical research due to the availability and accessibility of high-quality data. Histopathology archives also provide a valuable resource of clinical tissue for translational research. The aim was to construct a cohort of patients diagnosed with breast cancer and treated with adjuvant chemotherapy and to validate the associated clinical data, by determining if the clinical prognostic markers of tumour grade, lymph node status and molecular subtypes of breast cancer and chemotherapy regimens corresponded with outcomes in terms of disease-free survival and disease-specific survival.

Methods: A single-centre retrospective cohort of 305 women diagnosed with primary breast cancer and treated with adjuvant chemotherapy between 2006 and 2010 was constructed. Archival tumour tissue availability was confirmed. Data collection from electronic medical records comprised of patient demographics, pathological, surgical and oncological data, disease-free survival and disease-specific survival, with a follow-up period of between 11 to 166.4 months. Validation of tumour grade, lymph node status and molecular subtype as prognostic markers within the cohort was performed. Survival outcomes of different adjuvant chemotherapy regimens were also determined.

Results: Kaplan-Meier survival analyses were performed, which identified statistically significant better outcomes in terms of disease-free survival and disease-specific survival with breast cancers of lower grade and no nodal disease. Whereas, with molecular subtypes of breast cancer, only disease-specific survival was statistically significant, with triple negative breast cancers having the worse outcome followed by HER2-positive/ER-negative breast tumours and ER-positive breast cancers having the best outcome. Although, the same trend was followed for disease-free survival, the result was not statistically significant ($p=0.056$). Kaplan-Meier survival analyses were also performed separately on the patients who received either taxanes with anthracyclines ($n=116$), or anthracyclines without taxanes ($n=149$). The findings were of longer disease-free survival ($p=0.001$) and disease-specific survival ($p=0.001$) in the group of patients that received anthracycline based adjuvant chemotherapy only.

Conclusion: Following the identification of a large cohort of women diagnosed with primary breast cancer, treated with surgery and adjuvant chemotherapy and the validation of breast

cancer prognostic markers with outcomes in terms of disease-free survival and disease-specific survival, I constructed TMAs for use in subsequent analysis of specific candidate markers of chemotherapy response.

3.2. Introduction

Use of electronic medical records (EMR) is common when collecting data in clinical research due to the availability of high-quality data and the easy accessibility to clinicians [229]. Data contained within EMR are often used as the only source of information, or alternatively can be used to supplement other sources of data extraction such as questionnaire surveys. EMR are invaluable resources that record patients' demographics, medical history, laboratory, imaging, histopathology findings and clinical appointments in a chronological order that can be used to track the clinical course of both inpatients and outpatients [230]. However, extracting data from EMR does come with some disadvantages, the main one being it can become very time consuming to find and extract the data from different databases within the EMR. Also, in some instances there may be incomplete data or poor accuracy in the way the data is recorded, extracted and collected by healthcare professionals as reliability and validity checks are seldom performed. However, with the increase in usage of EMR, the movement away from the more old-fashioned handwritten records and the increase in use of coding and standardised entry of data, there has been a reduction in the recording of inaccurate data which has improved data extraction efforts [231].

I, therefore aimed to identify a large cohort of women diagnosed with primary breast cancer within Leeds Teaching Hospitals NHS Trust (LTHT), treated with adjuvant chemotherapy, and for whom tumour samples were available from histopathology archives, and to validate the clinical data obtained from the cohort prior to constructing TMAs containing the tumour tissues.

3.3. Results

3.3.1. Construction of a cohort of patients with primary breast cancer treated with adjuvant chemotherapy

My main aim was to identify a large cohort of women diagnosed with primary breast cancer and treated with adjuvant chemotherapy with an adequate follow-up time to determine outcome measures of DFS and DSS against four potential markers of chemotherapy response, IFNβ1, MX1, ITGA7 and NR4A1.

My approach was firstly to arrange a meeting with the breast oncology nurses as I had been informed that they keep handwritten hardcopy diaries of patients who receive chemotherapy for primary breast cancer in chronological order, which included the names of the patients, hospital unit numbers and chemotherapy regimens received. To allow sufficient time post-treatment for determination of meaningful DFS and DSS, I opted for the diaries that included patients that were diagnosed with primary breast cancer and received their adjuvant chemotherapy between 2006 and 2010. Exclusion criteria were individuals who received NACT, individuals with metastatic breast cancer, individuals with recurrences of breast cancer with their primary breast cancer being diagnosed prior to 2006, and males with breast cancer. Between 2006 and 2010, 542 patients were recorded in the diaries as having been diagnosed with primary breast cancer and having been seen by the oncology nurses. Figure 3.1 shows how this number was reduced to 379 patients, for whom I proceeded to data collection from the EMR. Comprehensive data including patient demographics (age at diagnosis, date of diagnosis, breast laterality), pathological data (tumour histological subtype, tumour grade, hormone receptor and HER2 status as well as lymph node status), surgical data (date of surgery and surgical procedure performed), oncological data (chemotherapy regimen, radiotherapy, endocrine treatment), outcome data, (progression to local and metastatic recurrence) were collected from operation notes, histopathology reports, breast multi-disciplinary team meeting records, breast surgery and oncology clinical letters using the LTHT computer database, PPM. DFS was defined as the time between the diagnosis date and the occurrence of either locoregional or distant metastasis and for those patients without an event, the last disease-free follow-up appointment. 19 patients were lost to follow-up with 12 patients moving to different parts of the country or abroad and 7 patients received their treatment in the private sector and therefore, data was not accessible. 360 patients were taken to the next stage, which was cellular pathology block and slide retrieval from the Department of Cellular Pathology. From looking at the histopathology report on PPM for each patient, up to 5 cellular

pathology slides and corresponding blocks containing the tumour were requested. 33 patients were then excluded due to cellular pathology block and/or slide being unavailable and a further 22 patients were found to be unsuitable as the cellular pathology slides did not contain suitable invasive cancer cells after review by Dr E. Verghese (Consultant Breast Histopathologist, LTHT). My final patient cohort comprised of 305 women diagnosed with primary invasive breast cancer between 2006 to 2010 who underwent surgical resection and treatment with adjuvant chemotherapy at LTHT (Figure 3.1). A summary of the clinico-pathological and pharmacological features of the cohort of primary breast cancers treated with adjuvant chemotherapy is shown in Chapter 2, Table 2.1, with a median follow-up of 120.4 months. Within this time frame, 22% of women had recurrences and 19% died from breast cancer.

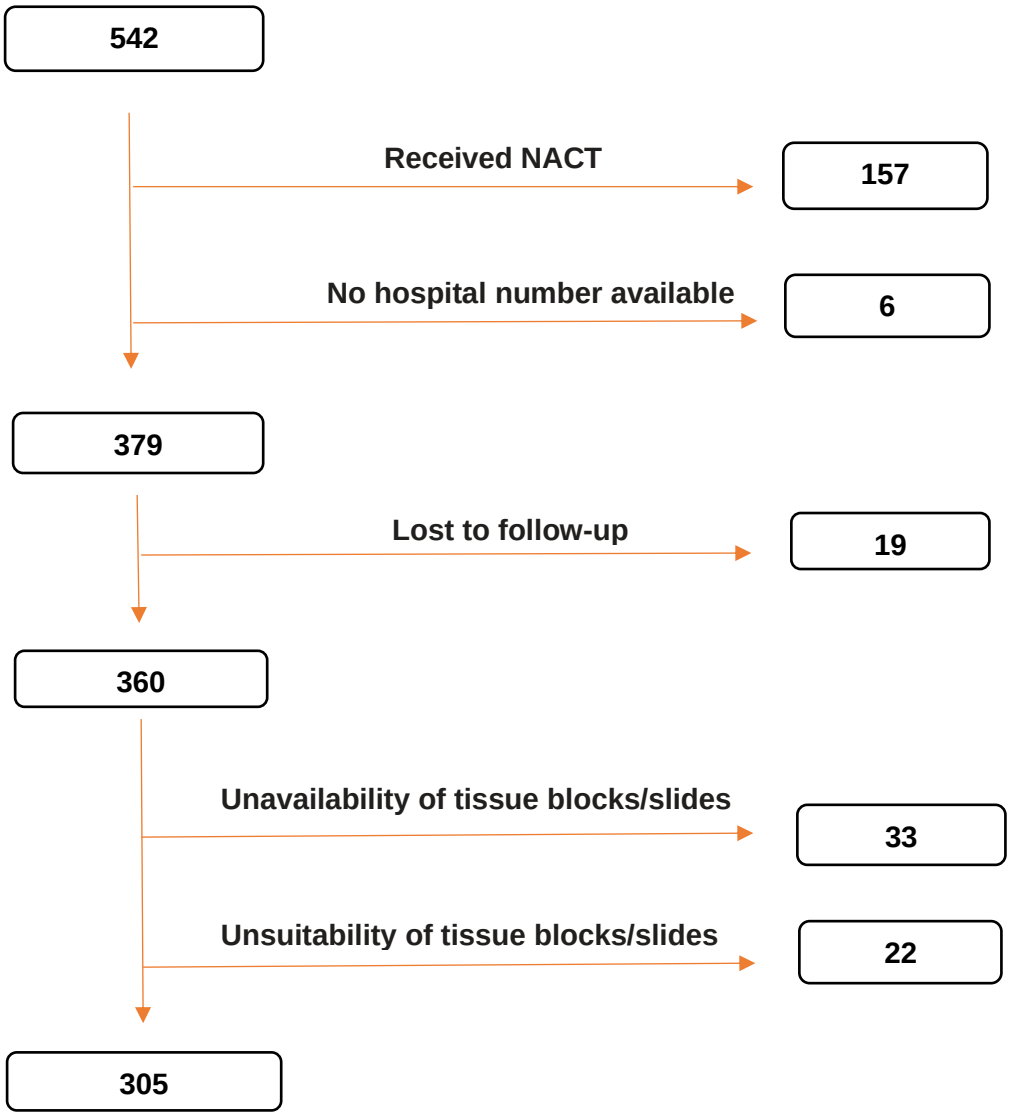


Figure 3.1 A flow diagram outlining case selection, starting from 542 breast cancer patients diagnosed within LTHT and treated with chemotherapy, and narrowing down to a final cohort of 305 cases

3.3.2. Validation of tumour grade, lymph node status and molecular subtype as prognostic markers in a retrospective cohort of patients diagnosed with breast cancer and treated with adjuvant chemotherapy

My next aim was to validate the clinical data, by determining if the clinical prognostic markers of tumour grade, lymph node status and molecular subtypes of breast cancer corresponded with outcomes in terms of DFS and DSS. To determine whether these prognostic markers were significantly associated with either DFS, for which the event was local or distant recurrence, or DSS, for which the event was death from breast cancer, Kaplan-Meier survival analyses were performed. Tumour grade was found to correlate significantly with both DFS and DSS, with lower grade having improved survival (Table 3.1 and Figure 3.2). Lymph node status was found to correlate significantly with DFS only, when assessed simply as positive or negative for metastases (Table 3.2 and Figure 3.3), but with both measures of survival (DFS and DSS) when assessed more quantitatively using the clinical definitions of N0 through to N3 (Table 3.3 and Figure 3.4). Whereas on analysis of, the molecular subtypes of breast cancer, only DSS showed a statistically significant influence, with triple negative breast cancers having the worse outcome followed by HER2-positive/ER-negative breast tumours and ER-positive breast cancers having the best outcome (Table 3.4, Figure 3.5). The same trend was followed for DFS, however the result was not statistically significant (p=0.056).

Tumour Grade	Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
Grade 1	4107 (3569-4645)	0.035*	4420 (4025-4816)	0.004*
Grade 2	4214 (4002-4425)		4340 (4156-4524)	
Grade 3	3678 (3432-3923)		3802 (3583-4020)	

Table 3.1 Comparison of mean disease-free survival (DFS) and disease-specific-survival (DSS) between tumour grades; Grade 1 (n=18), Grade 2 (n=124) and Grade 3 (n=163) in breast cancer. 95% confidence intervals and log rank p values stated. *represents significant log rank test (p≤0.05)

A. **B.**

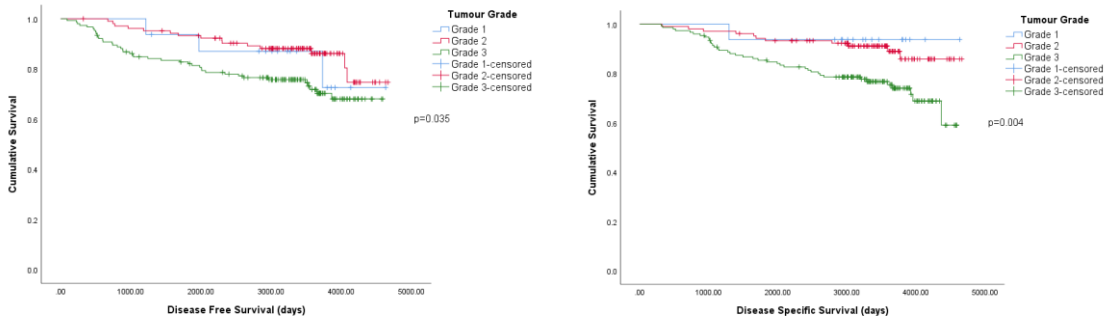


Figure 3.2 Kaplan-Meier survival analysis of tumour grade in patients with breast cancer treated with adjuvant chemotherapy. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient. Grade 1(n=18), Grade 2 (n=124) and Grade 3 (n= 163)

Lymph Node Status	Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
Negative	4203 (3993-4413)	0.007*	4240 (4048-4432)	0.068
Positive	3751 (3518-3985)		3952 (3746-4158)	

Table 3.2 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between negative (n=117) and positive (n=188) lymph node status in breast cancer. 95% confidence intervals and log rank p values stated. *represents significant log rank test (p≤0.05)

A. **B.**

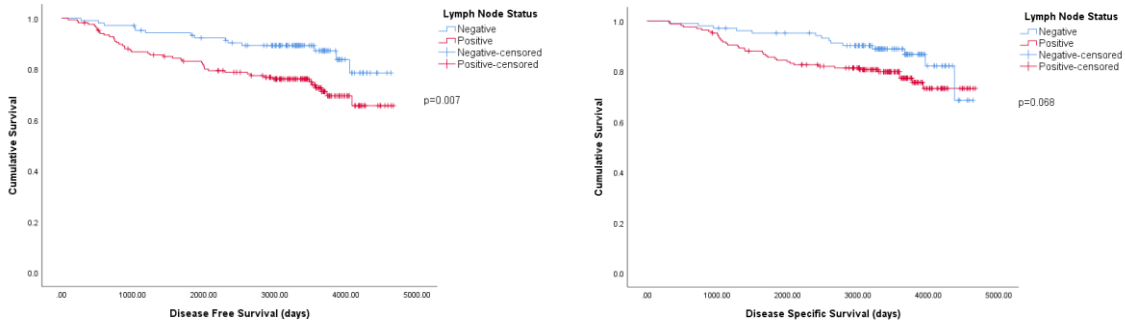


Figure 3.3 Kaplan-Meier survival analysis of lymph node status in patients with breast cancer treated with adjuvant chemotherapy. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient. Negative lymph node status (n=117), positive lymph node status (n=188)

Lymph Node Status	Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
N0	4148 (3933-4363)	0.002*	4216 (4026-4406)	0.001*
N1	3890 (3642-4137)		4132 (3929-4336)	
N2	2923 (2414-3432)		3075 (2626-3524)	
N3	3918 (3375-4461)		3969 (3421-4517)	

Table 3.3 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between lymph node status; N0 (n=117), N1 (n=120), N2 (n=42) and N3 (n=26) in breast cancer. 95% confidence intervals and log rank p values stated. *represents significant log rank test (p≤0.05)

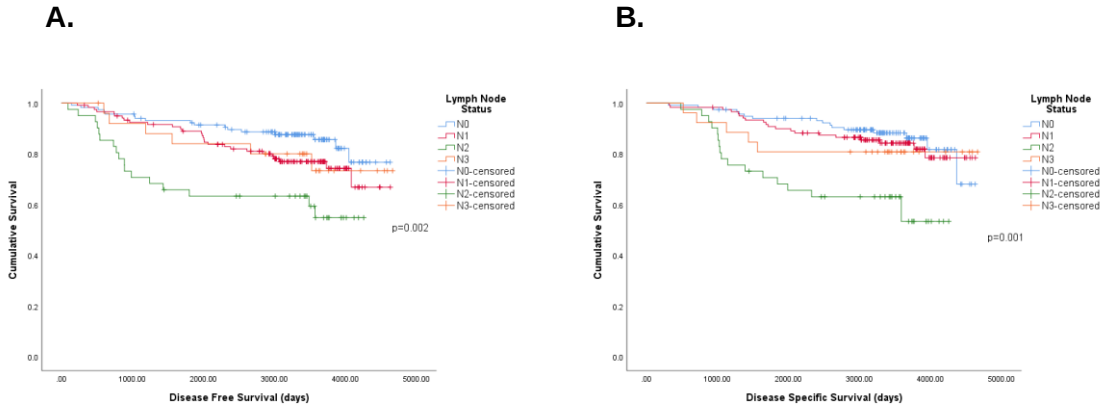


Figure 3.4 Kaplan-Meier survival analysis of N0, N1, N2 and N3 lymph node status in patients with breast cancer treated with adjuvant chemotherapy. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient.
N0 (n=117), N1 (n =120), N2 (n=42) and N3 (n=26)

Breast Tumour Subtypes	Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
ER-Positive	4154 (3977-4332)	0.056	4332 (4181-4482)	0.001*
Triple Negative	3465 (3033-3897)		3525 (3138-3912)	
HER2-Positive/ER-Negative	3636 (3767-4103)		3825 (3485.4-4164)	

Table 3.4 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between molecular subtypes; ER-Positive (n=207), Triple Negative (n=68) and HER2-Positive/ER-Negative (n=30) of breast cancer. 95% confidence intervals and log rank p values stated. *represents significant log rank test (p≤0.05)

A. **B.**

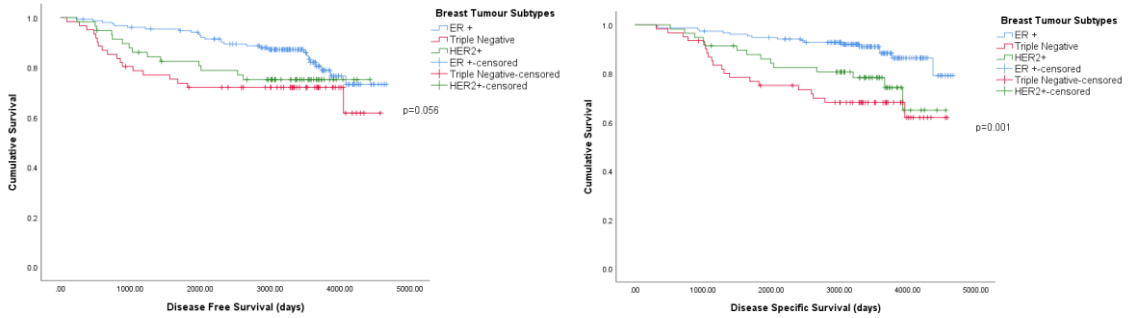


Figure 3.5 Kaplan-Meier survival analysis of ER-Positive (n=207), Triple Negative (n=68) and HER2-Positive/ER-Negative (n=30) molecular subtypes of breast cancer treated with adjuvant chemotherapy. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

3.3.3. Comparison of patient demographics and tumour characteristics of triple negative and the other molecular subtypes of breast cancer

Next, my aim was to perform a comparative analysis of the clinical features of the TNBCs compared to all the other histologies, as the triple negatives tend to have the poorest prognosis and are therefore of particular clinical interest. Patient demographics and breast tumour characteristics were compared between the triple negative subgroup and the other tumour subtypes using T-tests for means and chi squared tests for frequencies. 68 of 305 patients (22.3%) within the cohort were found to be triple negative breast tumours. The average age at diagnosis was slightly older for the triple negative group compared with the non-triple negative breast tumour subtypes (53.5 vs 51.9 years respectively), although this was not significant. Women with TNBCs were more likely to have grade 3 tumours (86.4% vs 47.7%; p=0.001) and showed a trend towards higher rates of recurrence (27.1% vs 20.1%; p=0.246) and being more likely to die from breast cancer than women that did not have the triple negative subtype (23.7% vs 17.3%; p=0.149).

3.3.4. Breast cancers treated with adjuvant anthracyclines and taxanes have poorer survival compared to those without taxanes

Since responses to chemotherapy are the subject of my subsequent work, I was also interested to determine whether survival outcomes correlated with different adjuvant chemotherapy regimens, independent of tumour subtype. Kaplan-Meier survival analyses were performed after separating the cohort into patients who received either taxanes with anthracyclines (n=116), or anthracyclines without taxanes (n=149). Use of taxanes with anthracyclines was associated with reduction in DFS of a mean of 836 days (p=0.001) and reduction in DSS of mean 738 days (p=0.001) as compared to anthracyclines without taxanes (Table 3.5 and Figure 3.6).

Chemotherapy Regimens	Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
Anthracyclines without Taxanes	4272 (4118-4426)	0.001*	4361 (4227-4495)	0.001*
Anthracyclines with Taxanes	3436 (3123-3750)		3623 (3339-3907)	

Table 3.5 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) of breast cancers that received anthracycline without taxane based adjuvant chemotherapy (n=149) compared with anthracycline with taxane based adjuvant chemotherapy (n=116). 95% confidence intervals and log rank p values stated. *represents significant log rank test (p≤0.05)

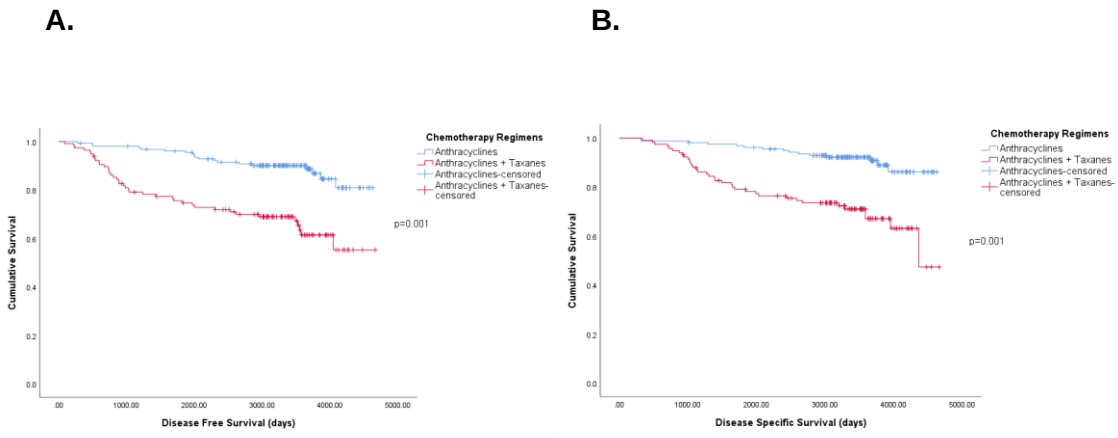


Figure 3.6 Kaplan-Meier survival analysis of breast cancers that received anthracycline without taxane based adjuvant chemotherapy (n=149) compared with anthracycline with taxane based adjuvant chemotherapy (n=116). Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

Both taxanes with anthracyclines, and anthracyclines without taxanes are established and effective treatment regimens [11], therefore I next compared the distributions of prognostic factors within these two groups in an effort to establish the potential cause of the poorer outcomes in the first group. To determine correlations between taxane chemotherapy regime and clinical prognostic markers, which comprised of tumour grade and lymph nodes status Chi squared analyses were performed. High tumour grade (grade 3) correlated with the use of taxanes with anthracyclines (68.1% vs 48.4%; p=0.001) as did positive axillary nodes (69% vs 56.1%; p=0.03). I concluded that anthracyclines with taxanes had been used to treat patients with poorer prognoses, potentially explaining the relatively poor outcomes in this group.

3.3.5. Construction of Tissue MicroArrays representative of 305 patients diagnosed with breast cancer and treated with adjuvant chemotherapy

Following the validation of the cohort, my aim was to construct tissue micro-arrays (TMAs). TMAs are a well-known method to evaluate the expression of protein markers on a large scale and their use can be both cost and time effective by saving on reagents and making the process of staining and scoring quicker [232]. Following retrieval of tumour slides and corresponding FFPE tissue blocks, 305 cases were suitable for TMA construction. Each case had three separate cores of breast cancer tissue from different areas within the breast tumour to achieve representation of intra-tumour heterogeneity, while additionally increasing the chances of having a successful core of breast tumour scored for each case, as a well-documented issue with TMA studies is the loss of tissue cores during the sectioning and staining process [232]. A representative image of a stained TMA slide is shown below (Figure 3.7).

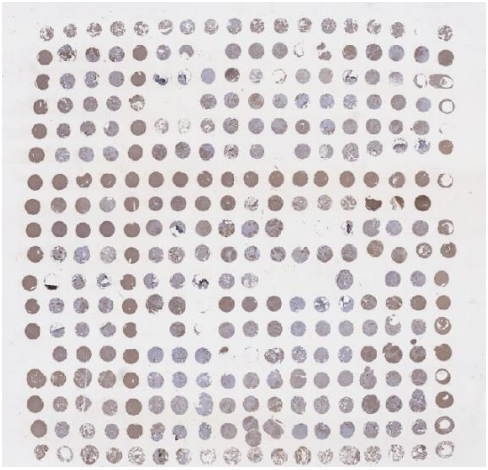


Figure 3.7 Representative image of a tissue microarray

3.4. Discussion

The aim of this chapter was firstly to construct a retrospective cohort of women diagnosed with primary breast cancer in a large teaching hospital in the UK that received adjuvant chemotherapy. Electronic medical records were used to obtain comprehensive data comprising of patient demographics (age at diagnosis, date of diagnosis, breast laterality), pathological data (tumour subtype, tumour grade, hormone receptor and HER2 status as well as lymph node status), surgical data (date of surgery and surgical procedure performed), oncological data (chemotherapy regimen, radiotherapy, endocrine treatment) and outcome data (progression to local and metastatic recurrence) on 305 patients from 2006 to 2010 with a follow up period of between 11 to 166 months.

Several multi-centre retrospective cohorts evaluating breast cancer outcomes have been documented in the literature [233, 234]. However, of the single-centre studies within the literature, a cohort comprising of 305 patients is similar in size. Gunasekaran et al who investigated the impact of modifications to the chemotherapy regimen in patients diagnosed with primary breast cancer in a retrospective single-centre study in a hospital in Malaysia over a 4-year period with a follow up period of 12-72 months had a much smaller sample size of 171 patients [235]. Whereas, a single-centre study by Oprean et al in Romania, comprised of a larger number of 721 patients over a 13-year period. However, the study focused only on post-menopausal women with breast cancer and performed correlation studies with regards to age, tumour location, stage of breast cancer, molecular subtype and living environment (rural vs urban), rather than assessing novel molecular markers [236].

My second aim was to describe patients breast cancer characteristics in terms of age at diagnosis, tumour histopathology, grade, lymph node status and molecular subtypes and to validate my clinical data by determining if the aforementioned clinical prognostic markers corresponded with outcomes in terms of DFS and DSS. In this study, women were diagnosed with breast cancer between 25 and 74 years with a mean age of diagnosis of 52 years. This is similar to a study evaluating survival outcomes in Iraqi women with breast cancer where the average age of diagnosis was 51 years [237] whereas, the AMAZONA retrospective cohort study evaluating the characteristics and prognosis of breast cancer in Brazil found a slightly higher mean age at diagnosis of 54 years [234]. Comparable with Al-Asadi and Al-Mayah the most common histopathological subtype was that of invasive ductal carcinoma in 72.8% of cases [237]. This was also observed in the AMAZONA study [234]. However, with regards to tumour grade in our study more than half (53.4%) were of the highest tumour grade (grade 3),

followed by grade 2 in 40.7% of cases and lastly grade 1 in 5.9% of cases. This is in contrast to other studies whereby, grade 2 tumours were the most represented, followed by grade 3 then grade 1 [234, 237]. With regards to molecular subtype distribution, our study demonstrated that the majority 38.0% were of Luminal B subtype. This was followed by Luminal A in 29.8% of cases. Triple negative cases made up 22.3% and lastly, the HER2-positive subtype was present in only 9.8% of women. These findings are similar to other studies with regards to the triple negative and HER2-positive subtype distribution [234, 238, 239] although in contrast many studies, report a larger proportion of Luminal A breast cancers than the Luminal B subtype [234,240, 241]. These findings are as expected as my cohort was selected to consist of only patients who were treated with adjuvant chemotherapy for their breast cancer. Therefore, my cohort has a poorer prognosis profile overall, with tumours of higher grade and less of the luminal A molecular subtype of breast cancers.

To determine whether these prognostic markers were significantly associated with either DFS or DSS, Kaplan-Meier survival analyses were performed. With regards to tumour grade, grade 3 had a statistically significant worse DFS (p=0.035) and DSS (p=0.004) compared with grade 1 and 2 irrespective of molecular subtype, which has been shown previously [234, 237, 242]. This was also true for DFS in the group with positive axillary lymph nodes (p=0.007) when compared with node negative breast cancers, irrespective of molecular subtype. Whereas, with molecular subtypes of breast cancer, only DSS was statistically significant, with TNBCs having the worse outcome followed by HER2-positive breast tumours and ER-positive breast cancers having the best outcome. Although, the same trend was followed for DFS, the result was not statistically significant (p=0.056). The same trend has also been described in the literature [234]. Our data showed that women with TNBCs were more likely to have grade 3 tumours (86.4% vs 47.7%; p=0.001) and were more likely to die from breast cancer than women that did not have a triple negative subtype (30.5% vs 15.4%; p=0.008). These findings are also echoed in a study by Dent et al who explored the prognostic significance of TNBCs in a cohort of 1601 women diagnosed over a 10-year period [238]. However, in our cohort the average age at diagnosis was slightly older for the triple negative group compared with the non-triple negative group (53.5 vs 51.9 years). Albeit this was not statistically significant, one would normally expect women with triple negative breast cancers to be of a younger age. However, younger women with triple negative breast cancers may have been more likely to receive NACT. Also, the BRCA1/2 mutation status is unknown in this cohort which could also contribute to these findings.

To determine whether survival outcomes were associated with different adjuvant chemotherapy regimens, Kaplan-Meier survival analyses were performed separately on the

patients who received either taxanes with anthracyclines (n=116), or anthracyclines without taxanes (n=149). The findings were of a prolonged DFS by 836 days; p=0.001 and DSS by 738 days; p=0.001 in the group of patients that received anthracycline based adjuvant chemotherapy. However, this may be due to combination chemotherapy being routinely administered in patients with larger tumours, increased tumour grade and with positive nodal disease, therefore may indicate it is in fact the severity of the breast cancer itself which is responsible for the higher mortality rate [243]. Both tumour grade (p=0.01) and lymph node status (p=0.03) also showed statistically significant correlations with taxane based chemotherapy regimens on chi squared analyses within the study.

3.5. Conclusion

I identified a cohort of primary breast cancers that were treated with adjuvant chemotherapy and have created TMAs containing cancer tissue supported by extensive, validated, clinicopathological data including survival follow up (n=305). However, it is important to acknowledge that this cohort is of a highly selected group of breast cancer patients, that are generally of high risk disease thereby, requiring treatment with adjuvant chemotherapy. Also, the women within this cohort are probably of a younger age and have fewer co-morbidities than the average breast cancer cohort, to tolerate the side effect profile of adjuvant chemotherapy. Therefore, this cohort may not be generally representative of the breast cancer population more broadly.

4. PARACRINE IFN SIGNALLING BETWEEN CANCER ASSOCIATED FIBROBLASTS AND CANCER CELLS CORRELATES WITH POOR SURVIVAL AFTER CHEMOTHERAPY IN TRIPLE NEGATIVE BREAST CANCER PATIENTS

4.1. Abstract

Background: It has been reported that the presence of cancer associated fibroblasts is associated with relative resistance of cancers to chemotherapy. One potential molecular pathway driving this resistance is paracrine activation of interferon signalling. My hypothesis was that expression levels of IFNβ1 in cancer associated fibroblasts, and of MX1 – a downstream marker of interferon activity - in cancer cells would be predictive markers of chemotherapy response and subsequent overall survival.

Methods: Breast cancer tissue was obtained from tissue microarrays from two separate cohorts of patients; an all-molecular subtypes cohort (cohort 1) and a triple negative cohort (cohort 2). Expression of IFNβ1 and MX1 was firstly determined by immunohistochemistry in 305 patients with primary breast cancer of all molecular subtypes, treated with adjuvant chemotherapy. Expression of IFNβ1 and MX1 was then further determined by immunohistochemistry in 109 patients with triple negative breast cancer treated with adjuvant chemotherapy. The triple negative cohort was further subdivided into claudin low and high subgroups, based on immunohistochemical assessment of relative expression of claudin-3.

Results: In cohort 1, IFNβ1 in fibroblasts was significantly, weakly positively associated with MX1 expression in the tumour cells (Spearman's correlation r=0.119; p=0.05) implying that signalling between the cell types was present. However, expression of IFNβ1 in fibroblasts, or MX1 in tumour cells was not significantly associated with disease-free survival (p=0.75; 0.71) or disease-specific survival (p=0.40; 0.06).

Similarly, in cohort 2, IFNβ1 in fibroblasts was significantly, positively associated with MX1 expression in the tumour cells (Spearman's correlation r=0.210; p=0.028). However, in this case, high expression of IFNβ1 in fibroblasts, and of MX1 in tumour cells were each significantly associated with poorer disease-free survival (p=0.01). Dividing the cohort into claudin-low (claudin-3 negative; n=49) and claudin-high (claudin-3 positive; n=60), resulted in a stronger correlation between fibroblast IFNβ1 and tumour cell MX1 in the claudin-low group (r=0.375; p=0.008), whereas it was lost in the claudin-high group (r=0.113; p=0.389). Likewise, correlations between survival and expression of each of IFNβ1 in fibroblasts and MX1 in tumour cells were maintained in claudin-low cases ($p<0.05$) but lost in claudin-highs.

Conclusion: IFNβ1 and MX1 may be of value as predictive biomarkers of chemo-response in breast cancer patients. These correlations imitate associations with chemoresistance identified in vitro using cancer associated fibroblasts and claudin-low breast cancer cell lines. Therefore, inhibiting the paracrine cross talk-between the cell types might be a viable strategy for chemo-sensitisation of breast cancers.

4.2. Introduction

The tumour microenvironment in breast cancer, comprising substantially of cancer associated fibroblasts, plays a role in chemotherapy resistance and breast cancer development via modifications of the extra-cellular matrix resulting in reduced permeability of therapeutic agents [189], as well as paracrine signalling pathways involving FGF, TGFβ [174], SPF-1 and IL6 [173] (Chapter 1, section 1.75).

Breast cancer has a range of molecular subtypes that differ greatly in incidence, treatment sensitivity and OS. Luminal A subtype cancers generally have an excellent prognosis despite often not needing to receive chemotherapy, whereas the HER2-positive subtypes are sensitive to HER2 targeted agents such as trastuzumab and pertuzumab (Chapter 1, section 1.3.3). TNBCs comprise up to 20% of all breast cancers [30] and lack the expression of oestrogen, progesterone and HER2 receptors, thereby limiting the systemic therapeutic options to cytotoxic chemotherapy [32,62]. Chemotherapy has been shown to enhance DFS and OS in breast cancer by the treatment of micro metastatic disease [48]. However, approximately 30% of individuals will develop resistance to chemotherapy during the course of their treatment [4]; with TNBCs having a graver outcome regardless of aggressive treatment in comparison to all other molecular subtypes [32]. Thus, the prospect of identifying markers that predict response to specific therapies in all molecular subtypes, but especially the triple negative subtype, would be beneficial and have the potential to be used widely.

Identification of molecular markers involved in cross-talk between CAFs and TNBC cancer cells gives rise to the possibility of overcoming chemoresistance through the inhibition of these interactions and ultimately prolonging disease free and overall survival in TNBC. The expression of IFNβ1 in breast CAFs, and of MX1, a marker of active IFN-signalling, in the cancer cells were identified in vitro as potential markers of chemoresistance (Chapter 1, section 1.8.3).

I therefore aimed to investigate whether IFNβ1 and MX1 could represent predictive markers of chemo-response and subsequently overall survival, as to my knowledge there is paucity of the literature with regards to the paracrine influence of CAFs and the protein expression levels of IFNβ1 and MX1 on chemo-response in breast cancer.

4.3. Results

4.3.1. Optimisation and analysis of IFNβ1 and MX1 expression in breast cancer

The first aims were to first optimise the staining methods for IFNβ1 and MX1 and the development of scoring protocols. Methods were optimised by varying conditions for antigen retrieval, blocking, and antibody concentrations so as to achieve staining that appeared specific (within expected cells and subcellular locations, but not elsewhere) and demonstrating a range of patterns (present in some tumours and less prevalent / absent in others).

