Interferon-dependant chemoresistance in breast cancer

Louis Francis Vaughan McLoughlin

Submitted in accordance with the requirements for the degree of Master's by Research

The University of Leeds

School of Medicine and Health

December 2022

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

I would firstly like to thank my supervisors Dr Thomas Hughes and Dr Milene Volpato who have both been exceedingly helpful and supportive throughout my degree and without whom this research would not have been possible. I would also like to thank the members of the Hughes lab including Ritika Rampal and Arindam Pramanik who have also been very supportive and generous with their time. In addition, I would like to thank many of the WTBB level 9 researchers including Ailsa Rose and Thomas Maisey for their guidance and support in cell culture and immunohistochemistry.

Abstract

Chemoresistance is a major obstacle in the treatment of primary and metastatic breast cancer (BC). Identifying chemoresistance mechanisms utilised by cancer cells is therefore an important step towards sensitising tumours to chemotherapy and improving treatment outcomes. The Hughes group has recently identified an IFN-dependent resistance pathway in claudin-low triple negative breast cancer (TNBC).

In this thesis, the relevance of IFN-dependent epirubicin resistance was assessed in multiple BC cell lines representing claudin-low TNBC (MDA-MB-231), HER2-enriched (AU565), luminal A (MCF-7) and luminal B (BT-474) BC through MTT and colony forming assays. The ability of IFN to induce docetaxel resistance was also assessed. The effect of IFN on epirubicin-induced DNA double stranded break formation was investigated in MDA-MB-231 through a γ-H2Ax immunofluorescence assay. In addition, the clinical relevance of IFN signalling was assessed in a cohort of 27 metastatic TNBC patients through immunohistochemical evaluation of IFNβ1 and MX1 expression in cancer and stromal cells.

Viability assays showed IFNα1 induced a dose-dependent epirubicin resistance in MDA-MB-231 but did not induce resistance in AU565, MCF-7 or BT-474 cells. Moreover, IFN did not induce resistance to docetaxel in any cell lines tested. In MDA-MB-231 cells, the addition of IFNα1 significantly reduced epirubicin-induced expression of γ-H2Ax. Analysis of the cohort of metastatic TNBC patients showed a significant correlation between IFNβ1 expression in lymphocytes and MX1 expression in cancer cells, however this was not associated with survival.

In conclusion, this thesis has shown IFN-dependent resistance to be subtype and chemotherapy dependant in BC. Moreover, this thesis has identified paracrine IFN signalling within the TNBC metastatic tumour microenvironment. Further study into the inhibition of this pathway may lead to chemosensitising treatments and improved patient outcomes in claudin-low TNBC. Additionally, investigation of the clinical relevance of this pathway in metastatic BC should be performed using a larger cohort.

Abbreviations

ABC	ATP-Binding Cassette
BC	Breast Cancer
Bcl	B-cell lymphoma
BCS	Breast Conserving Surgery
BRCA 1/2	Breast Cancer Gene 1/2
CAF	Cancer Associated Fibroblast
CDK	Cyclin-Dependant Kinase
Cxcl10	C-X-C motif chemokine ligand 10
CYP	Cytochrome P450
DAMP	Damage Associated Molecular Pattern
DCIS	Ductal Carcinoma in situ
DFS	Disease Free Survival
DMEM	Dulbecco's Modified Eagle Medium
DSB	Double Stranded Break
ER	Estrogen Receptor
FCS	Foetal Calf Serum
GAS	IFN-γ-activated site
HER2	human epidermal growth factor receptor 2
IDC	Invasive Ductal Carcinoma
IFN	Interferon
IFNAR	Interferon alpha receptor
IL	Interleukin

IRDS IFN-related DNA-damage resistance signature

IRF IFN-Related Factor

ISGF3 Interferon Stimulated Gene Factor

ISRE Interferon Stimulated Response Element

JAK Janus Kinase

LN Lymph Node

MAPK Mitogen-Activated Protein Kinase

MDA5 Melanoma Differentiation-Associated Protein 5

mTOR Mammalian Target of Rapamycin

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MX1 MX Dynamin Like GTPase 1

NACT Neoadjuvant Chemotherapy

NF-κB Nuclear Factor-kappa B

NGS Nottingham Grading System

NST No Special Type

OS Overall Survival

PAMP Pathogen Associated Molecular Pattern

PBS Phosphate Buffered Saline

PFS Progression Free Survival

P-gp P-Glycoprotein

PI3K Phosphoinositol 3-Kinase

PMS Post Metastasis Survival

PR Progesterone Receptor

qPCR Quantitative Polymer Chain Reaction

RIG-I retinoic acid-inducible gene I

ROS Reactive Oxygen Species

RPMI Roswell Park Memorial Institute

STAT signal transducer and activator of transcription

TAM Tumour Associated Macrophage

TBS Tris-Buffered Saline

TGFβ Transforming Growth Factor β

TIL Tumour infiltrating Lymphocyte

TIN Tumour Infiltrating Neutrophil

TLR Toll-Like Receptor

TME Tumour Microenvironment

TNBC Triple Negative Breast Cancer

TNM Tumour Node Metastasis

T-reg Regulatory T-Lymphocyte

TYK2 Tyrosine Kinase 2

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Breast cancer

1.1.1 Breast cancer incidence and mortality

Breast cancer (BC) is the most common cancer worldwide, accounting for 11.7 % of incidence and is the leading cause of cancer-related deaths in women [1]. Overall and disease-free survival have increased in breast cancer in the last few decades, largely due to increased awareness and implementation of screening strategies allowing early diagnosis, as well as the development of new and more effective targeted treatments. However, targeted treatments are not applicable in all cases and even when they are, innate and acquired resistance can limit their efficacy. As a result, treatment for many patients still involves more traditional systemic cytotoxic chemotherapies. Despite the range of treatments available, relapse and metastases are still major issues in breast cancer management [1-3]. The difficulty in breast cancer management is particularly exacerbated by the diverse nature of the disease meaning that individual patients can respond differently to treatments giving varying prognoses [4].

1.1.2 Breast cancer subtypes

Breast cancer is commonly categorised by histological and morphological assessment. In addition, due to the molecular heterogeneity of breast cancer, it is typically subdivided into groups by distinct prognostic and/or predictive markers that direct treatment strategies for patients [5, 6].

1.1.2.1 Breast cancer histological classification, grading and staging

Breast carcinomas are the most common form of breast cancer, accounting for 95 % of cases. The other 5 % largely consists of lymphomas and sarcomas, which have distinct prognosis and treatment from carcinomas and are therefore not discussed further in this thesis [7]. Carcinomas can be classified based on their histological appearance as being ductal or lobular as well as in situ or invasive. In situ carcinomas refer to those that have not penetrated the basement membrane of the duct or lobule epithelium from which they originate [8]. Most in situ carcinomas are considered ductal carcinoma in situ (DCIS) [9]. DCIS are considered precursor lesions, which if untreated, may lead to the development of invasive carcinoma [8]. However, improvements in BC screening strategies have resulted in an increase in early diagnosis of DCIS and subsequent treatment by surgery supplemented with radiotherapy [10]. As a result, prognosis for DCIS is very favourable, although there are concerns relating to

potential over-treatment of DCIS in some cases, as DCIS may not always progress to invasive breast carcinoma in a patient's lifetime [11]. This is currently being investigated in the on-going LORIS trial, in which the efficacy of close monitoring as compared to surgical intervention are being assessed in the context of DCIS [12].

Invasive carcinomas are defined by the invasion of cancer cells from the ducts or lobules into the stroma. Invasive ductal carcinoma (IDC) is the most common accounting for 55 % of all BC incidence [8]. IDC are very heterogenous and can be categorised into many different subtypes based on their morphological features, however most are defined as "no special type" (NST), in which no distinct morphological characteristics can be identified [6]. The prognosis of IDC varies greatly depending on grade, stage and metastasis as well as immunohistochemical profile (see below). As a result, the treatment of these carcinomas is dependent on evaluation of these factors [13].

Breast carcinomas are graded to determine the degree of differentiation, most typically through the Nottingham Grading System (NGS), a semiquantitative method which assesses several cellular and structural characteristics of breast cancer cells/tissue relative to normal breast tissue. These include: the formation of tubule structures, nuclear pleomorphism within cells and the mitotic frequency, each of which is given a classification from one to three. These grades are then combined to produce an overall grade between 3 and 9 (3-5 = Grade I, well differentiated; 6-7 = Grade II, moderately differentiated; 8-9 = Grade III, poorly differentiated) [14]. Histological grading has been shown to be of prognostic value irrespective of other factors within IDC in multiple studies [4]. A long-term follow-up study of 2,219 cases of operable breast cancer showed a strong association between both survival and disease-free survival with histological grade, indicating its prognostic value [15]. As a result, grading often helps to inform treatment decisions, such as whether patients are likely to benefit from adjuvant systemic therapies [4]. However, it has been suggested that inconsistencies can be found in grading both between and within laboratories, for example a major study identified a significant disagreement in the number of grade 1, 2 and 3 diagnoses in a retrospective analysis of patient results from 49 Dutch pathology laboratories. These methodological inconsistencies potentially had an impact on treatment implementation as tumour grade informed treatment in 29.9 % of cases studied [16, 17].

As well as grading, tumour staging is also used to assess prognosis and inform treatment, most often through the Tumour Node Metastasis (TNM) system. In the TNM system, patients are assessed for tumour size, presence of BC in the lymph nodes (LN) and metastasis, all of which are indicators of prognosis [18]. Multiple studies have shown an inverse correlation between tumour size and survival in node-negative and node-positive BC patients, which persists in both short and long-term follow-up [19-21]. Patients with larger node-negative tumours are therefore considered for adjuvant systemic chemotherapy [22]. Lymph node status is also used in assessing treatment options and is considered one the strongest indicators of prognosis [22, 23]. It is therefore used to assess the need of adjuvant therapy to attempt to eliminate micro-metastasis post-surgery [24].

The presence of metastasis is also considered in the classification of breast cancer. Metastasis is a complex multistep process in which tumour cells differentiate allowing them to escape the primary tumour and disseminate to distant organs resulting in the growth of secondary tumours [25]. BC metastasis most commonly occurs in the bone, lung, liver and brain, and frequency of metastasis at specific sites is influenced by the molecular subtype [26]. The presence of metastasis is associated with very poor prognosis in BC as metastatic tumours respond poorly to treatment. This resistance is in part due to characteristics acquired by metastatic cells, including increased drug efflux transporter expression, increased DNA-damage repair, differential expression of β-tubulin isotypes and altered expression of apoptotic signalling molecules [27, 28]. Moreover, metastatic breast cancer has high genetic diversity and complexity that further complicates treatment [29]. As a result, treatment is palliative, not curative, and metastasis is the main cause of BC related death [3, 27]. Metastases at first diagnosis is rare in the UK, probably due to relatively early diagnosis of primary disease resulting from the NHS breast cancer screening programme and to women carrying out self-examinations; therefore, metastases tend to be seen as recurrences from a previous primary cancer.