Scoring protocols for each antibody were developed after discussion with Prof. Hanby (Consultant Breast Pathologist, LTHT). It was found that IFNβ1 located to the cytoplasm of both cancer cells and the fibroblasts, while MX1 was located in the cytoplasm of only the cancer cells. For IFNβ1, scoring was based on intensity of staining independently in either tumour cells or fibroblast on a scale of 1-3 in both cases; 1 being weak, 2 intermediate, while 3 represented strong staining. The proportion of these cell types staining with this intensity was not taken into account, since this was almost always 100% and was therefore not informative (Figure 4.1). For MX1, scoring was based on cancer cell intensity (0-3); 0 being negative, 1 being weak, 2 being intermediate and 3 strong; again without taking into account the proportion of cells staining with this intensity since this was almost always 100% (Figure 4.2). Automated scoring of IHC was considered, but the setting-up process for the different components needing to be scored was thought to be too time consuming and excellent agreement between scorers was observed manually.

Having optimised staining and scoring protocols, I then proceeded to stain my experimental cohorts. Cohort one represented the cases I had collected previously (Chapter 3), encompassing a range of molecular subtypes, with use of adjuvant cytotoxic chemotherapy in common (n=305). These were collected in a tissue microarray. Cohort two represented an independent collection of triple negative cases (n=109), also in a tissue microarray format.

The TMAs were stained for both IFNβ1 and MX1 and scanned for scoring. Scoring was performed remotely using scanned slides accessed through the internet.

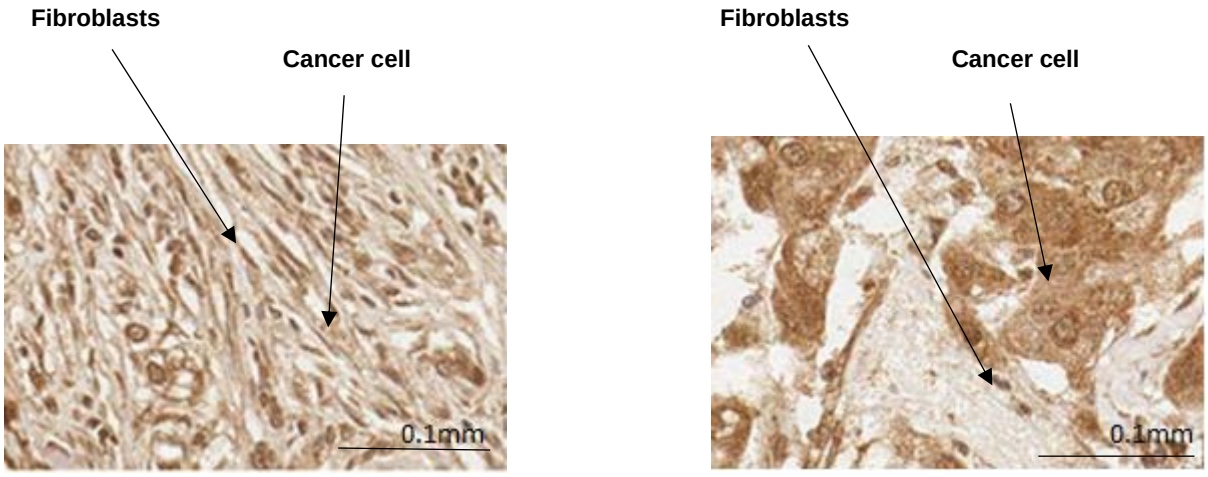


Figure 4.1 Representative images of immunohistochemical staining for IFNβ1 in breast cancers, demonstrating the scoring protocol. Breast cancer tissues were sectioned and treated to visualise IFNβ1 expression by immunohistochemistry (brown). IFNβ1 scoring was based on intensity in both cancer cells and in fibroblasts (1-3); 1 being weak, 2 intermediate, and 3 represented strong staining. Images are x20

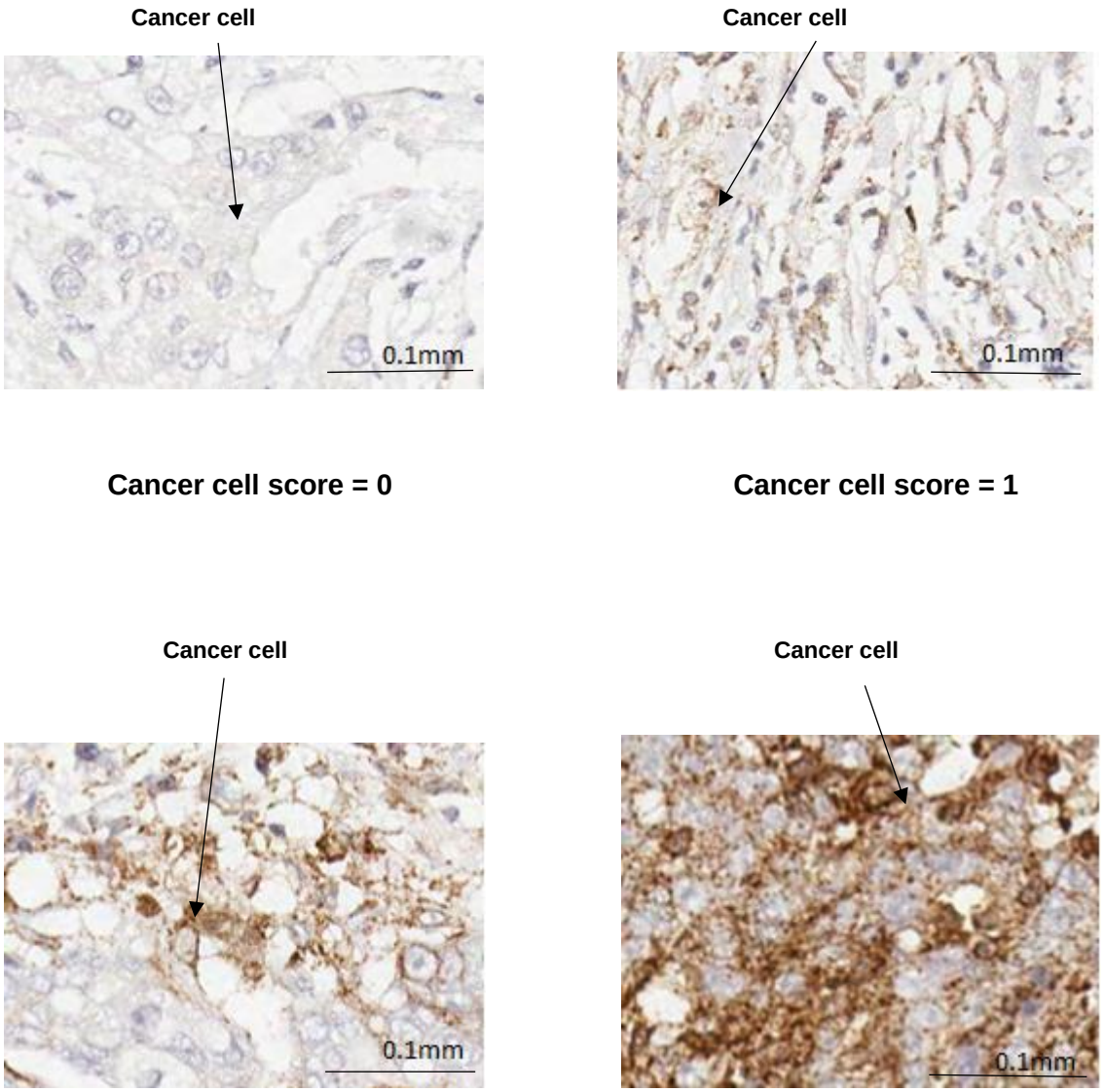


Figure 4.2 Representative images of immunohistochemical staining for MX1 in breast cancers, demonstrating the scoring protocol. Breast cancer tissues were sectioned and treated to visualise MX1 expression by immunohistochemistry (brown). They were also counterstained with Mayer's Haematoxylin to visualise nuclei (blue). MX1 scoring was based on cytoplasmic intensity (0-3); 0 being negative, 1 being weak, 2 being intermediate and 3 strong. Images are x20

4.3.2. Analysis of expression of IFNβ1 and MX1 in breast cancer patients of all molecular subtypes treated with adjuvant chemotherapy (Cohort 1)

To validate expression of IFNβ1 and MX1 and to allow for statistical analysis of scoring reproducibility some further analyses of the TMAs and their scores were performed.

10% of the tissue cores were also scored by a second independent scorer, Prof. Hanby (Consultant Breast Pathologist, LTHT). The inter-scorer concordance was determined using the Cohen's Kappa statistic which takes into consideration chance agreement among scorers. The Kappa scores concluded near perfect agreement between scorers for MX1 (0.725), and IFNβ1 cancer cell (0.769) and excellent for IFNβ1 fibroblast (0.672) [244], indicating that scoring was robust and reproducible.

As each case within the TMAs was represented by three tumour cores, it was also necessary to determine the variability amongst the cores from individual cases to both support the use of TMAs, and the decision concerning by which method core scores would be combined to provide scores for each case.

Spearman's rho correlation coefficients were established to assess correlations between scores, which were strongly and significantly correlated in all cases (Table 4.1). Therefore, the mean score of cores were taken as there was little evidence of intra-tumoral heterogeneity.

Spearman's Correlation Coefficient		Core 1	Core 2	Core 3
IFNβ1 Fibroblast Expression	**Core 1**	1.0	0.964	0.863
	Core 2		1.0	0.942
	Core 3			1.0
IFNβ1 Cancer Cell Expression	**Core 1**	1.0	0.926	0.894
	Core 2		1.0	0.925
	Core 3			1.0
MX1 Cancer Cell Expression	**Core 1**	1.0	0.884	0.868
	Core 2		1.0	0.896
	Core 3			1.0

Table 4.1 Core to core correlation of primary breast cancer cores for IFNβ1 and MX1 using Spearman's rho correlation. TMAs containing up to three primary breast cancer cores per case were stained for IFNβ1 and MX1 expression and quantified. Spearman's rho correlation analysis was used to compare the expression scores of cores within the same case. All correlations were statistically significant, $p<0.001$

4.3.2.1 IFNβ1 and MX1 proteins are expressed at a range of levels across invasive breast cancers

To determine the level of protein expression of IFNβ1 and MX1 among the breast cancer cohort (cohort 1), the mean protein expression scores for IFNβ1 (fibroblast, and separately, in cancer cells) and MX1 for each case successfully scored were tallied and rounded to the nearest whole number for ease of data presentation, as illustrated in Figures 4.3 and 4.4. For each protein, a range of expression levels were seen from negative expression for MX1 or weak expression for IFNβ through to strong expression.

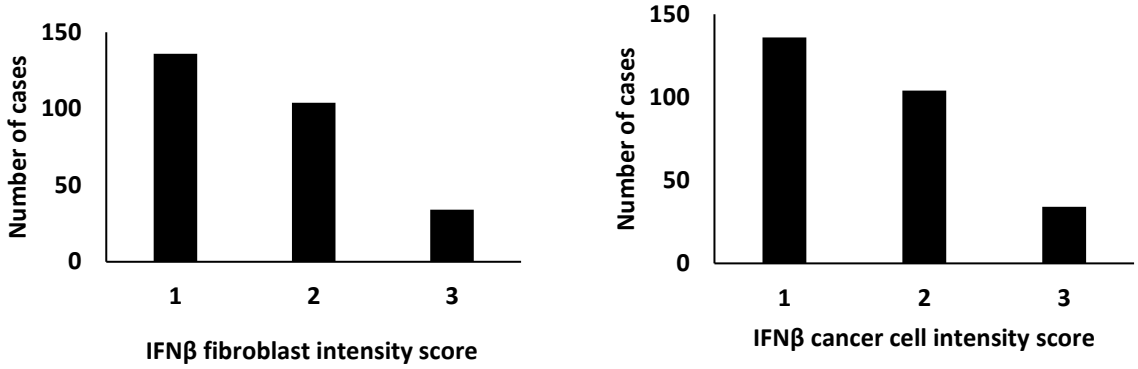


Figure 4.3 Score distributions for IFNβ1 in breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of all molecular subtypes of primary breast cancer cases were scored for IFNβ1 fibroblast and cancer cell cytoplasmic intensity expression on a scale of 1 to 3 and the mean scores calculated for each case from combining multiple core scores

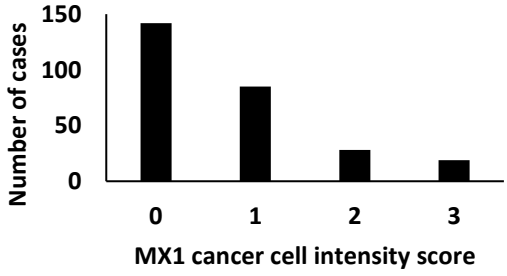


Figure 4.4 Score distributions for MX1 in breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of all molecular subtypes of primary breast cancer cases were scored for MX1 expression on a scale of 0 to 3 and the mean scores calculated for each case from combining multiple core scores

4.3.2.2. Expression of IFNβ1 in fibroblasts correlates with MX1 expression in cohort containing all molecular subtypes of breast cancer

From here forward for IFNβ1, I have focused on expression in fibroblasts only, as my hypothesis related to paracrine IFN signalling from these cancer associated fibroblasts to the cancer cells.

To determine if IFNβ1 in fibroblasts correlated with MX1 expression in the whole cohort, or in four different subgroups of breast cancer, Spearman's rho correlation analyses were performed.

Protein expression of fibroblast IFNβ1 was found to correlate weakly with MX1 expression in the whole cohort consisting of all molecular subtypes (Spearman's rho r= 0.119; p=0.050). However, the ER-negative subgroup (Spearman's rho r=0.216; p=0.047) and the triple negative subgroup (Spearman's rho r=0.257; p=0.050) produced stronger statistically significant correlations despite these groups consisting of fewer patients (Table 4.2).

Breast Cancer Subtype	Spearman's rho Correlation
All Subtypes	r=0.119; p=0.050
ER-Positive	r=0.087; p=0.234
ER-Negative	r=0.216; p=0.047
HER2-Positive	r=0.070; p=0.597
Triple Negative	r=0.257; p=0.050

Table 4.2 Correlation of IFNβ1 in fibroblasts with MX1 expression in cancer cells. All Subtypes (n=305), ER-Positive (n=207), ER-Negative (n=98), HER2-Positive (n=68) and Triple Negative (n=68). r = Spearman's rho coefficient and p = p-value. * represents significant log rank test (p<0.05)

4.3.2.3. Protein expression of MX1 correlated with grade of tumour in the whole cohort and all breast cancer subtypes, ER-positive, ER-negative, HER2-positive and triple negative

To determine correlations between IFNβ1 or MX1 protein expressions and clinical prognostic markers, which comprised of tumour grade and lymph nodes status (positive or negative) Spearman's rho analyses were performed. A sub-analysis of all breast cancer subtypes was performed paying particular attention to the TNBCs, since the IFN-MX1 correlation was

strongest in this group, suggesting that IFN signalling between cancer associated fibroblasts and cancer cells may be most relevant in this group. This molecular subtype has a poorer prognosis and a higher metastatic potential and commonly receive chemotherapy as part of adjuvant systemic therapy [32]. Table 4.3 shows the correlations coefficients and the associated p values. MX1 cancer cell cytoplasmic expression showed weak correlations with tumour grade which were statistically significant in each subtype.

Expression		Grade of Tumour	Lymph Node Status
Whole Cohort			
IFNβ1 Fibroblast	**r**	-0.081	0.056
	p	0.181	0.354
MX1 Cancer Cell Cytoplasm	**r**	0.326	-0.104
	p	0.001*	0.087
ER-Positive Subtype			
IFNβ1 Fibroblast	**r**	-0.093	0.100
	p	0.202	0.171
MX1 Cancer Cell Cytoplasm	**r**	0.333	-0.108
	p	0.001*	0.141
ER-Negative Subtype			
IFNβ1 Fibroblast	**r**	-0.009	-0.049
	p	0.933	0.658
MX1 Cancer Cell Cytoplasm	**r**	0.309	-0.021
	p	0.004*	0.847
HER2-Positive Subtype			
IFNβ1 Fibroblast	**r**	0.008	-0.110
	p	0.950	0.407
MX1 Cancer Cell Cytoplasm	**r**	0.257	0.009
	p	0.050*	0.948
Triple Negative Subtype			
IFNβ1 Fibroblast	**r**	-0.014	-0.034
	p	0.918	0.805
MX1 Cancer Cell Cytoplasm	**r**	0.309	-0.028
	p	0.020*	0.838

Table 4.3 MX1 cancer cell cytoplasm expression correlates with tumour grade in whole cohort and each breast cancer subtype. All Subtypes (n=305), ER-Positive (n=207), ER-Negative (n=98), HER2-Positive (n=68) and Triple Negative (n=68). Spearman's rho analyses were performed for IFNβ1 and MX1 expression levels against tumour grade. r = Spearman's rho coefficient and p = p-value. * represents significant result ($p<0.05$)

4.3.2.4. Kaplan-Meier survival analysis revealed no significant differences in survival relating to high or low IFNβ1 or MX1 protein expression in the whole cohort (cohort 1)

The main aim of this study was to investigate if expression of IFNβ1 or MX1 at the protein level could be utilised as predictive markers of survival outcomes following chemotherapy.

Firstly, to allow comparison between the two groups and to investigate survival by Kaplan-Meier analyses it was essential to dichotomise the cohort into individuals with high or low expression using suitable cut off values. These cut-offs were determined using ROC curve analyses, which suggests the optimum equilibrium between sensitivity and specificity for prediction of clinical outcomes in terms of recurrence or death [227].

To determine whether differential expression of IFNβ1 or MX1 was significantly associated with either DFS for which the event was recurrence, or DSS for which the event was death from breast cancer, Kaplan-Meier survival analyses were performed, which identified there were no statistically significant differences between the low and high expressing groups in terms of breast cancer survival after chemotherapy (Table 4.4, Figure 4.5 - 4.6).

Expression		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
IFNβ1 Fibroblast	**Low**	3957 (3779-4135)	0.751	4023 (3863-4183)	0.402
	High	3863 (3422-4304)		4107 (3708-4506)	
MX1 Cancer Cell Cytoplasm	**Low**	3973 (3578-4188)	0.711	4155 (3973-4337)	0.063
	High	3910 (3658-4161)		3904 (3672-4136)	

Table 4.4 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low protein expression of IFNβ1 and MX1. All Subtypes (n=305). 95% confidence intervals and log rank p values stated

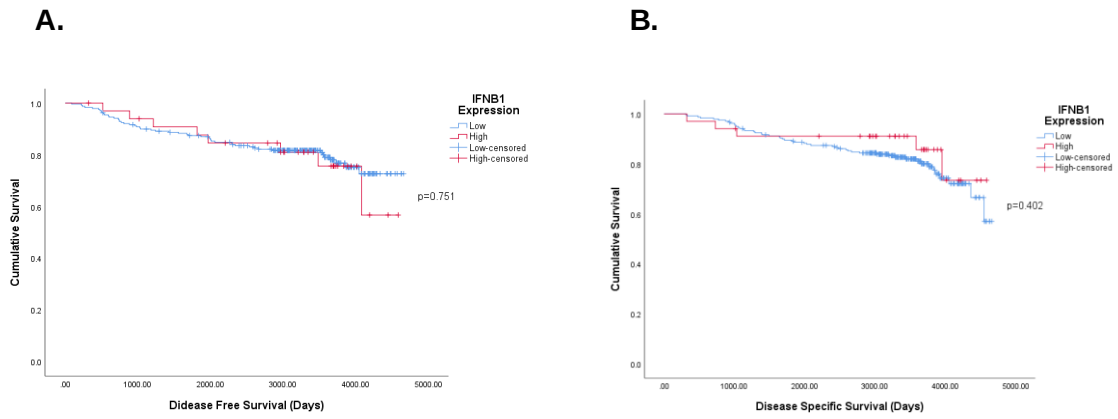


Figure 4.5 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression. Fibroblast IFNβ1 expression was determined in a cohort of 305 primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low fibroblast IFNβ1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

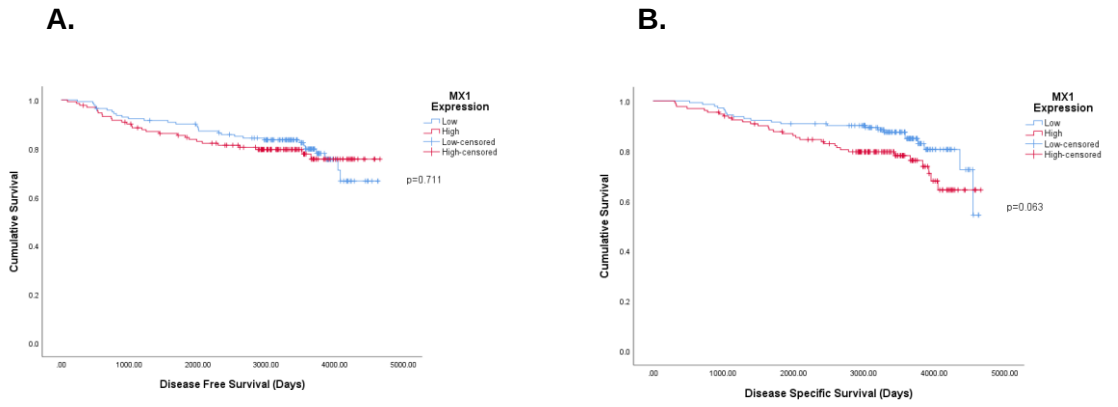


Figure 4.6 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression. Cytoplasmic cancer cell MX1 expression was determined in a cohort of 305 primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic cancer cell MX1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A sub-group analysis of the four-breast cancer sub-types within the primary breast cancer cohort was then performed using Spearman's rho (Table 4.5). The Kaplan-Meier plots can be found in Appendix 9.2. Although, the Kaplan-Meier survival analysis for triple negative breast cancer sub-type showed that high fibroblast IFNβ1 and MX1 cytoplasmic cancer cell expression had a worse DFS and DSS this was not statistically significant.

Expression		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
ER-Positive Subtype					
IFNβ1 Fibroblast	**Low** **High**	4101 4007	0.738	4223 4287	0.502
MX1 Cancer Cell Cytoplasm	**Low** **High**	4094 4082	0.800	4314 4130	0.170
ER-Negative Subtype					
IFNβ1 Fibroblast	**Low** **High**	3570 2826	0.670	3695 2886	0.689
MX1 Cancer Cell Cytoplasm	**Low** **High**	3405 3535	0.972	3812 3525	0.283
HER2-Positive Subtype					
IFNβ1 Fibroblast	**Low** **High**	4065 3309	0.402	3843 3362	0.975
MX1 Cancer Cell Cytoplasm	**Low** **High**	3800 4063	0.815	4161 3551	0.115
Triple Negative Subtype					
IFNβ1 Fibroblast	**Low** **High**	3537 3288	0.756	3579 3317	0.645
MX1 Cancer Cell Cytoplasm	**Low** **High**	3521 3419	0.648	3838 3349	0.153

Table 4.5 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of IFNβ1 in fibroblasts and MX1 cancer cell cytoplasm in ER-Positive (n=207), ER-Negative (n=98), HER2-Positive (n=68) and Triple Negative (n=68) subtypes of breast cancer. log rank p values stated

4.3.3. Analysis of the relevance of IFNβ1 and MX1 expression in a triple negative cohort treated with adjuvant chemotherapy

Next, I also carried out essentially the same analysis in a second cohort of triple negative cancers only (n=109). This is likely to have greater statistical power compared to the triple negative subgroup of the previous cohort (n=68). I persisted with analysis of triple negative cases as this had previously given the strongest correlation between fibroblast IFNβ1 and cancer cell MX1, and it was in this subtype that in vitro experiments had shown a functional interaction between fibroblasts and cancer cells (Chapter 1, section 1.8.3).

In addition, for this cohort, I optimised and stained the cores for claudin-3 with a view to classifying whether individual cases could be grouped as claudin-low or claudin-high subtypes

[245] as the in vitro data previously shown by the Hughes group [192] suggested that claudin-low subtype of breast cancer may be more susceptible to CAF-dependent chemoresistance.

As before, a scoring strategy was defined and based on cytoplasm/cell membrane staining with 0 = negative and 1 = positive, without taking into account the proportion of cells staining since this was almost always 100%. Representative images of staining of claudin-3 in triple negative cohort can be found in Figure 4.7. Scoring was robust and reproducible as 10% of the tissue cores were also scored by a second independent scorer Prof. Hanby (Consultant Breast Pathologist, LTHT), giving a Kappa score of 1 between the two scores, indicating perfect agreement [244].

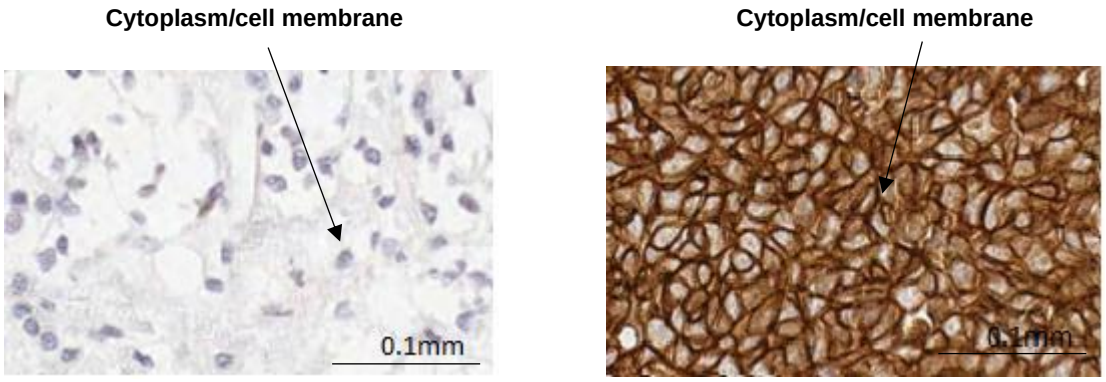


Figure 4.7 Representative images of immunohistochemical staining for Claudin-3 in triple negative breast cancers, demonstrating the scoring protocol. Breast cancer tissues were sectioned and treated to visualise claufin-3 expression by immunohistochemistry (brown). They were also counterstained with Mayer's Haematoxylin to visualise nuclei (blue). Claudin-3 scoring was based on cytoplasm/cell membrane staining, negative = 0 and positive =1. Images are x20

4.3.3.1. IFNβ1 and MX1 proteins are expressed at a wide range of levels across triple negative invasive breast cancers

To determine the range of expression of IFNβ1, MX1 and claudin-3 proteins across the triple negative cohort, protein expression scores, using the average scores of each successfully scored core for IFNβ1and MX1 and either positive or negative expression of claudin-3 were calculated. A range of expression levels were seen for IFNβ1 and MX1 (Figures 4.8 and 4.9), whereas for claudin-3 expression was either positive or negative (Positive = 60, Negative = 49).

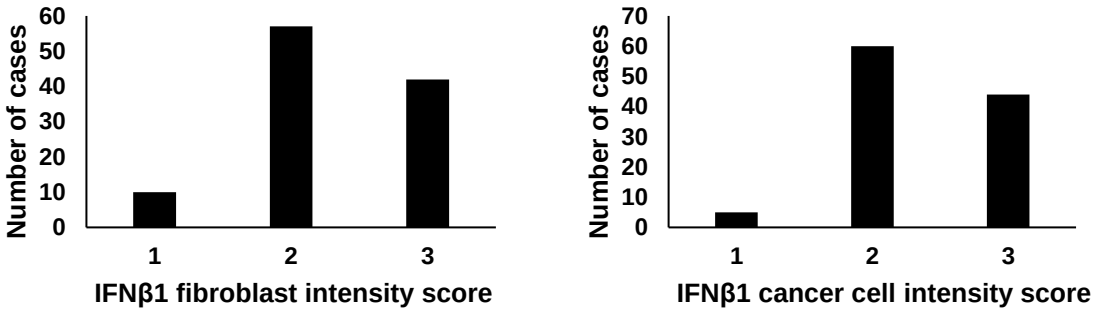


Figure 4.8 Score distributions for IFNβ1 in triple negative breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of triple negative primary breast cancer cases were scored for IFNβ1 fibroblast and cancer cell cytoplasmic intensity expression on a scale of 1 to 3 and the mean scores calculated for each case from combining multiple core scores

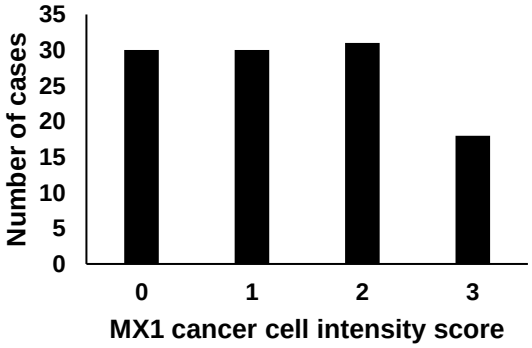


Figure 4.9 Score distributions for MX1 in triple negative breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of triple negative primary breast cancer cases were scored for MX1 cancer cell cytoplasmic intensity expression on a scale of 0 to 3 and the mean scores calculated for each case from combining multiple core scores

4.3.3.2. High expression of IFNβ1 in fibroblasts was weakly, but significantly, positively associated with high MX1 expression in the tumour cells in the triple negative cohort

To determine if IFNβ1 in fibroblasts correlates with MX1 expression in the triple negative cohort, a Spearman's rho correlation analysis was performed. High expression of IFNβ1 in fibroblasts was weakly, but significantly, positively associated with high MX1 expression in the tumour cells (Spearman's correlation r=0.210; p=0.028), suggesting that signalling between the cell types was active.

4.3.3.3. High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer disease-free survival

As with the previous cohort (cohort 1), the main aim was to investigate whether expression of IFNβ1 or MX1 at the protein level could be used as predictive markers for survival outcomes following chemotherapy. To allow comparison between the two groups and to investigate survival by Kaplan-Meier analyses it was essential to once again dichotomise the cohort into individuals with high or low expression using suitable cut off values. These cut-offs were determined using ROC analyses and the cut off values can be found in Appendix 9.3.

I initially performed Kaplan-Meier survival analysis for low expression versus high expression of IFNβ1 and MX1 for both DFS and DSS (Table 4.6 and Figures 4.10 and 4.11). High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer DFS, by means of 788 and 786 days respectively; p<0.01, although surprisingly this was not reflected in significant differences in DSS.

Triple Negative Breast Cancers (Whole cohort)		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
IFNβ1 Fibroblast Expression	**Low**	2751 (2449-3052)	0.011*	2890 (2637-3143)	0.088
	High	1963 (1561-2366)		2447 (2086-2808)	
MX1 Cancer Cell Expression	**Low**	3100 (2763-3437)	0.010*	3036 (2704-3367)	0.103
	High	2314 (2014-2615)		2653 (2401-2906)	

Table 4.6 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of IFNβ1 and MX1 in Triple Negative breast cancers (n=109). 95% confidence intervals and log rank p values stated. * represents significant log rank test (p<0.05)

A. **B.**

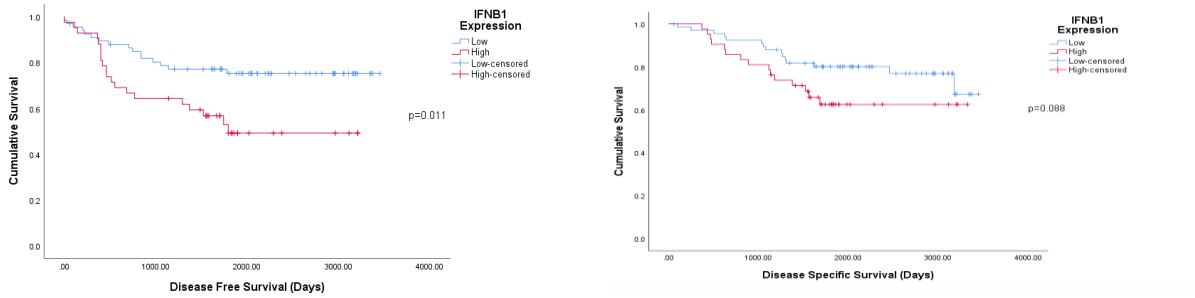


Figure 4.10 Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with Triple Negative breast cancer. IFNβ1 fibroblast expression was determined in a cohort of 109 primary Triple Negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low IFNβ1 fibroblast expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient
Low Group = 67 and High Group = 42

A. **B.**

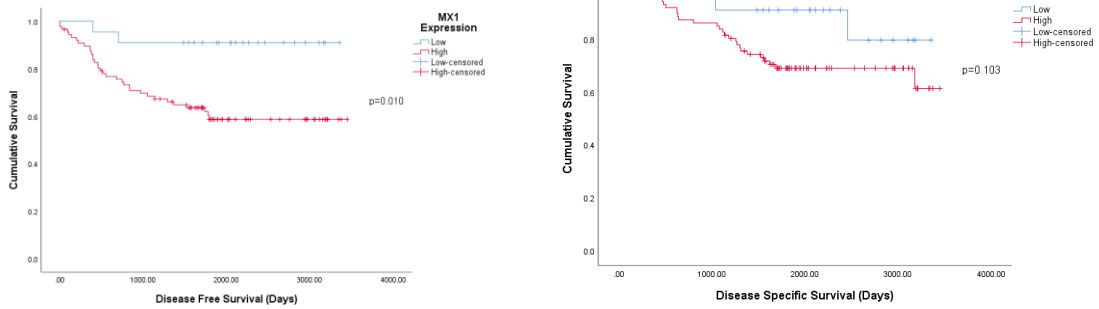


Figure 4.11 Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with Triple Negative breast cancer. Cytoplasmic cancer cell MX1 expression was determined in a cohort of 109 primary triple negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low cytoplasmic cancer cell MX1 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient. Low Group = 22 and High Group = 87

4.3.3.4. Correlation between fibroblast IFNβ1 and tumour cell MX1 was strengthened in the claudin-low group

Next, I wanted to determine if there was a correlation between fibroblast IFNβ1 and tumour cell MX1 in the separate claudin-low and claudin-high groups, since in vitro work had suggested this might be a factor (Chapter 1, section 1.8.3). I divided the cohort into claudin-low (negative for claudin-3; n=49) and claudin-high (positive for claudin-3; n=60). Fascinatingly, the expression of IFNβ1 was statistically significantly different amongst the subgroups, with claudin-low tumours expressing overall higher levels of IFNβ1 than the claudin-high subgroup (mean scores 2.6 [SD 0.59] vs 2.0 [SD 0.52]). I also performed Spearman's rho analyses. The findings were that the correlation between fibroblast IFNβ1 and tumour cell MX1 was strengthened in the claudin-low group (r=0.375; p=0.008) while it was lost in the claudin-high cohort (r=0.113; p=0.389).

4.3.3.5. IFNβ1 fibroblast expression and lymph node status were statistically significant independent predictors of disease-free survival in the claudin-low group

To determine whether IFNβ1 in fibroblasts and MX1 in tumour cells offered prognostic information that was independent of tumour grade and lymph node status a multivariate analysis was performed (Table 4.7). Within the whole triple negative cohort, IFNβ1 fibroblast expression and lymph node status were statistically significant independent predictors of DFS (HR of 2.99; p=0.001 and 2.24; p=0.007 respectively). In the claudin-low subgroup, the aforementioned factors remained significant, HR of 3.52; p=0.015 and 3.77; p=0.034 respectively. Lastly, in the claudin-high subgroup, not one of the standard prognostic factors were statistically significantly associated with outcome, although lymph node status was shown to be of borderline significance (HR 2.52; p=0.052).

	DFS		DSS	
	Hazard Ratio	**P-Value**	**Hazard Ratio**	**P-Value**
Whole Cohort				
IFNβ1 Fibroblasts	2.24	0.007*	1.69	0.099
MX1 Cancer Cell	0.90	0.509	0.92	0.662
Lymph Node Status	2.99	0.001*	3.94	0.001*
Tumour Grade	1.10	0.821	1.10	0.833
Claudin-low Subgroup				
IFNβ1 Fibroblasts	3.77	0.034*	1.99	0.214
MX1 Cancer Cell	0.77	0.286	0.64	0.142
Lymph Node Status	3.52	0.015*	3.51	0.026*
Tumour Grade	0.74	0.527	0.78	0.604
Claudin-high Subgroup				
IFNβ1 Fibroblasts	1.90	0.127	1.43	0.454
MX1 Cancer Cell	1.03	0.886	1.33	0.321
Lymph Node Status	2.52	0.052	4.28	0.010*
Tumour Grade	3.56	0.222	5.02	0.975

Table 4.7 Multivariate analysis to determine whether IFNβ1 in fibroblasts and MX1 in cancer cells offered prognostic information that was independent of tumour grade and lymph node status in Whole Cohort (n=109), Claudin-low Subgroup (n=49) and Claudin-high Subgroup (n=60). Hazard ratio and log rank p values stated. * represents significant log rank test ($p<0.05$)

4.3.3.6. High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer disease-free survival in the claudin-low subgroup of triple negative breast cancers

The aim was to investigate whether expression of IFNβ1 or MX1 at the protein level could be used as predictive markers for survival outcomes following chemotherapy in the claudin-low subgroup of triple negative breast cancers. I initially performed Kaplan-Meier survival analysis for low expression versus high expression of IFNβ1 in fibroblasts and MX1 in tumour cells for both DFS and DSS in the claudin-low subgroup. High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each significantly associated with poorer DFS by means of 989 and 941 days respectively; p=0.02 and p=0.047 (Table 4.8 and Figures 4.12 and 4.13).