1.1.2.2 Breast cancer molecular subtypes

In recent decades, molecular biomarkers, confirmed through microarray expression profiling, have also been identified for use in the treatment of breast cancer [30]. An early study to use microarray expression profiling identified groups of distinct hierarchical clustering of gene expression in breast cancer samples from 42 patients, and thereby determined 4 subtypes:

luminal-like, HER2-enriched, normal-like and basal-like [31]. A follow-up paper subdivided the luminal-like subtype into both luminal A and B, showing distinct clinical outcomes for each subtype [32]. These subtypes and their clinical relevance were subsequently confirmed using 3 independent data sets of breast tumour gene expression [33]. Moreover, these subtypes were further validated using a much larger sample size (n = 501 in total) [34].

Four distinct breast cancer subtypes are now typically recognised and used to inform patient treatments including: luminal A, luminal B, HER2-enriched and triple negative breast cancer (TNBC), which can be further categorised into claudin-low or claudin-high TNBC [30]. Clinically, these subtypes could be identified by microarray assessments of the expression of many genes, but more typically this is achieved by immunohistochemical assessment of a much more limited selection of molecules [35]. Microarray subtyping is considered more accurate than immunohistochemistry and clinical trials have shown this to have better prognostic value [36, 37]. Moreover, inconsistencies in the application of immunohistochemistry between labs can cause variation in results and therefore patient treatment [38]. However, microarray technology is not readily available or affordable and therefore immunohistochemical evaluation is typically used [37]. Using immunohistochemistry, subtypes are identified by evaluating expression of oestrogen receptor alpha (ERα), progesterone receptor (PR), Human Epidermal growth factor receptor-2 (HER2) and Ki-67 (Table 1.1) [39].

Table 1.1. Expression of biomarkers within the different breast cancer subtypes

Molecular Subtype	Receptor Status			
	ER	PR	HER2	Ki67
Luminal A	+	+	-	Low
Luminal B	+	+	+/-	High
HER2-Enriched	-	-	+	Low/High
Triple Negative	-	-	-	Low/High

1.1.2.2a Luminal A

Luminal A breast cancer is the most frequently diagnosed subtype, comprising 40 % of all incidences, and is defined as ER-positive or PR-positive and HER2-negative with low Ki-67 expression [39]. The Luminal A subtype is characterised by relatively slow proliferation, as indicated by relatively low expression of the proliferative marker Ki-67, and consistently shows favourable clinical outcomes compared to other subtypes, as shown in multiple clinical trials [36, 40, 41]. A clinical trial of 1,951 node-negative breast cancer patients showed patients with Luminal A breast cancer to have significantly better 10-year disease-free survival (DFS) (86 %) and overall survival (OS) (89 %) when compared to all other subtypes [41]. Favourable outcomes are likely due to the slow proliferative nature of luminal A BCs, as well as the use of therapies targeting expression/activity of the hormone receptors that have improved efficacy over cytotoxic chemotherapies in this subtype. These therapies include tamoxifen and the aromatase inhibitors. However, patients with large luminal A tumours may still benefit from systemic chemotherapy [42].

1.1.2.2b Luminal B

This subtype is the second most prevalent, comprising around 15-20 % of breast cancer incidence. Luminal B tumours express the hormone receptors ER and/or PR but typically have lower PR expression and higher ki-67 expression as compared to luminal A tumours [43]. These tumours are more aggressive than luminal A tumours and are often of a higher grade with greater risk of recurrence, resulting in poorer prognosis in this subtype [44]. Due to the greater instances of recurrence, identification of this subtype can inform length of endocrine therapy to reduce this risk. Moreover, luminal B tumours respond more favourably to systemic chemotherapy than luminal A, making identification of the subtype crucial for informing treatment strategies. ER-positive breast cancers that also over-express HER2 are usually classified as luminal B, and typically have higher proliferation rates as indicated by high Ki-67 expression; patients with the carcinomas can also benefit from HER2-targeting therapies (see section 1.1.2.2c below) [43].

1.1.2.2c HER2-enriched

HER2-enriched BC accounts for 15-20 % of BC incidence and is characterised by low/no ER or PR hormone receptor expression with over-expression of the human epidermal growth receptor, HER2 [43]. This subtype is associated with aggressive tumour progression resulting

in worse prognosis than luminal BCs [41]. Additionally, recurrence and metastasis occur in around 20 % of patients treated with adjuvant targeted and systemic therapies [45]. Moreover, metastasis to the brain is more common in HER2-enriched BC which presents difficulties in treatment due to inability of many therapies to cross the blood brain barrier [46]. Evaluation of HER2 expression is important for determining treatment strategy, as targeted therapies such as trastuzumab, which specifically target cells that display HER2 overexpression, have been shown to significantly improve survival in this subtype [47]. However, it should be noted resistance to trastuzumab still remains a critical issue in treatment and systemic therapies are often still necessary [48].

1.1.2.2d Triple-negative breast cancer

TNBC consists of 10-20 % of incidence and is defined as having no hormone receptor overexpression and no HER2 amplification [49]. This subtype is considered the most aggressive and is often of a high grade with poor differentiation [50]. As a result, TNBC has the worst overall survival and disease-free survival as well as an earlier onset of recurrence and metastasis [51]. Moreover, this subtype does not respond to current targeted therapies, due to the absence of hormone receptors and HER2 overexpression and is therefore treated with aggressive systemic chemotherapy [52].

Treatment difficulties are furthered by the heterogeneity of this subtype [52]. TNBC can be further separated into claudin-high and claudin-low TNBC based on differential expression of claudins and other cell-cell adhesion proteins [53]. The less common claudin-low TNBC has been shown to express epithelial-to-mesenchymal transition and immune response gene markers as well as having cancer stem cell-like features [54]. Claudin-low tumours are therefore more aggressive with a greater rate of metastasis and chemotherapy resistance [55].

1.2 Breast cancer treatment

Breast cancer treatment requires a multidisciplinary approach and is often personalised based on stage, grade and molecular subtype of the tumour. Treatment can consist of surgery, radiotherapy, chemotherapy, targeted therapies, and immunotherapies. The order of systemic treatment is also dependant on the characteristics of the tumour and therapies may be given pre- (neoadjuvant) or post- (adjuvant) surgery [56].

1.2.1 Surgery

Surgery is used in the treatment of most non-metastatic breast cancers to remove cancerous tissue. This can be done via mastectomy, often followed by reconstructive surgery, or by breast conserving surgery (BCS). BCS focuses on removing the tumour with only a small margin of normal tissue to allow conservation of the breast. This is followed by radiotherapy to reduce risk of local recurrence [57]. Improvements in BCS in recent years have led to comparable overall survival, disease free survival and risk of recurrence to that of mastectomy, resulting in this being the favoured approach for patients and physicians [57, 58]. However, mastectomies are still considered an important part of BC treatment and patients have input on the decision between these two treatment options [59].

1.2.2 Radiotherapy

Radiotherapy is the application of high-energy radiation to tumours, resulting in DNA damage, cell cycle arrest and programmed cell death in cancerous cells. This strategy effects tumour cells more potently than most healthy cells due to their rapidly dividing nature [60]. In breast cancer, radiotherapy is most often applied in the adjuvant setting to eliminate micro-metastasis and reduce the risk of local recurrence [61]. Multiple clinical trials have shown adjuvant radiotherapy to significantly improve DFS, translating into greater OS [62, 63]. Use of radiotherapy in a neoadjuvant context is less common and research into its efficacy is largely lacking [64].

1.2.3 Chemotherapy

Systemic chemotherapies can be used in both neo-adjuvant and adjuvant treatment, as well in the inoperable and metastatic settings. The decision to treat with chemotherapy is often dependant on grade, stage, and molecular subtype [65]. Moreover, due to the potential of innate and acquired resistance to chemotherapeutics, they are often given as a combination of multiple active agents and/or combined with targeted therapies to improve response [66].

Neo-adjuvant chemotherapy (NACT) is typically used when treating currently inoperable or large locally advanced BC to reduce tumour size, making them operable or better candidates for BCS [67]. NACT also allows clinical response to therapy to be monitored through longitudinal evaluation of tumour size during therapy, and post-surgery assessment of residual tumour burden by histopathology; pathological complete response has been shown to be a good indicator of prognosis in this context [68]. Recent retrospective studies have also

shown a comparable OS rate in those treated with neo-adjuvant and adjuvant chemotherapy in the context of early breast cancer, although local recurrence was higher in the NACT treated group [69]. Pathological complete response varies between molecular subtypes with luminal BC (particularly luminal A) surprisingly showing worse response than HER2-enriched and TNBC , despite luminal A having the better overall prognosis [67, 70].

Most commonly, chemotherapy is used post-surgery to eliminate micro-metastasis and prevent recurrence [65]. Cytotoxic chemotherapies have been well established to reduce recurrence and improve 10- and 15-year survival rates through multiple clinical trials and retrospective studies [71]. Adjuvant therapies often combine chemotherapeutics with targeted therapies or other cytotoxic agents to reduce the effects of therapy resistance. Chemotherapy-based combination treatments commonly consist of anthracyclines and taxanes which are most active in the treatment of early and advanced breast cancer, however other chemotherapeutics can also be used including platinum-based drugs and alkylating agents [65].

Cytotoxic chemotherapy is also utilised in the treatment of metastatic breast cancer; however, the aim of this treatment is to extend and improve qualitive of life and is not curative [72]. Chemotherapeutic regimes have been well established in improving time to progression in metastatic breast cancer particularly with the incorporation of anthracyclines and taxanes [73, 74]. Both mono- and poly-chemotherapeutic treatments are effective, although poly-chemotherapy treatments have been associated with more frequent side effects [75].

Although multiple chemotherapeutics are used in the treatment of breast cancer, only anthracyclines and taxanes will be discussed further as they are the subjects of this thesis.

1.2.3.1 Anthracyclines

Anthracyclines, including doxorubicin and epirubicin, are a family of widely used chemotherapeutic agents consisting of an amino sugar and anthraquinone aglycone [76]. These drugs are thought to have multiple mechanisms of action resulting in DNA damage, oxidative stress, and subsequent apoptosis [77]. Anthracyclines have been shown to form DNA adducts resulting in disruption of replication and transcription. Moreover, DNA-intercalated anthracyclines form stable ternary structures with topoisomerase II, inhibiting

the enzyme's activity [77]. Topoisomerase II is responsible for regulating supercoiled DNA during replication, transcription, and recombination through cleavage, unwinding and subsequent recombination of DNA [78]. Binding of anthracyclines stabilises topoisomerase II in its DNA cleavage conformation preventing reformation of phosphodiester bonds resulting in permanent double-stranded breaks [76]. Anthracyclines have also been shown to increase the production of reactive oxygen species (ROS), which subsequently induce protein alkylation and DNA damage, further contributing to pro-apoptotic signalling [76].