Claudin-low Subgroup		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
IFNβ1 Fibroblast Expression	**Low**	2902 (2407-3397)	0.021*	2843 (2370-3316)	0.240
	High	1913 (1439-2386)		2426 (1999-2854)	
MX1 Cancer Cell Cytoplasmic Expression	**Low**	3086 (2584-3588)	0.047*	2968 (2486-3450)	0.254
	High	2145 (1699-2590)		2492 (2103-2880)	

Table 4.8 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of IFNβ1 and MX1 in Claudin-low Subgroup of Triple Negative Breast Cancers (n=49). 95% confidence intervals and log rank p values stated. * represents significant log rank test ($p<0.05$)

A. B.

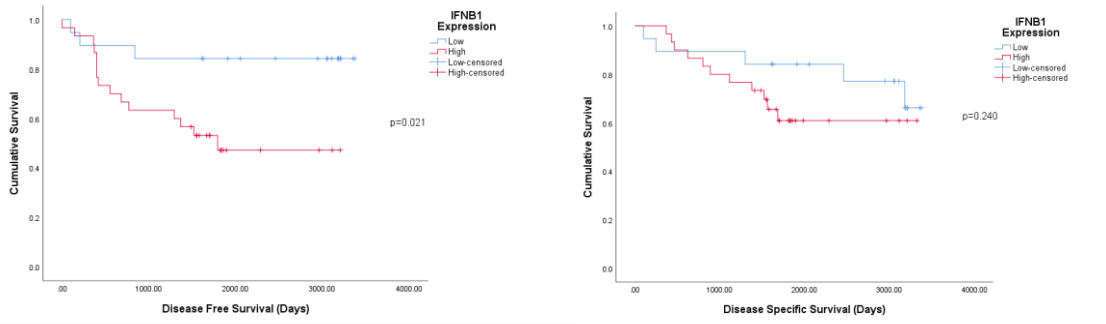


Figure 4.12 Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with Claudin-low Subgroup of Triple Negative Breast Cancers (n=49). IFNβ1fibroblast expression was determined in a cohort of 49 Claudin-low Triple Negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low IFNβ1 fibroblast expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient
Low Group = 19 and High Group = 30

A. B.

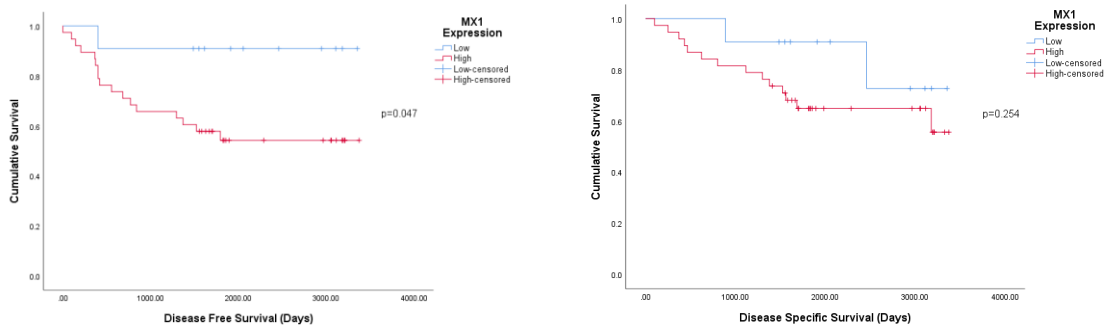


Figure 4.13 Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with Claudin-low Subgroup of Triple Negative Breast Cancers (n=49). Cytoplasmic cancer cell MX1 expression was determined in a cohort of 49 Claudin-low Triple Negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low cytoplasmic cancer cell MX1 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient. Low Group = 11 and High Group = 38

Next, I wanted to investigate whether expression of IFNβ1 in fibroblasts or MX1 in tumour cells at the protein level could be used as predictive markers for survival outcomes following chemotherapy in the claudin-high subgroup of triple negative breast cancers. I initially performed Kaplan-Meier survival analysis for low expression versus high expression of IFNβ1 and MX1 for both DFS and DSS in the claudin-high subgroup. High expression of IFNβ1 in fibroblasts and MX1 in tumour cells were each not significantly associated with poorer DFS (Table 4.9 and Figures 4.14 and 4.15).

Claudin-high Subgroup		**Mean DFS (days) (95% CI)**	**Log Rank**	**Mean DSS (days) (95% CI)**	**Log Rank**
IFNβ1 Fibroblast Expression	**Low**	2678 (2316-3040)	0.205	2941 (2658-3224)	0.192
	High	1574 (1021-2126)		1810 (1356-2265)	
MX1 Cancer Cell Cytoplasmic Expression	**Low**	2939 (2521-3356)	0.093	2969 (2608-3330)	0.220
	High	2425 (2030-2820)		2767 (2448-3086)	

Table 4.9 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of IFNβ1 fibroblasts and MX1 tumour cell in Claudin-high Subgroup of Triple Negative Breast Cancers (n=60). 95% confidence intervals and log rank p values stated

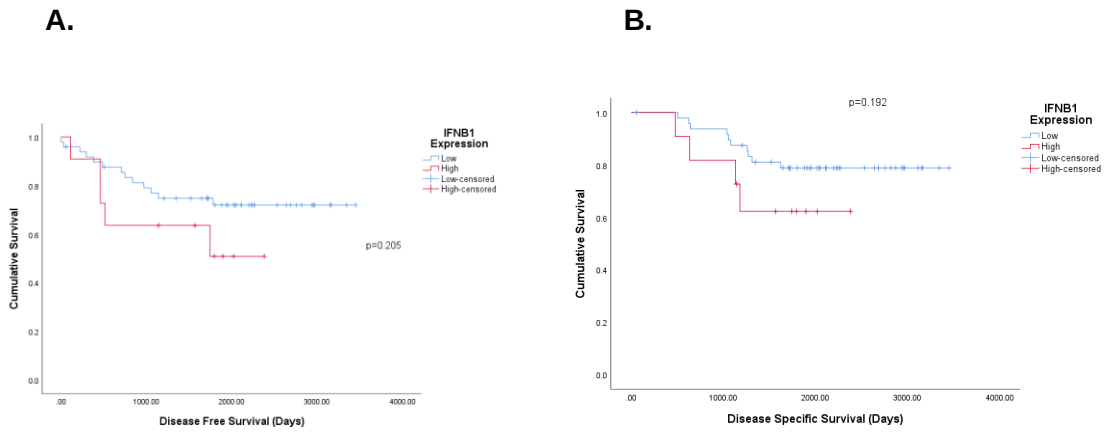


Figure 4.14 Kaplan-Meier survival analysis of high versus low IFNβ1 fibroblast expression in patients with Claudin-high Subgroup of Triple Negative Breast Cancers. IFNβ1 fibroblast expression was determined in a cohort of 60 Claudin-high Triple Negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low fibroblast IFNβ1 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient.
Low Group = 49 and High Group = 11

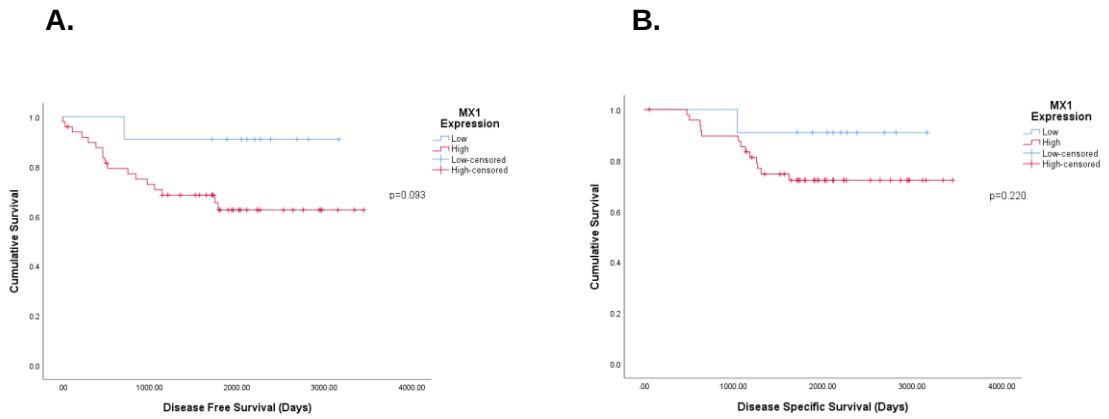


Figure 4.15 Kaplan-Meier survival analysis of high versus low cytoplasmic cancer cell MX1 expression in patients with Claudin-high Subgroup of Triple Negative Breast Cancers. Cytoplasmic cancer cell MX1 expression was determined in a cohort of 60 Claudin-high triple negative breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high and low cytoplasmic cancer cell MX1 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient. Low Group = 11 and High Group = 49

4.4. Discussion

4.4.1. IFNβ1 and MX1 as prognostic markers in Claudin-3 low subtype of triple negative breast cancers

The key aim of this chapter was to firstly, determine if IFNβ1 and MX1 can be utilised as predictive indicators to guide chemotherapy usage and enhance survival outcomes in individuals with breast cancer. Secondly, to support *in vitro* findings of IFNβ1 and MX1 as drivers of chemo-response and subsequently used as prognostic markers of treatment.

TNBCs tend to have the worse prognosis of all breast cancer molecular subtypes [31] and much current research is aimed at finding new drug targets and chemotherapeutic agents as systemic chemotherapy is currently the only available option [32]. Alternatively, we have the option to utilise chemotherapeutic agents already fully established, more successfully. However, in order to do this we must first fully understand the mechanisms driving chemoresistance [128].

Previous in-vitro models by the Hughes group demonstrated that breast CAFs protect a subgroup of TNBCs known as claudin-low from the anthracycline agent, epirubicin. CAF induced protection is thought to be due to the secretion of IFNβ1 which ultimately results in the paracrine stimulation of IFN signalling in the cancer cell, as represented by an increased production of MX1 [192].

I then set about confirming this using two patient cohorts. I firstly wished to determine if IFNβ1 expression in fibroblasts correlated with tumour cell expression of MX1, a marker of active IFN signalling in a cohort of all molecular subtypes of breast cancer (Cohort 1) and secondly, in a TNBC patient cohort (Cohort 2) in particular the claudin low subtype. Lastly, to determine prognosis in terms of DFS and DSS with the aim that inhibition of IFN signalling may give rise to new therapeutic targets that may help overcome chemotherapy resistance.

Kaplan-Meier survival analysis was performed for low versus high protein expression of IFNβ1 and MX1 in the two separate cohorts as well as the Claudin-low and Claudin-high subgroups of the triple negative cohort.

A key finding of this analysis is the correlation in expression of IFNβ1 in fibroblasts and the expression of a marker of IFN signalling; MX1 in the tumour cell. Also, in the triple negative cohort, a high IFNβ1 fibroblast expression and MX1 tumour cell expression were associated with a reduced DFS in comparison to patients with low expression (log rank test, p=0.01)

following adjuvant chemotherapy. The same pattern in DFS was maintained in the claudin-low patients (p=0.02 and p=0.047 respectively). However, this was not the case in the claudin-high subset of patients. This supports in-vitro findings that showed that inhibition of IFN receptors by antibodies in cell-lines representative of the claudin-low subset of patients (MDA-MB-231 and MDA-MB-157) reduced CAF-dependent protection. Thereby, validating that an up-regulation in interferon signalling is required for the protection induced by CAFs [192].

The findings of the survival analysis in cohort 1 in both the whole cohort and triple negative subset were in agreement with the trend of in vitro findings but were not statistically significant. However, it is important to state that only 22% of patients had a recurrence with 19% of patients dying from breast cancer, which ultimately would affect DFS and DSS respectively. Therefore, an increased number of cases were censored in the Kaplan-Meier survival analysis, resulting in decreased statistical power.

However, our findings contribute to the literature as Zhou et al also concluded that CAFs correlate with worse survival in patients with TNBC [246] and a study by Aljohani et al demonstrated that the expression of MX1 in breast tumour cells is also associated with a graver outcome [201]. Previous in-vitro studies involving breast cancer cells have also concluded that the secretion of IFNβ1 by breast CAFs can stimulate IFN signalling thereby, affecting breast cancer cell behaviour [37]. However, in dissimilarity to the majority of the literature which believe that CAFs influence breast cancer prognosis by encouraging growth, invasion and tumour metastasis [5,6] we believe that the alteration in the function of IFN signalling can affect chemotherapy treatment and resistance. These differences are key as it gives rise to the potential development of therapeutic agents that could potentially inhibit CAF-induced chemoresistance [39]. Whereby, the alternative explanation of CAF-induced tumour growth and metastasis is a more difficult obstacle to overcome as just under 10% of women present with metastatic disease [247].

These findings give rise to the potential possibility of enhancing DFS and DSS in TNBCs in particular the claudin low subgroup by sensitising tumour cells to chemotherapeutic agents by preventing cross-talk between CAFs and tumour cells. One potential chemotherapeutic agent is that of a JAK1/2 kinases inhibitor known as ruxolitinib which has been used in combination with capecitabine in a phase 2 randomised study in patients with metastatic breast cancer [248].

The claudin-low subtype of breast cancer was discovered by Herschkowitz et al [38] in 2007 and makes up approximately 14% of invasive breast tumours and has been associated with

poor outcomes in terms of DFS and DSS [39]. Although, the research on claudins in various cancers is increasingly expanding [249], as of yet claudin expression levels are not determined as part of breast cancer management as to date they have not yielded adequate treatment options to enhanced survival [250].

It is not yet fully clear why CAFs protect the breast cancer claudin-low subtype from chemotherapy but not the claudin-high subgroup. However, one proposed explanation from our patient cohort is that the claudin-high breast cancers express lower levels of CAF IFNβ1 levels to stimulate IFN signalling. An alternative explanation could be a defect in IFN signalling as in-vitro studies by the Hughes group in claudin-high cancer cells demonstrated that the level of IFNβ1 present doesn't correlate with IFN-target gene expression.

To date literature on Type 1 signalling induced by IFNβ1 expression is sparse. However, Odnokoz et al who investigated the role of Type 1 interferon receptors (IFNAR1) in breast cancer in both murine and human tissue found that non-degradable IFNAR1 stimulated the IFN1 pathway [251]. Unlike, other cancers such as colorectal adenocarcinomas [252] and melanomas [253] an increased expression of IFNAR1 in breast tumours was associated with a graver outcome [251]. These findings together with outcomes from other studies [254, 255] who concluded an association of increased expression of IFN-stimulated genes (ISGs) with treatment resistance and decreased prognosis supports the fact that the IFNβ1 signalling pathway in breast cancer has pro-tumorigenic consequences.

Expression of interferon regulatory factors (IRFs), in particular IRF-1 and IRF-2 which play a role in the modification of IFN-target gene activation have been shown to be altered in breast tumours. IRF-1 was first discovered over three decades ago and has tumour suppressor activities whereby, IRF-2 which was discovered shortly afterwards was found to possess oncogenic properties [256]. Doherty et al who stained both human invasive ductal breast cancer tissue and normal breast tissue with IRF-1 and IRF-2 antibodies found that the majority of normal breast tissue expressed IRF-1 but not IRF-2. Whereas, the invasive ductal cancers were less likely to express IRF-1 and more likely to express IRF-2 than normal tissue [257]. Further work is required to identify therapeutic interventions that target the aforementioned mechanisms of CAF-dependent chemoresistance. As well as the need to look at IFNβ1 expression in patients with breast cancer that did not receive chemotherapy.

4.5. Conclusion

In this chapter I conclude based upon the correlations found that breast CAFs protect a subtype of TNBCs known as claudin-low from chemotherapeutic agents via the production of IFNβ which activates IFN signalling in tumour cells, as represented by an up-regulation of MX1 expression and ultimately worse survival.

5. EXPRESSION OF ITGA7 PREDICTS SURVIVAL FROM BREAST CANCER FOLLOWING ADJUVANT CHEMOTHERAPY

5.1. Abstract

Background: ITGA7 encodes an alpha chain member of the integrin family, which is known to form a heterodimer with integrin β1 in the plasma membrane that then acts as a receptor for the attachment of laminin 1 and 2. Numerous studies have demonstrated ITGA7's involvement in cell proliferation, migration and invasion in a range of cancers. However, the functional impact of ITGA7 in breast cancer is unclear. The aim of this chapter was to investigate the relevance of ITGA7 expression with respect to clinical prognostic markers, and disease-free survival and disease-specific survival after chemotherapy in breast cancer.

Methods: Breast cancer tissue was obtained from a tissue microarray comprising of all tumour subtypes, with the common factor of use of adjuvant cytotoxic chemotherapy (cohort 1; n=305). Expression of ITGA7 was determined by immunohistochemistry. Relative expression of ITGA7 was assessed.

Results: ITGA7 protein was located in both the cytoplasm and nuclear compartments, and expression was assessed in these locations separately. Overall, it was concluded that ITGA7 levels were independent of the clinical prognostic markers, tumour grade, lymph nodes status (positive or negative), oestrogen receptor status (positive or negative) and molecular subtype (TNBC vs other subtype). Kaplan-Meier survival analyses showed that high ITGA7 nuclear protein expression was associated with longer disease-free survival, by a mean of 647 days ($p=0.036$) in the whole cohort. The same trend was visible for ITGA7 cytoplasmic protein expression albeit that this was not significant. A sub-analysis of ER-positive breast cancers also showed that high ITGA7 nuclear protein expression was associated with both a longer disease-free survival and disease-specific survival; 682 days, $p=0.05$ and 604 days, $p=0.005$ respectively. Kaplan-Meier survival analysis was performed separately on the patients who received either taxanes with anthracyclines or anthracyclines without taxanes. This demonstrated a significant increase in disease-free survival of 806 days; $p=0.004$ in primary breast cancers that had high ITGA7 nuclear protein expression in the group that received anthracycline-based adjuvant chemotherapy alone. No statistically significant difference in disease-free survival or disease-specific survival was noted between high and low ITGA7 expression in the group that received anthracycline and taxane based adjuvant chemotherapy.

Conclusion: ITGA7 has been identified as impacting upon disease-free survival and disease-specific survival in breast cancer potentially via the modification of chemoresponse.

5.2. Introduction

Increased understanding of the molecules and molecular pathways that define the responses of cancer cells to cytotoxic chemotherapy will potentially allow more effective stratification of patients to chemotherapy treatments, and development of novel chemo-sensitisers.
ITGA7 was identified as a candidate gene that could be involved in chemoresistance in breast cancer from a genomics screen previously performed by the Hughes lab [191] (Chapter 1, Section 1.8.1). The ITGA7 gene contained somatic mutations in one third of the cohort that were eliminated after therapy, suggesting these mutations defined chemosensitive cells. However, without knowing whether mutations were gain of function or loss of function further work was required.

Independently of this work, the Hughes lab also examined transcriptome profiling of stem-like cells primary breast cancers, fluorescently labelled using the Aldefluor assay [258]. Transcriptomes of breast cancer stem-like cells and matched non-stem cancer cells were defined using RNA-seq and it was shown that BCSCs expressed ~20 fold significantly reduced levels of ITGA7 as compared to the matched bulk cancer cells, again hinting at a role in defining response to chemotherapy as cancer stem-like cells are thought to be relatively chemoresistant (Chapter 1, Section 1.8.2).

ITGA7 encodes the α-7 integrin protein, which belongs to the integrin family [191] and forms a heterodimer with integrin β1 in the plasma membrane which then acts as a receptor for the attachment of laminin 1 and 2 [204]. Many studies have shown ITGA7's involvement in cell proliferation, migration and invasion in a range of cancers [205-210]. ITGA7 as a tumour suppressor gene is supported by a study by Guan et al who showed that ITGA7 knockdown in papillary thyroid cancer prompted invasion of tumour cells via epithelial-mesenchymal transition (EMT) as a consequence of elevated vimentin and N-cadherin and low E-cadherin expression [210]. Whereas Tan et al concluded that ITGA7 behaved as a tumour suppressor in prostate cancer by interacting with TIMP3 and causing a pause in cell growth at the G0/G1 phase within the cell cycle as a consequence of the TIMP3/NF-KB/cyclin D1 pathway which resulted in the low levels of cyclin D1 [211].

However, with respect to ITGA7 specifically, the literature on its function and mechanism of action in breast cancer is lacking. One proposed mechanism by Bhandari et al was that ITGA7 possesses an oncogenic role by encouraging tumour invasion by enhancing c-met and vimentin activity, which enhances the EMT process and ultimately breast cancer progression [203].

ITGA7 has also been associated with chemotherapy resistance, however, not in the context of breast cancer. Oesophageal squamous cell carcinoma cells that became resistant to cisplatin treatment demonstrated the capability to form spheroids as well as an increased expression of CSC associated genes in comparison to the tumour cells that did not develop resistance to cisplatin. ITGA7 is thought to encourage chemotherapy resistance by the focal adhesion kinase (FAK)/ Akt (protein kinase B)/ Caspase-9/Caspase-3 pathway [206].

Considering all the aforementioned findings I therefore aimed to investigate ITGA7 expression and effect in all subtypes of breast cancer in order to identify predictive markers of chemo-response and subsequently overall survival in a large cohort of human breast cancers following adjuvant chemotherapy.

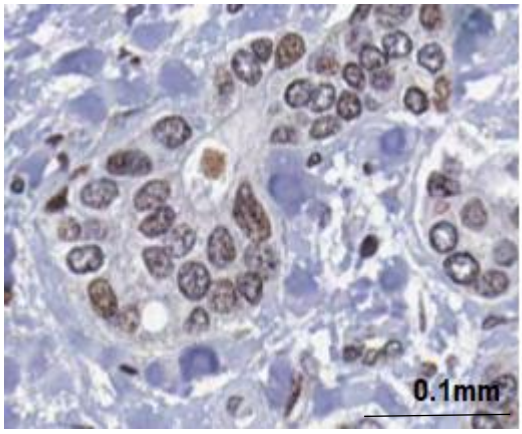
5.3. Results

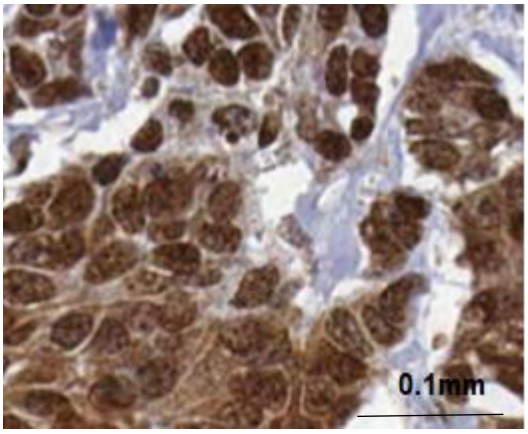
5.3.1. Optimisation and analysis of ITGA7 expression in breast cancer

The aim was to firstly achieve the best possible staining methods for ITGA7 and secondly to develop a representative scoring protocol. Adequate staining methods were obtained by adjusting conditions for antigen retrieval, blocking, and antibody concentrations which produced staining that appeared specific and demonstrated a range of patterns.

Following discussion with Dr Millican-Slater (Consultant Breast Pathologist, LTHT) a scoring protocol for ITGA7 was developed following observation that ITGA7 located to both nucleus and cytoplasm. Staining was scored taking into account intensity and proportion of cells staining positively using the Allred system (scores of 0 to 8), quantifying cytoplasmic and nuclear compartments separately. Representative images of staining, and the scores they were given, are shown in Figure 5.1.

Having optimised staining and scoring protocols, I then proceeded to stain and score my experimental cohort (Cohort 1). Cohort one represented the cases I had collected (Chapter 3), encompassing 305 primary breast cancers with a range of tumour subtypes and histological features, that each received adjuvant cytotoxic chemotherapy alone or combined with other treatments based upon individual tumour subtype. These were collected in a tissue microarray, with three tissue cores representing each case.





Nuclear intensity = 1

Proportion of nuclear staining = 4

Total score = 5

Nuclear intensity = 3

Proportion of nuclear staining = 5

Total score = 8

Cytoplasmic intensity = 0

Proportion of cytoplasmic staining = 0

Cytoplasmic intensity = 2

Proportion of cytoplasmic staining = 5

Figure 5.1 Representative images of immunohistochemical staining for ITGA7 in breast cancers, demonstrating the scoring protocol. Breast cancer tissues were sectioned and treated to visualise ITGA7 expression by immunohistochemistry (brown). They were also counterstained with Mayer's Haematoxylin to visualise nuclei (blue). ITGA7 scoring was based on cytoplasmic and nuclear staining, intensity (0-3), 0 being negative, 1 being weak, 2 intermediate and 3 strong and proportion (0-5); (0=0%, 1=<1%, 2=1-10%, 3=11-33%, 4=34-66% and 5=67-100%). Images are x20

5.3.2. Analysis of ITGA7 expression in breast cancer patients of all tumour subtypes treated with adjuvant chemotherapy

To authenticate ITGA7 expression and to assess the analysis for reproducibility of tumour core scores, 10% of the tumour cores were also scored by a second independent scorer, Dr Millican-Slater (Consultant Breast Pathologist, LTHT). Cohen's Kappa statistic was employed to determine the inter-scorer concordance and the reliability between scorers. This statistical test accounts for uncertainty amongst scorers and is more representative and accurate than other approaches, for example percentage scoring. The Kappa scores concluded near perfect agreement between scorers, 0.826 for nuclear ITGA7 and 0.878 for cytoplasmic ITGA7 expression [239], indicating that scoring was robust and reproducible.

As performed previously, there was a requirement to determine the variability amongst the three tumour cores that make up each case within the TMAs, in order to firstly, verify TMAs usage and secondly the decision relating to which approach core scores would be amalgamated to provide scores for each case. Analysis on TMAs may be unsuitable if there is large variability amongst the cores, which may imply that expression was heterogeneous. The various methods available in determining a score for each case include taking the highest or lowest score among tumour cores, using the mean or median score value of the triplicate tumour cores.

Spearman's rho correlation coefficients were determined to provide correlations between scores, which were strongly and significantly correlated in all cases (Table 5.1). Therefore, once again, the mean score of cores were taken as there was little evidence of intra-tumoral heterogeneity.

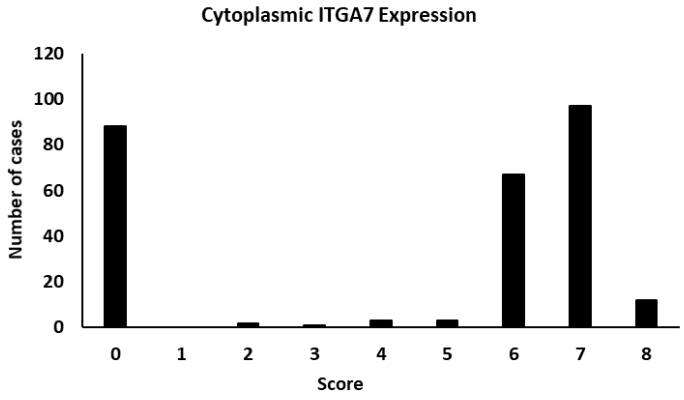
Spearman's Correlation Coefficient		**Core 1**	**Core 2**	**Core 3**
ITGA7 Cytoplasmic Expression	**Core 1**	1.0	0.926	0.889
	Core 2		1.0	0.948
	Core 3			1.0
ITGA7 Nuclear Expression	**Core 1**	1.0	0.878	0.934
	Core 2		1.0	0.872
	Core 3			1.0

Table 5.1 Core to core correlation of primary breast cancer cores for ITGA7 using Spearman's rho correlation. TMAs containing up to three primary breast cancer cores per case were stained for ITGA7 expression and quantified. Spearman's rho correlation analysis was used to compare the expression scores of cores within the same case. All correlations were statistically significant, p-value< 0.001

5.3.2.1. ITGA7 proteins are expressed at a wide range of levels across invasive breast cancers

To determine the level of protein expression of ITGA7 among the breast cancer cohort the mean protein expression scores for each case successfully scored were tallied and rounded to the nearest whole number. Distributions of protein expression scores are illustrated in Figures 5.2, displaying a dualistic distribution in the cytoplasm (Figure 5.2A), leaning towards being either absent or with a medium or strong expression in the majority of cells. In comparison, ITGA7 nuclear expression was more evenly distributed across the scores (Figure 5.2B). Despite the differences in the protein expression distributions , cytoplasmic and nuclear expression significantly correlated (Spearman's coefficient 0.66, p=0.01).

A.



B.

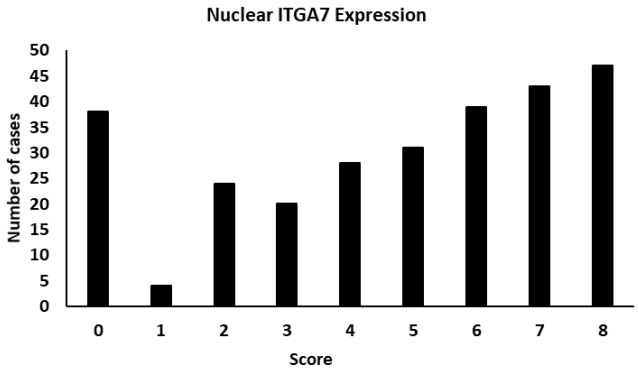


Figure 5.2 Score distributions for ITGA7 in breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of all molecular subtypes of primary breast cancer cases were scored for ITGA7 expression. Individual tumour cores were scored to quantify A. Intensity (0-3) of and proportion (0-5) of cells showing nuclear staining, sum of (0-8). B. Intensity (0-3) of and proportion (0-5) of cells showing cytoplasm staining, sum of (0-8). Mean scores calculated for each case were rounded to the nearest whole number

5.3.2.2. Protein expression of nuclear ITGA7 weakly correlated with tumour grade in breast cancer cases treated with adjuvant chemotherapy

To determine correlations between ITGA7 protein expression and clinical prognostic markers, which comprised of tumour grade, lymph nodes status (positive or negative), oestrogen receptor status (positive or negative) and molecular subtype (TNBC vs other subtype) Spearman's rho analyses were performed. A sub-analysis of TNBCs was performed since, as mentioned previously, this molecular subtype has the worst prognosis of all the molecular subtypes [32]. Table 5.2 shows the correlation scores and their associated p values. The only statistically significant correlations between protein expression of ITGA7 and any of the breast cancer prognostic markers, was an extremely weak negative correlation with tumour grade and ITGA7 nuclear expression, which was statistically significant at p=0.044, although this significance did not pass stringent correction for multiple testing. Overall, it was concluded that ITGA7 levels were independent of these factors.

		Grade of Tumour	Lymph Node Status	Oestrogen Expression	Triple Negative Subtype
ITGA7 Cytoplasmic Expression	**r**	-0.104	-0.010	0.114	-0.090
	p	0.086	0.989	0.059	0.137
ITGA7 Nuclear Expression	**r**	-0.122	0.004	0.045	0.008
	p	0.044*	0.944	0.456	0.891

Table 5.2 ITGA7 nuclear expression correlates with tumour grade in whole cohort. Spearman's rho analysis was performed for ITGA7 expression levels against tumour grade. r = Spearman's rho coefficient, p = p-value and * represents significant value ($p<0.05$)

5.3.2.3. Kaplan-Meier survival analysis revealed high ITGA7 protein expression is associated with improved survival after chemotherapy in breast cancer

One of the main objectives of this study was to determine if ITGA7 protein expression was significantly related to survival outcomes and could be used as predictive markers in patients with primary breast cancer following treatment with adjuvant chemotherapy.

To allow for the comparison between the two groups and to investigate survival by Kaplan-Meier analyses, cut-off values obtained from ROC curve analysis were applied to dichotomise

the cohort into patients regarded as having high or low expression. These cut-offs were based on the values that gave the best balance between sensitivity and specificity for prediction of clinical outcome. Further details and the ROC graphs can be found in Appendix 9.4.

To determine if the difference in ITGA7 expression was significantly associated with either DFS, for which the event was recurrence, or DSS, for which the event was death from breast cancer, Kaplan-Meier survival analyses were performed. High ITGA7 nuclear protein expression was associated with longer DFS, by a mean of 647 days; p=0.036. This trend was also demonstrated for cytoplasmic expression, although this was not significant and the difference in mean DFS was less, at 341 days. For DSS, neither cytoplasmic or nuclear ITGA7 expression were significantly associated with outcome, although the same trend for high expression being associated with longer survival was shown (p=0.065 and 0.063 respectively) (Table 5.3 and Figures 5.3 and 5.4).

		Mean DFS (days) (95% CI)	**Log Rank**	**Mean DSS (days) (95% CI)**	**Log Rank**
ITGA7 Cytoplasmic Expression	**Low**	3787 (3556-4018)	0.116	3935 (3730-4139)	0.065
	High	4128 (3902-4353)		4237 (4034-4441)	
ITGA7 Nuclear Expression	**Low**	3400 (3054-3745)		3759 (3427-4091)	
	High	4047 (3869-4226)	0.036*	4148 (3987-4309)	0.063

Table 5.3 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of ITGA7 in a cohort of 305 primary breast cancer patients treated with adjuvant chemotherapy. 95% confidence intervals and log rank p values stated. * represents significant log rank test (p<0.05)

A. **B.**

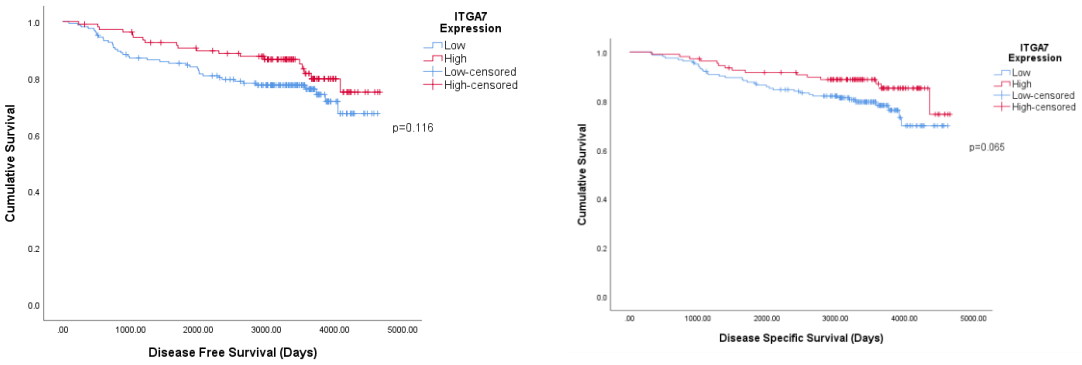


Figure 5.3 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression. Cytoplasmic ITGA7 expression was determined in a cohort of 305 primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic ITGA7 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

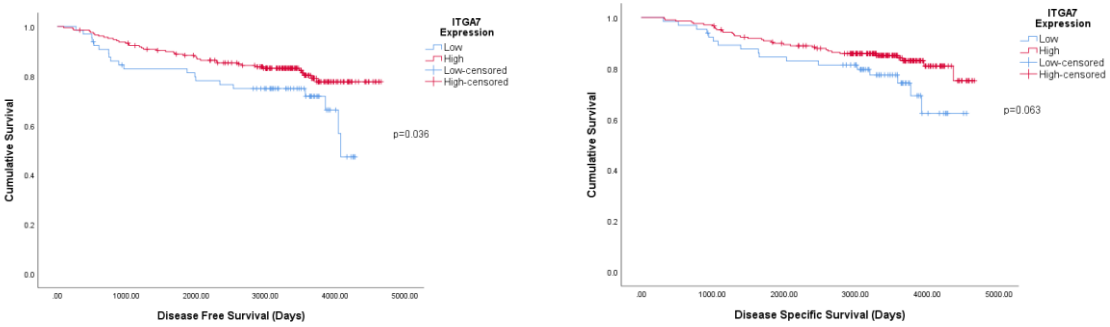


Figure 5.4 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear ITGA7 expression. Nuclear ITGA7 expression was determined in a cohort of 305 primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear ITGA7 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

5.3.2.4. High ITGA7 nuclear protein expression is associated with longer disease-free survival and disease-specific survival in ER- positive breast cancers

Next, I wanted to determine if the expression of ITGA7 in any of the breast cancer subtypes; ER-positive, ER-negative, HER2-positive or triple negative within the cohort correlated with DFS or DSS. Sub-group analyses were performed using Kaplan-Meier survival analysis.

This showed that high ITGA7 nuclear expression had longer DFS and DSS in ER-positive breast cancers (n=207) by 682 days; p=0.05 and 604 days; p=0.005 respectively (Table 5.4 and Figures 5.5 and 5.6).

ER-Positive Breast Cancers		**Mean DFS (days) (95% CI)**	**Log Rank**	**Mean DSS (days) (95%CI)**	**Log Rank**
ITGA7 Cytoplasmic Expression	**Low**	3989 (3740-4237)	0.205	4129 (3907-4351)	0.070
	High	4281 (4081-4482)		4414 (4236-4593)	
ITGA7 Nuclear Expression	**Low**	3548 (3160-3936)	0.05*	3777 (3379-4176)	0.005*
	High	4230 (4061-4400)		4381 (4238-4523)	

Table 5.4 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of ITGA7 in ER-Positive breast cancers (n=207). 95% confidence intervals and log rank p values stated. * represents significant log rank test ($p<0.05$)

A. **B.**

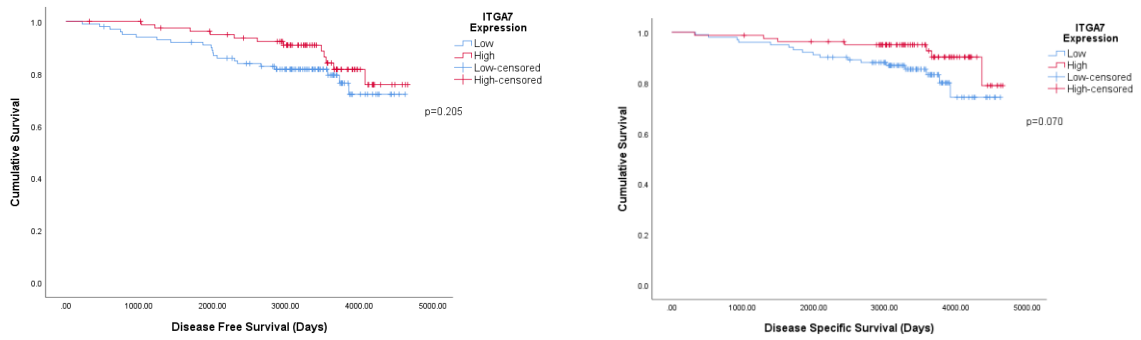


Figure 5.5. Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression. Cytoplasmic ITGA7 expression was determined in a cohort of 207 ER-Positive primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

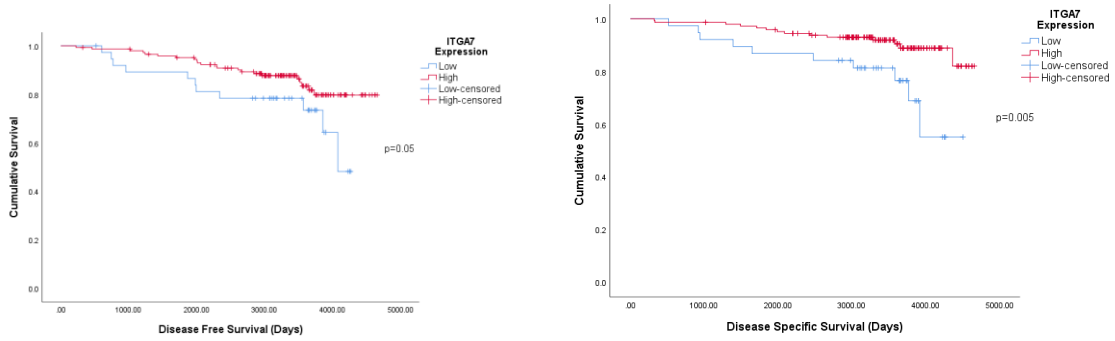


Figure 5.6 Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low nuclear ITGA7 expression. Nuclear ITGA7 expression was determined in a cohort of 207 ER-Positive primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

However, the sub-group analyses of the HER2-positive (n=68), triple negative (n=68) or ER-negative (n=98) subtypes demonstrated no significant differences in DFS or DSS relating to high or low ITGA7 protein expression (Table 5.5, while the Kaplan-Meier plots can be found in Appendix 9.5).

		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
HER2-Positive Subtype					
ITGA7 Cytoplasmic Expression	**Low**	3547	0.500	3778	0.584
	High	3637		3719	
ITGA7 Nuclear Expression	**Low**	2778	0.199	3316	0.240
	High	3754		3935	
Triple Negative Subtype					
ITGA7 Cytoplasmic Expression	**Low**	3415	0.737	3472	0.832
	High	3428		3488	
ITGA7 Nuclear Expression	**Low**	3364	0.936	3686	0.403
	High	3473		3451	
ER-Negative Subtype					
ITGA7 Cytoplasmic Expression	**Low**	3431	0.645	3581	0.874
	High	3432		3499	
ITGA7 Nuclear Expression	**Low**	3191	0.513	3701	0.640
	High	3564		3560	

Table 5.5 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of ITGA7 in HER2-Positive (n=68), Triple Negative (n=68) and ER-Negative (n=98) breast cancers. 95% confidence intervals and log rank p values stated

5.4. Kaplan-Meier survival analysis revealed a significant increase in disease-free survival in primary breast cancers that had high ITGA7 nuclear protein expression and that received anthracycline-based adjuvant chemotherapy

To determine whether the influence of ITGA7 was dependent on different adjuvant chemotherapy regimens, Kaplan-Meier survival analyses were performed separately on the patients who received either taxanes with anthracyclines or anthracyclines without taxanes. The findings were that high ITGA7 nuclear expression was associated with an extended DFS, by 806 days; p=0.004, in the group of patients that received anthracycline based adjuvant chemotherapy (Table 5.6 and Figures 5.7 and 5.8). No statistical significant difference in DFS or DSS was noted between high and low ITGA7 expression in the group that received taxane based adjuvant chemotherapy (Table 5.7) (Appendix 9.6).

		Mean DFS (days) (95% CI)	**Log Rank**	**Mean DSS (days) (95% CI)**	**Log Rank**
ITGA7 Cytoplasmic Expression	Low	4164 (3935-4393)	0.102	4278 (4080-4476)	0.151
	High	4418 (4239-4598)		4461 (4292-4631)	
ITGA7 Nuclear Expression	Low	3603 (3188-4019)	0.004*	4012 (3645-4378)	0.071
	High	4409 (4276-4542)		4429 (4300-4559)	

Table 5.6 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of ITGA7 in whole cohort of breast cancers that received anthracycline based adjuvant chemotherapy (n=149). 95% confidence intervals and log rank p values stated

A. **B.**

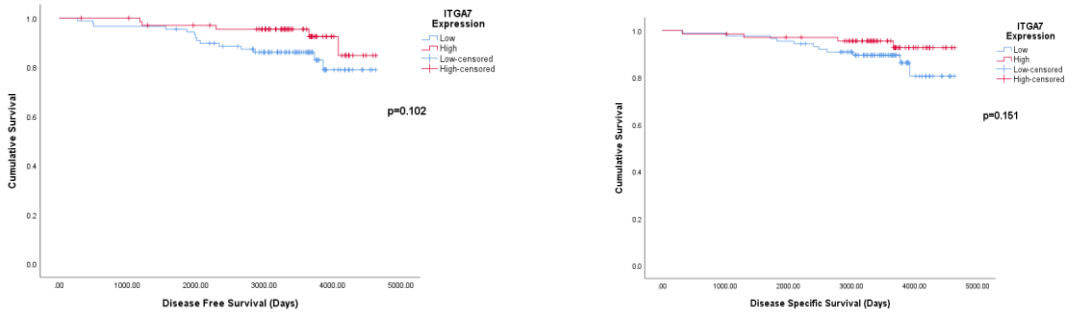


Figure 5.7 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression in patients that received Anthracycline adjuvant chemotherapy. Cytoplasmic ITGA7 expression was determined in a cohort of 149 primary breast cancer patients treated with anthracycline adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

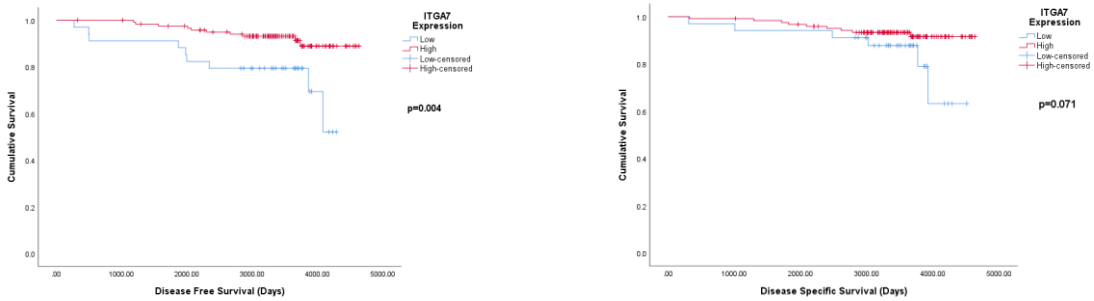


Figure 5.8 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear ITGA7 expression in patients that received Anthracycline adjuvant chemotherapy. Nuclear ITGA7 expression was determined in a cohort of 149 primary breast cancer patients treated with anthracycline adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
ITGA7 Cytoplasmic Expression	Low	3249 (2863-3634)	0.721	3476 (3126-3826)	0.372
	High	3591 (3115-4067)		3781 (3350-4213)	
ITGA7 Nuclear Expression	Low	3154 (2600-3709)	0.821	3407 (2858-3955)	0.467
	High	3485 (3127-3842)		3671 (3345-3996)	

Table 5.7 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of ITGA7 in whole cohort of breast cancers that received taxane based adjuvant chemotherapy (n=116). 95% confidence intervals and log rank p values stated

5.5. Discussion

5.5.1. ITGA7 as a prognostic marker in breast cancer following adjuvant chemotherapy

The main aim of this chapter was firstly to determine if ITGA7 protein expression was associated with survival outcomes following adjuvant chemotherapy for breast cancer and can be utilised as a predictive indicator to guide chemotherapy usage and enhance survival outcomes. Secondly, to support initial observations regarding therapy-induced selection of ITGA7 somatic mutations and reduced expression in cancer stem cells, that implicated ITGA7 as a mediator of chemotherapy response (Chapter 1; Section 1.81 and 1.82).

The quality of antibodies is vital for IHC analysis and therefore, the ITGA7 antibody was tested for specificity and validated by a member of the Hughes lab with the aim of confidently using this antibody in the IHC analysis. The commonly used breast cancer cell line MCF7 was transfected with siRNA targeting ITGA7 or with non-targeting siRNAs used as a control. ITGA7 expression using this antibody was assessed by both Western blot and immunofluorescence (Figures 5.9 A and B). The main ITGA7 species of ~26kDa was identified by the antibody and also corresponds with the size of the protein's C-terminal fragment [259]. The known epitope of this antibody is also found within the C-terminal. Additionally, a small fragment ~15Da was also identified. Both fragments were specific to ITGA7 as following targeted knock-down there was reduced expression of the bands. Following immunofluorescence ITGA7 was identified both within the cytoplasm and more unexpectedly within the nuclear component of cells. Importantly, expression in both the cytoplasm and nucleus was demonstrated as being specific to ITGA7 as there was a marked decrease in expression of both cytoplasmic and nuclear components following a targeted knockdown (Figure 5.9 B).

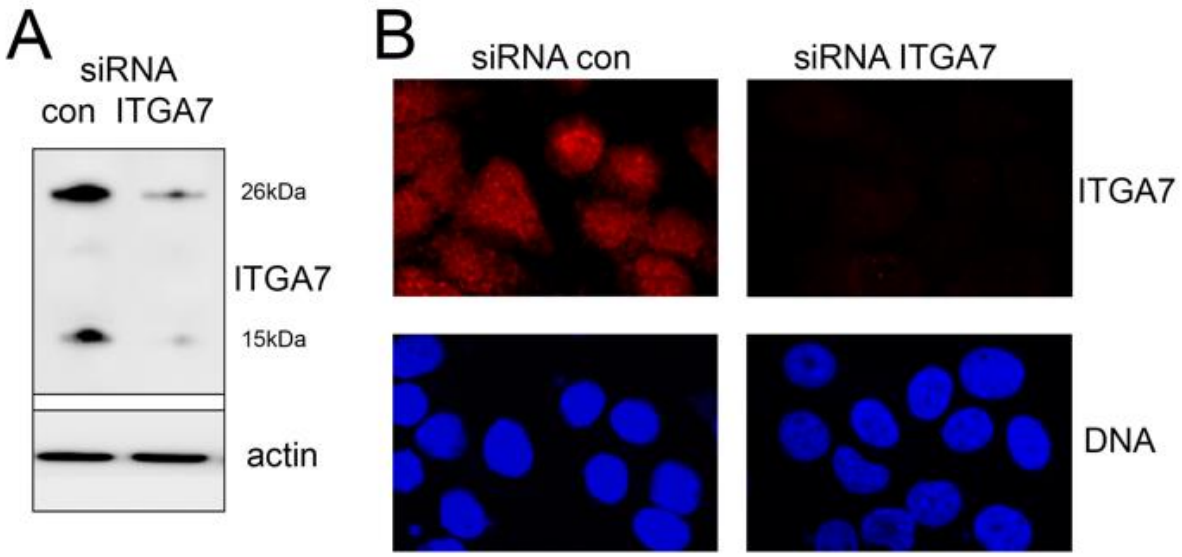


Figure 5.9 A and B. The ITGA7 antibody used in this study is specific for ITGA7. MCF7 cells were transfected with siRNA targeted against ITGA7 or control siRNA (con). ITGA7 expression was analysed using Western blots (A) or immunofluorescence (B) using actin as a house-keeping gene or the DNA stain DAPI as a counter-stain. This work was performed by Arindam Pramanik (University of Leeds)

Following the finding of antibody specificity for ITGA7, I then set about examining the expression of ITGA7 in both cytoplasmic and nuclear compartments in a cohort of 305 breast cancers comprising of a variety of subtypes that all received adjuvant chemotherapy, as well as a combination of other treatments according to individual tumour subtypes.

I firstly wished to determine if ITGA7 expression correlated with clinical prognostic markers, which comprised of tumour grade, lymph nodes status (positive or negative), oestrogen receptor status (positive or negative) and molecular subtype (TNBC vs other subtype) in the whole cohort and to determine prognosis in terms of DFS and DSS in the whole cohort as well as each of the four tumour subtypes with the aim of identifying new therapeutic targets that may help overcome chemotherapy resistance.

Kaplan-Meier survival analysis was performed for low versus high protein expression of ITGA7. A key finding of this analysis is that a relatively low ITGA7 expression in nuclei was significantly associated with shorter survival, by a mean of 647 days (p=0.036). This trend was also visible for cytoplasmic expression, although this was not significant, and the difference in survival was less; 341 days. Also, in the ER-positive sub-analysis a high ITGA7 nuclear expression was associated with a longer DFS (682 days; p=0.05) and a longer DSS (604 days; p=0.005) than low expression following adjuvant chemotherapy.

In contrast to our findings, Bai et al has shown that a high expression of ITGA7 in breast cancer, correlated with worse clinicopathological characteristics in terms of size and grade of tumour, TNM stage and poor OS. Whereas knockdown of ITGA7 in MCF7 cell lines resulted in increased apoptosis and inhibition of cell growth and migration of breast cancer cells [200]. Unlike, our study ITGA7 expression was determined by intensity categories from 0, no staining to 3, intense staining and percentage of cells staining using the histological score. The authors did not differentiate between the expression of cytoplasmic or nuclear staining. Other studies have confirmed the association of upregulation of ITGA7 with adverse outcome in oesophageal cancer [206] and pancreatic cancer [205]. Knockdown of ITGA7 in hepatocellular cancer prevented cell invasion [203] and ITGA7 knockdown in glioblastoma cells blocked cell proliferation [209].

Whereas, Bhandari et al concluded that ITGA7 functions as a tumour suppressor in breast cancer as ITGA7 expression was low in breast cancer tissue in comparison to normal mammary tissue using RT-qPCR. Knocking down ITGA7 in MDA-MB-231 and BT-549 cell lines using siRNA was thought to encourage tumour invasion by enhancing c-met and vimentin activity which enhances the EMT process and ultimately breast cancer progression [203]. ITGA7 as a tumour suppressor gene was also supported by a study that demonstrated the downregulation of ITGA7 in papillary thyroid cancer resulted in increased growth, motility and invasion of tumour cells and ITGA7 knockdown prompted invasion of tumour cells via EMT as a consequence of elevated vimentin and N-cadherin and low E-cadherin expression [210].

Remarkably, it was demonstrated that ITGA7 was expressed in both the plasma membrane/cytoplasm and in the nucleus, which has been documented for many integrins [260, 261], although there is paucity in the literature for ITGA7 nuclear expression. Although, the function of nuclear ITGA7 at the molecular level is unknown it is evident from our findings that nuclear expression of ITGA7 significantly correlated with survival outcomes and thus potentially functional in nature. A study by Seraya-Bareket et al highlights a good example of the presence of nuclear integrins, albeit ITGαvβ3, in high grade ovarian malignancies which were not present in normal tissue and encouraged proliferation independently of the plasma-membrane adherence function [260].

The findings of the unusual ITGA7 nuclear expression in breast cancer tissue contests the fact that integrins exclusively utilise their actions at the plasma membrane/cytoplasm. This finding may enhance our knowledge of breast cancer pathogenesis at the molecular level.

Lastly, Kaplan-Meier survival analysis revealed the impact of ITGA7 to be particularly notable in patients who received anthracyclines without taxanes (significant increase in DFS of 806

days; p=0.004) as opposed to those who received taxanes (no significant impact on DFS). This was also supported by in-vitro studies performed by the Hughes lab which concluded that knock down of ITGA7 was associated with significant protection of MCF7 cells from the effects of the anthracycline epirubicin. This is the first study to explore ITGA7 expression and chemotherapy response. These findings are likely due to the fact that both anthracyclines and taxanes have different mechanisms of actions. Anthracyclines limit cancer growth via multiple mechanisms, such as intercalation between DNA base pairs leading to nucleic acid damage and ultimately interfering with the synthesis of DNA and RNA in highly proliferating cells thereby, inhibiting transcription and replication. They can also form a DNA-topoisomerase II complex thereby, preventing the repair of double-strand DNA breaks-which leads to growth arrest and programmed cell death and lastly, they form free radicals destroying cell membranes [66]. Whereas taxanes bind to the β-tubulin of the microtubule and suppress microtubules dynamic instability during the mitotic stage of the cell cycle, which leads to mitotic arrest and induces apoptosis in cells during division [69]. ITGA7 controls interactions between the cell membranes and the extracellular matrix and therefore can greatly influence the effects of epirubicin in comparison to taxanes.

5.6. Conclusion

In this chapter I have shown that high ITGA7 nuclear protein expression correlates with longer DFS and DSS in ER-positive breast cancers treated with adjuvant chemotherapy. Also, high ITGA7 nuclear expression correlates with increased DFS and DSS in breast cancer after anthracycline based chemotherapy and these findings suggest that ITGA7 could act as a possible predictive marker or, more excitingly, a target for chemo-sensitizing drugs that aim to enhance ITGA7 expression or activity.

6. HIGH NUCLEAR NR4A1 EXPRESSION IS ASSOCIATED WITH POOR PROGNOSIS IN BREAST CANCER PATIENTS

6.1. Abstract

Background: The orphan nuclear receptor 4A1 (NR4A1) belongs to the nuclear receptor family and acts as a transcription factor that regulates downstream gene expression. It plays a key role in a wide range of cellular behaviours including cell growth and differentiation, migration, DNA repair and apoptosis. However, within the current literature NR4A1's involvement in carcinogenesis is inconsistently reported. The aim of this chapter is to evaluate the relevance of NR4A1 expression with respect to clinical prognostic markers and outcomes in my breast cancer cohort, as well as in the METABRIC dataset.

Methods: Breast cancer tissue was obtained from a tissue microarray comprising of all tumour subtypes, with the common factor of use of adjuvant cytotoxic chemotherapy (n=305). Expression of NR4A1 was determined by immunohistochemistry. METABRIC data were accessed via cBioPortal. Records with suitable NR4A1 expression data and clinical data of relevance were obtained (n=1886). For both cohorts, cases were then dichotomised into high or low NR4A1 expression by performing receiver operator curve analyses, and relationships between expression and survival were tested.

Results: Using immunohistochemistry, NR4A1 was detected in all breast cancer cases, with cytoplasmic expression of varying degrees in all cases, and nuclear expression in approximately half. Kaplan-Meier survival analyses showed that high NR4A1 nuclear protein expression was associated with a poorer disease-free survival and disease-specific survival, by means of 487 days (p=0.005) and 621 days (p=0.001) respectively in the whole cohort. A sub-analysis of triple negative and ER-negative breast cancers also showed that high NR4A1 nuclear protein expression was associated with both a shorter disease-free survival (by 1452 days, p=0.002 and 1141 days, p=0.006 respectively) and disease-specific survival (by 1603 days, p=0.001 and 1187 days, p=0.001 respectively). In contrast, analyses of the METABRIC dataset demonstrated no significant influence of NR4A1 mRNA expression on disease-free survival (p=0.978) or disease-specific survival (p=0.288) in the whole cohort, or in the different molecular subgroups, or depending on use of chemotherapy.

Conclusion: High nuclear NR4A1 expression correlates with decreased disease-free survival and disease-specific survival after chemotherapy in breast cancer and these findings suggest that NR4A1 could act as a potential target for inhibitor drugs in breast cancer.

6.2. Introduction

The orphan nuclear receptor 4A1 (NR4A1) was identified as a candidate gene potentially involved in breast cancer chemoresistance from a genomics screen previously performed by the Hughes lab [190] (Chapter 1, Section 1.8.1).

NR4A1 belongs to the nuclear receptor family of ligand-dependent transcription factors, and has roles in controlling gene expression, thereby modifying cell growth, and apoptosis. It is classified as an orphan receptor, as its endogenous ligand(s) have not yet been identified, which makes its potential use in targeted therapy more difficult than better characterised receptors [212].

The roles of NR4A1 in breast cancer reported within the current literature do not allow consistent conclusions. Many studies have described NR4A1 as a tumour suppressor [213, 214], likely due to function of reducing activity of the JNK-AP-1-cyclin D1 pathway [213]. In contrast, other studies have shown that NR4A1 expression encouraged tumour invasion and disease progression in breast cancer by initiating TGF-β/SMAD signalling [216]. Similarly, Hedrick and Safe supported these findings but also reported that this TGF-β signalling induced mitogen-activated protein kinase 14 (MAPK14) that was crucial in the metastasis-related process EMT [217].

Taking into consideration the findings to date, I therefore aimed to investigate NR4A1 expression in a range of breast cancer subtypes, and to assess its potential correlation with cancer outcomes. In particular, I aimed to assess it as a predictive marker of chemo-response. I had also planned to test experimentally whether NR4A1 expression impacted on chemo-response using tissue culture models, however this work was cancelled due to laboratory closures associated with the COVID19 pandemic in 2020-21. Therefore, I attempted to further assess the impact of NR4A1 expression by mining publicly available data on-line; specifically, using the METABRIC dataset through cBioPortal [228], which is an accessible web resource that allows for investigation and analyses of cancer genomic/transcriptomic data from multiple sources [262].

6.3. Results

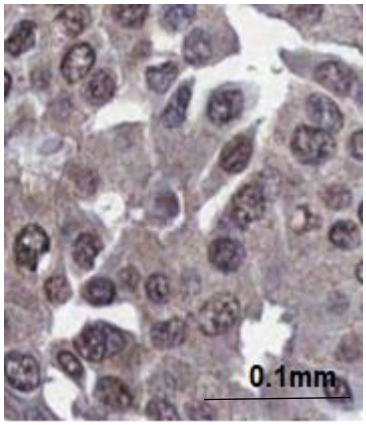
6.3.1. Optimisation and analysis of NR4A1 expression in breast cancer

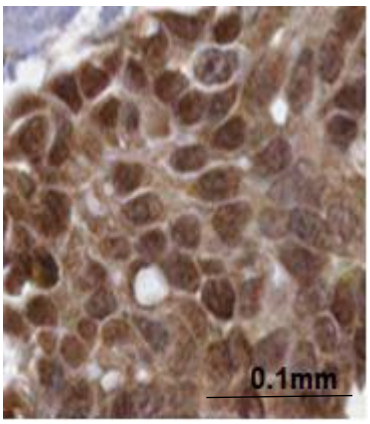
Firstly, there was a requirement to optimise the immunohistochemical staining methods for NR4A1 and to develop a representative scoring protocol. Staining was improved by altering the conditions for antigen retrieval, blocking, and antibody concentrations in order to obtain a staining pattern that appeared specific and representative of an assortment of patterns.

NR4A1 staining was seen in both the nucleus and cytoplasm of tumour cells and the intensity of staining was highly uniform amongst all cores of a particular case. Representative images are shown in Figure 6.1.

A scoring protocol for the stained epithelial cancer cells was developed after discussion with Dr Millican-Slater (Consultant Breast Pathologist, LTHT) and was based upon intensity and proportion of cells staining positively using the Allred system (scores of 0 to 8), quantifying cytoplasmic and nuclear compartments separately.

Once staining methods of NR4A1 were optimised and a scoring protocol devised, I then proceeded to stain my experimental cohort comprising of 305 cases of primary breast cancer treated with adjuvant chemotherapy that were collected in a tissue microarray, as previously (see Chapter 3 for a more detailed description of cohort selection).





Nuclear intensity = 0

Proportion of nuclear staining = 0

Total score = 0

Nuclear intensity = 3

Proportion of nuclear staining = 5

Total score = 8

Cytoplasmic intensity = 1

Proportion of cytoplasmic staining = 5

Total score = 6

Cytoplasmic intensity = 3

Proportion of cytoplasmic staining = 5

Total score = 8

Figure 6.1 Representative images of immunohistochemical staining for NR4A1 in breast cancers, demonstrating the scoring protocol. Breast cancer tissues were sectioned and treated to visualise NR4A1 expression by immunohistochemistry (brown). They were also counterstained with Mayer's Haematoxylin to visualise nuclei (blue). NR4A1 scoring was based on cytoplasmic and nuclear staining, intensity (0-3), 0 being negative, 1 being weak, 2 intermediate and 3 strong and proportion (0-5); (0=0%, 1=<1%, 2=1-10%, 3=11-33%, 4=34-66% and 5=67-100%). Images are x20

6.3.2. Analysis of expression of NR4A1 in breast cancer patients treated with adjuvant chemotherapy

I scored NR4A1 expression in all tissue cores. To confirm scoring reproducibility, 10% of the tissue cores were also scored by Dr Millican-Slater (Consultant Breast Pathologist, LTHT). Cohen's Kappa scores of inter-scorer concordance were calculated, indicating near perfect agreement between scorers for NR4A1 cytoplasmic expression (0.771) and NR4A1 nuclear expression (0.801) [244], signifying that scoring was reproducible. Yet again, there was a requirement to determine the inconsistencies between the three tumour cores that made up each case within the TMAs to defend TMA usage as well as to allow a suitable explanation for the method used to pool scores for individual tumour cores into a score for each case.

Assessment of correlations between scores was performed by Spearman's rho correlation coefficients analysis; scores were strongly and significantly correlated in all cases (Table 6.1). Therefore, as there was little evidence of intra-tumoral heterogeneity, the mean score of cores was again taken to provide a case score for each tumour.

		Core 1	**Core 2**	**Core 3**
NR4A1 Cytoplasmic Expression	**Core 1**	1.0	0.874	0.858
	Core 2		1.0	0.888
	Core 3			1.0
NR4A1 Nuclear Expression	**Core 1**	1.0	0.934	0.914
	Core 2		1.0	0.934
	Core 3			1.0

Table 6.1 Core to core correlation of primary breast cancer cores for NR4A1 using Spearman's rho correlation. TMAs containing up to three primary breast cancer cores per case were stained for NR4A1 expression and quantified. Spearman's rho correlation analysis was used to compare the expression scores of cores within the same case. All correlations were statistically significant, $p<0.001$

6.3.2.1. NR4A1 protein is expressed at a range of levels across invasive breast cancers

To establish the degree of protein expression of NR4A1 amongst the breast cancer cohort, the average scores for protein expression of NR4A1 for each case successfully scored were tallied and rounded to the nearest whole number for simplicity of data representation, as shown in Figure 6.2. Approximately half the cohort was negative for nuclear protein expression, with the remainder exhibiting a range of expression levels from weak expression through to strong expression. In comparison, NR4A1 cytoplasmic expression comprised of low, intermediate and strong expression but no negative expression. NR4A1 cytoplasmic and nuclear expression were found to be statistically significantly associated with each other with a Spearman's rho of 0.257 (p=0.001), indicating a relatively weak association.

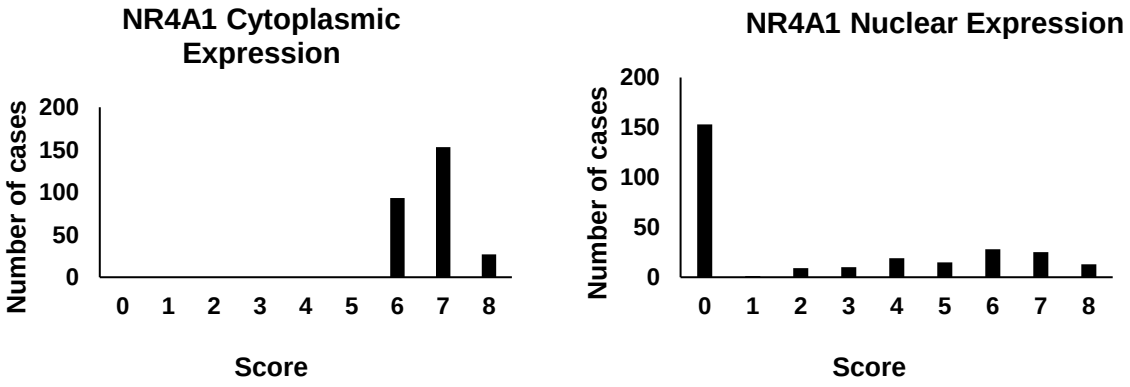


Figure 6.2 Score distributions for NR4A1 in breast cancer patients treated with adjuvant chemotherapy. TMAs comprising of all tumour subtypes of primary breast cancer cases were scored for NR4A1 expression. Individual tumour cores were scored to quantify A. Intensity (0-3) of and proportion (0-5) of cells showing cytoplasmic staining, sum of (0-8). B. Intensity (0-3) of and proportion (0-5) of cells showing nuclear staining, sum of (0-8). The mean scores calculated for each case from combining multiple core scores rounded to the nearest whole number are shown

6.3.2.2. Protein expression of NR4A1 did not correlate with any clinical prognostic markers in breast cancer cases that received adjuvant chemotherapy

To establish correlations between NR4A1 protein expression and clinical prognostic markers, comprising of tumour grade, lymph nodes status (positive or negative), oestrogen receptor status (positive or negative) and molecular subtype (TNBC vs other subtype), Spearman's rho analyses were performed. Table 6.2 shows the correlations coefficients and the associated p values. There were no statistically significant correlations between protein expression of NR4A1 and any of the aforementioned breast cancer prognostic markers. I concluded that NR4A1 expression was unrelated to standard prognostic factors, and therefore that any prognostic or predictive information the marker offered would potentially be additional to that available already through standard markers.

		Grade of Tumour	Lymph Node Status	Oestrogen Expression	Triple Negative Subtype
NR4A1 Cytoplasmic Expression	**r**	-0.013	-0.062	-0.096	0.042
	p	0.833	0.311	0.114	0.485
NR4A1 Nuclear Expression	**r**	0.036	0.007	-0.116	0.040
	p	0.555	0.910	0.056	0.515

Table 6.2 NR4A1 expression did not correlate with any clinical prognostic markers. Spearman's rho analysis was performed for NR4A1expression levels against tumour grade, lymph node status, oestrogen expression and triple negative subtype. r = Spearman's rho coefficient, p = p-value

6.3.2.3. High NR4A1 nuclear protein expression is associated with poorer survival after chemotherapy in breast cancer

The key purpose of this study was to establish if NR4A1 expression at the protein level could be used as a predictive marker for DFS and DSS after adjuvant chemotherapy for the treatment of breast cancer.

To permit comparison between both groups and to examine survival by Kaplan-Meier analyses it was a requirement to dichotomise the cohort into patients regarded as having either a high or low expression of NR4A1 using suitable cut off values which were made available by performing ROC curve analyses. Cut off values can be found in Appendix 9.7.

To establish if differences in protein expression of NR4A1 was significantly associated with either DFS or DSS, Kaplan-Meier survival analyses were performed. Cytoplasmic NR4A1 expression was not significantly associated with survival (Table 6.3, Figure 6.3). However, high NR4A1 nuclear protein expression was associated with a poorer DFS and DSS, by means of 487 days; p=0.005 and 621 days; p=0.001 respectively (Table 6.3, Figure 6.4).

		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95% CI)	Log Rank
NR4A1 Cytoplasmic Expression	**Low**	3817 (3513-4124)	0.334	3964 (3695-4234)	0.183
	High	3970 (3772-4168)		4106 (3931-4281)	
NR4A1 Nuclear Expression	**Low**	4133 (3950-4316)	0.005*	4331 (4190-4472)	0.001*
	High	3646 (3353-3940)		3710 (3438-3982)	

Table 6.3 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low protein expression of NR4A1 in the Whole Cohort (n=305). 95% confidence intervals and log rank p values stated**.** * represents significant log rank test (p<0.05)

A. **B.**

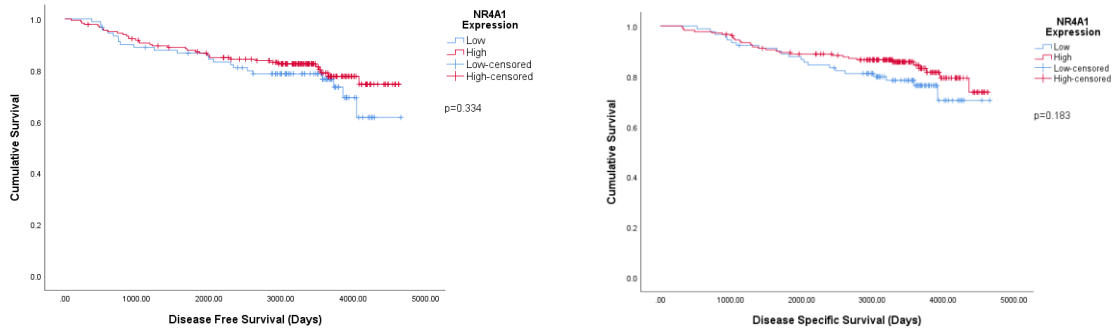


Figure 6.3 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic NR4A1 expression. Cytoplasmic NR4A1 expression was determined in a cohort of 305 primary breast cancer patients treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic NR4A1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

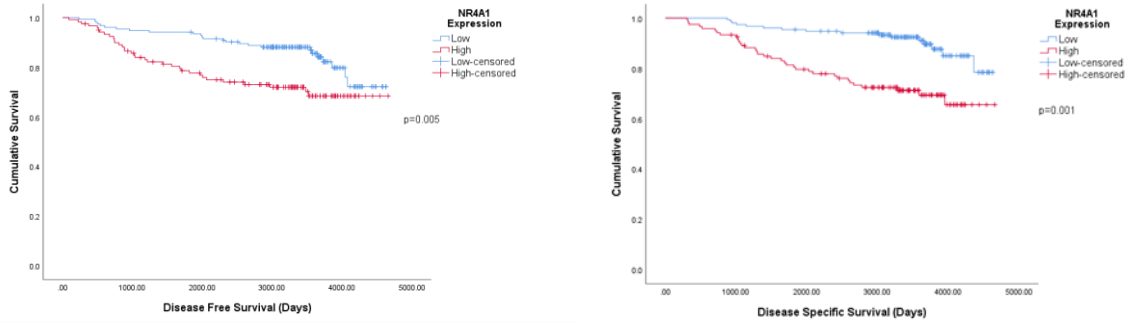


Figure 6.4 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear NR4A1 expression. Nuclear NR4A1 expression was determined in a cohort of 305 primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

6.3.2.4. High NR4A1 nuclear protein expression is associated with poorer disease-free survival and disease- specific survival in triple negative and ER-negative breast cancer subtypes

Subsequently, I sought to clarify if the expression of NR4A1 correlated with DFS or DSS in any of the breast cancer subtypes; triple negative, ER-negative, ER-positive and HER2-positive within the cohort. A sub-group analysis was carried out using Kaplan-Meier survival analysis. This analysis demonstrated that cytoplasmic NR4A1 expression was not significantly associated with survival in any group (Table 6.4). However, high NR4A1 nuclear expression had poorer DFS and DSS in triple negative breast cancers (n=68), by 1452 days; p=0.002 and 1552 days; p=0.001 respectively. With regards to the ER-negative subtype (n=98) this also demonstrated a poorer DFS and DSS by 1141 days; p=0.006 and 1187 days; p=0.001 respectively. However, for ER-positive tumour subtype (n=207) only DSS had a statistically poorer outcome with high nuclear expression, by 537 days (p=0.012), although the trend for DFS also showed a poorer outcome with high nuclear expression albeit not statistically significant (p=0.110). Similarly, high NR4A1 nuclear expression in HER2-positive breast cancers followed the same trend of a worse outcome in terms of DFS and DSS but again was not statistically significant (Table 6.4, Figures 6.5-6.7). Kaplan-Meier plots for survival outcomes in groups with high versus low cytoplasmic NR4A1 expression in all breast cancer subtypes can be found in Appendix 9.8.