Anthracyclines are commonly used in both the adjuvant and neo-adjuvant treatment of breast cancer [79]. Neo-adjuvant anthracycline-based regimens have been shown to induce pathological complete response in 20.5 % of operable or locally advanced breast cancer patients, increasing to 57 % in those under 40 [80]. In the adjuvant setting, anthracyclines significantly reduce mortality and recurrence over a 10-year period, with higher-dose anthracycline regimes inferring greater benefits when compared to cyclophosphamide, methotrexate, and fluorouracil polychemotherapy regimes [81].

Despite this, anthracyclines are associated with both short- and long-term toxicities. Most notably, anthracyclines can induce severe cardiotoxicity, through the generation of ROS and inhibition of topoisomerase II in cardiomyocytes, which can lead to cardiac disfunction and heart failure [82]. This results in dosage limitations and often prevents the use of secondary anthracycline-based treatment regimes in the metastatic context, as the cardiotoxic effects are cumulative [72, 83].

1.2.3.2 Taxanes

Taxanes, including paclitaxel and docetaxel, are used in the treatment of many cancers including ovarian, prostate, and small-cell lung cancer, and are considered one of the most active chemotherapeutics against breast cancer, alongside anthracyclines [84]. Taxanes are microtubule stabilising agents which act through the binding of the β-tubulin subunit preventing microtubule depolymerisation [85]. This inhibits many processes which rely on the dynamic nature of microtubule structures including cell signalling and migration [84]. However, their cytotoxicity is usually associated with the inhibition of mitosis, in which microtubules are essential in the formation of mitotic spindles. This leads to G2/M cell cycle arrest and subsequent apoptosis [86].

In breast cancer, taxanes have been shown to improve both OS and DFS in the adjuvant treatment of early breast cancer. Additionally, taxanes are used in the treatment of metastatic breast cancer, either as a second line therapy when anthracycline based regimes fail or when recurrence occurs within 6 months of adjuvant treatment [87, 88]. Neoadjuvant paclitaxel treatment was shown to significantly improve pathological complete response rates relative to cyclophosphamide, doxorubicin and 5-fluorouracil treatment regimes in locally advanced breast cancer [89]. Like anthracyclines, taxanes are subject to multiple toxicities including pulmonary- and neuro-toxicity [90, 91]. A study of 4,554 women with operable breast cancer showed neuropathy as a side effect in 13 and 15 % of patients treated with docetaxel weekly or every three weeks, respectively [92]. The high frequency of these toxicities and their impact on patient quality of life have resulted in dosage limitations in the use of taxanes [92].

1.3 Chemoresistance

As well as toxicities, chemotherapeutics are also subject to chemoresistance, in which cancer cells fail to respond to cytotoxic therapy. This presents a major obstacle in the treatment of breast cancer, as approximately one third of breast cancer patients develop local recurrence or distant metastasis caused by therapy resistance [93]. Resistance can be both intrinsic, in which cancer cells are inherently resistant to chemotherapy before exposure to treatment; or acquired, in which cancer cells develop a resistance phenotype during treatment [94]. In both cases, chemoresistance is incredibly diverse and may result from increased drug efflux, DNA damage resistance, drug metabolism and modulation of apoptotic signalling molecules. Moreover, chemoresistance may be induced through a range of cellular mechanisms including, mutation, epigenetic regulation, microRNA expression, tumour heterogeneity and tumour microenvironment involvement [95].

1.3.1 Drug efflux

Enhanced cellular drug efflux is one of the most common forms of drug resistance and results in the decrease in intracellular drug concentration through the active transport of chemotherapeutics out of the cells [96]. This mechanism is typically facilitated by a group of transmembrane ATP-binding cassette (ABC) transporters which bind and efflux multiple drugs including anthracyclines and taxanes resulting in multidrug resistance. Of these transporters P-glycoprotein (P-gp) is the most studied in breast cancer and its high expression is associated

with chemoresistance in all BC subtypes [97]. Induced paclitaxel resistance in the TNBC cell line HCC1806 resulted from increased expression of P-gp as well as cross-resistance to doxorubicin. The resistant cell line also displayed a decrease in intracellular drug concentration relative to the parent cell line during treatment confirming that P-gp activity was responsible for increased resistance [98]. Additionally, meta-analysis of thirty-one studies found P-gp expression to be associated with a three-fold higher risk of treatment failure [99].

1.3.2 Drug inactivation

Cancer cells may also resist treatment through increased drug metabolism, resulting in less active drug and therefore reduced anti-cancer activity. Drug metabolism is largely mediated by members of the cytochrome P450 (CYP) superfamily [100]. For example, CYP1B1 is responsible for the metabolism of multiple chemotherapeutics including doxorubicin, paclitaxel, docetaxel, cisplatin, and 5-fluorouracil [101]. Breast cancer patients with a CYP1B1 4326G allele have been found to have reduced PFS and OS when treated with paclitaxel [102]. Additionally, several other drug metabolising CYPs have been associated with chemotherapy efficacy in breast cancer including CYP1A2, CYP2A6, CYP2B [100].

1.3.3 Apoptosis resistance

Most chemotherapeutics used in breast cancer treatment induce cellular damage resulting in the activation of intrinsic apoptosis through increased apoptotic signalling. Whether intrinsic apoptosis is initiated is dependent on the ratio of pro- and anti-apoptotic proteins known as the Bcl-2 family members. A high ratio of pro- to anti-apoptotic proteins results in mitochondrial membrane polarisation, cytochrome C release and subsequent activation of caspases 9 and 3 inducing cell death. In cancerous cells, anti-apoptotic Bcl-2 family members are often over-expressed resulting in resistance to pro-apoptotic signalling induced by chemotherapy [103]. Overexpression of anti-apoptotic proteins Bcl-2 or Bcl-xL in MCF7 breast cancer cells induced a significant increase in resistance to cisplatin treatment [104]. Furthermore, overexpression of microRNA let-7i in cisplatin resistant MCF7 cells, resulted in a decrease in Bcl-2 expression and increase in chemosensitivity [105]. In the clinical setting, high expression of Bcl-2 was significantly associated with worse BC-specific and disease-free survival in TNBC patients treated with adjuvant chemotherapy, as well as worse pathological complete response rates in those treated with neo-adjuvant therapy [106].

Mutations in the transcription factor p53 have also shown to induce resistance to apoptosis in breast cancer treatment. The TP53 gene is one of the most frequently mutated in breast cancer and is responsible for inducing cell cycle arrest, DNA damage repair and senescence. Mutations in this gene can result in aberrant expression and downregulation of the pro-apoptotic protein Bax [107]. These mutations have been shown to cause chemotherapy resistance and induction of senescence in breast cancer cells, and breast cancer patients with TP53 mutations have significantly worse OS during chemotherapy treatment [108].

1.3.4 DNA-damage resistance

Many cancer treatments, including radiotherapy, platinum-based agents, anthracyclines and alkylating agents function through inducing DNA-damage. DNA-damage may be induced through several mechanisms including directly, through formation of DNA adducts, single-stranded breaks and double-stranded breaks, or indirectly through the production of ROS and inhibition of DNA-influencing enzymes such as topoisomerase II [77]. Typically, DNA-damage results in the induction of senescence and DNA-damage repair mechanisms which attempt to restore genomic integrity. Failure of these mechanisms to repair DNA damage will ultimately result in apoptosis [109]. Due to the importance of DNA-damage induction to the efficacy of many cancer treatments, modulation of DNA-damage repair mechanisms is often seen in resistant cancers [110].

The role of DNA damage repair in therapy resistance has largely been investigated in the setting of radiotherapy resistance [110]. A population of MCF-7 cells identified as radiotherapy resistant were shown to have increased expression of proteins associated with single-stranded break repair as well as decreased ROS levels [111]. In the context of chemotherapy, increased expression of DNA damage repair proteins before treatment was associated with poor response to neo-adjuvant epirubicin-cyclophosphamide treatment in a cohort of 60 breast cancer patients [112].

Anthracyclines have been shown to induce cell death, in part, through inhibition of topoisomerase II resulting in double stranded breaks (see section 1.1.1.3). Reduced expression of topoisomerase IIα has been observed in breast cancer and is associated with decreased patient survival and lymph node metastasis [113]. Moreover, comparison between the sensitivity of MDA-MB-231 and MCF-7 to doxorubicin showed the more resistant MDA-

MB-231 cells to express topoisomerase II at lower levels. Lower levels of DNA double stranded breaks were also observed in this cell line after 24 h of doxorubicin treatment [114].

1.4 The tumour microenvironment of breast cancer

The complexity of breast cancer is further enhanced by the involvement of the tumour microenvironment (TME), which has a strong influence on tumour progression and response to treatment. The TME includes a number of non-malignant cell types including tumour-associated macrophages (TAM), tumour infiltrating lymphocytes (TIL) and cancer-associated fibroblasts (CAF) [115].

CAFs are typically the most abundant non-malignant cells of the TME and are responsible for the modulation of the extracellular matrix and secretion of growth factors and inflammatory molecules [115]. High density of CAFs in BC tumours has been associated with worse overall and disease-free survival through multiple studies and meta-analysis [116, 117]. CAFs have been found to contribute to both metastasis and chemoresistance through modulation of the extracellular matrix, cell-cell interactions and paracrine signalling [118]. Primary CAFs from resistant tumours have been shown to increase resistance to docetaxel in coculture with MCF7 and SK-BR3 cell lines through up-regulation of CD10 and GPR77 membrane proteins [119]. Additionally, CAF-dependant paracrine IFN signalling has been shown to induce chemoresistance in claudin-low TNBC cell lines MDA-MD-231 and MDA-MB-157 in co-culture models [120].

TILs are mononucleated immune cells which can be found in varying abundance in breast carcinomas [121]. High abundance of TILs is typically associated with good prognosis, particularly in HER2-enriched and TNBC [115]. This effect is in part due to improving the sensitivity of cancer cells to chemotherapy, which is used extensively in both subtypes, and high levels of TILs have been shown to correlate with improved pathological complete response rates in TNBC treatment [122]. This anti-tumour activity is most commonly associated with CD8+ and CD4+ T-lymphocytes, which can kill cancer cells and/or release pro-inflammatory cytokines leading to improved anti-cancer activity of innate immune cells [115].

Although TILs are broadly associated with positive chemotherapy response, other immune cells of the TME display chemoresistance-inducing properties [123]. TAMs are typically abundant in breast cancer tumours and have been associated with increased angiogenesis,

suppression of anti-cancer immunity and resistance to therapy [124]. Paracrine IL-6 signalling between TAMs and breast cancer cells has been shown to induce resistance to doxorubicin through upregulation of the detoxifying enzyme glutathione S-transferase P1 [125]. Secretion of IL-10 from TAMs has also been shown to induced paclitaxel resistance in BT-549 and T47D breast cancer cell lines [126].

The TME also plays a role in breast cancer metastasis both in the establishment and progression of metastatic tumours [127]. CAFs can be found in the metastatic TME and are either produced through activation of metastatic site stromal cells or from migration of CAFs from the primary tumour [128]. CAFs from the metastatic site have been shown to increase tumour growth and doxorubicin resistance with a greater activity than CAFs from the primary site in TNBC cell lines [129]. Moreover, these 'metastatic' CAFs have been shown to mediate hormone therapy resistance through the transfer of microRNA containing microvesicles [130]. Immune cells are also present in the metastatic TME, however enrichment of each cell type differs between metastatic sites [131]. TILs are present in the metastatic TME, although typically in lesser abundance [132, 133]. Currently, little is known about the prognostic relevance of TILs in the metastatic setting, however, regulatory T-lymphocyte abundance has been associated with poor post-recurrence survival in distant metastatic breast cancer [134].

1.5 The involvement of type-I IFN in cancer

1.5.1 Type-I interferon

IFNs are cytokines that play an important role in inflammation, the immune response to viruses, and modulation of both the innate and acquired immune system [135]. As well as this, IFN has been shown to have conflicting and context dependant roles in the progression of several malignancies, including breast cancer [136].

There are three types of IFNs, type I, II and III, with distinct structures, receptors, and physiological roles. There are 5 type-I IFN subtypes in humans (IFNα, IFNβ, IFNε, IFNκ, IFNω) that share structural homology and physiological function, although IFNα and IFNβ are the most widely expressed and clinically relevant [136]. Type-I IFNs can be expressed by most cell types but during the early stages of viral infection their expression is largely associated with dendritic cells [137]. These IFNs modulate the expression of a vast array of genes influencing many physiological processes including cell proliferation, antigen presentation and immune cell activation [138]. However, in terms of pathology, type-I IFNs have also been implicated in

the modulation of tumour cell behaviours and in the progression of autoimmune disease underlining that their influence on gene expression and cellular behaviour is context dependant [138].

1.5.1.1 Type-I IFN expression

IFN expression can be induced by a range of pattern recognition receptors (PRRs) including, Toll-like receptor (TLR) 3, 4, 8 and 9, RIG-I and MDA5. These receptors can be found on the cell surface or in endosomal compartments and are activated by pathogen or self-DNA/RNA fragments known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [139]. In the context of cancer, PRR activation occurs in cells of the TME due to the presence of tumour associated antigens such as ssDNA produced by cytotoxic chemotherapy and radiotherapy [140].

Activation of these receptors results in a range of intracellular signalling pathways, which ultimately result in the activation of interferon-related factor 3 (IRF3) and NF-κB. Activation of these two transcription factors results in their nuclear translocation and subsequent increased expression of IFNα and IFNβ. Moreover, this pathway also induces the transcription of IRF7 which contributes to further expression of type-I IFNs in a positive feedback loop [141].

1.5.1.2 Type-I IFN-induced signaling pathways

IFN-induced signalling has been shown to occur through multiple signalling pathways resulting in the altered expression of hundreds of genes [135]. The canonical signalling pathway, first identified in the 1990s, occurs through the janus activated kinase (JAK) and signal transducer and activator of transcription (STAT) families (Figure 1.1) [135, 142]. Once secreted type-I IFN binds its cell membrane receptor consisting of two transmembrane subunits IFNAR1 and IFNAR2 which are associated with the tyrosine kinases, tyrosine kinase 2 (TYK2) and JAK1, respectively. Upon ligation, these subunits form a heterodimer and the resulting conformational change indues the autophosphorylation and activation of TYK2 and JAK1 [143]. These kinases recruit and phosphorylate STAT1 and 2, which bind IRF9 to form the transcription factor complex, IFN-stimulated gene factor 3 (ISGF3). Once formed, ISGF3 translocates to the nucleus where it binds IFN-stimulated response elements (ISRE) in the promoter region of IFN stimulated genes (ISG), resulting in the activation of transcription [143].

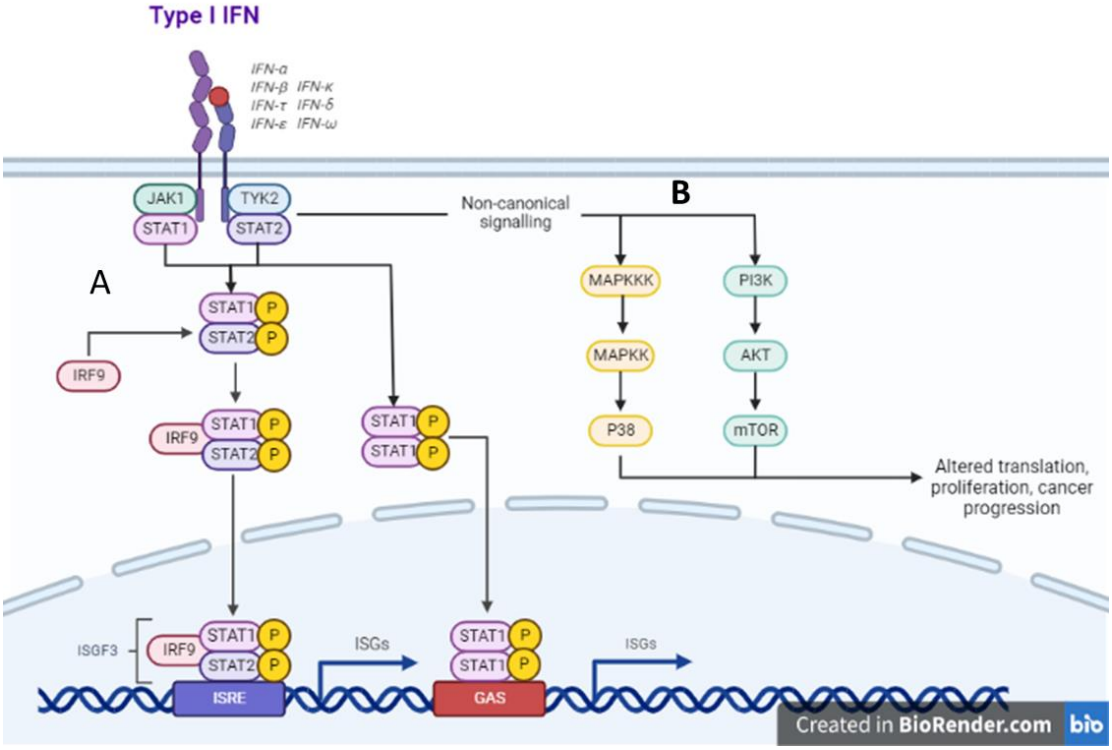

Figure 1.1. A Diagram showing both the (A) canonical and (B) non-canonical intracellular signalling pathways induced by type-I IFNs (created with BioRender.com).

Type-I IFNs are also responsible for the phosphorylation of other members of the STAT family including STAT 3-6 which form alternative transcription factors with affinity for different gene promotors. Moreover, STATs may form various hetero- and homodimers such as phosphorylated-STAT1 (pSTAT1) homodimers, which bind to IFN-γ-activated site (GAS) in the promotors of ISGs [144]. ISG promoters can contain ISRE and/or GAS elements and therefore, differential expression, modulation, phosphorylation, and regulation of STAT family members can alter the expression profile of IFN signalling [145, 146].

As well as activation of STAT-dependant ISG expression, IFN also signals through non-canonical pathways such as MAPK and PI3K/mTOR pathways, adding to the complexity of IFN-induced biological response [147]. IFN-dependant activation of the p38 MAP kinase pathway has been shown to influence the translation of ISGs as well as directly effecting their expression [135, 148]. This pathway has been shown to act independently of STAT phosphorylation and ISRE binding and has been implicated in the growth inhibitory effects of type-I IFN both in healthy and malignant cells [149-151].

IFN has also been shown to activate the PI3K/mTOR pathway, which effects biological processes including translation, proliferation, and metabolism [147]. IFN-induced activation of this pathway was shown to have a critical role in the translation of ISG proteins from their mRNAs [152]. Additionally, this non-canonical pathway has been implicated in the epithelial to mesenchymal transition in several cancer cell lines highlighting its potential importance in malignancy [153].

1.5.2 The roles of type-I IFN in cancer

The involvement of IFN in cancer has been well researched in recent years, however, many studies have found contradictory roles in both cancer progression and inhibition likely due to the complex and context-dependent pleiotropisms of IFN signalling [154].

The anti-tumour effects of IFN on cancer were first characterised in 1969, where high dose IFN treatment was shown to improve the survival of tumour inoculated mice [155]. Subsequent clinical trials showed improved survival in IFNα treated patients with multiple haematological malignancies and solid tumours leading to the broad use of IFNα as a cytostatic therapy [141]. However, continuous high-dose treatment regimens led to frequent toxicities leading to cessation of treatment in many patients. Moreover, later clinical trials showed reduced efficacy compared to newer therapeutic agents and IFN treatment in cancer has been discontinued [156].

Since the discovery of IFN's involvement in cancer, research has shown both pro- and anti-tumorigenic roles in multiple types of cancer. Furthermore, IFN has been shown to modulate a range of pathways relating to cancer progression including tumour growth, apoptosis, differentiation, epithelial to mesenchymal transition, migration, antigen presentation and immune cell interactions [154].

The influence of IFN on therapy response has been characterised in several cancers. Type-I IFN has been shown to increase sensitivity to chemotherapy through up-regulation of the chemokine Cxcl10 in sarcomas *in vivo* [157]. However, contrary to this, several studies have found an association between IFN signalling and resistance to radio and chemotherapy. This resistance has been largely associated with the increased expression of several ISGs known as the IFN-related DNA-damage resistance signature (IRDS) [158]. Selecting human squamous carcinoma cells for radiation resistance, resulted in a radioresistant cell line which

differentially expressed 49 genes associated with the IRDS. Inhibition of STAT1 restored sensitivity of this cell line *in vivo*. Moreover, this resistance extended to the DNA-damaging agent doxorubicin, and knockdown of STAT1 restored chemosensitivity in resistant cells. Up-regulation of STAT1 also induced doxorubicin resistance in the HER2-enriched BC cell line SK-BR-3 [159]. This was additionally confirmed in B16F1 cells, in which clones highly expressing ISGs showed increased radiotherapy and doxorubicin resistance coupled with increased activation of caspases 3/7 compared to those with low ISG expression [160]. Clinical analysis has shown increased expression of IRDS genes was predictive of increased risk of metastasis in a data set of 295 early breast cancer patients. This increased risk was greater in those treated with adjuvant chemotherapy, further suggesting its involvement in chemotherapy resistance [159]. Moreover, increased expression of MX1, a common marker of IFN signalling, was significantly associated with reduced overall survival and distant metastasis-free survival in a cohort of 845 BC patients. MX1 expression also differed between BC subtypes and was specifically associated with TNBC [161]. Similarly, increased expression of a 7 gene IRDS was significantly associated with reduced distant metastasis-free survival and local-regional relapse-free survival in breast cancer patients treated with adjuvant chemotherapy. This correlation did not persist in the cohort treated without adjuvant chemotherapy [159].