		Mean DFS (days) (95% CI)	Log Rank	Mean DSS (days) (95%CI)	Log Rank
Triple-Negative Subtype					
NR4A1 Cytoplasmic Expression	**Low**	3227 (2587-3867)	0.940	3475 (2807-4142)	0.813
	High	3474 (2946-4002)		3558 (3076-4020)	
NR4A1 Nuclear Expression	**Low**	4095 (3662-4529)	0.002*	4254 (3911-4597)	0.001*
	High	2643 (1996-3289)		2651 (2093-3209)	
ER-Negative Subtype					
NR4A1 Cytoplasmic Expression	**Low**	3084 (2505-3663)	0.442	3542 (2987-4098)	0.595
	High	3563 (3148-3977)		3635 (3261-4008)	
NR4A1 Nuclear Expression	**Low**	4024 (3616-4431)	0.006*	4189 (3867-4512)	0.001*
	High	2883 (2390-3377)		3002 (2571-3433)	
ER-Positive Subtype					
NR4A1 Cytoplasmic Expression	**Low**	4090 (3805-4374)	0.665	4119 (3835-4404)	0.177
	High	4162 (3966-4358)		4319 (4155-4482)	
NR4A1 Nuclear Expression	**Low**	4228 (4060-4396)	0.110	4363 (4218-4507)	0.012*
	High	3786 (3368-4204)		3826 (3425-4228)	
HER2-Positive Subtype					
NR4A1 Cytoplasmic Expression	**Low**	3098 (2488-3708)	0.370	3422 (2897-3946)	0.496
	High	3749 (3324-4173)		3885 (3488-4282)	
NR4A1 Nuclear Expression	**Low**	3779 (33014257)	0.718	3870 (3408-4332)	0.652
	High	3433 (2910-3955)		3485 (3027-3944)	

Table 6.4 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of NR4A1 in Triple Negative (n=68), ER-Negative (n=98), ER-Positive (n=207) and HER2-Positive (n=68) breast cancers. 95% confidence intervals and log rank p values stated

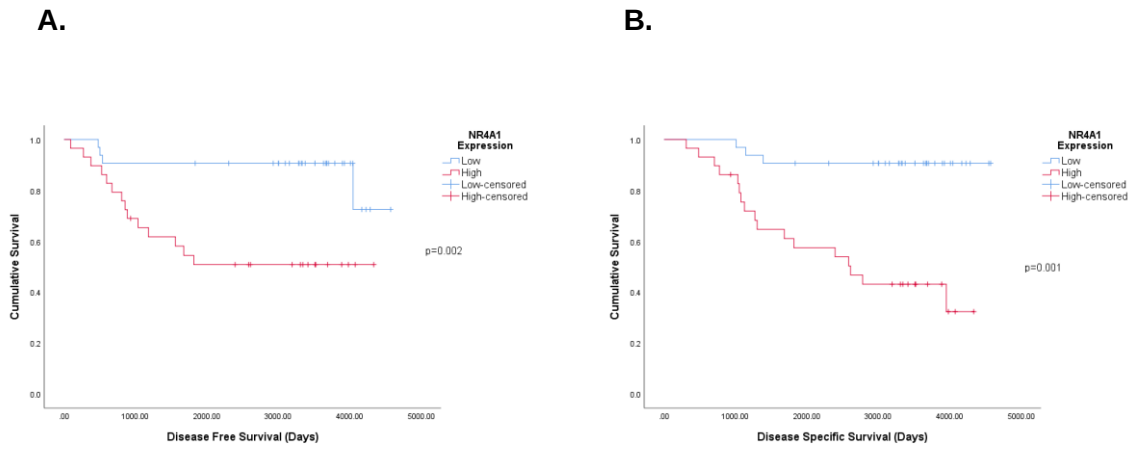


Figure 6.5 Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low nuclear NR4A1 expression. Nuclear NR4A1 expression was determined in a cohort of 68 Triple Negative primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

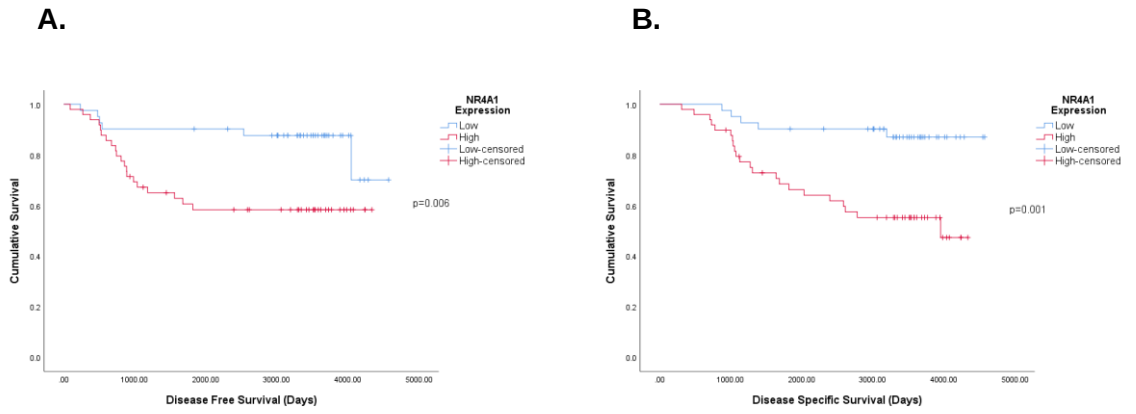


Figure 6.6 Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low nuclear NR4A1 expression. Nuclear NR4A1 expression was determined in a cohort of 98 ER-Negative primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

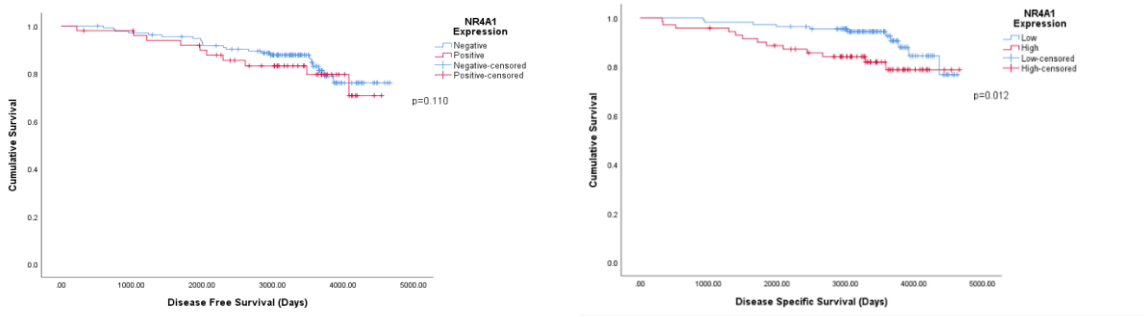


Figure 6.7 Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low nuclear NR4A1 expression. Nuclear NR4A1 expression was determined in a cohort of 207 ER-Positive primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

6.3.3. NR4A1 mRNA levels correlate very weakly with prognostic factors, and not with outcomes in the METABRIC dataset

My aim now was to support independently the findings from my cohort that high nuclear NR4A1 was associated with worse outcomes in women with primary breast cancer treated with adjuvant chemotherapy. I aimed to test this using public data from the METABRIC study; however, a key difference with the METABRIC data set is that data available are for NR4A1 mRNA levels and therefore, this does not allow for separate analyses of protein locations. The METABRIC database was accessed via the cBioPortal for Cancer Genomics [237] (see chapter 2, section 2.8). 1886 cases with NR4A1 expression data and appropriate clinical information were obtained. Comprehensive data including patients age at diagnosis, pathological data (tumour subtype, tumour grade, hormone receptor and HER2 status as well as lymph node status), oncological data (chemotherapy, radiotherapy, endocrine treatment received) and DFS and DSS were collected. Table 6.5 provides a summary of the clinico-pathological and pharmacological features for breast cancers within the cohort selected from the METABRIC dataset.

Characteristics	n=1886 (100%)
Age: 61 (22-96) years	
Tumour Histopathology	
Ductal NST	1500 (79.5)
Lobular	142 (7.5)
Mixed Ductal/Lobular	207 (11)
Mixed Mucinous	22 (1.2)
Other	15 (0.8)
Tumour Grade	
1	185 (9.8)
2	758 (40.2)
3	943 (50)
Lymph Node Status	
N0	981 (52)
N1	602 (31.9)
N2	200 (10.6)
N3	103 (5.5)
Molecular Subtype	
Luminal A	816 (43.3)
Luminal B	456 (24.2)
Triple Negative	393 (20.8)
HER2- Positive	221 (11.7)
Chemotherapy	
Yes	394 (20.9)
No	1492 (79.1)

Table 6.5 Summary of the clinico-pathological and pharmacological features for breast cancers within the METABRIC dataset

To determine correlations between NR4A1 expression and clinical prognostic markers, comprising of tumour grade, lymph nodes status, oestrogen receptor status and molecular subtype (TNBC vs other subtype) Spearman's rho analyses were performed. Table 6.6 shows the correlations coefficients and the associated p values. Statistically significant correlations between expression of NR4A1 and breast cancer prognostic factors, lymph node status, oestrogen expression and Triple negative subtype were noted, although in every case these correlations were extremely weak (consistently $r<0.06$) and therefore could be regarded as unimportant in biological or clinical terms.

		Grade of Tumour	Lymph Node Status	Oestrogen Expression	Triple Negative Subtype
NR4A1 Expression	**r**	-0.039	-0.059	-0.048	0.052
	p	0.091	0.010*	0.039*	0.025*

Table 6.6 NR4A1 expression correlated with the clinical prognostic markers; lymph node status, oestrogen expression and triple negative subgroup. Spearman's rho analysis was performed for NR4A1expression levels against tumour grade, lymph node status, oestrogen expression and triple negative subtype. r = Spearman's rho coefficient, p = p-value

Next, influences on survival were assessed. To allow for Kaplan-Meier analyses it was necessary to dichotomise the cohort into patients regarded as having either a high or low mRNA expression of NR4A1 using suitable cut off values by ROC curve analyses [263].

Kaplan-Meier survival analyses were performed comparing low and high NR4A1 mRNA expression groups for both DFS or DSS; no statistically significant differences were identified in terms of breast cancer DFS and DSS in this cohort (Table 6.7, Figure 6.8).

		Mean DFS (months) (95% CI)	Log Rank	Mean DSS (months) (95% CI)	Log Rank
NR4A1 Expression	**Low**	164 (154-174)	0.288	191 (179-203)	0.978
	High	171 (163-179)		200 (188-212)	

Table 6.7 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low mRNA expression of NR4A1 (n=1886). 95% confidence intervals and log rank p values stated

A. **B.**

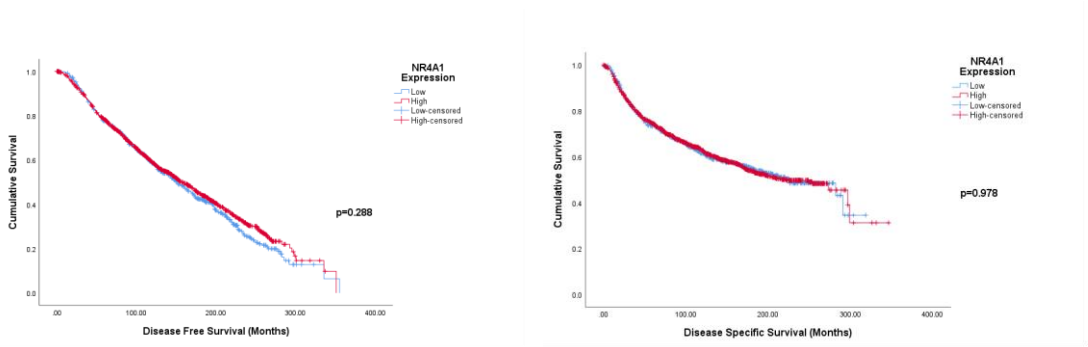


Figure 6.8 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset. NR4A1 expression was determined in 1886 patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

6.3.3.1. NR4A1 mRNA levels do not correlate with outcomes in the METABRIC datasets in individual subgroup analyses

Next, I wanted to determine if NR4A1 expression in any of the breast cancer subtypes; triple negative, ER-negative, ER-positive and HER2-positive within the METABRIC dataset correlated with DFS or DSS. An analysis of each breast cancer subtype was performed using Kaplan-Meier survival analysis; as for the whole cohort, no significant impact on survival was identified in any subgroup (Table 6.8, Figures in Appendix 9.9).

		Mean DFS (months) (95% CI)	Log Rank	Mean DSS (months) (95%CI)	Log Rank
Triple Negative Subtype					
NR4A1 Expression	**Low**	177.0 (151.4-202.6)	0.962	192.9 (169.3-216.6)	0.556
	High	171.7 (156.1-187.3)		191.1 (174.6-207.6)	
ER-Negative Subtype					
NR4A1 Expression	**Low**	170.2 (147.5-192.8)	0.667	187.5 (163.2-211.8)	0.601
	High	157.9 (145.1-170.7)		178.5 (164.2-192.7)	
ER-Positive Subtype					
NR4A1 Expression	**Low**	163.5 (152.6-174.2)	0.110	186.5 (173.9-199.1)	0.574
	High	174.6 (166.0-183.2)		202.0 (188.3-215.6)	
HER2-Positive Subtype					
NR4A1 Expression	**Low**	160.6 (141.4-179.9)	0.726	192.5 (169.2-215.8)	0.446
	High	159.4 (133.5-185.4)		168.4 (134.9-201.8)	

Table 6.8 Comparison of mean disease-free survival (DFS) and disease specific-survival (DSS) between high and low expression of NR4A1 in Triple Negative (n=393), ER-Negative (n=614), ER-Positive (n=1272) and HER2-Positive (n=221) breast cancers. 95% confidence intervals and log rank p values stated

6.3.3.2. NR4A1 mRNA levels do not correlate with outcomes after chemotherapy in the METABRIC dataset

A further key difference between the METABRIC cohort and my cohort is that my cohort was selected to include only patients receiving adjuvant chemotherapy. Next, I also separated analysis of METABRIC patients to those who received chemotherapy (n=394) and those that did not receive chemotherapy (n=1492). ROC analyses were then performed to dichotimise the data into high or low NR4A1 expression. Kaplan-Meier survival analyses were then performed. The findings revealed no statistically significant difference between high or low NR4A1 expression in either the group that received and did not receive chemotherapy (Table 6.9 and Figure 6.9 and 6.10). Further subgroup analyses (triple negative, ER-negative, ER-positive, HER2-positive) were performed on the breast cancer subtypes within those that received chemotherapy, but this again did not reveal any statistically significant findings (Table 6.10). Kaplan-Meier plots can be found in Appendix 9.10.

		Mean DFS (months) (95% CI)	Log Rank	Mean DSS (months) (95% CI)	Log Rank
Chemotherapy Group					
NR4A1 Expression	**Low**	158 (131-185)	0.778	147 (122-172)	0.273
	High	141 (127-156)		152 (138-166)	
No Chemotherapy Group					
NR4A1 Expression	**Low**	194 (182-206)	0.737	168 (158-179)	0.346
	High	211 (198-224)		174 (166-182)	

Table 6.9 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of NR4A1 in breast cancers within the METABRIC dataset that received (n=394) and did not receive chemotherapy (n=1492). 95% confidence intervals and log rank p values stated

A. **B.**

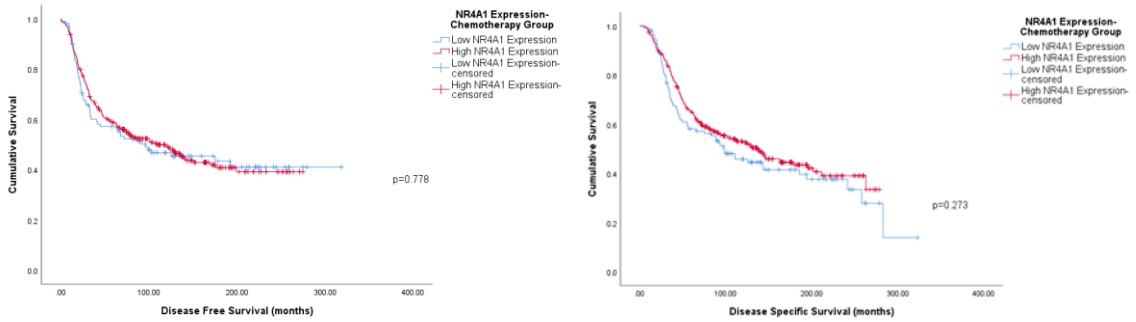


Figure 6.9 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset. NR4A1 expression was determined in a cohort of 394 primary breast cancers treated with chemotherapy. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**

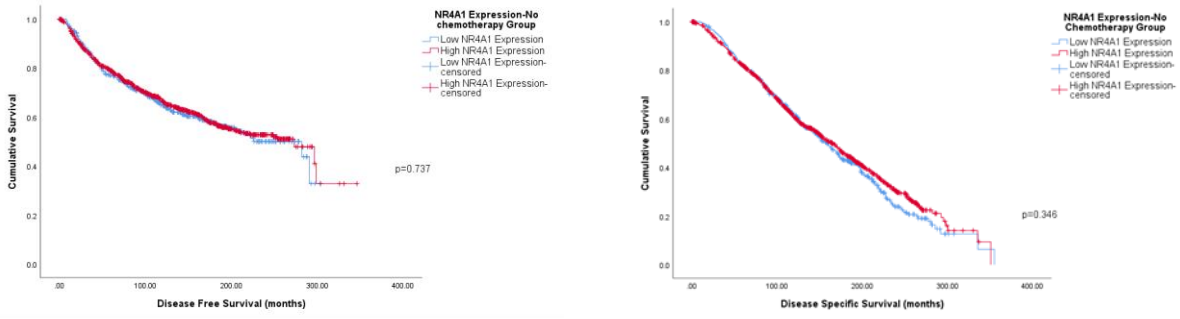


Figure 6.10 Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low NR4A1 expression in patients that did not receive chemotherapy within the METABRIC dataset. NR4A1 expression was determined in a cohort of 1492 primary breast cancers treated with chemotherapy. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

Chemotherapy Group		Mean DFS (months) (95% CI)	Log Rank	Mean DSS (months) (95%CI)	Log Rank
Triple Negative Subtype					
NR4A1 Expression	**Low**	151.8 (115.6-188.0)	0.797	141.5 (107.5-175.4)	0.175
	High	154.4 (132.4-176.3)		166.7 (145.4-188.0)	
ER-Negative Subtype					
NR4A1 Expression	**Low**	158.6 (125.0-192.2)	0.815	142.1 (112.3-171.9)	0.315
	High	140.0 (121.7-158.2)		148.7 (131.2-166.1)	
ER-Positive Subtype					
NR4A1 Expression	**Low**	124.8 (91.6-158.0)	0.779	136.4 (104.6-168.1)	0.765
	High	140.0 (117.2-162.7)		151.0 (126.6-175.3)	
HER2-Positive Subtype					
NR4A1 Expression	**Low**	138.7 (73.0-204.3)	0.762	161.5 (94.6-228.4)	0.295
	High	93.4 (57.7-129.2)		116.4 (86.5-146.3	

6.10 Comparison of mean disease-free survival (DFS) and disease-specific survival (DSS) between high and low expression of NR4A1 in Triple Negative (n=175), ER-Negative (n=253), ER-Positive (n=140) and HER2-Positive (n=29) subtypes of breast cancers within the METABRIC dataset that received receive chemotherapy. 95% confidence intervals and log rank p values stated

6.4. Discussion

6.4.1. NR4A1 as a marker of prognosis in breast cancer following adjuvant cytotoxic chemotherapy

One of the aims of this chapter was to establish if NR4A1 protein expression correlated with DFS and DSS outcomes following adjuvant cytotoxic chemotherapy for breast cancer and therefore could be used as a predictive marker to guide chemotherapy administration and prolong survival. Secondly, to assess whether expression levels of NR4A1 correlate with survival outcomes in the METABRIC dataset, a large breast cancer genomics and transcriptomics dataset.

Therefore, I initially analysed the expression of NR4A1 in both cytoplasmic and nuclear compartments using the Allred scoring, to determine if NR4A1 expression correlated with clinical prognostic markers and survival outcomes in a cohort of 305 breast cancer patients treated with adjuvant chemotherapy. The whole cohort also underwent a sub-analysis of the four tumour subtypes. One of the main conclusions from this analysis was that high NR4A1 nuclear protein expression was significantly associated with poorer survival (Table 6.4, Figure 6.5 - 6.6), a result that was most striking in the ER-negative and particularly the triple negative subgroups, although it was still detectable within the larger ER-positive group.

Our findings that NR4A1 behaves as an oncogene are supported by Zhou et al who concluded that NR4A1 expression behaves as an oncogene by encouraging tumour invasion and disease progression in breast cancer by initiating TGF-β/SMAD signalling. Functionally a decreased expression of NR4A1 prevents the activation of TGF-β which stimulates EMT and tumour progression [216]. A further study by Hedrick and Safe also found that NR4A1 expression also behaves as an oncogene as TGF-β induces mitogen-activated protein kinase 14 which results in the phosphorylation and export of the NR4A1 receptor from the nucleus. This is crucial for EMT, cell migration and β-catenin induction in TNBC cell lines, SUM159, H5587T and MDA-MB-231 expressing NR4A1 [217]. Whereas, Xie et al identified that TNF-α has the potential to induce expression of NR4A1 in MCF-7 breast tumour cells via the stimulation of IκB kinase and JNK. Downregulation of NR4A1 led to TNF-α dependent apoptosis [264].

In addition to these studies, NR4A1 has been shown to behave as an oncogenic driver in a wide range of malignancies such as that of the pancreas, lung and colon [265-267]. In contrast, many studies have concluded that NR4A1 acts as a tumour suppressor. A study by

Alexopoulou et al concluded that low cytoplasmic NR4A1 expression of primary breast tumours correlated with increased tumour grade and metastasis. Whereas, high expression in PMC42 and ZR-75-1 cell lines resulted in decreased cell motility following transwell migration assays [214]. A further study concluded that following NR4A1 protein expression analysis by immunohistochemistry, a low expression correlated with the growth of basal-like breast tumours in murine models as well as in tissue microarrays containing 148 human TNBC tissues. Whereas, increased NR4A1 expression correlated with decreased tumour stage as only 4% of tumours expressing high levels of NR4A1 were of stage T3 or T4 in comparison to 15% of tumours that expressed low levels of NR4A1. With regards to DFS, around 17% of women with high expressing NR4A1 tumours had a recurrence in comparison to 30% of women with low expressing NR4A1 tumours. Lastly, expression of NR4A1 in the MDA-MB-231 cell line was shown to result in inhibition of cell growth and migration via alteration in the JNK1-AP-cyclin D1 pathway. However, the authors did not classify NR4A1 expression into nuclear or cytoplasmic [213]. NR4A1 has also been shown to behave as a tumour suppressor in a range of malignancies such as acute myeloid leukaemias [215], lymphomas [268] and prostate [269].

Therefore, it is clearly seen from the literature that NR4A1 has the potential to behave as an oncogene or a tumour suppressor depending upon the tumour type. However, with regards to breast cancer the literature is inconsistent in particular with regards to triple negative breast cancer with some studies reporting that NR4A1 expression behaves as an oncogene whereas other studies confirm that NR4A1 behaves as a tumour suppressor.

Findings of the METABRIC dataset analysis using the cBioPortal for cancer genomics was not as encouraging. cBioPortal is an online resource that allows for the analyses of cancer genomic data from numerous sources and was initially designed to enhance access to data sets to facilitate the transfer of genomic findings into new discoveries at the biological level as well as the development of new cancer therapies without having to be an expert in bioinformatics [262]. In contrast to my primary breast cancer cohort, the analysis using the METABRIC dataset determined that high NR4A1 expression did not have a significant relationship with DFS and DSS. However, a key point is that within the METABRIC dataset analyses, mRNA expression rather than protein expression was assesses and it was not possible to differentiate between nuclear and cytoplasmic NR4A1 expression – with mRNA expression levels that presumably reflect overall expression without reference to sub-cellular compartment of the protein.

6.4.2. Is NR4A1 a potential therapeutic target?

NR4A1 receptors exhibit a diverse range of roles in carcinogenesis such as apoptosis, DNA repair, tumour proliferation and motility and angiogenesis and their effects are both context and tissue specific, subjective to their expression level (Figure 6.11) [270].

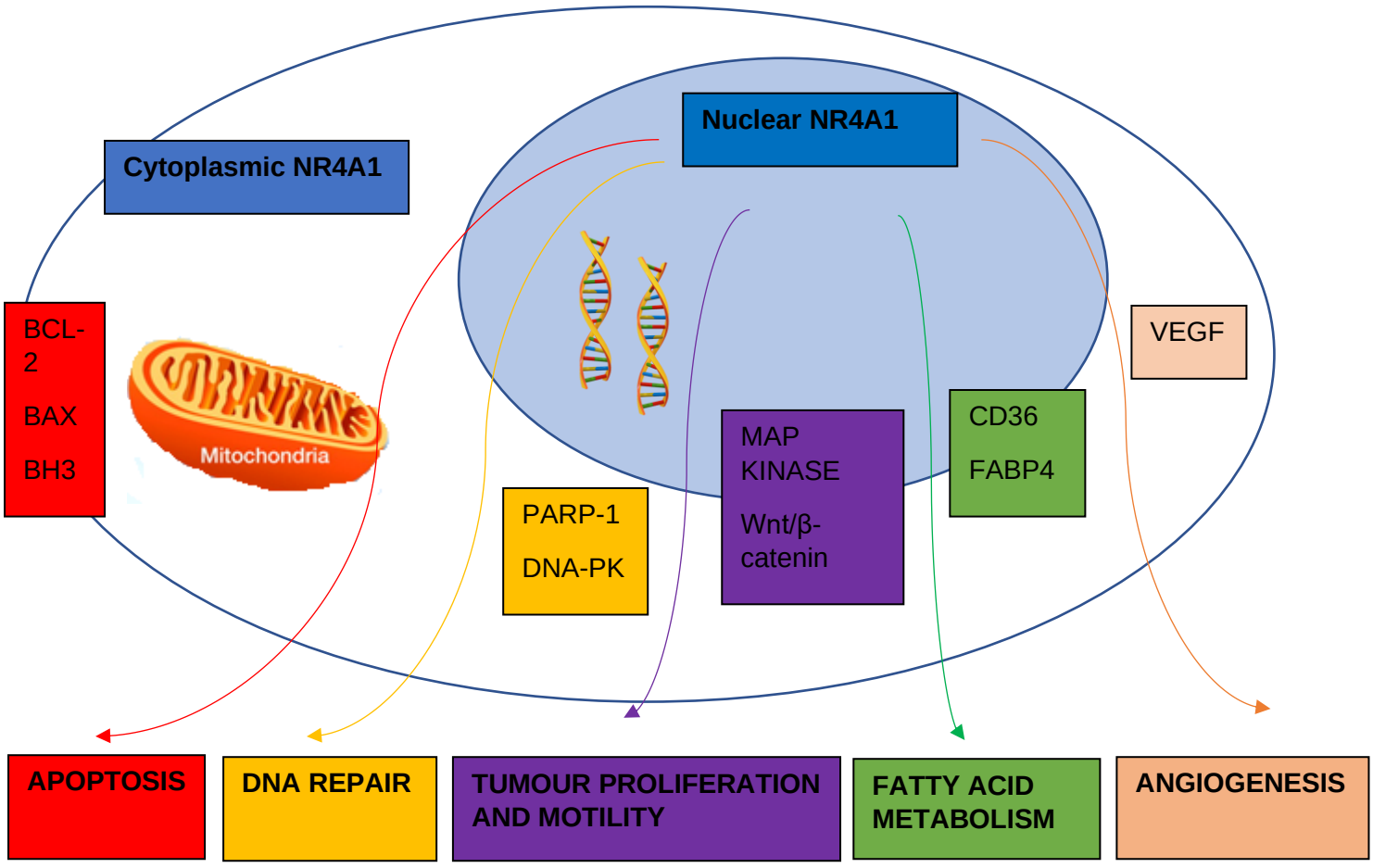


Figure 6.11 NR4A1 receptors play a key role in many cellular functions that lead to breast cancer. These include apoptosis, DNA repair, tumour proliferation and motility, fatty acid metabolism and angiogenesis. Adapted from [270]

As NR4A1 receptors have been shown to alter response to chemotherapeutic agents, this may represent an opportunity for chemotherapeutic intervention [221]. Despite the many challenges faced in part due to they are orphan nuclear receptors and therefore may lack a

natural ligand they also exhibit a cumbersome ligand-binding domain and vary greatly in their tumour cell and context-specific roles [212]. In spite of these obstacles, novel chemotherapeutic agents targeting these receptors are under development or have already been developed, with the main emphasis on altering NR4A1 expression, nuclear export and interference with cell-signalling pathways such as protein kinase A, NF-κB, AKT, Wnt and MAPK [270].

NR4A1 receptors have been shown to possess the capabilities to both inhibit apoptosis [271, 272] as well as being proapoptotic. Export of NR4A1 from the nucleus to the cytosol is key as cytosolic NR4A1 has been shown to play a proapoptotic function in vitro by interacting with Bcl-2 within mitochondria resulting in the exposure of BH3 a proapoptotic or alternatively by indirectly activating the apoptotic cascade by stimulating the attachment of BAX a cytosolic proapoptotic protein to the mitochondria [273, 274]. Whereas, we found within our study that NR4A1 cytoplasmic expression was not of great importance. Thus, NR4A1 nuclear export is a key target for chemotherapeutic agents and many agents such as 5-fluorouracil are already in use that are capable of inducing export of NR4A1 from the nucleus [275]. Therapeutic agents which simulate the mechanism of NR4A1 to induce apoptosis via its action on mitochondrial Bcl-2 such as the nanopeptide nu-BCP9 [274] have also been explored and with advancements in the understanding of both the function and action of NR4A1 receptors, new and different peptides may be developed to either imitate or inhibit the tissue specific actions of NR4A1 receptors on a nongenomic or transcriptional basis [276]. A third group of chemotherapeutic agents that can encourage apoptosis either by inhibiting or inducing the transcription of NR4A1 as well as inducing cell death by endoplasmic reticular stress are known as diindoylmethane derivatives (CDIM) [270]. Based on the fact that NR4A1 expression exhibits the role of a tumour driver, Hendrick et al focused their research on both the function of NR4A1 receptor by RNAi with siNR4A1 and the impact of CDIM/NR4A1 antagonist, p-carbomethyoxyphenyl[1,1-bis(3'-indolyl)-1-(p-carboxymethylphenyl)methane (DIMCpPhCO_2Me) analog in breast cancer cell lines MDA-MB-231, MCF-7 and SKBR3. Following transfection of the breast cancer cells with siNR4A1, the cells demonstrated a reduced proliferative capacity and increased apoptosis. Additionally, there was reduced expression of bcl-2 and activation of endoplasmic reticulum stress. Thereby, displaying the great possibilities of the role of NR4A1 inhibitors in individuals that are diagnosed with increased expression levels of NR4A1 [277]. Further work by the authors also found that the NR4A1 antagonist, DIM-C-pPhCO_2Me prevented the migration of MDA-MB-231 cells induced by TGF-β signalling by the inhibition of NR4A1 nuclear export as well as blocking both β1 and β3 integrin expression which gives rise to a treatment method for breast cancers dependent on integrins [218]. Xie et al evaluated the effects of TNF-α on the function and expression of

NR4A1 both in vitro studies as well as in human breast cancer tissues and concluded that NR4A1 expression in breast cancer cells was induced by TNF-α via the stimulation of IκB kinase and JNK. Knocking down NR4A1 led to TNF-α dependent apoptosis. A small polyphenol known as honokiol was also shown to be a candidate for treating breast cancer due to its ability to sensitise tumour cells to TNF-α induced apoptosis by preventing TNF-α induced NR4A1 mRNA expression [264].

Malewicz et al have also shown that NR4A receptors play a role in DNA double-stand break repair, by translocating to the specific area of the DNA that needs to be repaired by a mechanism dependent on the action of poly (ADP-ribose) polymerase-1 (PARP-1). NR4A is then phosphorylated by DNA-dependent protein kinase (DNA-PK) and repair of DNA double-strand break occurs. Thus, repair of DNA double-stand break may give rise to chemoresistance and alteration of the NR4A1 double-stand break binding could be a potential new approach to enhancing the sensitivity of breast tumour cells to chemotherapeutic agents [278]. NR4A receptors also play a key role in the association between inflammation and malignancy. An in vivo study of carcinogenesis by Zheng et al found that secretion of prostaglandin E2 (PGE2) by tumour cells led to the activation of NR4A1 which then heterodimerise to form an NR4A-RXR complex which results in the secretion of prolactin which ultimately acts on tumour cells enhancing their proliferation and resulting in carcinogenesis [279] and a study by Holla et al found that PGE2 activates NR4A2 expression in colon cancer which can be inhibited by COX-2 inhibitors [280].

NR4A1 has also been identified as playing a role in fatty acid metabolism and Yang et al confirmed that NR4A1 attaches and engages a corepressor to both CD36 and FABP4 promoter domains resulting in suppression of transcription which ultimately hinders the uptake of fatty acids resulting in decreased breast tumour cell proliferation and growth [281].
Lastly, NR4A1 has been shown to be involved in VEGF-A induced angiogenesis an important element of tumour progression [282, 283] which demonstrates that NR4A1 has potential as a chemotherapeutic drug target for breast cancer by targeting the vasculature within the tumour microenvironment.

In summary, the evidence within the current literature mostly supports the use of NR4A1 inhibitors as being potential therapies. As our work has previously demonstrated that increased expression of nuclear NR4A1 leads to worse survival outcomes, the further development and use of agents that are inhibitors of NR4A1 would be beneficial in treating breast cancers that overexpress NR4A1.

6.5. Conclusion

In this chapter I have demonstrated that high nuclear NR4A1 expression correlates with decreased DFS and DSS in breast cancer. NR4A1 receptors possess a wide range of functions depending on their cellular microenvironment and could act as a potential chemotherapeutic target in breast cancer.

7. DISCUSSION

7.1. Prognostic and predictive markers in breast cancer

Upon being diagnosed with breast cancer, patients firstly wish to know their prognosis as well as the most beneficial adjuvant systemic treatment options available to them. Prognosis can be determined by the more conventional clinicopathological prognostic factors such as lymph node status, tumour size and tumour grade. However, it is widely known that the aforementioned factors alone are unsatisfactory in determining the optimal treatment for the patient [284].

Subsequently, over recent years a large proportion of breast cancer research has focused on the progression and development of molecular biomarkers that provide prognostic information in terms of disease recurrence as well as predictive biomarkers that predict response to therapy. Both ER and PR status have been critical in predicting response to endocrine therapy over the last four decades whereas, HER2 tumour status became important in predicting response to Herceptin nearly 20 years ago [284].

Gene expression profiling tests such as Oncotype DX, MammaPrint, EndoPredict and Prosigna are now in routine use in clinical practice to aid in the identification of patients diagnosed with breast cancer that are likely to have an increased risk of recurrence and therefore, more likely to benefit from chemotherapy. Although, all the aforementioned gene expression profiling tests have prognostic ability, only Oncotype DX has demonstrated its predictive ability and is the most widely used test [285].

However, it is evident that new prognostic and predictive biomarkers are required as in the adjuvant setting nearly 3 in 10 individuals with breast cancer become resistant to systemic cytotoxic therapy and ultimately develop a recurrence of their breast cancer [4].

In my study I have looked at the prognostic and predictive implications of four potential biomarkers, IFNβ1, MX1, ITGA7 and NR4A1.