In breast cancer, the involvement of IFN paracrine signalling between CAFs and BC cells has also been implicated in cancer progression and resistance. Co-culture with CAFs increased the expression of ISGs as well as increasing proliferation and radioresistance in claudin-low TNBC *in vivo* [162]. Knockdown of STAT1 further eliminated the radio and chemoresistance effects of CAFs cocultured with MDA-MB-231 *in vitro*. Moreover, cell lines which showed no IRDS gene expression did not acquire chemoresistance in CAF cocultures suggesting this signature is crucial for CAF-dependant chemoresistance [162]. An additional study has also shown indirect co-culture of MCF-7 and primary CAFs induced proliferation in cancer cells, which was inhibited by an IFNβ neutralising antibody [163]. Although this study did not investigate the chemoresistance potential of this paracrine signalling, the role of type-I IFN in DNA-damage resistance is supported by an increase in autophagy in MCF-7 cells treated with IFNβ1. Autophagy has been shown to increase both DNA-damage resistance and chemoresistance and inhibition of autophagy in IFN-treated cells resulted in an increase in apoptosis, suggesting this process is involved in evading cell death [164].

A recent study by Broad *et al.* has further shown IFN-dependant resistance in breast cancer which is both CAF-dependant and subtype specific [120]. CAFs were shown to protect claudin-low (MDA-MB-231 and MDA-MB-157), but not claudin-high (MDA-MB-468), TNBC cell lines from epirubicin. This protection coincided with an increase in IFNβ1 and ISG expression in CAFs and cancer cells respectively, upon introduction of epirubicin. Furthermore, inhibition of the type-I IFN receptor significantly reduced chemoprotection from CAFs in cocultures, and recombinant IFNα alone increased proliferation and epirubicin resistance in claudin-low TNBC cell lines with no effect on claudin-high TNBC. When analysing the clinical relevance of this pathway in a cohort of 109 TNBC patients, CAF IFNβ1 expression was significantly correlated with MX1 expression in cancer cells ($r = 0.21$, $P = 0.028$). Additionally, both high IFNβ1 expression in CAFs and high MX1 expression in cancer cells were significantly negatively associated with survival in claudin-low TNBC, suggesting this paracrine mechanism is clinically relevant in this subtype [120].

Although subtype-specific IFN-dependent chemoresistance has been identified in breast cancer, it is still unclear which subtypes are affected by this resistance pathway. Moreover, investigations into this chemoresistance mechanism have largely been in primary BC and its clinical implications in metastatic breast cancer are still unclear. Further exploration of these areas may elucidate the clinical relevance of this pathway and could potentially lead to targeted treatments to improve chemotherapy response.

1.6 Hypothesis and aims

My hypothesis is that type-I IFN is capable of inducing chemoresistance in breast cancer in a subtype dependant manner. I also hypothesise that paracrine IFN-dependant resistance previously observed in primary breast cancers persists in the metastatic TNBC setting.

Specific aims:

1. To identify breast cancer subtypes that are subject to IFN-dependant chemoresistance through viability screening assays.
2. To investigate the relevance of type-I IFN signalling in the extent of DNA damage during chemotherapy treatment in breast cancer.

3. To use immunohistochemistry to evaluate the expression of IFNβ and MX1 in metastatic breast cancer tissues and make preliminary assessments of their potential clinical relevance through correlations with survival.

2 Methods

2.1 Reagents

Human IFNα1 (Sigma Aldrich, Missouri, USA) was rehydrated in 0.5 % (w/v) BSA solution at a concentration of 0.1 mg/ml before aliquoting into 4 µL aliquots and freezing at -80 °C. Upon thawing, IFN was diluted to a 0.01 mg/ml intermediate stock and used immediately. IFN aliquots were not used more than once to avoid freeze-thaw cycles. Epirubicin hydrochloride (Sigma Aldrich, Missouri, USA) was dissolved in ultra-pure water to a stock concentration of 1 mg/ml and stored at -20 °C. Docetaxel (Alfa Aesar, Massachusetts, USA) was dissolved in DMSO to a stock concentration of 0.1 mg/ml and stored at -20 °C.

2.2 Ethical approval and patient samples

Ethical permission for the use of breast cancer tissue samples in this project was granted by Leeds East Research Ethics Committee (reference: 06/A1206/180). Formalin-fixed paraffin-embedded tissue blocks were collected from the LTH pathology archive representing 31 breast cancer metastases from 31 patients, along with 13 matched primary breast cancer samples from 13 patients.

2.3 Haematoxylin and eosin staining

5 µm tissue sections were taken from formalin fixed paraffin embedded tissue using a microtome and were collected on X-tra adhesive glass microscope slides (Leica Biosystems, Wetzlar, Germany). Slides were then heated at 70 °C for one hour before dewaxing and dehydration with xylene (5 min in 3 fresh xylene solutions) and 100 % ethanol (5 min in 3 fresh ethanol washes), respectively. Slides were then stained with haematoxylin for 30 seconds before rinsing in tap water for 1 minute. Slides were then transferred to Scott's tap water for 1 min before an additional rinse in tap water for 1 minute. Next slides were stained with eosin for 1 minute before again rinsing in tap water. After staining, slides were dehydrated in ethanol (5 min in 3 fresh ethanol washes) and xylene (5 min in 3 fresh xylene washes) before mounting under coverslips using depex (SERVA, Oklahoma, USA).

2.4 Immunohistochemistry

2.4.1 Optimisation

Dilution of antibodies was initially optimised using primary breast tumour tissues, starting with dilutions optimised in a previous study [120]. Further confirmation of these dilutions was performed on sections of tissue microarrays containing breast tumour tissue. Staining was

then expertly assessed by Dr Eldo Verghese (Consultant Breast Histopathologist) before proceeding to perform IHC on the full cohort. Optimisation of IHC was performed as described in 2.4.2.

2.4.2 Immunohistochemistry

FFPE tissues were sectioned, dewaxed and hydrated as in section 2.3. They were then washed under running tap water before antigen retrieval by submersion in 10 mM citric acid buffer (pH 6.0) and heating for 10 min in a microwave (900W, high power). Slides were left in citric acid buffer to cool for 20 min before an additional wash under running tap water. Blocking was then performed by submersion in 1 % (v/v) hydrogen peroxide for 10 min. Slides were washed under running tap water and in Tris-buffered saline (TBS) before incubation in antibody diluent (Life Technologies, California, USA) for 10 min. Diluent was removed and antibodies were added to cover each sample at their optimal dilutions of 1:50 and 1:800 for MX1 (37849S, Cell Signal Technologies, Massachusetts, USA) and IFNβ (PA5-20390, Thermofisher Scientific, Massachusetts, USA), respectively. Negative controls were treated with diluent only at this stage. Slides were incubated in primary antibody or control at 4 °C overnight in a humidified chamber. Next, slides were washed twice in TBS-T (TBS with 0.1 % Tween-20) and once in TBS before application of 40 - 120 µL the anti-rabbit SignalStain Boost IHC Detection Reagent (Cell Signal Technologies, Massachusetts, USA) and incubation in a humidified chamber at room temperature for 30 min. IHC detection reagent was removed and slides were further washed in TBS-T and TBS. DAB substrate (Cell Signal Technologies, Massachusetts, USA) was made up to its working concentration following the manufacturer's instructions and 60 µL added to slides for 10 min (room temperature). After washing under running water, samples were counter-stained with haematoxylin for 1 min before emersion in Scott's tap water for 1 minute. This was followed by an additional wash in running water followed by a wash in Scott's water and further running water. Once counter-stained, slides were mounted with cover slips using depex (SERVA, Oklahoma, USA).

2.4.3 Scoring protocol

Scoring protocols for the expression of IFNβ1 and MX1 were developed in discussion with Dr Eldo Verghese (Consultant Breast Histopathologist). In cancer cells, scoring of IFNβ1 was based on both intensity of staining (a scale of 1-3, where 1 = weak, 2 = intermediate and 3 = strong staining) and proportion of cells stained (1 = < 50 %, 2 = 51-75 % and 3 = 75-100 %).

These scores were then combined to produce a final expression score between 2 and 6. For MX1 expression of cancer cells, scoring was also based on intensity (0-3) and proportion of cells stained (0 = 0 %, 1 = 1-15 %, 2 = 16-30 % and 3 = > 30 %). Scores were then combined to produce a final expression score between 0 and 6. The expression of IFNβ1 and MX1 in CAFs and lymphocytes was scored independently for each cell type based on the proportion of stained cells within the tumour (0 = 0 %, 1 = < 50 % and 2 = > 50 %).

2.5 Cell culture

Breast cancer cell lines were acquired from ATCC (ATCC, Virginia, USA). MDA-MB-231 and BT-474 and MCF-7s where grown in Dulbecco's Modified Eagle Media (DMEM) (Thermofisher Scientific, Massachusetts, USA) and AU565 were grown in Roswell Park Memorial Institute (RMPI) media (ATCC, Virginia, USA). All media was completed with 10 % (v/v) FCS and 0.1 % (v/v) penicillin-streptomycin before use. All cells were incubated at 37 °C in humidified air / 5 % CO2; incubation for tissue culture will refer to these conditions unless otherwise specified.

2.6 MTT assays

Breast cancer cell lines were seeded in 96-well plates in complete media and incubated for 24 h. Cells were pre-treated with human IFNα1 or a media control before further incubation for 24 h. Cells were then treated with chemotherapeutics or media control for 24 h. Treatments were removed and fresh media added before further incubation for 72 h. A 5 mg/ml MTT stock (dissolved in PBS) was diluted 1:10 in complete media. Media was removed from wells and 100 µL of MTT (0.5 mg/ml) was added before incubation for 3 hr. Media/MTT was removed from wells and replaced with 100 µL of DMSO to dissolve formazan crystals. Absorbance was read using the Mithras LB 940 plate reader Berthold, Baden-Württemberg, Germany) at 620 nm.