IFNβ1 in fibroblasts and MX1 in tumour cells have been found to be promising candidates for prognostic biomarkers in triple negative breast cancers in particular the claudin low subgroup in the presence of chemotherapy. In the triple negative cohort, a high IFNβ1 fibroblast expression and MX1 tumour cell expression were associated with a reduced DFS in comparison to patients with low expression ($p=0.01$) following adjuvant chemotherapy. The same pattern in DFS was maintained in the claudin-low patients ($p=0.02$; $p=0.047$) but lost in the claudin-high subset of patients ($p=0.205$; $p=0.093$). However, in order to confirm that

IFNβ1 in fibroblasts and MX1 in tumour cells are predictive of chemotherapy benefit, one would need to demonstrate that IFNβ1 in fibroblasts and MX1 in tumour cells were not prognostic in the absence of chemotherapy.

The claudin-low subtype of breast cancer consists of around 14% of invasive breast tumours and has been linked with poor outcomes [39]. Despite an increase in the research on claudins in different types of cancers [249], claudin expression levels do not play a role in the breast cancer management to date as there are currently no treatment options available that target claudin-low breast tumours to enhance survival [250].
Thus, the prospect of identifying markers that predict response to specific therapies in TNBCs, but especially the claudin-low subtype, would be beneficial and have the potential to be used widely.

Previous in-vitro models by the Hughes group showed that inhibition of IFN receptors by antibodies in cell lines representative of the claudin-low subset of patients (MDA-MB-231 and MDA-MB-157) reduced CAF-dependent chemoprotection. It was also demonstrated that breast CAFs protect a subgroup of TNBCs, the claudin-lows, from the anthracycline agent, epirubicin by the secretion of IFNβ1 which ultimately results in the paracrine stimulation of IFN signalling in the cancer cell, as represented by an increased production of MX1 [192] and ultimately worse survival.

Therefore, identification of molecular markers involved in cross-talk between CAFs and TNBC cells gives rise to the possibility of overcoming chemoresistance through the inhibition of these interactions and ultimately prolonging disease free and overall survival in triple negative breast cancers.

My third biomarker of interest, ITGA7, also shows promising potential as both a prognostic and predictive biomarker in ER-positive breast cancers. High ITGA7 nuclear protein expression was associated with both a longer DFS and DSS than low expression following adjuvant chemotherapy (by 682 days, p=0.05, and 604 days, p=0.005, respectively).

The impact of ITGA7 was particularly notable in patients who received anthracyclines without taxanes (an improvement in DFS of 806 days; p=0.004) as opposed to those who received taxanes (no significant impact on DFS). This was also supported by in-vitro studies performed by the Hughes lab which concluded that knock down of ITGA7 was associated with significant protection of MCF7 cells from the effects of the anthracycline epirubicin.

Therefore, it has been shown that high nuclear ITGA7 expression correlates with increased DFS and DSS in breast cancer after chemotherapy and these findings suggest that ITGA7 could act as a possible predictive biomarker or, more excitingly, a target for chemo-sensitizing drugs that aim to enhance ITGA7 expression or activity.

My last biomarker of interest, NR4A1, has demonstrated its potential as a prognostic biomarker however, its usefulness as a predictive biomarker needs further validation. High nuclear NR4A1 expression was associated with a poorer prognosis in breast cancer patients following chemotherapy. Kaplan-Meier survival analyses showed that high NR4A1 nuclear protein expression was associated with a poorer DFS (by 487 days; p=0.005) and DSS (by 621 days; p=0.001) in the whole cohort. A sub-analysis of triple negative and ER-negative breast cancers also showed that high NR4A1 nuclear protein expression was associated with both shorter DFS (by 1452 days, p=0.002 and 1141 days, p=0.006 respectively) and DSS (by 1603 days, p=0.001 and 1187 days, p=0.001 respectively).

The limitations of this work include the lack of a control cohort comprising of women diagnosed with breast cancer that did not receive chemotherapy. By having a control cohort it may aid in confirming that the specific markers are predictive of chemotherapy benefit. It should also be noted that this cohort is now quite historic and that the systemic therapy of breast cancer has evolved in this time period to include varying chemotherapy regimens to include the use of platinum based therapy as well as the use of dual-anti-HER2 therapies such as trastuzumab and pertuzumab and the increasing use of NACT. Therefore, further validation in the context of contemporary chemotherapy regimens as well as in the NACT setting will need to be considered. It's also important to note the relatively small number of cases in each of the molecular subgroups of breast cancers. An insufficient sample size can decrease statistical power and increase the margin error of the results. However, due to time constraints of the work and to obtain adequate follow-up data women diagnosed with primary breast cancer and treated with adjuvant chemotherapy between 2006 and 2010 were included only. Lastly, there were challenges in correlating the outcomes of NR4A1 expression at the protein level with NR4A1 mRNA expression levels from the METABRIC dataset, as with the METABRIC dataset analyses it was not possible to differentiate between nuclear and cytoplasmic NR4A1 expression, with mRNA levels reflecting overall expression without reference to the sub-cellular compartment of the protein. To address this and to determine if NR4A1 expression impacted on chemo-response tissue culture models could be used (Section 7.3).

7.2. Potential drug targets

This study has identified two potential clinical drug targets, IFNβ1 and NR4A1.

Prognostic outcome may be enhanced in the claudin-low subgroup of TNBCs by sensitising tumour cells to chemotherapeutic agents by preventing cross-talk between CAFs and tumour cells as demonstrated by in-vitro models within the Hughes group, which showed that inhibition of IFN receptors by antibodies reduced CAF-dependent protection [192]. Also, a potential chemotherapeutic agent known as ruxolitinib, a JAK1/2 kinases inhibitor has been used in a few breast cancer clinical trials. IFN β signals via JAKs which are downstream of the IFN receptor in the canonical IFN signalling pathway. Activated JAKs then lead to the tyrosine phosphorylation of signal transducer and activator of transcription (STAT1/STAT2) which interacts with IFN regulatory factor 9 (IRF9) to activate gene expression. Ruxolitinib induces its chemotherapeutic effects as inhibition of JAK will ultimately result in the failure of IFN β signalling [286]. A phase 2 trial that evaluated the efficacy and side effect profile of ruxolitinib alone in women diagnosed with metastatic TNBC concluded a poor progression free survival with a median of 1.2 months. However, no unexpected side effects were noted apart from the known, headaches, bruising, dizziness and thrombocytopenia [287]. The poor efficacy of ruxolitinib in this study may be explained by the fact that it was administered alone and not in combination with any other chemotherapeutic agents as Han et al demonstrated in preclinical studies on their work involving ovarian tumours that ruxolitinib synergistically increases the chemotherapeutic actions of paclitaxel [10]. In comparison, a phase 1 study investigating the combination of ruxolitinib and weekly paclitaxel for the treatment of women diagnosed with HER2-negative metastatic breast cancer concluded that both agents used in combination were well tolerated. With regards to survival outcomes, 21% of women obtained a partial response to treatment, 63% of the women's breast cancer remained stable and 16% had disease progression [286]. Based upon the safety profile of ruxolitinib and paclitaxel used in combination within this study, has lead to the development and ongoing recruitment to a new phase 2 randomised trial by Translational Breast Cancer Research Consortium (TBCRC) investigating these chemotherapeutic agents in combination for the treatment of tiple negative inflammatory breast cancer [288]. Therefore, as safety and dosing regimens are already established [286] and ruxolitinib is already available on the market and approved by the US FDA for use in myelofibrosis and polycythemia vera [289, 290], it could be used in the treatment of IFNβ/MX1 positive breast cancers in the near future.

However, although further work is required to identify therapeutic interventions that target the CAF-dependent chemoresistance pathway. We do have preliminary data that shows that ruxolitinib inhibits CAF-dependent chemoprotection in cell lines [192].

NR4A1 is another potential therapeutic drug target as NR4A1 receptors exhibit a diverse range of roles in carcinogenesis and their effects are both context and tissue specific, subjective to their expression level [270]. Currently, novel chemotherapeutic agents targeting these receptors are under development or have already been developed, with the main emphasis on altering NR4A1 expression, nuclear export and interference with cell-signalling pathways such as protein kinase A, NF-κB, AKT, Wnt and MAPK [270].

Based on the fact that NR4A1 expression exhibits the role of a tumour driver, Hendrick et al focused their research on both the function of NR4A1 receptor by RNAi with siNR4A1 and the impact of CDIM/NR4A1 antagonist, p-carbomethyoxyphenyl[1,1-bis(3'-indolyl)-1-(p-carboxymethylphenyl)methane (DIMCpPhCO_2Me) analog in breast cancer cell lines MDA-MB-231, MCF-7 and SKBR3. Following transfection of the breast cancer cells with siNR4A1, the cells demonstrated a reduced proliferative capacity and increased apoptosis. Additionally, there was reduced expression of bcl-2 and activation of endoplasmic reticulum stress. Thereby, displaying the great possibilities of the role of NR4A1 inhibitors in individuals that are diagnosed with higher expression levels of NR4A1 [277].

Further work by the authors also found that the NR4A1 antagonist, DIMCpPhCO_2Me prevented the migration of MDA-MB-231 cells induced by TGF-β signalling by the inhibition of NR4A1 nuclear export as well as blocking both β1 and β3 integrin expression which gives rise to a treatment method for breast cancers dependent on integrins [218].

In summary, the evidence within the current literature mostly supports the use of NR4A1 inhibitors as being potential therapies. As our work has previously demonstrated that increased expression of nuclear NR4A1 leads to worse survival outcomes, the further development and use of agents that are inhibitors of NR4A1 would be beneficial addition to cytotoxic chemotherapy in treating breast cancers that overexpress NR4A1.

7.3. Impact of COVID19

Taking into consideration the findings to date, I aimed to investigate NR4A1 expression in a range of breast cancer subtypes, and to assess its potential correlation with cancer outcomes. In particular, I aimed to assess it as a predictive marker of chemo-response. I had also planned to test experimentally whether NR4A1 expression impacted on chemo-response using tissue culture models. This would have firstly involved checking which cancer cell lines express NR4A1 by using the Cancer Cell Line Encyclopaedia (CCLE) data set. Next, I would have taken cell lines that do express NR4A1 and are representative of the main intrinsic subtypes of breast cancer, luminal A (MCF7 or T47D), luminal B (BT474 or MDA-MB-361), HER2 subtype SKBR3 or AU565), triple negative (MDA-MB-231 or MDA-MB-468) and transfect with NR4A1 siRNA or control siRNA. Western blots analysis would have been performed to check for knock down and lastly chemosensitivity with either epirubicin or docetaxel after targeted or control transfection would have been compared. However, this work was cancelled due to laboratory closures associated with the COVID19 pandemic in 2020-21. Therefore, as an alternative I learnt how to perform, analyse and evaluate the relevance of NR4A1 expression with respect to outcomes in the METABRIC dataset.

Therefore, this is the reason why I can't say with certainty that the biomarker NR4A1 is predictive in nature as opposed to prognostic.

7.4. Conclusion

Although I have demonstrated the four biomarkers IFNβ1, MX1, ITGA7 and NR4A1 to be potential clinical markers of chemo-response, further work is required to convert these findings into better patient outcomes. This can be achieved by thorough validation in several clinical cohorts and the clinical trial setting. Only then the amalgamation of both the conventional prognostic factors, nodal status, tumour size and tumour grade together with validated prognostic and predictive biomarkers, can be used clinically to predict progression of the disease as well as chemo-response and begin the journey towards a more tailored adjuvant chemotherapy regimen that can ultimately enhance both chemo-response and patient survival.

8. REFERENCES

1. Cancer Research UK. *Breast cancer incidence statistics*. 2015 [cited 10/07/20]
2. WCRF.ORG. *American Institute for cancer research*. [cited 07/08/20]
3. Youlden, D., et al., *The descriptive epidemiology of female breast cancer: An international comparison of screening, incidence, survival and mortality*. Cancer Epidemiology. 2012. **36**(3): p. 237-248
4. Rivera, E. and Gomez, H, *Chemotherapy resistance in metastatic breast cancer: the evolving role of ixabepilone*. Breast Cancer Research. 2010. **12**(2): p.1-12
5. Koo, M., et al., *Typical and atypical presenting symptoms of breast cancer and their associations with diagnostic intervals: Evidence from a national audit of cancer diagnosis*. Cancer Epidemiology. 2017. **48**: p.140-146
6. Ough, M., Velasco, J. and Hieken, T, *A comparative analysis of core needle biopsy and final excision for breast cancer: histology and marker expression*. The American Journal of Surgery. 2011. **201**(5): p. 692-694
7. Makki, J, *Diversity of Breast Carcinoma: Histological subtypes and clinical relevance*. Clin Med Insights Pathol. 2015. **8**: p. 23-31
8. van Seijen, M., et al., *Ductal carcinoma in situ: to treat or not to treat, that is the question*. Br J Cancer. 2019.**121**(4): p. 285-292
9. Hong, Y., et al., *Ductal carcinoma in situ current trends, controversies, and review of literature*. Am J Surg. 2018. **216**(5): p. 998-1003
10. Han, M. and Khan, S, *Clinical Trials for Ductal Carcinoma in Situ of the Breast*. J Mammary Gland Biol Neoplasia. 2018. **23**(4):293-301
11. NICE.ORG [cited 08/08/20]
12. Birmingham.ac.uk/research/crctu/trials/loris/index.aspex [cited 08/12/21]
13. Wen, H. and Brogi, E, *Lobular Carcinoma in Situ*. Surg Pathol Clin. 2018.**11**(1): p.123-145
14. King, T., et al., *Lobular Carcinoma in Situ: A 29-Year Longitudinal Experience Evaluating Clinicopathologic Features and Breast Cancer Risk*. J Clin Oncol. 2015.**20**(33): p. 3945-52
15. Liao, H., et al., *The Clinicopathological Features and Survival Outcomes of Different Histological Subtypes in Triple-negative Breast Cancer*. J Cancer. 2018.**9**(2): p. 296-303
16. Viale, G, *The current state of breast cancer classification*. Annals of Oncology. 2012. **23**(10): p. 207-210
17. Veronesi, U., et al., *Rethinking TNM: A Breast Cancer Classification to Guide to Treatment and Facilitate Research*. The Breast Journal. 2009. **15**(3): p. 291-295
18. Bansal, C., et al., *Comparative evaluation of the modified Scarff-Bloom-Richardson grading system on breast carcinoma aspirates and histopathology*. Cytojournal. 2012. **9**: p. 4
19. Gray, E., et al., *Survival estimates stratified by the Nottingham Prognostic Index for early breast cancer: a systematic review and meta-analysis of observational studies*. Systematic Reviews. 2018. **7**: p.142
20. Weledji, E. and Tambe, J, *Breast cancer detection and screening*. Med Clin Rev, 2018. **4**(2): p. 8
21. Perou, C., et al., *Molecular portraits of human breast tumours*. Nature. 2002.**406**: p. 747-752
22. Sørlie. T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications*. 2001. **98** (19) p. 10869-10874
23. Koboldt, D., et al., *Comprehensive molecular portraits of human breast tumours*. Nature. 2012. **490**: p. 61–70
24. Prat, A., et al., *Clinical implications of the intrinsic molecular subtypes of breast cancer*. Breast. 2015. **24**(2): p. 26-35
25. Herr, D., *Does chemotherapy improve survival in patients with nodal positive luminal A breast cancer? A retrospective Multicenter Study*. PLoS One. 2019.**14**(7): e0218434

26. Metzger-Filho, O., et al., *Patterns of Recurrence and outcome according to breast cancer subtypes in lymph node-negative disease: results from international breast cancer study group trials VIII and IX*. J Clin Oncol. 2013. **31**(25): p. 3083-90
27. Li, Z., *Luminal B breast cancer: patterns of recurrence and clinical outcome*. Oncotarget. 2016. **7**(40): p. 65024-65033
28. Mitri, Z., Constantine, T. and O'Regan, R, *The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy*. Chemother Res Pract. 2012. **2012**: p. 743193
29. von Minckwitz, G., et al., *Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer*. N Engl J Med. 2017.**377**(2): p. 122-131
30. Morris, G., et al., *Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution complication compared with the National Cancer Institute's surveillance, Epidemiology and end results database*. Cancer. 2007. **110**(4): p. 876-884
31. Haffty, B., et al., *Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer*. J Clin Oncol. 2006. **24**(36): p. 5652-5657
32. Pandy, J., et al., *Triple negative breast cancer and platinum-based systemic treatment: a meta-analysis and systematic review*. BMC Cancer. 2019.**19**: p.1065
33. Goldhirsch, A., et al., *Thresholds for therapies: highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer*. Ann Oncol. 2009. **20**: p. 1319-1329
34. Untch, M., et al., *13th St. Gallen International Breast Cancer Conference 2013: Primary Therapy of Early Breast Cancer Evidence, Controversies, Consensus – Opinion of a German Team of Experts*. Breast Care. 2013.**8**: p.221-229
35. Sparano, J., et al., *Adjuvant Chemotherapy Guided by a 21-Gene Expression Assay in Breast Cancer.* N Engl J Med. 2018. **379**(2): p. 111-121
36. Kalinsky, K., et al., *21-Gene Assay to Inform Chemotherapy Benefit in Node-Positive Breast Cancer.* N Engl J Med. 2021. **385**(25): p. 2336-2347
37. Lehmann, B., et al., *Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies*. *J Clin Invest*. 2011.**121**(7): p. 2750-2767
38. Herschkowitz, J., et al., Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumours. Genome Biol. 2007. **8**(5): p. 76
39. Dias, K., *Claudin-low breast cancer; clinical and pathological characteristics*. PLoS ONE. 2017. **12**(1): p. 0168669
40. Sabatier, R., et al., *Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization*. Mol Cancer. 2014.**13**: p. 228
41. Prat, A., et al., *Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer*. Breast cancer Res. 2010. **12**: p, 68
42. Fougner, C., et al., *Re-definition of claudin-low as a breast cancer phenotype*. Nat Commun. 2020.**11**(1): p. 1787
43. Lu, S., et al., *Claudin expression in high-grade invasive ductal carcinoma of the breast: correlation with the molecular subtype*. Mod Pathol. 2013. **26**(4): p. 485-95
44. Creighton, C., et al., *Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features*. Proc. Natl. Acad. Sci. USA. 2009.**106**: p. 13820 –13825
45. Romani, C., et al., *Development and characterization of a human single-chain antibody fragment against claudin-3: A novel therapeutic target in ovarian and uterine carcinomas*. Am. J. Obstet. Gynecol. 2009. **201**: p: 71–79
46. Suzuki, M., et al., *Therapeutic antitumor efficacy of monoclonal antibody against Claudin-4 for pancreatic and ovarian cancers*. Cancer Sci. 2009. **100**: p. 1623 –1630
47. Kato-Nakano, M., et al., *Characterization and evaluation of the antitumour activity of a dual-targeting monoclonal antibody against claudin-3 and claudin-4*. Anticancer Res. 2010. **30**: p. 4555–4562

48. Moo, T., et al., *Overview of Breast Cancer Therapy.* PET Clin. 2018.**13**(3): p. 339-354
49. https://associationofbreastsurgery.org.uk [cited 03/12/21]
50. Chen, A., et al*., Disparities and Trends in Sentinel Lymph Node Biopsy Among Early-Stage Breast Cancer Patients (1998–2005)*, JNCI. 2008. **100**: p. 462-474
51. Peintinger, F., et al., *Comparison of quality of life and arm complaints after axillary lymph node dissection vs sentinel lymph node biopsy in breast cancer patients*. Br J Cancer. 2003. **89**: p. 648–652
52. Ahmed, M., et al., *Meta-analyses of tumour burden in pre-operative axillary ultrasound positive and negative breast cancer patients*. Breast Cancer Res Treat. 2017. **166**: p. 329-336
53. Krag, D., et al., *Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial*. Lancet Oncol. 2010. **11**: p. 927-33
54. Giuliano, A., et al., *Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial*. JAMA. 2011. **305**: p. 569-575
55. Dixon, J. and Cartlidge, C, *Twenty-five years of change in the management of the axilla in breast cancer*. Breast J. 2020. **26**: p. 22-26
56. Poggi, M., et al., *Eighteen-year results in the treatment of early breast carcinoma with mastectomy versus breast conservation therapy: The National Cancer Institute Randomized Trial*. Cancer. 2003. **98**: p. 697-702
57. Kirwan, C., Coles, C. and Bliss, J, *It's PRIMETIME. Postoperative avoidance of radiotherapy: Biomarker selection of women at very low risk of local recurrence*. Clinical Oncology. 2016. **28**: p. 594-596
58. Bonito, F., et al., *Radiation-induced angiosarcoma of the breast: A review*. Breast J. 2020. **26**: p. 458-463
59. Chew, H., *Adjuvant therapy for breast cancer: who should get what?* West J Med. 2001. **174**: p. 284-287
60. Gnant, M., Thomssen, C. and Harbeck, N, *St. Gallen/Vienna 2015: a brief summary of the consensus discussion*. Breast Care. 2015. **10**: p. 124-130
61. Wang, J. and Xu, B, *Targeted therapeutic options and future perspectives for HER2-positive breast cancer*. Signal Transduction and Targeted Therapy. 2019. **4**: p. 34
62. Wahba, H. and El-Hadaad, H, *Current approaches in treatment of triple-negative breast cancer*. Cancer Biol Med. 2015.**12**(2): p. 106-116
63. Fisusi, F. and Akala, E, *Drug Combinations in Breast Cancer Therapy*. Pharm Nanotechnol. 2019.**7**(1): p. 3-23
64. www.breast.predict.nhs.uk/tool [cited 05/12/21]
65. Shah, A. and Gradishar, W, *Adjuvant Anthracyclines in Breast Cancer: What Is Their Role?* Oncologist. 2018. **23**(10): p.1153-1161
66. Hortobagyl, G, *Anthracyclines in the treatment of cancer*. Drugs. 1997. **54**(4):1-7
67. Peto, R., et al., *Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Comparisons between different polychemotherapy regimens for early breast cancer: Meta-analyses of long-term outcome among 100,000 women in 123 randomised trials*. Lancet. 2012. **379**: p. 432–444
68. Hagiwara, H. and Sunada, Y, *Mechanism of taxane neurotoxicity*. Breast cancer. 2004. **11**(1): p. 82-85
69. Azarenko, O. et al., *Antiproliferative Mechanism of Action of the Novel Taxane Cabazitaxel as Compared with the Parent Compound Docetaxel in MCF7 Breast Cancer Cells*. Mol Cancer Ther. 2014. **13**(8): p. 2092-2103

70. Ghoreishi, Z., et al., *Risk factors for paclitaxel-induced peripheral neuropathy in patients with breast cancer*. *BMC Cancer*. 2018.**18**(1): p. 958
71. Zajączkowska, R., et al., *Mechanisms of Chemotherapy-Induced Peripheral Neuropathy*. Int J Mol Sci. 2019. **20**(6): p. 1451
72. Fleming, R, *An overview of cyclophosphamide and ifosfamide pharmacology*. Pharmacotherapy. 2012. **17**(5): p. 146-154
73. Mackey, J., et al., *Long-term outcomes after adjuvant treatment of sequential versus combination docetaxel with doxorubicin and cyclophosphamide in node-positive breast cancer: BCIRG-005 randomized trial*. *Ann Oncol*. 2016. **27**(6): p.1041-1047
74. Koukourakis, G., et al., *Efficacy of the oral fluorouracil pro-drug capecitabine in cancer treatment: a review*. Molecules. 2008.**13**(8):1897-1922
75. Longley, D., Harkin, D. and Johnston, P, *5-Fluorouracil: mechanisms of action and clinical strategies*. Nature Reviews Cancer. 2003. **3**: p. 330–338
76. Natori, A., et al., *Capecitabine in early breast cancer: A meta-analysis of randomised controlled trials*. Eur J Cancer. 2017. **77**: p. 40-47
77. Dasari, S. and Tchounwou, P. *Cisplatin in cancer therapy: molecular mechanisms of action*. European Journal of Pharmacology. 2014. **740**: p. 364-378
78. Tutt, A., et al., *Carboplatin in BRCA1/2-mutated and triple-negative breast cancer BRCAness subgroups: the TNT Trial*. Nat Med. 2018. **24**(5): p. 628-637
79. Moudi, M., et al., *Vinca alkaloids*. Int J Prev Med. 2013. **4**(11): p.1231- 1235
80. Vu, T. and Claret, F, *Trastuzumab: Updated mechanism of action and resistance in breast cancer*. Front Oncol. 2012. **2**: p. 62
81. Cameron, D., et al., *11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial* Lancet. 2017. **389**(10075): p. 1195-1205
82. NICE.ORG. Lapatinib for the treatment of women with previously treated advanced or metastatic breast cancer [cited 13/07/20]
83. von Minckwitz, G., et al., *APHINITY Steering Committee and Investigators. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer.* N Engl J Med. 2018. **379**(16): p:1585
84. Opdam, F., et al., *Lapatinib for advanced or metastatic breast cancer*. Oncologist. 2012.**17**(4): p. 536-542
85. Geyer, C., et al., *Lapatinib plus Capecitabine for HER2-positive advanced breast cancer*. New England Journal of Medicine. 2006. **355**: p. 2733-2743
86. Gril, B., et al., *Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain*. J Natl Cancer Inst. 2008.**100**(15): p.1092-1103
87. Morgan, J., et al., *Surgery versus primary endocrine therapy for operable primary breast cancer in elderly women (70 years plus).* Cochrane Database of Systematic Reviews 2014. **5**: p.4272
88. YU, F. and Bender, W, *The mechanism of tamoxifen in breast cancer prevention*. Breast cancer research. 2001. **3**(1):74
89. Fisher, B., et al., *Treatment of lymph-node-negative, oestrogen-receptor-positive breast cancer: long-term findings from National Surgical Adjuvant Breast and Bowel Project randomised clinical trials.* The Lancet. 2004. **364**(9437): p. 858-868
90. Howell, A., et al., *Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer*. Lancet. 2005. **365**(9453): p. 60-62
91. Tremont, A., Lu, J, and Cole, J, *Endocrine Therapy for Early Breast Cancer: Updated Review*. Ochsner J. 2017.**17**(4): p. 405-411
92. Pistelli, M., et al., *Aromatase inhibitors in premenopausal women with breast cancer: the state of the art and future prospects*. Curr Oncol. 2018. **25**(2): p.168-175

93. Kümmel, S., Holtschmidt, J. and Loibl, S, *Surgical treatment of primary breast cancer in the neoadjuvant setting*. Br J Surg. 2014.**101**(8): p. 912-924
94. Reinert, T., Gonçalves, R. and Ellis, M, *Current Status of Neoadjuvant Endocrine Therapy in Early Stage Breast Cancer*. Curr. Treat. Options in Oncol. 2018. **19**: p. 23
95. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). *Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials*. Lancet Oncol. 2018.**19**(1): p. 27-39
96. Thompson, A. and Moulder-Thompson, S, *Neoadjuvant treatment of breast cancer*. Annals of Oncology, 2012. **23**(10): p. 231-236
97. Schott, A. and Hayes, D, *Defining the benefits of neoadjuvant chemotherapy for breast cancer*. Journal of clinical oncology. 2012. **30**(15): p. 1747-1749
98. Samiei, Sanaz., et al., *Correlation Between Pathologic Complete Response in the Breast and Absence of Axillary Lymph Node Metastases After Neoadjuvant Systemic Therapy*. Annals of Surgery. 2020. **271**(3): p. 574-580
99. Pinder, S., et al., *Macroscopic handling and reporting of breast cancer specimens pre- and post-neoadjuvant chemotherapy treatment: review of pathological issues and suggested approaches.* Histopathology. 2015. **67**(3): p. 279-293
100. Zujewski, J. and Rubinstein, L, *CREATE-X a role for capecitabine in early-stage breast cancer: an analysis of available data.* NPJ Breast Cancer. 2017. **20**(3): p. 27
101. von Minckwitz, G., et al., *KATHERINE Investigators. Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer.* N Engl J Med. 2019. **380**(7): p. 617-628
102. McCann, K. and Hurvitz, S, *Advances in the use of PARP inhibitor therapy for breast cancer*. Drugs Context. 2018. **7**: p. 212540
103. Robson, M., et al., *Olaparib for metastatic breast cancer in patients with a germline BRCA mutation*. New England Journal of Medicine. 2017. **377**: p. 523-533
104. O'Donnell, J., Teng, M. and Smyth, M, *Cancer immunoediting and resistance to T cell-based immunotherapy*. Nat Rev Clin Oncol. 2019.**16**(3): p. 151-167
105. García-Aranda, M. and Redondo, M, *Immunotherapy: A Challenge of Breast Cancer Treatment*. Cancers (Basel). 2019.**11**(12):1822
106. Emens, L, *Breast Cancer Immunotherapy: Facts and Hopes*. Clin Cancer Res. 2018. **24**(3): p. 511-520
107. *Atezolizumab Combo Approved for PD-L1-positive TNBC*. Cancer Discov. 2019. **9**(5): OF2
108. Lee, H., et al., *Molecular mechanism of PD-1/PD-L1 blockade via anti-PD-L1 antibodies atezolizumab and durvalumab*. Sci Rep. 2017. **7**(1): p. 5532
109. Schmid, P., et al., *Atezolizumab and nab-paclitaxel in advanced triple-negative breast Cancer*. N Engl J Med. 2018. **379**(22): p. 2108–21
110. Malumbres, M, *Cyclin-dependent kinases*. *Genome Biol*. 2014.**15**(6): p. 122
111. Goel, S., et al., *CDK4/6 Inhibition in Cancer: Beyond Cell Cycle Arrest*. Trends Cell Biol. 2018. **28**(11): p. 911-925
112. Du, Q., et al., *The application and prospect of CDK4/6 inhibitors in malignant solid tumors*. J Hematol Oncol. 2020.**13**: p. 41
113. Sobhani, N., et al., *Updates on the CDK4/6 Inhibitory Strategy and Combinations in Breast Cancer*. Cells. 2019. **8**(4): p. 321
114. Goel, S., et al., *CDK4/6 inhibition triggers anti-tumour immunity*. Nature. 2017. **548**(7668): p. 471-475
115. Deng, J., et al., *CDK4/6 Inhibition Augments Antitumor Immunity by Enhancing T-cell Activation*. Cancer Discov. 2018. **8**(2): p. 216-233
116. Pandey, K., et al *Molecular mechanisms of resistance to CDK4/6 inhibitors in breast cancer: A review*. Int J Cancer. 2019. **145**(5): p. 1179-1188

117. Beaver, J., et al., *FDA Approval: Palbociclib for the Treatment of Postmenopausal Patients with Estrogen Receptor-Positive, HER2-Negative Metastatic Breast Cancer*. Clin Cancer Res. 2015. **21**(21): p. 4760-4766
118. Finn, R., et al., *The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study*. Lancet Oncol. 2015.**16**(1): p. 25-35
119. Walker, A., et al., *FDA Approval of Palbociclib in Combination with Fulvestrant for the Treatment of Hormone Receptor-Positive, HER2-Negative Metastatic Breast Cancer*. Clin Cancer Res. 2016. **22**(20): p. 4968-4972
120. Cristofanilli, M., et al., *Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial*. Lancet Oncol. 2016.**17**(7): p. *425-439*
121. Hortobagyi, GN., et al., *Updated results from MONALEESA-2, a phase III trial of first-line ribociclib plus letrozole versus placebo plus letrozole in hormone receptor-positive, HER2-negative advanced breast cancer*. Ann Oncol. 2018. **29**(7): p. 1541-1547
122. Shah, A., et al., *FDA Approval: Ribociclib for the Treatment of Postmenopausal Women with Hormone Receptor-Positive, HER2-Negative Advanced or Metastatic Breast Cancer*. Clin Cancer Res. 2018. **24**(13): p. 2999-3004
123. Kim, E, *Abemaciclib: First Global Approval*. Drugs. 2017.**77**(18): p. 2063-2070
124. Sledge, G., et al., *MONARCH 2: Abemaciclib in Combination with Fulvestrant in Women With HR+/HER2- Advanced Breast Cancer Who Had Progressed While Receiving Endocrine Therapy*. J Clin Oncol. 2017. **35**(25): p. 2875-2884
125. Goetz, M., et al., *MONARCH 3: Abemaciclib As Initial Therapy for Advanced Breast Cancer*. J Clin Oncol. 2017.**35**(32): p. 3638-3646
126. Finn, R., Aleshin, A. and Slamon, D, Targeting the cyclin-dependent kinases (CDK) 4/6 in estrogen receptor-positive breast cancers. Breast Cancer Res. 2016. **18**(1):17
127. O'Shaughnessy, J, *Extending survival with chemotherapy in metastatic breast cancer*. Oncologist. 2005. **10**(3): p. 20-29
128. Wang, X., Zhang, H. and Chen, X, *Drug resistance and combating drug resistance in cancer*. Cancer Drug Resist. 2019. **2**: p. 141-160
129. Robey, R., et al., *Revisiting the role of ABC transporters in multidrug-resistant cancer*. Nat Rev Cancer. 2018. **18**(7): p. 452-464
130. Vasiliou, V., Vasiliou, K. and Nebert, D, *Human ATP-binding cassette (ABC) transporter family*. Hum Genomics. 2009.**3**(3): p. 281-90
131. Liang, Y., Li, S. and Chen, L, *The physiological role of drug transporters*. Protein Cell. 2015. **6**: p.334–350
132. Wilkens, S, *Structure and mechanism of ABC transporters*. F1000Prime Rep. 2015. **7**: p. 14
133. ter Beek, J., Guskov, A. and Slotboom, D, *Structural diversity of ABC transporters*. J Gen Physiol. 2014. **143**(4): p. 419-35
134. Němcová-Fürstová, V., et al., *Characterization of acquired paclitaxel resistance of breast cancer cells and involvement of ABC transporters*. Toxicol Appl Pharmacol. 2016. **1**:(310): p. 215-228
135. Jiao, Q., et al., *Advances in studies of tyrosine kinase inhibitors and their acquired resistance*. Mol Cancer. 2018.**17**(1): p. 36
136. Hata, A., et al., *Re-biopsy of non-small cell lung cancer patients with acquired resistance to epidermal growth factor receptor-tyrosine kinase inhibitor: Comparison between*

T790M mutation-positive and mutation-negative populations. Cancer. 2013.**119**(24): p. 4325-4332
137. Yun, C., et al., *The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP*. Proc Natl Acad Sci U S A. 2008.**105**(6): p. 2070-2075
138. Ali, S., et al., *Molecular mechanisms and mode of tamoxifen resistance in breast cancer. Bioinformation*. 2016. **12**(3): p. 135-139
139. Ranganathan, S., et al., *Altered beta-tubulin isotype expression in paclitaxel-resistant human prostate carcinoma cells*. Br J Cancer. 1998. **77**(4): p. 562-6
140. Chen, S. and Chang, J, *New Insights into Mechanisms of Cisplatin Resistance: From Tumor Cell to Microenvironment.* Int J Mol Sci. 2019.**20**(17): p. 4136
141. Rocha, C., et al., *DNA repair pathways and cisplatin resistance: an intimate relationship*. Clinics (Sao Paulo). 2018. **73**(1): p. 478
142. Li, Q., et al., *Association between the level of ERCC-1 expression and the repair of cisplatin-induced DNA damage in human ovarian cancer cells*. Anticancer Res. 2000. **20**(2): p. 645-652
143. Chen, S., et al., *O (6) -methylguanine DNA methyltransferase repairs platinum-DNA adducts following cisplatin treatment and predicts prognoses of nasopharyngeal carcinoma*. Int J Cancer. 2015.**137**(6): p. 1291-1305
144. Mansoori, B., et al., *The Different Mechanisms of Cancer Drug Resistance: A Brief Review.* Adv Pharm Bull. 2017.**7**(3): p. 339-348
145. Pistritto, G., et al., *Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies*. Aging (Albany NY). 2016. **8**(4): p. 603-19
146. Qin, M., et al., Let-7i reduces chemotherapy resistance in breast cancer cells through down-regulation of K-Ras and Bcl2 expression. 2019. **35**(11): p. 992-999
147. Zhao, T., et al., *TRIM32 promotes proliferation and confers chemoresistance to breast cancer cells through activation of the NF-κB pathway*. J Cancer. 2018. **9**(8): p. 1349-1356
148. Hientz, K., et al., *The role of p53 in cancer drug resistance and targeted chemotherapy*. Oncotarget. 2017. **8**(5): p. 8921-8946
149. Housman, G., et al., *Drug resistance in cancer: an overview*. Cancers (Basel). 2014. **6**(3): p. 1769-92
150. Pan, S., et al., *Molecular mechanisms for tumour resistance to chemotherapy*. Clin Exp Pharmacol Physiol. 2016. **43**(8):723-737
151. Justenhoven, C., et al., *CYP2B6*6 is associated with increased breast cancer risk.* International Journal of Cancer. 2014.**134**(2): p. 426-430135
152. Goetz, M., et al., *The impact of cytochrome P450 2D6 metabolism in women receiving adjuvant tamoxifen.* Breast Cancer Res Treat. 2007.**101**(1): p. 113-121136
153. Levin, M., et al., *Surmounting Cytarabine-resistance in acute myeloblastic leukaemia cells and specimens with a synergistic combination of hydroxyurea and azidothymidine.* Cell Death Dis. 2019.**10**: p. 390137
154. Kantharidis, P., et al., *Altered methylation of the human MDR1 promoter is associated with acquired multidrug resistance*. Clin Cancer Res. 1997.**3**(11): p. 2025-2032
155. Magee, P., Shi, L. and Garofalo, M, *Role of microRNAs in chemoresistance.* Ann Transl Med. 2015. **3**(21): p. 332139
156. Si, W., et al., *The role and mechanisms of action of microRNAs in cancer drug resistance*. Epigenet, 2019. **11**: p. 25
157. Wang, Z., et al., *MicroRNA-21 modulates chemosensitivity of breast cancer cells to doxorubicin by targeting PTEN*. Arch Med Res. 2011. **42**(4): p. 281-290
158. Gong. C., et al., *Up-regulation of miR-21 mediates resistance to trastuzumab therapy for breast cancer*. *J Biol Chem*. 2011. **286**(21): p. 19127-19137