2.7 Colony forming assays

Breast cancer cell lines were seeded in 6-well plates and incubated for 24 h to allow adherence. Cells were then pre-treated with IFN or a media control and incubated for a further 24 h. Cells were then treated with chemotherapy or a media control for an additional 24 h in the continued presence of IFN or control. Each well was washed in PBS, and cells were trypsinised and reseeded in 10 cm dishes (300-750 cells per plate) before incubation for 14-21 days in incubators with minimal disturbance. Fixing and staining of cells was performed

using 50 % methanol/ 20 % ethanol (crystal violet 0.5 %). Colonies were then counted manually, aiming to consider a group of ~40 cells as a colony.

2.8 qPCR

RNA extraction was performed using the ReliaPrep™ RNA Miniprep System (Promega, Wisconsin, USA), following the manufacturer's protocol. RNA was then quantified using the Nanodrop 8000 (Thermofisher Scientific, Massachusetts, USA). Reverse transcription was then performed using 1 µg of RNA per sample using the GoScript™ Reverse Transcriptase kit (Promega, Wisconsin, USA) as per the manufacture's protocol. Negative controls for each RNA sample were also generated by preparing parallel reactions lacking reverse transcriptase, and these controls were taken forward for qPCR. qPCR was performed using the Luna® Universal qPCR SYBR green Master Mix (New England Biolabs, Massachusetts, USA) and QuantStudioTM 3 Real Time PCR machine (Thermofisher Scientific, Massachusetts, USA) with the SYBR setting. Primers used during qPCR were as follows; MX1 (Hs.PT.58.40261042), ACTB (Hs.PT.39a.22214847) and were provided by Integrated DNA Technologies. qPCR was also performed on negative controls, containing ultra-pure water in place of cDNA.

2.8 γ-H2Ax immunofluorescence assay

Breast cancer cell lines were seeded on glass cover slips in 6-well plates (200,000 cells seeded) before incubation for 24 h to allow cells to adhere. Cells were then pre-treated with IFNα1 or a vehicle control and incubated for a further 24 h. Cells were treated with chemotherapy and IFNα1 or a vehicle control, in the continued presence of IFN or control, before incubation for 2 h. Cover slips were then washed in PBS and cells were fixed in 4 % formaldehyde at room temperature for 10 min. Cover slips were washed in PBS and then incubated on ice in 0.2 % Triton X-100 solution (10 min) to allow permeabilisation. Cells were further washed with PBS and then incubated in 5 % FBS blocking solution for 1 h on ice. After washing in washing buffer (0.5 % FBS, 0.05 % Tween-20), cover slips were transferred to a humidified chamber and treated with γ-H2Ax primary antibody at a dilution of 1:800 (#2577, Cell Signal Technologies, Massachusetts, USA) and incubated at 4 °C overnight. Coverslips were then washed in wash buffer before treatment with Anti-rabbit IgG, F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) (#4412, Cell Signal Technologies, Massachusetts, USA) at a dilution of 1:1000 and incubated for 1 h in a dark humid environment. Once incubated, coverslips were further washed with wash buffer before mounting on superfrost plus slides (Epredia, Michigan, USA) in mounting

media consisting of 50 % ultrapure water, 50 % glycerol and 10 µg/ml Hoechst 33342 (Thermofisher Scientific, Massachusetts, USA). Coverslips were then sealed with nail varnish. Images were taken using a Nikon A1R confocal laser scanning microscope (Nikon Instruments Inc., New York, USA). ImageJ was then used to quantify foci in individual cell nuclei.

3. IFN-induced chemoresistance is not a consistent feature of breast cancers, across cell lines representing different subtypes

3.1 ABSTRACT

Resistance to chemotherapy is a major obstacle in the treatment of breast cancer and one third of patients develop resistance, often resulting in local recurrence or metastasis. Identification of the resistance pathways utilised by breast cancer is therefore necessary to improve chemotherapy response and patient survival. Recent work within the Hughes group has identified an IFN-dependant resistance pathway in claudin-low TNBC which can be inhibited to improve response to chemotherapy. However, the relevance of this pathway in other breast cancer subtypes has not yet been explored.

The effects of IFNα1 on the anticancer activity of epirubicin was assessed in 4 breast cancer cell lines representing luminal A (MCF-7), luminal B (BT-474), HER2-enriched (AU565) and claudin-low TNBC (MDA-MB-231) through colony forming and MTT viability assays. The ability of IFNα1 to induce resistance to docetaxel was also assessed in these cell lines. The effects of IFNα1 on epirubicin-induced DNA DSB formation was also assessed in the claudin-low TNBC cell line MDA-MB-231 using a γ-H2Ax immunofluorescence assay. IFNα1 induced epirubicin resistance in a dose-dependent manner in MDA-MB-231 cells in viability assays but did not induced resistance to epirubicin in AU565, MCF-7 or BT-474. Docetaxel resistance was also not induced by IFNα1 in any breast cancer cell lines tested. The introduction of IFNα1 to epirubicin treatment in MDA-MB-231 resulted in a decrease in epirubicin-induced DNA DSB formation. This chapter has shown type-I IFNs induce breast cancer resistance to chemotherapy in a subtype and chemotherapeutic dependent manner *in vitro*, likely through the reduction of chemotherapeutic-induced DSB formation.

3.2 Introduction

Type-I IFNs have been shown to induce anthracycline resistance in TNBC cells through a paracrine CAF-dependant resistance mechanism, which could be induced through the addition of IFNα1 alone. This resistance was shown to occur in claudin-low but not claudin-high TNBC suggesting it may be subtype specific [120]. Although the role of IFN-dependant resistance has been established in breast cancer, the specific breast cancer subtypes effected by this mechanism have still not been explored.

Cytotoxic chemotherapy is used in the treatment of all breast cancer subtypes, particularly in locally advanced disease, and resistance to chemotherapy is a major cause of recurrence and metastasis [66]. The considerable heterogeneity of breast cancer means subtypes respond differently to therapy and may induce chemoresistance through different pathways [165]. Moreover, chemotherapeutics, such as anthracyclines and taxanes commonly used in the treatment of breast cancer, function via distinct mechanisms and are affected by different resistance pathways [166, 167]. Identifying which chemotherapeutics and BC subtypes are affected by chemoresistance pathways may provide opportunity for additional therapies to combat chemoresistance and reduce the frequency of treatment failure.

In this chapter, I therefore aimed to identify which breast cancer subtypes may be susceptible to IFN-dependant resistance using four breast cancer cell lines representing luminal A, luminal B, HER2-enriched and claudin-low TNBC. In addition, I investigated the effects of IFN on the efficacy of two different chemotherapeutics, representing the anthracyclines and taxanes.

3.3 Results

3.3.1. Breast cancer subtypes show differential responses to IFNα1 in combination with chemotherapy treatment in short-term viability/growth assays

To assess whether IFN-dependant chemoresistance occurs in breast cancer subtypes other than the claudin-low triple negative subtype previously reported, cancer cell lines were selected as representatives of different subtypes and were screened for IFN-dependant chemoresistance using a short-term viability/growth assay. The breast cancer cell lines selected were AU565, BT-474 and MCF7 that are representative of HER-enriched, luminal B and luminal A breast cancer respectively [168]. MDA-MB-231 cells, representing claudin-low TNBC were also assessed as a positive control, since IFN-dependant chemoresistance has been seen in these cells previously. Claudin-high TNBC was not examined as the resistance pathway has been shown to be absent in this subtype in the previously study [120].

An MTT assay was designed for use to assess IFN-induced chemoresistance, including effects both at the levels of cytotoxicity and growth inhibition. In order to take account of any growth inhibition effects correctly, it was first important to ensure each cell line was in the log or near log phase of growth during treatment and subsequent recovery. Growth kinetics experiments were therefore performed for each cell line to determine the duration of log phase and establish an appropriate seeding density for MTTs (Figure 3.1). Time points for MCF-7 growth kinetics are limited to days 1, 4, 7 and 10.

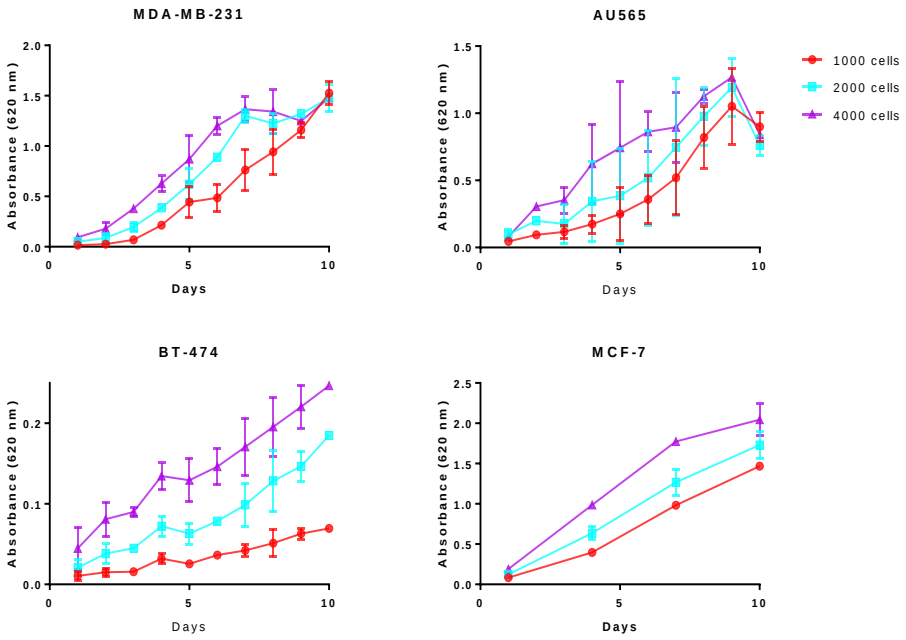

Figure 3.1. Breast cancer cell line growth curves in 96 well plates. Cells were seeded at 1000, 2000 and 4000 cells per well and growth was assessed through MTT assay. Data represents the mean ± SD of two biological repeats.

MDA-MB-231, AU565 and BT-474 breast cancer cell lines showed little to no lag phase at least one, typically the highest number of cells, while the lowest seeding density resulted in a notable lag phase of up to 3 days. Lag phase could not be assessed in MCF-7 cells as data was not available at days 1-3.