159. Gan, R., et al., *Downregulation of miR-221/222 enhances sensitivity of breast cancer cells to tamoxifen through upregulation of TIMP$_3$.* Cancer Gene Ther 2014. **21**: p. 290–296
160. Bao, L., et al., *Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298*. Am J Pathol. 2012.**180**(6): p. 2490-2503
161. Hill, C. and Wang, Y, *The importance of epithelial-mesenchymal transition and autophagy in cancer drug resistance.* Cancer Drug Resist. 2020. **3**(1): p. 38-47
162. Xu, X., et al., *TGF-β plays a vital role in triple-negative breast cancer (TNBC) drug-resistance through regulating stemness, EMT and apoptosis.* Biochem Biophys Res Commun. 2018. **502**(1): p. 160-165
163. Wu, Y., et al., *Expression of Wnt3 activates Wnt/β-catenin pathway and promotes EMT-like phenotype in trastuzumab-resistant HER2-overexpressing breast cancer cells*. Mol Cancer Res. 2012.**10**(12): p. 1597-1606
164. Wang, L., et al., *Association between Twist and multidrug resistance gene-associated proteins in Taxol®-resistant MCF-7 cells and a 293-cell model of Twist overexpression.* Oncol Lett. 2018.**15**(1): p. 1058-1066
165. Prasetyanti, P. and Medema, J, *Intra-tumor heterogeneity from a cancer stem cell perspective.* Mol Cancer. 2017.**16**(1): p. 41
166. De Angelis, M., et al., *Breast Cancer Stem Cells as Drivers of Tumor Chemoresistance, Dormancy and Relapse: New Challenges and Therapeutic Opportunities.* Cancers (Basel). 2019.**11**(10): p. 1569
167. Britton, K., et al., *Breast cancer, side population cells and ABCG2 expression*. Cancer Lett. 2012.**323**(1): p. 97-105
168. Zhang, M., et al., *Identification of tumor-initiating cells in a p53-null mouse model of breast cancer*. Cancer Res. 2008. **68**(12): p. 4674-4682
169. Palmieri, M., et al., *PIK3CA-CDKN2A clonal evolution in metastatic breast cancer and multiple points cell-free DNA analysis*. Cancer Cell Int. 2019. **19**: p. 274
170. Balko, J., et al., *Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets*. Cancer Discov, 2014. **4**: p. 232-45
171. Al Amri, W., *Genomic and Expression Analyses Define MUC17 and PCNX1 as Predictors of Chemotherapy Response in Breast Cancer*. Mol Cancer Ther. 2020. **19**(3): p. 945-955
172. Kim, C., et al., *Chemoresistance evolution in triple-negative breast cancer delineated by single-cell sequencing*. Cell. 2018. **173**: p. 879-93
173. Senthebane, D., et al., *The Role of Tumor Microenvironment in Chemoresistance: To Survive, Keep Your Enemies Closer.* International journal of molecular sciences. 2017. **18**(7): p.1586
174. Son, B., et al., *The role of tumor microenvironment in therapeutic resistance*. Oncotarget. 2017. **8**(3): p. 3933-3945
175. Morin, P, *Drug resistance and the microenvironment: nature and nurture*. Drug Resist Update. 2003. **6**(4): p. 169-172
176. Santoni, M., et al., *Emerging role of tumor-associated macrophages as therapeutic targets in patients with metastatic renal cell carcinoma.* Cancer Immunol Immunother. 2013. **62**(12): p. 1757-1768
177. Jinushi, M. and Komohara, Y, *Tumor-associated macrophages as an emerging target against tumors: Creating a new path from bench to bedside.* Biochim Biophys Acta. 2015.**1855**: p. 123–130
178. Shree, T., et al. *Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer*. Genes Dev. 2011. **25**(23): p. 2465-2479

179. Wu, L., et al., *Tumor-Associated Neutrophils in Cancer: Going Pro*. Cancers (Basel). 2019.**11**(4): p. 564
180. Masucci, M., Minopoli, M. and Carriero, M, *Tumor Associated Neutrophils*. *Their Role in Tumorigenesis, Metastasis, Prognosis and Therapy.* Front Oncol. 2019.**15**(9): p. 1146
181. Beury, D., et al., *Cross-talk among myeloid-derived suppressor cells, macrophages, and tumor cells impacts the inflammatory milieu of solid tumors*. J Leukoc Biol. 2014. **96**: p. 1109–1118
182. Ostrand-Rosenberg, S., et al., *Cross-talk between myeloid-derived suppressor cells (MDSC), macrophages, and dendritic cells enhances tumor-induced immune suppression.* Semin Cancer Biol. 2012. **22**: p. 275–281
183. Katoh, M. and Nakagama, H, *FGF receptors: cancer biology and therapeutics*. Med Res Rev. 2014. **34**(2): p. 280-300
184. Straussman, R., et al., *Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion*. Nature. 2012. **487**: p. 500–504
185. Sun, X., et al., *Retraction Note: IL-6 secreted by cancer-associated fibroblasts induces tamoxifen resistance in luminal breast cancer*. Oncogene. 2014. **33**: p. 4450
186. Zhang, H. et al., *Interference of Frizzled 1 (FZD1) reverses multidrug resistance in breast cancer cells through the Wnt/β-catenin pathway*. Cancer Lett. 2012. **323**(1): p. 106-113
187. Yu, D., et al., *Exosomes in development, metastasis and drug resistance of breast cancer*. *Cancer Sci*. 2015. **106**(8): p. 959-964
188. Yeung, C., et al., *Exosomal transfer of stroma-derived miR21 confers paclitaxel resistance in ovarian cancer cells through targeting APAF1*. Nat Commun. 2016. **7**: p. 11150
189. Cirri, P. and Chiarugi, P, *Cancer associated fibroblasts: the dark side of the coin.* Am J Cancer Res. 2011.**1**(4): p. 482-97
190. Al Amri, W., et al., *Identification of candidate mediators of chemoresponse in breast cancer through therapy-driven selection of somatic variants.* Breast Cancer Res Treat. 2020. **183**(3): p. 607-616
191. Gwili, N., et al., *Transcriptome profiles of stem-like cells from primary breast cancers allow identification of ITGA7 as a predictive marker of chemotherapy response.* Br J Cancer. 2021. **125**(7): p.983-993
192. Broad, R., et al., *Inhibition of interferon-signalling halts cancer-associated fibroblast-dependent protection of breast cancer cells from chemotherapy*. Br J Cancer. 2021. **124**(6): p. 1110-1120
193. Borden, E., *Interferons α and β in cancer: therapeutic opportunities from new insights.* Nat Rev Drug Discov. 2019. **18**: p. 219–234
194. Hosein, A., et al., *A functional in vitro model of heterotypic interactions reveals a role for interferon-positive carcinoma associated fibroblasts in breast cancer*. BMC Cancer. 2015. **15**(15): p. 130
195. Franci, C., et al., *Biomarkers of residual disease, disseminated tumor cells, and metastases in the MMTV-PyMT breast cancer model.* PLoS One. 2013. **8**(3): p. 58183
196. Boelens, M., et al., *Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways.* Cell. 2014. **159**(3): p. 499-51
197. Yu, Y., et al., *Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling*. Br J Cancer.2014. **110**: p. 724-732
198. Rhodes, L., et al., *Cytokine receptor CXCR4 mediates estrogen-independent tumorigenesis, metastasis, and resistance to endocrine therapy in human breast cancer*. Cancer Res. 2011. **71**: p. 603-613
199. Soon, P., et al., *Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells*. Endocr Relat Cancer. 2013. **20**: p. 1-12

200. Dittmer, J. and Leyh, B, *The impact of tumor stroma on drug response in breast cancer.* Semin Cancer Biol. 2015. **31**: p. 3-15
201. Aljohani, A., et al., *Myxovirus resistance 1 (MX1) is an independent predictor of poor outcome in invasive breast cancer*. Breast Cancer Res Treat. 2020. **181**(3): p. 541-551
202. Croner, R., et al., *Quantitative proteome profiling of lymph node-positive vs. -negative colorectal carcinomas pinpoints MX1 as a marker for lymph node metastasis*. Int J Cancer. 2014.**135**(12): p. 2878-86
203. Bhandari, A., et al., *ITGA7 functions as a tumor suppressor and regulates migration and invasion in breast cancer*. Cancer Management and Research. 2018:**10**: p. 969–976
204. Zhu, Z., et al., *Integrin alpha 7 interacts with high temperature requirement A2 (HtrA2) to induce prostate cancer cell death*. Am J Pathol. 2010. **177**(3): p. 1176-1186
205. Bai, X., et al., *Integrin α7 high expression correlates with deteriorative tumor features and worse overall survival, and its knockdown inhibits cell proliferation and invasion but increases apoptosis in breast cancer*. J Clin Lab Anal. 2019. **33**(8): p. 22979
206. Ming, X., et al., *Integrin α7 is a functional cancer stem cell surface marker in oesophageal squamous cell carcinoma*. Nat Commun. 2016. **7**: p. 13568
207. Shi, W., et al., *Identification of liver metastasis-related genes in a novel human pancreatic carcinoma cell model by microarray analysis*. Cancer Lett. 2009. **283**(1): p. 84-91
208. Zhang, Y., et al., *Targetable Molecular Chaperone Hsp27 Confers Aggressiveness in Hepatocellular Carcinoma*. Theranostics. 2016. **6**(4): p. 558-70
209. Haas, T., et al., *Integrin α7 is a Functional Marker and Potential Therapeutic Target in Glioblastoma*. Cell Stem Cell. 2017.**21**(1): p. 35-50
210. Guan, Y., et al., *Downregulating integrin subunit alpha 7 (ITGA7) promotes proliferation, invasion, and migration of papillary thyroid carcinoma cells through regulating epithelial-to-mesenchymal transition.* Acta Biochimica et Biophysica Sinica*.* 2020. **52**(2): p. 116–124
211. Tan, L., et al., *Integrin α7 binds tissue inhibitor of metalloproteinase 3 to suppress growth of prostate cancer cells*. Am J Pathol. 2013. **183**(3): p. 831-40
212. Pawlak, A., Strzadala, L. and Kalas, W, *Non-genomic effects of the NR4A1/Nur77/TR3/NGFIB orphan nuclear receptor*. Steroids. 2015. **95**: p. 1-6
213. Wu, H., et al., *Nuclear receptor NR4A1 is a tumour suppressor down-regulated in triple-negative breast cancer*. Oncotarget. 2017. **2**(33); p. 54364-54377
214. Alexopoulou, A., et al., *Dissecting the transcriptional networks underlying breast cancer: NR4A1 reduces the migration of normal and breast cancer cell lines.* Breast Cancer Res. 2010.**12**(4): p. 51
215. Mullican, S., et al., *Abrogation of nuclear receptors Nr4a3 and Nr4a1 leads to development of acute myeloid leukaemia*. Nat Med. 2007.**13**(6): p. 730-735
216. Zhou, F., et al., *Nuclear receptor NR4A1 promotes breast cancer invasion and metastasis by activating TGF-β signalling*. Nature communications. 2013. **5**(3388): p. 1-13
217. Hedrick, E. and Safe, S, *Transforming Growth Factor β/NR4A1-Inducible Breast Cancer Cell Migration and Epithelial-to-Mesenchymal Transition Is p38α (Mitogen-Activated Protein Kinase 14) Dependent.* Molecular and Cellular Biology. 2017. **37** (18): p. 306-17
218. Hedrick, E., et al., *NR4A1 Antagonists Inhibit β1-Integrin-Dependent Breast Cancer Cell Migration.* Mol Cell Biol. 2016. **36**(9): p. 1383-1394
219. Hedrick, E, Lee, S. and Safe, S, *The nuclear orphan receptor NR4A1 regulates β1-Integrin expression in pancreatic and colon cancer cells and can be targeted by NR4A1 antagonists*. Mol Carcinog. 2017. **56**(9): p. 2066-2075
220. Delgado, E., et al., *High expression of orphan nuclear receptor NR4A1 in a subset of ovarian tumors with worse outcome*. Gynecol Oncol. 2016. **141**(2): p. 348-356

221. Wilson, A., et al., *TR3 modulates platinum resistance in ovarian cancer.* Cancer Res. 2013. **73**(15): p. 4758-69
222. https://www.thermofisher.com/antibody/product/NR4A1-Antibody-Polyclonal/PA5-32949 [cited 08/06/21]
223. Mukherjee, P., et al., *Transgenic tomatoes expressing the 6F peptide and ezetimibe prevent diet-induced increases of IFN-β and cholesterol 25-hydroxylase in jejunum.* J Lipid Res. 2017.**58**(8): p. 1636-1647
224. Demaria, O., et al., *STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity*. Proc Natl Acad Sci U S A. 2015. **112**(50): p. 15408-13
225. www.cellsignal.co.uk/products/primary-antibodies/mx1 [cited 03/02/21]
226. www.thermofisher.com/antibidy/product/Claudin-3-Antibody-Polyclonal/PA5-16867 [cited 03/02/21]
227. Hajian-Tilaki, K, *Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation*. Caspian J Intern Med. 2013. **4**(2): p. 627-35
228. Cbioportal https://www.cbioportal.org/ [cited 08/08/21]
229. Lin, J., et al., *Application of electronic medical record data for health outcomes research: a review of recent literature*. Expert Rev Pharmacoecon Outcomes Res. 2013. **13**(2): p. 191-200
230. Saczynski, J., McManus, D. and Goldberg. R, *Commonly used data-collection approaches in clinical research*. Am J Med. 2013.**126**(11): p. 946-50
231. Aaronson, L. and Burman M, *Use of health records in research: reliability and validity issues.* Res Nurs Health. 1994. **17**(1): p. 67-73
232. Kumar, S., Puja, S. and Ranjana, G, *Making and using inexpensive manually constructed tissue micro-array: Experience of a tertiary care hospital in India*. Indian Journal of pathology and microbiology. 2009. **52**(3): p. 304-309
233. Williams, L., et al., *Retrospective cohort study of breast cancer incidence, health service use and outcomes in Europe: a study of feasibility*. Eur J Public Health. 2018. **28**(2): p. 327-332
234. Simon, S., et al., *Characteristics and prognosis of stage I-III breast cancer subtypes in Brazil: The AMAZONA retrospective cohort study.* Breast. 2019. **44**: p. 113-119
235. Gunasekaran, G., et al., H., *Impact of chemotherapy schedule modification on breast cancer patients: a single-centre retrospective study*. Int J Clin Pharm. 2020. **42:** p. 642–651
236. Oprean, C., *Postmenopausal Breast Cancer in Women, Clinical and Epidemiological Factors Related to the Molecular Subtype: A Retrospective Cohort Study in a Single Institution for 13 Years. Follow-Up Data*. Int J Environ Res Public Health. 2020. **17**(23): p. 8722
237. Al-Asadi, J. and Al-Mayah, S, *Three-year Survival of Women with Breast Cancer in Basrah, Iraq*. Oman Med J. 2020. **35**(4): p. 147
238. Dent, R., et al., *Triple-negative breast cancer: clinical features and patterns of recurrence*. Clin Cancer Res. 2007.**13**(15): p. 4429-34
239. Boyle, P, *Triple-negative breast cancer: epidemiological considerations and recommendations*. Ann Oncol. 2012. **6**(6): p. 7-12
240. Spitale, A., et al., *Breast cancer classification according to immunohistochemical markers: clinicopathologic features and short-term survival analysis in a population-based study from the South of Switzerland*. Ann Oncol. 2009. **20**(4): p. 628-35
241. Vallejos, C., et al., *Breast cancer classification according to immunohistochemistry markers: subtypes and association with clinicopathologic variables in a peruvian hospital database*. Clin Breast Cancer. 2010.**10**(4): p. 294-300

242. Kumar, S., et al., *Presenting features, treatment patterns and outcomes of patients with breast cancer in Pakistan: Experience at a university hospital*. Indian J Cancer. 2016.**53**(2): p. 230-234
243. *Early and locally advanced breast cancer: diagnosis and management*. NICE Guideline, No. 101. National Guideline Alliance (UK). London: National Institute for Health and Care Excellence (UK); 2018
244. McHugh, M, Interrater reliability: the kappa statistic. Biochem Med (Zagreb). 2012. 22(3): p. 276-282
245. Jääskeläinen, A., et al., *High-level cytoplasmic claudin 3 expression is an independent predictor of poor survival in triple-negative breast cancer*. BMC Cancer. 2018. **18**(1):223
246. Zhou, J., et al., *Cancer associated fibroblasts correlate with tumour associated macrophages infiltration and lymphatic metastasis in triple negative breast cancer patients*. J Cancer. 2018. **9**(24): p. 4635-4641
247. https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer/incidence-invasive [cited 02/05/21]
248. O'Shaughnessy, J., et al., *A randomized, double-blind, phase 2 study of ruxolitinib or placebo in combination with capecitabine in patients with advanced HER2-negative breast cancer and elevated C-reactive protein, a marker of systemic inflammation.* Breast Cancer Res Treat, 2018. **170**(3): p. 547-557
249. Salah-Eddin, A., et al., *FAST: An international, multicenter, randomized, phase II trial of epirubicin, oxaliplatin, and capecitabine (EOX) with or without IMAB362, a first-in-class anti-CLDN18.2 antibody, as first-line therapy in patients with advanced CLDN18.2+ gastric and gastroesophageal junction (GEJ) adenocarcinoma*. Journal of Clinical Oncology. 2016. **34**(18)
250. Logullo, A, *Immunoexpression of claudins 4 and 7 among invasive breast carcinoma subtypes: a large diagnostic study using tissue microarray*. Mol. Clin. Oncol. 2018. **9**(4): p. 377-388
251. Odnokoz, O., et al., *Malignant cell-specific pro-tumorigenic role of type I interferon receptor in breast cancers*. Cancer Biol Ther. 2020.**21**(7): p. 629-636
252. Katlinski, K., et al., *Inactivation of interferon receptor promotes the establishment of immune privileged tumor microenvironment*. Cancer Cell. 2017.**31**(2): p. 194–207
253. Katlinskaya, Y., et al., *Suppression of type i interferon signaling overcomes oncogene-induced senescence and mediates melanoma development and progression.* Cell Rep. 2016.**15**(1): p. 171–180
254. Weichselbaum, R., et al., *An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer*. Proc Natl Acad Sci U S A. 2008. **105**(47): p. 18490–18495
255. Post, A., et al., *Interferon-stimulated genes are involved in cross-resistance to radiotherapy in tamoxifen-resistant breast cancer*. Clin Cancer Res. 2018. **24**(14): p. 3397–3408
256. Alsamman, K. and El-Masry, O, *Interferon regulatory factor 1 inactivation in human cancer*. Biosci Rep. 2018. **38**(3): p. 20171672
257. Doherty, G., et al., *Interferon regulatory factor expression in human breast cancer*. Ann Surg. 2001. **233**(5): p. 623-629
258. Sultan, M., et al., *Epigenetic Silencing of TAP1 in Aldefluor^{+} Breast Cancer Stem Cells Contributes to Their Enhanced Immune Evasion*. Stem Cells. 2018. **36**(5):641-654
259. Song, W., et al., *H36-alpha 7 is a novel integrin alpha chain that is developmentally regulated during skeletal myogenesis.* The Journal of cell biology. 1992. **117**: p. 643-657

260. Seraya-Bareket, C., et al., Weisz, A., *The identification of nuclear alpha beta3 integrin in ovarian cancer: non-paradigmal localization with cancer promoting actions*. Oncogenesis. 2020. **9**: p. 69-69
261. Liu, S., et al., *A small molecule induces integrin β4 nuclear translocation and apoptosis selectively in cancer cells with high expression of integrin β4*. Oncotarget. 2016. **7**: p. 16282-16296
262. Gao, J., et al., *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal. 2013.**6**(269): p1
263. Zlobec, I., et al., *Selecting immunohistochemical cut-off scores for novel biomarkers of progression and survival in colorectal cancer*. J Clin Pathol. 2007. **60**: p. 1112-1116
264. Xie, L., et al., *Honokiol sensitizes breast cancer cells to TNF-α induction of apoptosis by inhibiting Nur77 expression*. British Journal of Pharmacology. 2016.**173**: p. 344-356
265. Cho, S., et al., *Nur77 agonists induce proapoptotic genes and responses in colon cancer cells through nuclear receptor-dependent and nuclear receptor-independent pathways*. Cancer Res. 2007. **67**(2): p. 674-83
266. Kolluri, S., et al., *Mitogenic Effect of Orphan Receptor TR3 and Its Regulation by MEKK1 in Lung Cancer Cells.* Molecular and Cellular Biology. 2003. **23** (23): p. 8651-8667
267. Lee, et al., *Inactivation of the orphan nuclear receptor TR3/Nur77 inhibits pancreatic cancer cell and tumor growth. Cancer Res.* 2010. **70**(17): p. 6824-36
268. Lenz, G., et al., *Lymphoma/Leukaemia Molecular Profiling Project. Stromal gene signatures in large-B-cell lymphomas*. N Engl J Med. 2008. **359**(22): p. 2313-23
269. Uemura, H. and Chang, C, *Antisense TR3 orphan receptor can increase prostate cancer cell viability with etoposide treatment*. Endocrinology. 1998.**139**(5): p. 2329-34
270. Mohan, H., et al., *Molecular pathways: the role of NR4A orphan nuclear receptors in cancer.* Clin Cancer Res. 2012. **18**(12): p. 3223-8
271. Chen, H., et al., *The orphan receptor TR3 suppresses intestinal tumorigenesis in mice by downregulating Wnt signalling*. Gut. 2012. **61**(5): p. 714-24
272. Sun, Z., et al., *Inhibition of β-catenin signaling by nongenomic action of orphan nuclear receptor Nur77*. Oncogene. 2012. **31**: p. 2653–2667
273. Thompson, J. and Winoto, A, *During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain*. J Exp Med. 2008. **205**(5): p. 1029-1036
274. Kolluri, S., et al., *A short Nur77-derived peptide converts Bcl-2 from a protector to a killer.* Cancer cell. 2008.**14**(4): p. 285–298
275. Wilson, A., et al., *TR3/Nur77 in colon cancer cell apoptosis.* Cancer Res. 2003. **63**(17): p. 5401-7
276. Xie, J., et al., *Macroporous organosilicon nanocomposites co-deliver Bcl2-converting peptide and chemotherapeutic agent for synergistic treatment against multidrug resistant cancer*. Cancer Lett. 2020. **28**(469): p. 340-354
277. Hedrick, E., et al., *Nuclear receptor 4A1 as a drug target for breast cancer chemotherapy.* Endocr Relat Cancer. 2015. **22**(5): p. 831-40
278. Malewicz, M., et al., *Essential role for DNA-PK-mediated phosphorylation of NR4A nuclear orphan receptors in DNA double-strand break repair*. Genes & development. 2011. ***25***(19): p. 2031–2040
279. Zheng, Y., et al., *COX-2 mediates tumor-stromal prolactin signaling to initiate tumorigenesis*. Proc Natl Acad Sci U S A. 2019. **116**(12): p. 5223-5232
280. Holla, V., et al., *Prostaglandin E2 regulates the nuclear receptor NR4A2 in colorectal cancer.* J Biol Chem. 2006. **281**(5): p. 2676-82
281. Yang, P., et al., *Blocking PPARγ interaction facilitates Nur77 interdiction of fatty acid uptake and suppresses breast cancer progression.* Proc Natl Acad Sci U S A. 2020. **117**(44): p. 27412-27422

282. Ye, T., et al., *Orphan nuclear receptor TR3/Nur77 differentially regulates the expression of integrins in angiogenesis*. Microvasc Res. 2019. **3**(122): p. 22-33
283. Chen, C., et al., *Orphan nuclear receptor TR3/Nur77 biologics inhibit tumor growth by targeting angiogenesis and tumor cells*. Microvasc Res. 2020. **3**(128): p. 103934
284. Nicolini, A., Ferrari, P. and Duffy, M, *Prognostic and predictive biomarkers in breast cancer: Past, present and future*. Semin Cancer Biol. 2018. **52**(1): p. 56-73
285. Smith A. and Farrah, K, *Gene Expression Profiling Tests for Breast Cancer: A Rapid Qualitative Review.* Canadian Agency for Drugs and Technologies in Health. 2019. **4**(18)
286. Lynce, F., et al. *Phase I study of JAK1/2 inhibitor ruxolitinib with weekly paclitaxel for the treatment of HER2-negative metastatic breast cancer*. Cancer Chemother Pharmacol. 2021. **87**(5): p. 673-679
287. Stover, D., et al., *Phase II study of ruxolitinib, a selective JAK1/2 inhibitor, in patients with metastatic triple-negative breast cancer.* NPJ Breast Cancer. 2018.**4**(4): p. 10
288. Clinicaltrials.gov/ct2/show/NCT02876302 [cited 16/07/2021]
289. Harrison, C., et al., *JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis*. N Engl J Med. 2012. **366**(9): p. 787-98
290. Verstovsek, S., et al., *A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis.* N Engl J Med. 2012. **366**(9): p. 799-807

9. APPENDICES

Appendix 9.1. Ethical approval documents from Leeds (East) research committee for the study

Leeds (East) Research Ethics Committee
Room 5.2, Clinical Sciences Building
St James's University Hospital
Beckett Street
Leeds
LS9 7TF
Telephone: 0113 2065652

Full title of study: Studies of the biological significance of breast cancer subtype, using molecular and cytogenetic profiling and in vivo models. 06/Q1206/180

REC reference number:

The Research Ethics Committee reviewed the above application at the meeting held on 7 November 2006.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to complete Part C of the application form or to inform Local Research Ethics Committees (LRECs) about the research. The favourable opinion for the study applies to all sites involved in the research.

Conditions of approval

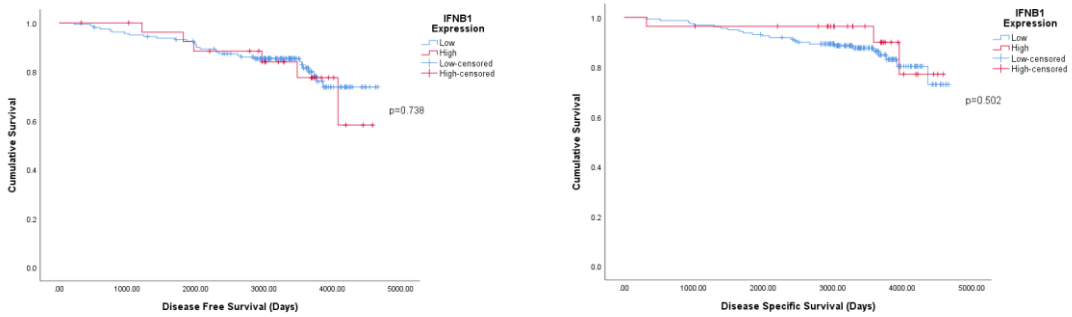
The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents
The documents reviewed and approved at the meeting were:
Document Version Date
Application 03 October 2006
Investigator CV .
An advisory committee to West Yorkshire Strategic Health Authority
06/Q1206/180 Page 2
Protocol 1 09 October 2006
Letter from Sponsor 26 October 2006

Research governance approval

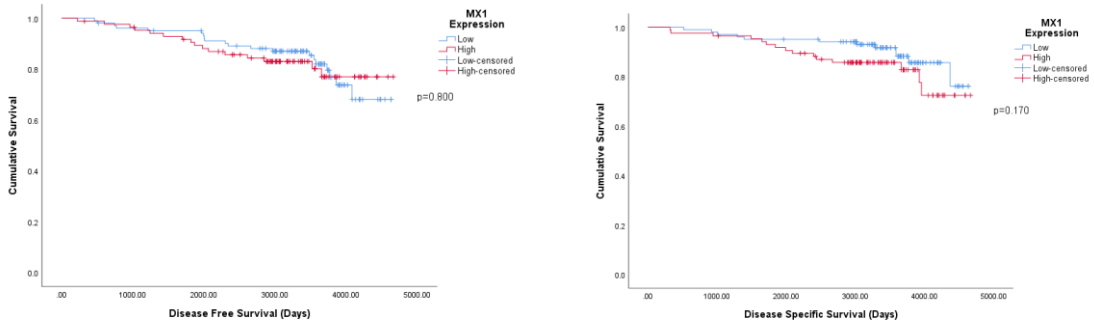
Appendix 9.2. Kaplan-Meier plots comparing mean disease-free survival and disease-specific survival between low or high expression of IFNβ1 in fibroblasts and MX1 cancer cell cytoplasm in all four subtypes of breast cancer, ER-Positive, ER-Negative, HER2-Positive and Triple Negative

A. **B.**



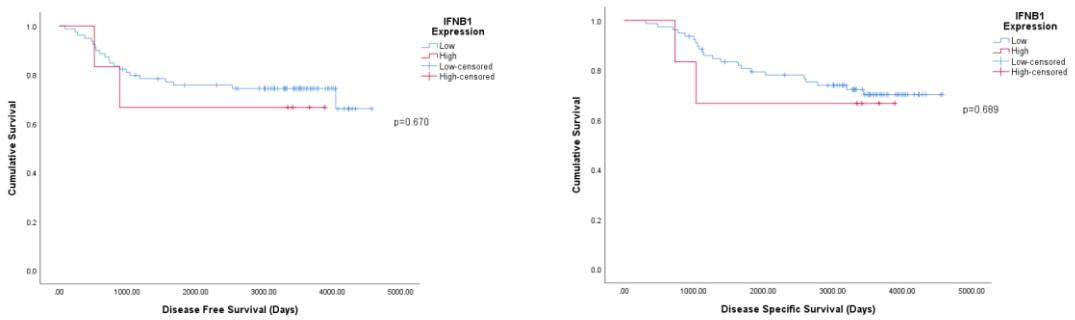
Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression. Fibroblast IFNβ1 expression was determined in a cohort of primary ER-Positive breast cancer patients treated with adjuvant chemotherapy (n=207). The cohort was dichotomised into high or low fibroblast IFNβ1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



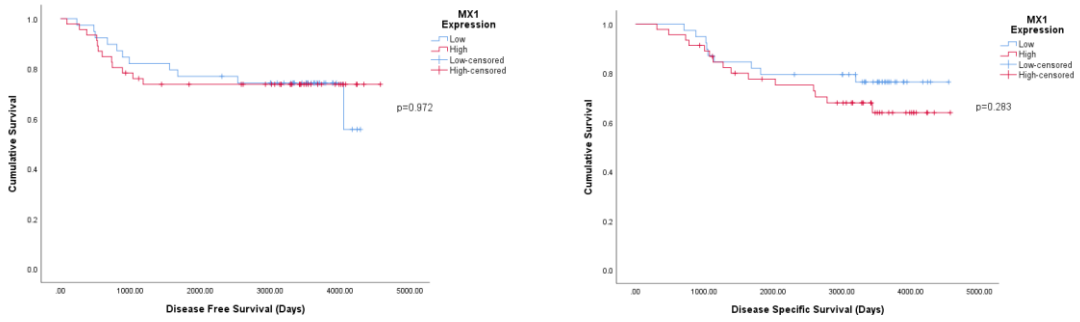
Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression. Cytoplasmic cancer cell MX1 expression was determined in a cohort of primary ER-Positive breast cancer patients treated with adjuvant chemotherapy (n=207). The cohort was dichotomised into high or low MX1 cancer cell cytoplasm expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



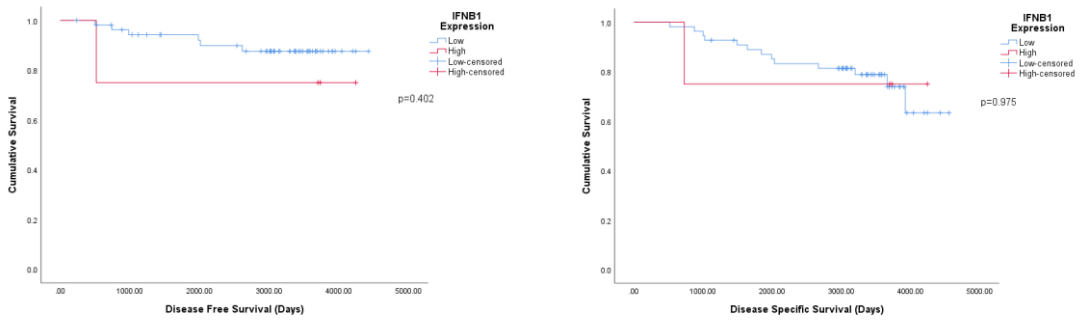
Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression. Fibroblast IFNβ1 expression was determined in a cohort of primary ER-Negative breast cancer patients treated with adjuvant chemotherapy (n=98). The cohort was dichotomised into high or low fibroblast IFNβ1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



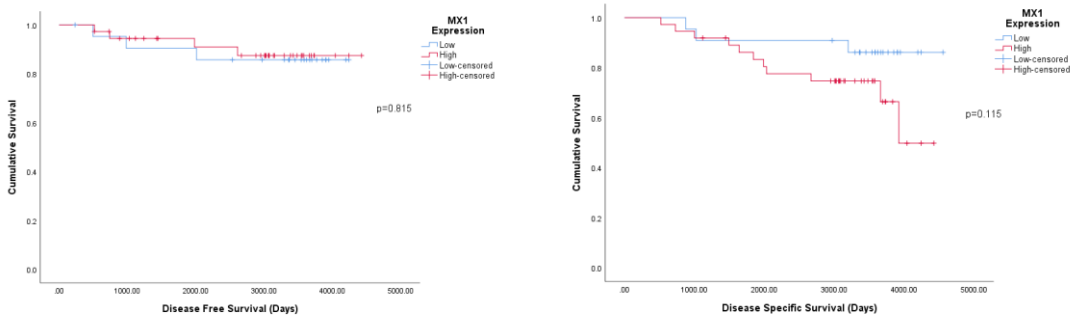
Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression. Cytoplasmic cancer cell MX1 expression was determined in a cohort of primary ER-Negative breast cancer patients treated with adjuvant chemotherapy (n=98). The cohort was dichotomised into high or low MX1 cancer cell cytoplasm expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



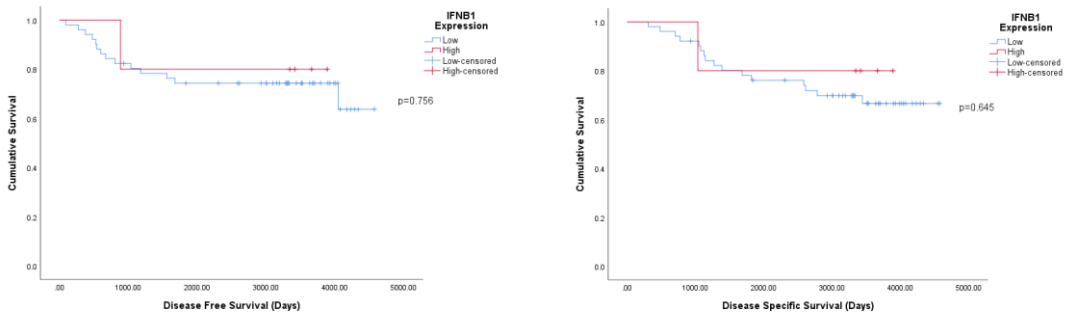
Kaplan-Meier survival analysis for HER2-Positive breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression. Fibroblast IFNβ1 expression was determined in a cohort of primary HER2-Positive breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low fibroblast IFNβ1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



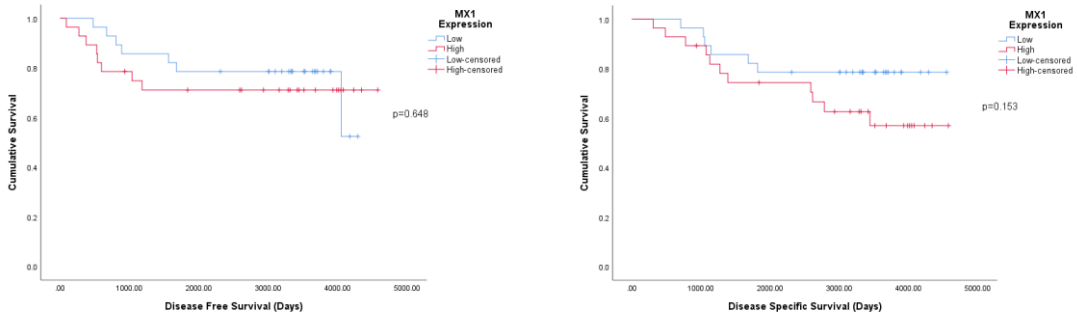
Kaplan-Meier survival analysis for HER2-Positive breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression. Cytoplasmic cancer cell MX1 expression was determined in a cohort of primary HER2-Positive breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low MX1 cancer cell cytoplasm expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



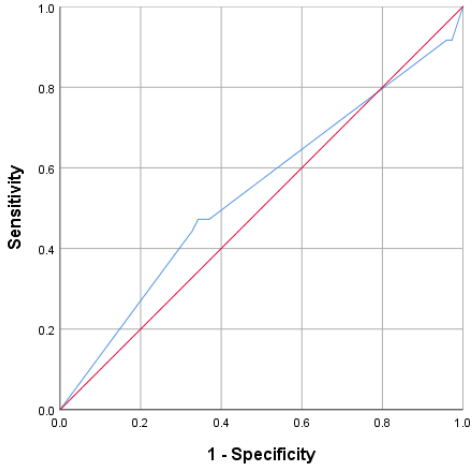
Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low fibroblast IFNβ1 expression. Fibroblast IFNβ1 expression was determined in a cohort of primary Triple Negative breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low fibroblast IFNβ1 expression groups based upon ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low cytoplasmic cancer cell MX1 expression. Cytoplasmic cancer cell MX1 expression was determined in a cohort of primary Triple Negative breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low MX1 cancer cell cytoplasm expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

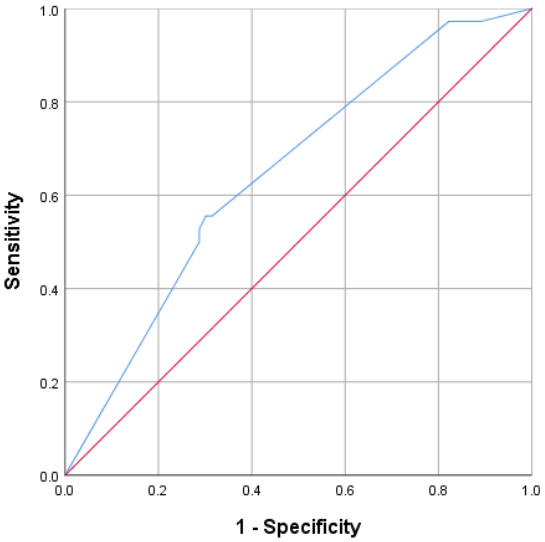
Appendix 9.3. Receiver operation curve (ROC) analysis was performed to establish cut-off scores for IFNβ1 and MX1 in the triple negative cohort (Cohort 2) that received adjuvant chemotherapy for breast cancer



Coordinates of the Curve

Test Result Variable(s): IFNβ1 Fibroblast

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
.0000	1.000	1.000
1.2500	.917	.973
1.7500	.917	.959
2.2500	.472	.370
2.6000	.472	.342
2.8500	.444	.329
4.0000	.000	.000

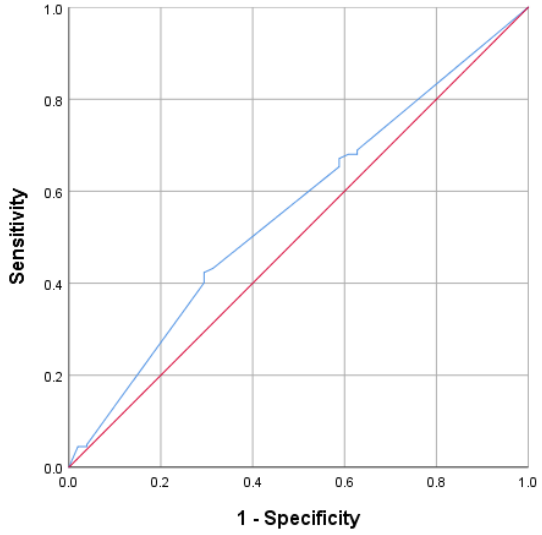


Coordinates of the Curve

Test Result Variable(s): MX1 Cancer Cell Cytoplasm

Positive if Greater Than or Equal To[a]	Sensitivity	1 – Specificity
.0000	1.000	1.000
1.1500	.972	.890
1.4000	.972	.877
1.7500	.972	.822
2.1500	.556	.315
2.4000	.556	.301
2.6000	.528	.288
2.8500	.500	.288
4.0000	.000	.000

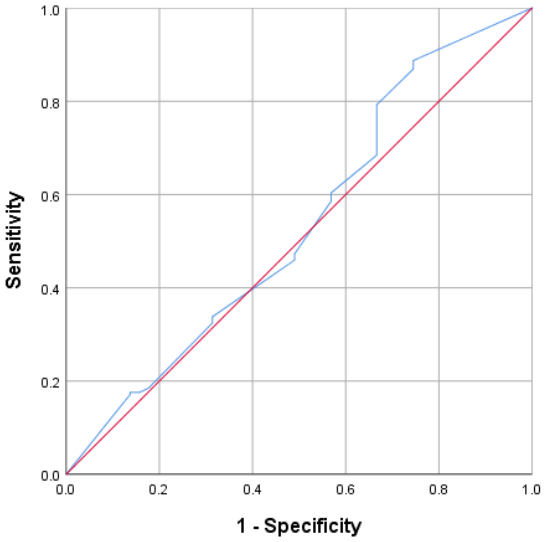
Appendix 9.4. Receiver operation curve (ROC) analysis was performed to establish cut-off scores for ITGA7 in the whole cohort (Cohort 1) that received adjuvant chemotherapy for breast cancer



Coordinates of the Curve

Test Result Variable(s): ITGA7cytoplasm

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-1.0000	1.000	1.000
.8500	.689	.627
1.8500	.685	.627
2.2500	.680	.627
3.2500	.680	.608
4.5000	.671	.588
5.3500	.658	.588
5.8500	.653	.588
6.1500	.432	.314
6.5000	.423	.294
6.8500	.401	.294
7.1500	.050	.039
7.4000	.045	.039
7.7500	.045	.020
9.0000	.000	.000



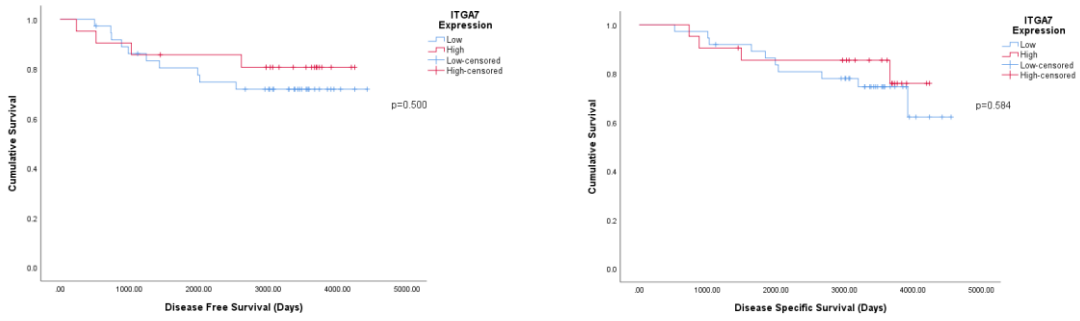
Coordinates of the Curve

Test Result Variable(s): ITGA7nucleus

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-1.0000	1.000	1.000
.3500	.887	.745
1.0000	.883	.745
1.6500	.869	.745
2.1500	.793	.667
2.5000	.784	.667
2.8500	.779	.667
3.1500	.698	.667
3.5000	.694	.667
3.8500	.685	.667
4.1500	.604	.569
4.5000	.590	.569
4.8500	.586	.569
5.1500	.473	.490
5.5000	.468	.490
5.8500	.459	.490
6.1500	.338	.314
6.5000	.333	.314
6.8500	.324	.314
7.1500	.185	.176
7.4000	.176	.157
7.6000	.176	.137

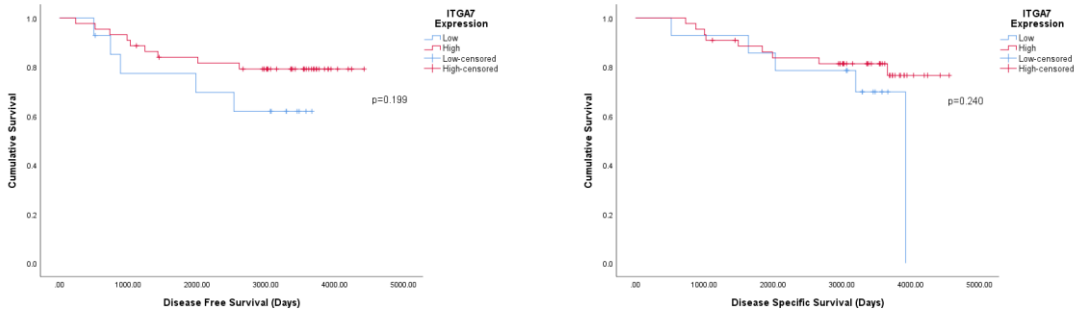
Appendix 9.5. Kaplan-Meier plots, comparing mean disease-free survival and disease-specific survival between low or high expression of cytoplasmic and nuclear ITGA7 in breast cancer subtypes, HER2-Positive, Triple negative and ER-Negative

A. **B.**



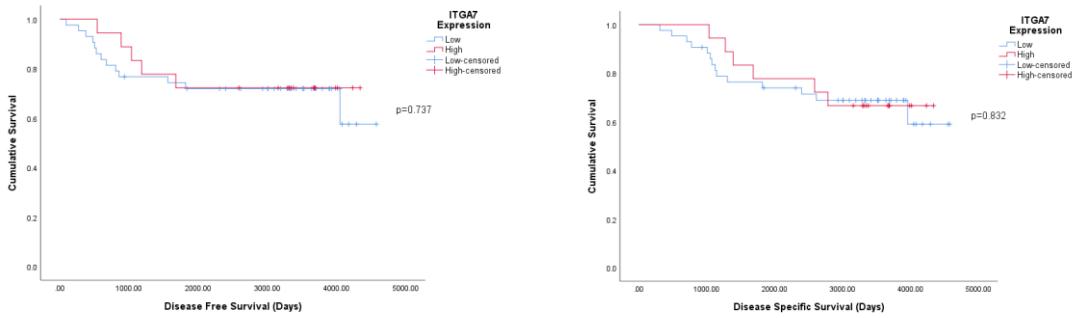
Kaplan-Meier survival analysis for HER2-Positive breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression. ITGA7 cytoplasmic expression was determined in a cohort of primary HER2-Positive breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low ITGA7 cytoplasmic expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



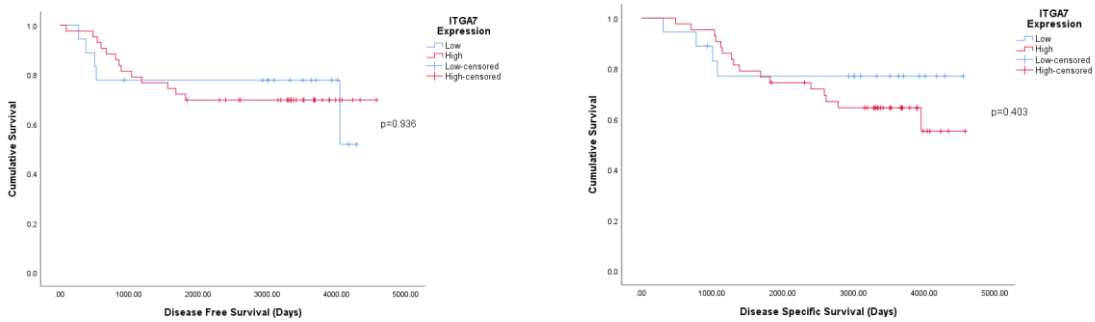
Kaplan-Meier survival analysis for HER2-Positive breast cancer outcomes in groups with high versus low nuclear ITGA7 expression. Nuclear ITGA7 expression was determined in a cohort of primary HER2-Positive breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low nuclear ITGA7 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



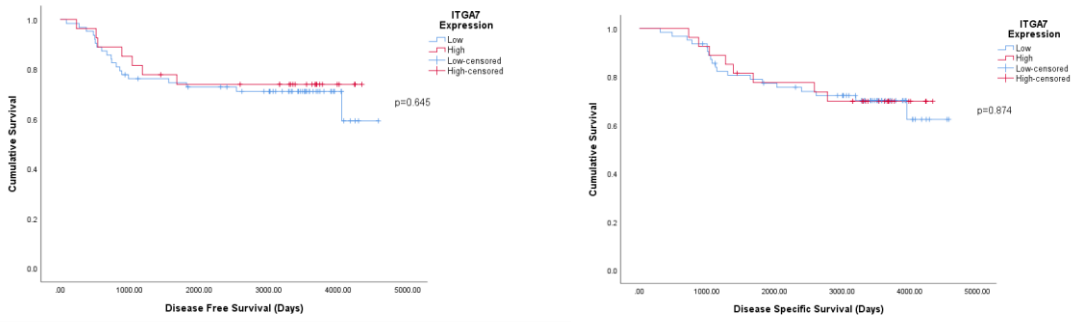
Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression. ITGA7 cytoplasmic expression was determined in a cohort of primary Triple Negative breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high or low ITGA7 cytoplasmic expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



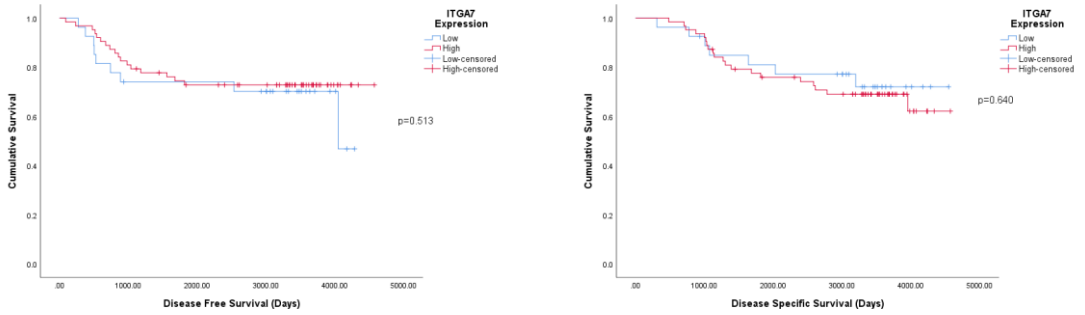
Kaplan-Meier survival analysis of high versus low nuclear ITGA7 expression in patients with Triple Negative breast cancers. Nuclear ITGA7 expression was determined in a cohort of primary Triple Negative breast cancer patients treated with adjuvant chemotherapy (n=68). The cohort was dichotomised into high and low nuclear ITGA7 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression. ITGA7 cytoplasmic expression was determined in a cohort of primary ER-Negative breast cancer patients treated with adjuvant chemotherapy (n=98). The cohort was dichotomised into high or low ITGA7 cytoplasmic expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

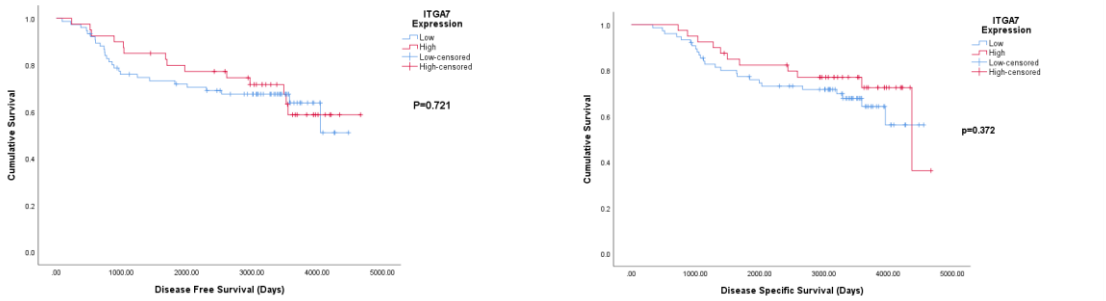
A. **B.**



Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low nuclear ITGA7 expression. Nuclear ITGA7 expression was determined in a cohort of primary ER-Negative breast cancer patients treated with adjuvant chemotherapy (n=98). The cohort was dichotomised into high or low nuclear ITGA7 expression groups based upon ROC analysis. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

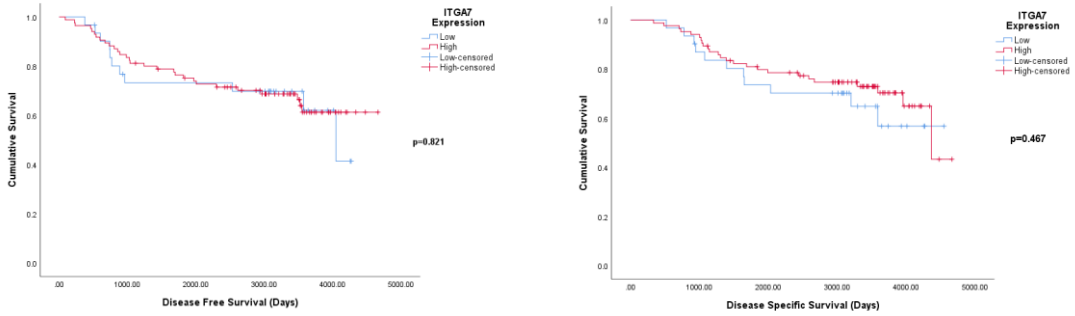
Appendix 9.6. Kaplan-Meier plots comparing mean disease-free survival and disease-specific survival between high versus low cytoplasmic and nuclear ITGA7 expression in patients that received taxane based adjuvant chemotherapy

A. **B.**



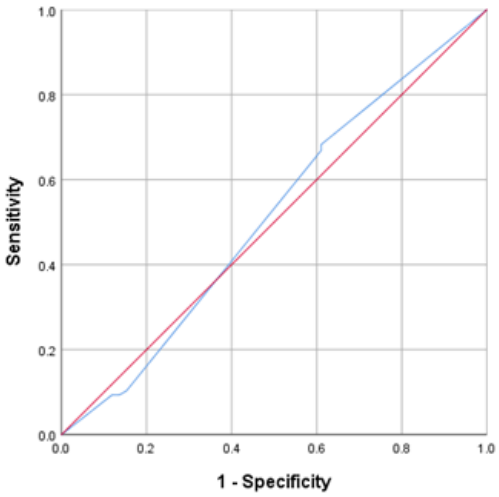
Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low cytoplasmic ITGA7 expression in patients that received taxane based adjuvant chemotherapy. Cytoplasmic ITGA7 expression was determined in a cohort of 116 primary breast cancer patients treated with taxane adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis for breast cancer outcomes in groups with high versus low nuclear ITGA7 expression in patients that received taxane based adjuvant chemotherapy. Nuclear ITGA7 expression was determined in a cohort of 116 primary breast cancer patients treated with taxane adjuvant chemotherapy. The cohort was dichotomised into high or low nuclear ITGA7 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

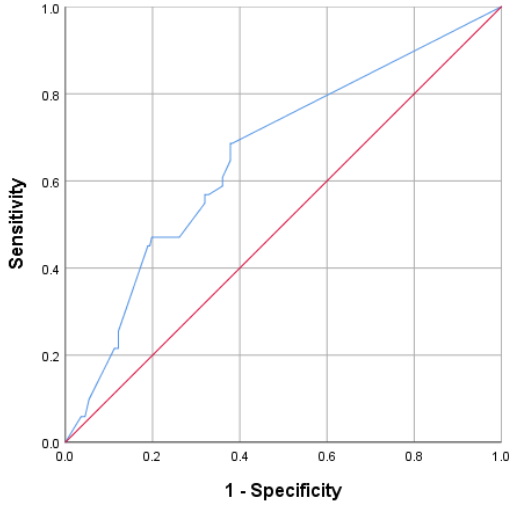
Appendix 9.7. Receiver operation curve (ROC) analysis was performed to establish cut-off scores for NR4A1 in the whole cohort (Cohort 1) that received adjuvant chemotherapy for breast cancer.



Coordinates of the Curve

Test Result Variable(s): NR4A1Cytoplasmic Expression

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
5.0000	1.000	1.000
→ 6.1500	.682	.610
6.5000	.678	.610
6.8500	.668	.610
7.1500	.103	.153
7.5000	.093	.136
7.8500	.093	.119
9.0000	.000	.000



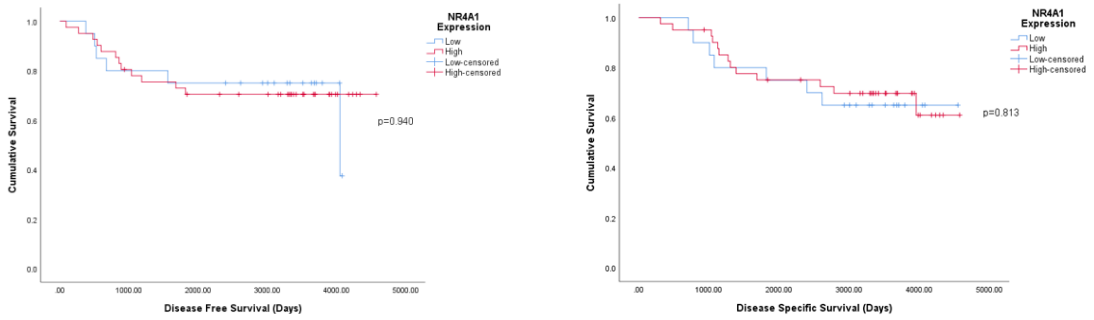
Coordinates of the Curve

Test Result Variable(s): **NR4A1Nucleus Expression**

Positive if Greater Than or Equal To[a]	Sensitivity	1 - Specificity
-1.0000	1.000	1.000
→ .6500	.686	.383
1.4000	.686	.378
1.6000	.667	.378
1.8500	.647	.378
2.1500	.608	.360
2.6500	.588	.360
3.1500	.569	.329
3.4000	.569	.320
3.7500	.549	.320
4.1500	.471	.261

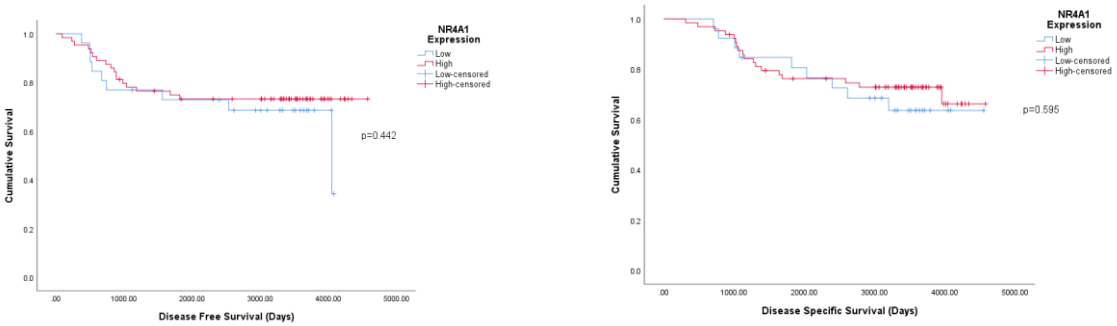
Appendix 9.8. Kaplan-Meier plots, comparing mean disease-free survival and disease-specific survival between low and high cytoplasmic NR4A1 expression in Triple Negative, ER-Negative and ER-Positive subtypes of breast cancers

A. **B.**



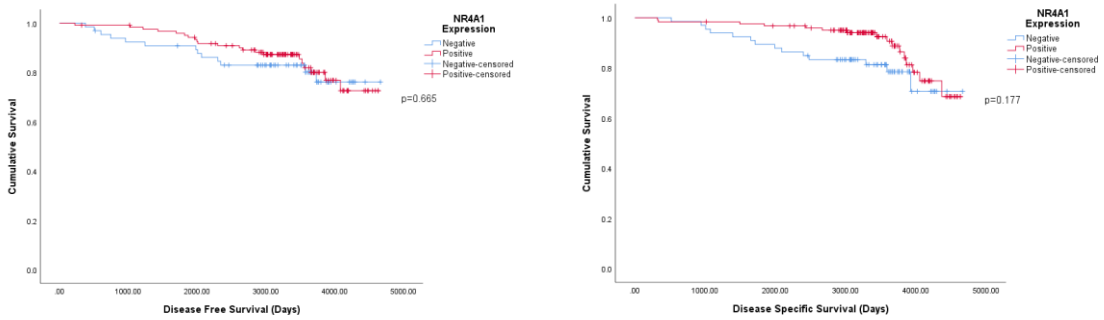
Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low cytoplasmic NR4A1 expression. Cytoplasmic NR4A1 expression was determined in a cohort of 68 Triple Negative primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low cytoplasmic NR4A1 expression. Cytoplasmic NR4A1 expression was determined in a cohort of 98 ER-Negative primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

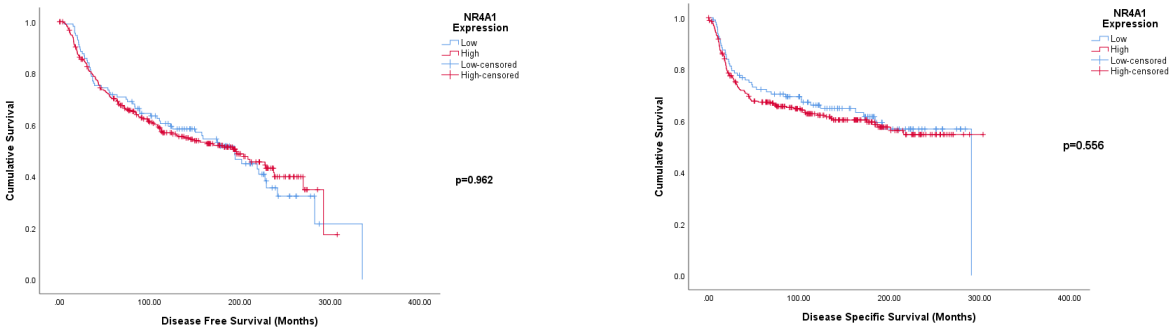
A. **B.**



Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low cytoplasmic NR4A1 expression. Cytoplasmic NR4A1 expression was determined in a cohort of 207 ER-Positive primary breast cancer patient treated with adjuvant chemotherapy. The cohort was dichotomised into high or low cytoplasmic NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

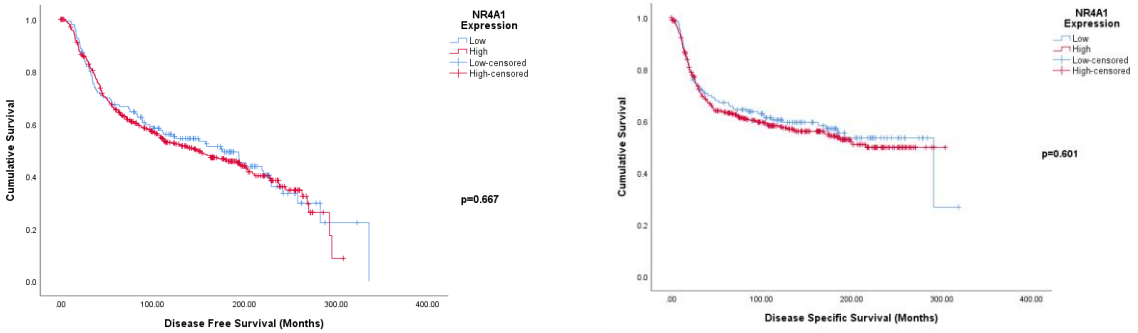
Appendix 9.9. Kaplan-Meier plots, comparing mean disease-free survival and disease-specific survival between low and high NR4A1 expression within the METABRIC dataset for all breast cancer subtypes, Triple Negative, ER-Negative, ER-Positive and HER2-Positive

A. **B.**



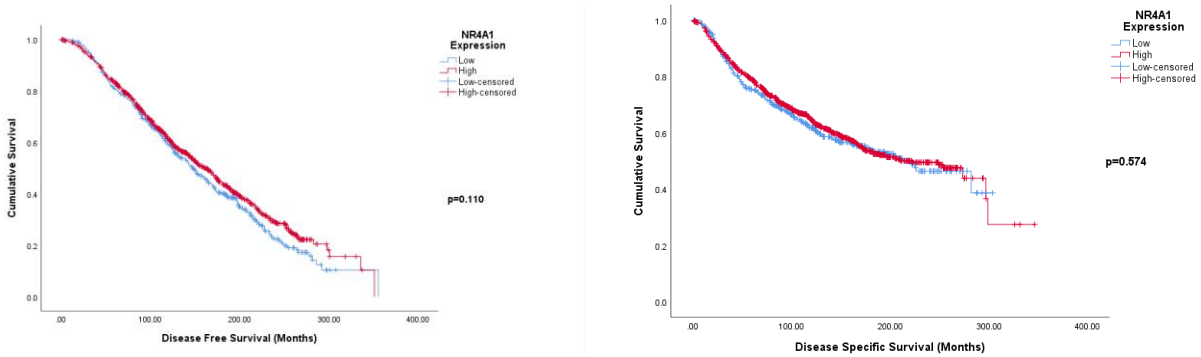
Kaplan-Meier survival analysis for Triple Negative breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset. NR4A1 expression was determined in 393 patients with Triple Negative primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



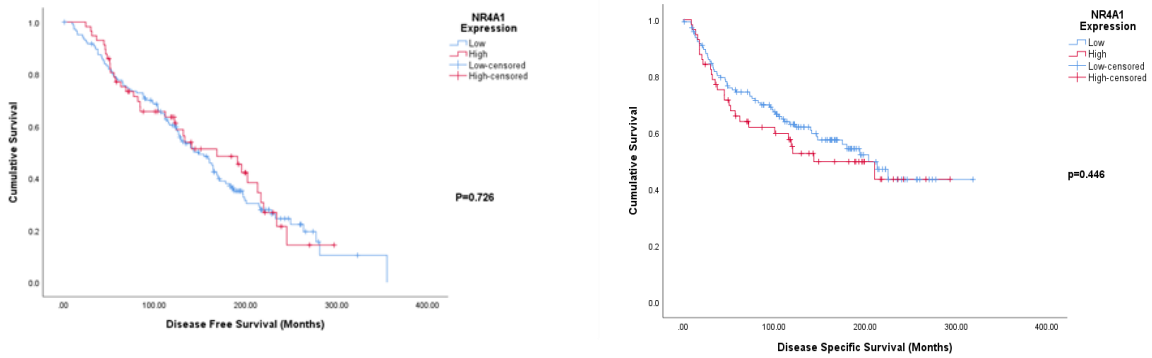
Kaplan-Meier survival analysis for ER-Negative breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset. NR4A1 expression was determined in 614 ER-Negative patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis for ER-Positive breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset. NR4A1 expression was determined in 1272 ER-Positive patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

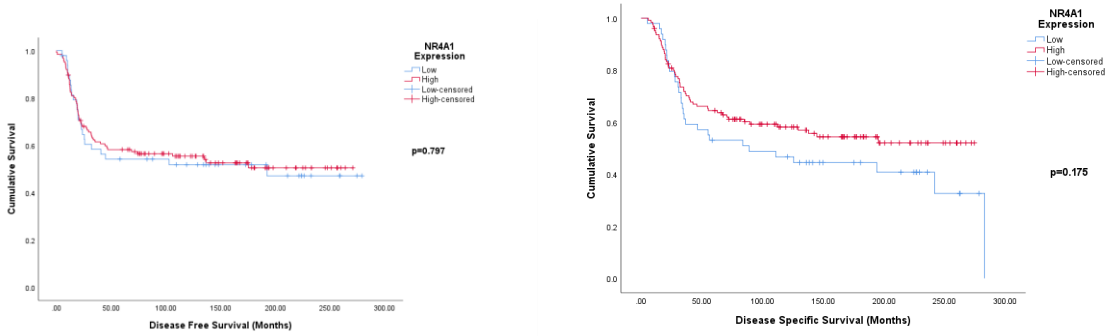
A. **B.**



Kaplan-Meier survival analysis for HER2-Positive breast cancer outcomes in groups with high versus low NR4A1 expression within the METABRIC dataset. NR4A1 expression was determined in 221 HER2-Positive patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease- specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

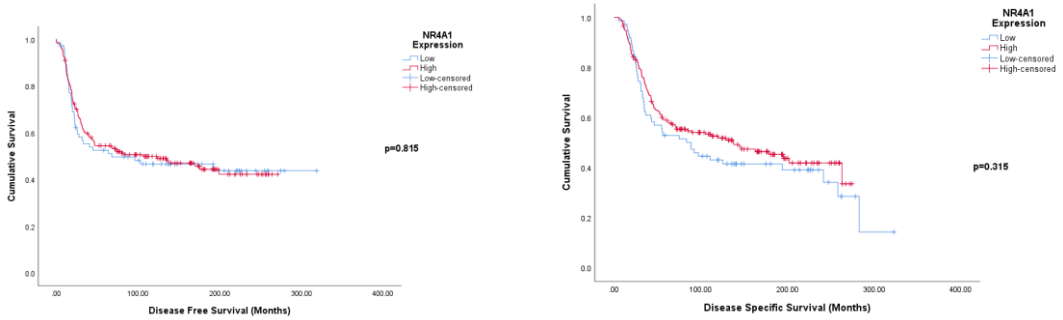
Appendix 9.10. Kaplan-Meier plots, comparing mean disease-free survival and disease-specific survival between low and high NR4A1 expression in patients that received chemotherapy within the METABRIC dataset for each breast cancer subtype, Triple Negative, ER-Negative, ER-Positive and HER2-Positive

A. **B.**



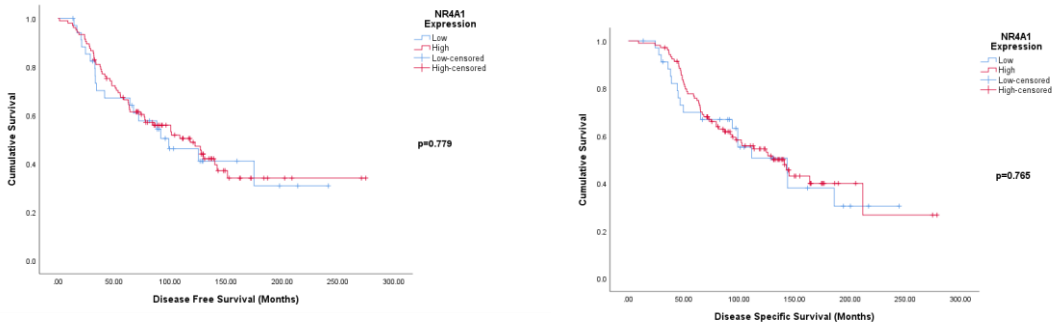
Kaplan-Meier survival analysis, for Triple Negative breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset. NR4A1 expression was determined in 175 Triple Negative patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



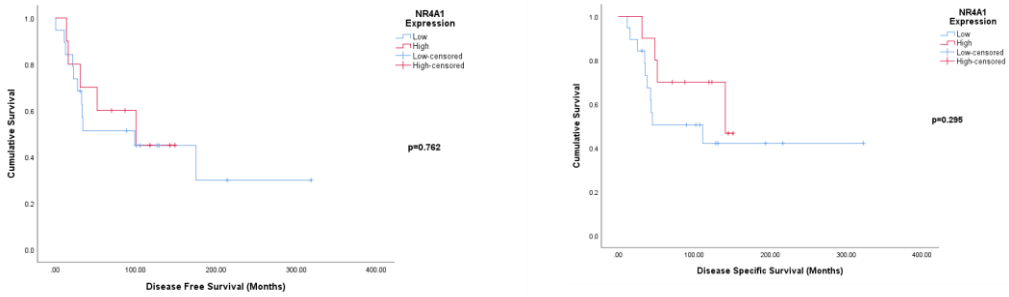
Kaplan-Meier survival analysis, for ER-Negative breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset. NR4A1 expression was determined in 253 ER-Negative patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis, for ER-Positive breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset. NR4A1 expression was determined in 140 ER-Positive patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient

A. **B.**



Kaplan-Meier survival analysis, for HER2-Positive breast cancer outcomes in groups with high versus low NR4A1 expression in patients that received chemotherapy within the METABRIC dataset. NR4A1 expression was determined in 29 HER2-Positive patients with primary breast cancer. The dataset was dichotomised into high or low NR4A1 expression groups using ROC analyses. Graph A shows disease-free survival and graph B shows disease-specific survival. End of follow-up (censor points) is indicated by the small coloured vertical lines for each patient