Although all cell lines showed a time dependant increase in absorbance, implying growth, this trend was not logarithmic in some cell lines, at some seeding densities, and exponential curves fit some data poorly, indicated by low R^2 values (Table 3.1). This prevented the accurate calculation of doubling times. Although exponential growth is expected during log phase theoretically under conditions where space and resources are unlimited for the cells, non-exponential growth during cell culture is not uncommon and continuous linear growth seen was deemed acceptable for the subsequent experiments. Each cell line showed distinct growth kinetics. MDA-MB-231 cells showed consistent growth until day 10 when seeded at 1000 cells per well, but plateaued at day 7 when seeded at both higher densities. Alternatively, AU565 continued to grow at all seeding densities until day 9 whereas MCF-7 cells grew continually up until day 10. BT-474 showed much slower, continuous, growth at all seeding densities (note that the y-axis scale for the BT-474 plot in Figure 3.1 is substantially different from the other plots). These findings were used to define the maximum time period for cell culture after treatment with IFN/chemotherapy (not to exceed 7 days) and appropriate seeding densities for the cells (1000 cells per well for both MDA-MB-231 and AU565; 4000 cells per well for BT-474 cells).

Table 3.1. Doubling times and R squared values of exponential curves for the growth of breast cancer cell lines in 96 well plates at different seeding densities.

	Seeding Density (cells per well)					
	1000		2000		4000	
	Doubling Time (Days)	R Squared	Doubling Time (Days)	R Squared	Doubling Time (Days)	R Squared
MDA-MB-231	2.332	0.941	3.329	0.851	4.315	0.773
AU565	2.687	0.801	3.831	0.574	5.817	0.535
BT-474	3.687	0.889	3.484	0.904	4.866	0.871
MCF-7	3.319	0.951	3.838	0.910	4.600	0.800

Next, it was necessary to define appropriate concentration ranges of chemotherapy agents for each cell line. Previous published experiments had focused on epirubicin; I intended to

use this, and also separately to examine responses to docetaxel (a representative taxane). To allow relative assessment of the effects of IFN on chemoresistance, an ideal range of chemotherapy concentrations would show between 20 - 80 % growth/survival inhibition compared with untreated controls, allowing scale for IFN to either increase or decrease this influence.

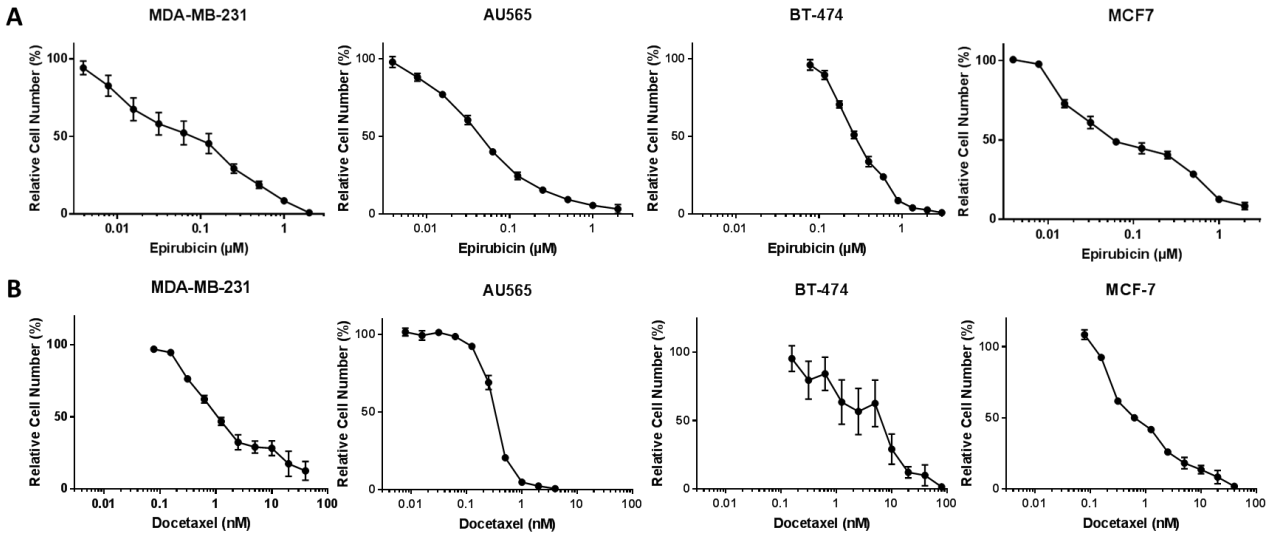

Figure 3.2. Dose response curves of breast cancer cell lines treated with (A) epirubicin or (B) docetaxel. Cells were treated with chemotherapy for 24 h before further incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance was then normalised to that of vehicle controls. Data represents the mean ± SE of 3 biological repeats.

As expected, both epirubicin and docetaxel induced dose-dependent growth/survival inhibition in all breast cancer cell lines (Figure 3.2). When treated with epirubicin, AU565 and MCF7 showed similar sensitivity with IC50s of 37.0 and 39.2 nM, respectively. MDA-MB-231 showed slightly higher innate resistance (IC50 = 86.9 nM) but produced a full dose-response curve within the same concentration range. BT-474 however, displayed the greatest resistance to epirubicin with an IC50 of 164.2 nM. Docetaxel showed dose-dependent growth inhibition at a much lower concentration range than epirubicin in all cell lines, demonstrating that it has a more potent chemotherapeutic effect in this assay. Relative sensitivity to docetaxel determined through the IC50 values was however similar to that of epirubicin, with

BT-474 being the most resistant followed by MDA-MB-231, MCF-7 and AU565, respectively (IC50s = 3.82 ± 0.94 nM, 1.49 ± 0.19 nM, 0.84 ± 0.09 nM, 0.32 ± 0.07 nM, respectively).

After cell growth conditions and chemotherapy dose ranges had been defined, the next step was to assess chemotherapy dose-response curves in the presence of increasing concentrations of IFNα1 (0, 250 and 500 U/ml). Concentrations of IFNα1 to be used were determined based on those found in the literature [120, 169, 170].

3.3.1a MDA-MB-231 cells: a positive control for IFN-induced chemoresistance

To assess whether the MTT assay to be used was sensitive enough to detect IFN-dependent resistance, the assay was first performed using our positive control cell line (MDA-MB-231) and epirubicin chemotherapy. It should be noted that IFN-dependant chemoresistance was not reported using a relatively short-term survival assay in the previously published work (which focused on colony forming assays), therefore this validation of the assay was particularly important. Cells were pre-treated with IFN α1 for 24 h before receiving additional treatment with the appropriate concentrations of chemotherapy.

Initially, MDA-MB-231 showed no significant difference in response to epirubicin in the presence of IFNα1 at either concentration tested (Figure 3.3). As a result, the experiment was repeated with a higher concentration of IFN α1 which also showed no difference in response relative to epirubicin alone (Figure 3.4).

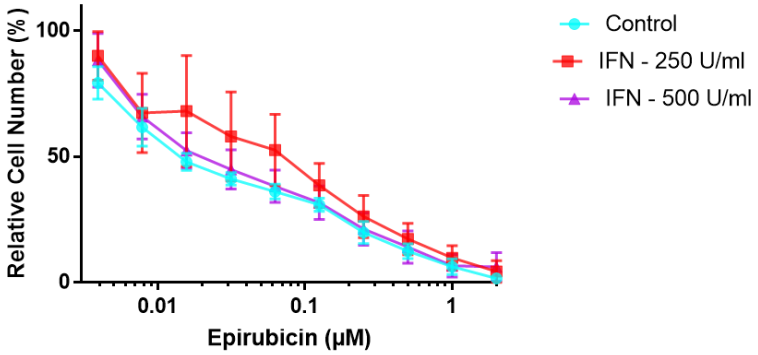

Figure 3.3. Dose response curves of MDA-MB-231 cells treated with epirubicin and IFNα1 at concentrations of 0 U/ml (Blue line), 250 U/ml (red line) and 500 U/ml (purple line). Cells were cultured with IFNα1 or vehicle control for 24 h before treatment with chemotherapy and IFNα1 for a further 24 h. cells were then incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance from each dose response was then normalised to vehicle controls treated with the same concentration of IFNα1. Data represents the mean ± SE of 3 biological repeats.

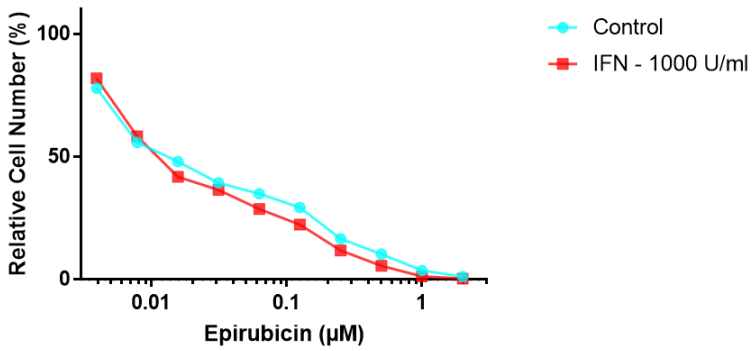

Figure 3.4. Dose response curves of MDA-MB-231 cells treated with epirubicin and IFNα1 at concentrations of 0 U/ml (Blue line), 1000 U/ml (red line). Cells were cultured with IFNα1 or vehicle control for 24 h before treatment with chemotherapy and IFNα1 for a further 24 h. cells were then incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance from each dose response was then normalised to vehicle controls treated with the same concentration of IFNα1. Data represents 8 technical repeats.

As the positive control cell line showed no observable IFN-dependant epirubicin resistance in MTT assays, I next assessed whether the IFNα1 I had used was able to induce signalling in these cells effectively or was potentially inactive, perhaps through degradation. This was assessed through measurement of the expression of MX1, an ISG which has been shown to increase in expression as a result of IFN signalling in multiple studies and therefore serves as a good indicator of IFN function [120, 171].

Cells were treated under two different conditions before MX1 expression analysis by qPCR; (1) high-dose IFN treatment (1000 U/ml) for 24 h; (2) lower-dose IFN treatment (250 U/ml) using a dose schedule and cell seeding density exactly as for the MTT chemoresistance assays in Figure 3.3. High-dose IFNα1 treatment resulted in a significant 5538 (± 4169)-fold ($P = 0.05$) increase in MX1 expression relative to untreated controls (Figure 3.5). Additionally, at the lower dose, MX1 expression was only observed in IFNα1 treated cells. These results demonstrate that the IFN stock was functional and that the MDA-MB-231 cells were capable of receiving and responding to the signal.

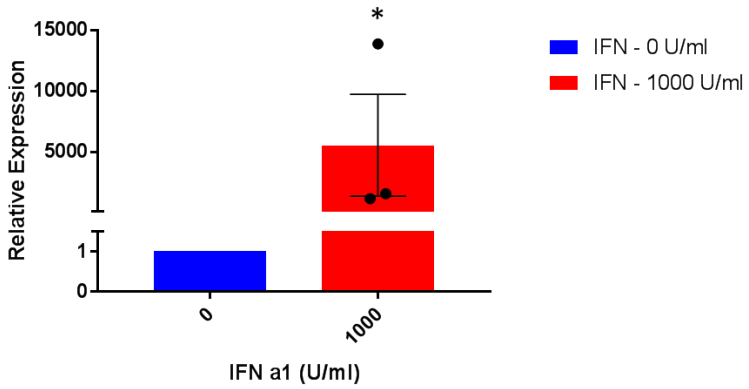

Figure 3.5. Relative expression of MX1 in MDA-MB-231 cells treated with IFNα1 or vehicle control. Cells were seeded and treated with IFNα1 or vehicle control for 24 h. RNA was then extracted and MX1 expression was quantified by qPCR. MX1 expression was normalised to the expression of ACTB and data is shown as MX1 expression relative to vehicle control. Data represents the mean ± SE of 3 biological repeats and significance was determined using the Mann-Whitney U test (*p = 0.05).

Next, I evaluated whether I was unable to observe IFN-induced chemoresistance because of genetic drift of my cells from the MDA-MB-231 genotype and behaviour. Cells were analysed through short-tandem repeat (STR) profiling for the genotype of 16 hypervariable genetic loci which were compared with the genotype of MDA-MB-231 reference samples. STR analysis showed an 88 % similarity between my MDA-MB-231 stock and reference samples, confirming that they originate from same source material but have developed some slight genetic drift. Accordingly, replacement, low-passage, MDA-MB-231 cells were bought, and the assay to detect IFN-induced chemoresistance was repeated.

IFNα1 induced no change in epirubicin dose-response at a dose of 250 U/ml as compared to the non-IFN treated control cells (Figure 3.6). However, at both 500 and 1000 U/ml, IFNα1 induced an increase in resistance to epirubicin. Non-linear regression analysis showed that when treated with 0 or 250 U/ml IFN, MDA-MB-231 cells show a biphasic dose-response to epirubicin. This implies epirubicin is functioning through two distinct anti-cancer mechanisms in this cell line and therefore produce two IC50 values ($IC50_1$ = 11.5 ± 1.0 nM or 9.3 ± 2.5 nM; $IC50_2$ = 496.7 ± 104.3 nM or 377.7 ± 92.1 nM for 0 or 250 U/ml IFN treated groups respectively). When treated at the two higher doses of IFNα1 (500 and 1000 U/ml) biphasic non-linear regression curves could not accurately fit the epirubicin dose-response data which

instead fit more accurately in non-biphasic non-linear regression models. This suggests that IFN may eliminate the biphasic nature of epirubicin dose-response in MDA-MB-231 cells. To further investigate this, the epirubicin concentrations required to induce an equivalent decrease in relative cell number to $IC50_1$ and $IC50_2$ of non-IFN treated cells was compared between treatment groups. The statistical significance of these differences were determined by Mann Witney U tests. IFNα1 induced a significant 5.6-fold and 9.7-fold increase in $IC50_1$ at concentrations of 500 and 1000 U/ml, respectively ($p = 0.05$) ($IC50_1 = 72.7 \pm 22.3$nM and 155.5 ± 85.1 nM, respectively). However, at the equivalent of $IC50_2$, epirubicin concentrations were similar to those of non-IFN treated controls in 500 and 1000 U/ml treated cells (563.0 ± 239.0 and 436.8 ± 169.4 nM, respectively). This suggests IFN induces resistance to the initial mechanism of epirubicin anti-cancer activity in a concentration dependant manner but has less or no effect on the mechanism induced at higher concentrations. Furthermore, this clear IFN-dependant resistance response in the new MDA-MB-231 stock, coupled with the previously published IFN-dependant resistance in this cell line, suggests the lack of IFN response in the initial MDA-MB-231 cells may have been a result of phenotypic drift.

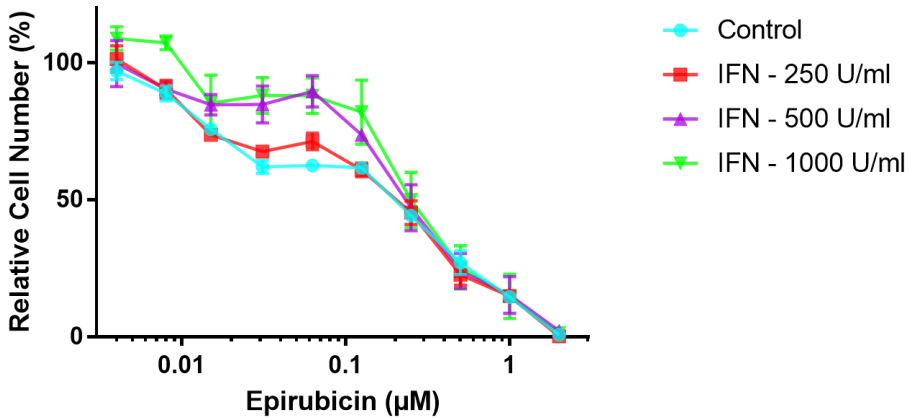

Figure 3.6. Dose response curves of MDA-MB-231 cells treated with epirubicin and IFNα1 at concentrations of 0 U/ml (Blue line), 250 U/ml (red line), 500 U/ml (purple line) and 1000 U/ml (green line). Cells were cultured with IFNα1 or vehicle control for 24 h before treatment with chemotherapy and IFNα1 for a further 24 h. cells were then incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance from each dose response was then normalised to vehicle controls treated with the same concentration of IFNα1. Data represents the mean ± SE of 3 biological repeats.

3.3.1b AU565, BT-474 and MCF-7 breast cancer cell lines show differential IFN-dependent chemoresistance to epirubicin in short-term viability/growth assays

After confirming IFN-dependant epirubicin resistance can be detected through short-term viability/growth assays in the positive control cell line (MDA-MB-231), assays were repeated at all concentrations of IFNα1 with each other breast cancer cell line (Figure 3.7).

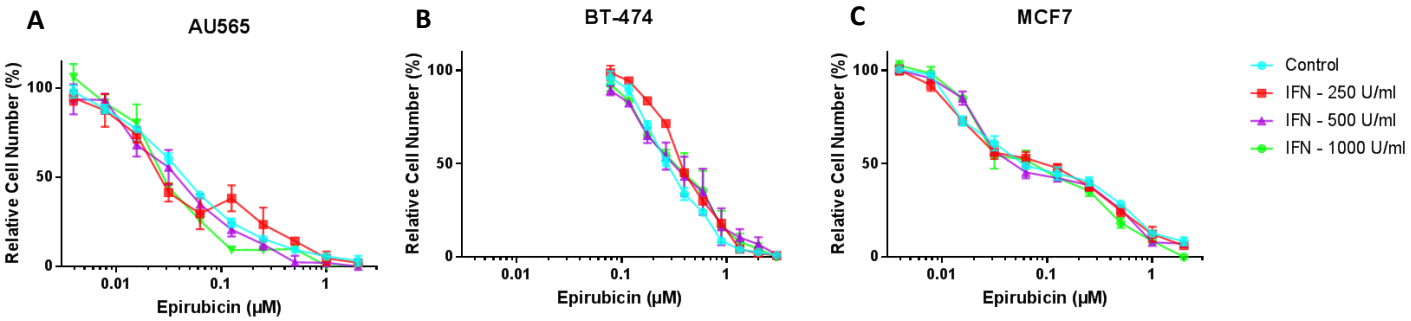

Figure 3.7. Dose response curves of (A) AU565, (B) BT-474 and (C) MCF-7 cells treated with epirubicin and IFNα1 at concentrations of 0 U/ml (Blue line), 250 U/ml (red line), 500 U/ml (purple line) and 1000 U/ml (green line). Cells were cultured with IFNα1 or vehicle control for 24 h before treatment with chemotherapy and IFNα1 for a further 24 h. cells were then incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance from each dose response was then normalised to vehicle controls treated with the same concentration of IFNα1. Data represents the mean ± SE of 3 biological repeats.

Like MDA-MB-231 cells, MCF-7 produced a biphasic response to epirubicin. This biphasic curve persisted at all IFNα1 treatment concentrations, however a slight significant increase in $IC50_1$ is seen when treated with 500 U/ml IFN relative to the control (1.3-fold $p = 0.05$). Treatment with 1000 U/ml induced a similar fold increase in $IC50_1$ but this increase was not significant (1.4-fold). No difference was seen in $IC50_2$ at any concentration of IFNα1 in this cell line. AU565 and BT-474 did not show a biphasic response to epirubicin. Additionally, AU565 showed no change in response to epirubicin at any concentration of IFNα1. BT-474 however, showed a significant 1.4-fold increase in IC75 when treated with IFNα1 at only the 250 U/ml ($p = 0.05$), although, this increase was not maintained at higher concentrations of IFNα1.

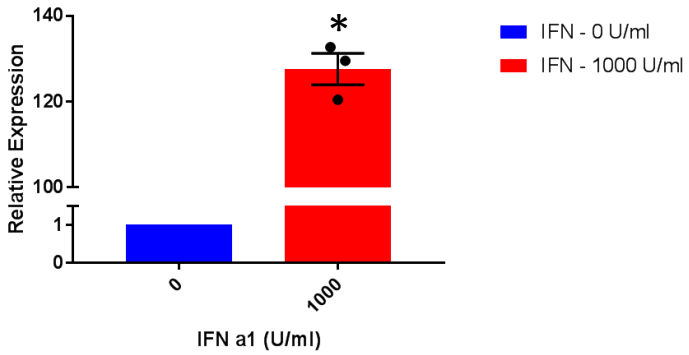

Figure 3.8. Relative expression of MX1 in AU565 cells treated with IFNα1 or vehicle control. Cells were seeded and treated with IFNα1 or vehicle control for 24 h. RNA was then extracted and MX1 expression was quantified by qPCR. MX1 expression was normalised to the expression of ACTB and data is shown as MX1 expression relative to vehicle control. Data represents the mean ± SE of 3 biological repeats and significance was determined using the Mann-Whitney U test (*p = 0.05).

As AU565 was the only cell line to show no significant response to IFNα1 whatsoever, qPCR was performed to determine whether IFNα1 was capable of inducing signalling in this cell line. As previously, this was assessed through the expression of MX1. IFN α1 induced a significant 127.5 ± 3.7 -fold increase in MX1 expression relative to untreated controls, suggesting this cell line is capable of IFN signalling in response to IFNα1 (Figure 3.8). AU565 was therefore taken forward for the investigation of IFN signalling in docetaxel treatment along with the other three cell lines.

3.3.1c IFN does not induce resistance to docetaxel in breast cancer cell lines in short-term viability-growth assays

To investigate whether IFN-dependant resistance persisted in treatment with other chemotherapeutics, MTT assays were repeated with docetaxel in place of epirubicin using only the highest dose of IFNα1 (1000 U/ml) or control (Figure 3.9). However, IFNα1 had no significant effect on IC50 values in any cell lines tested.

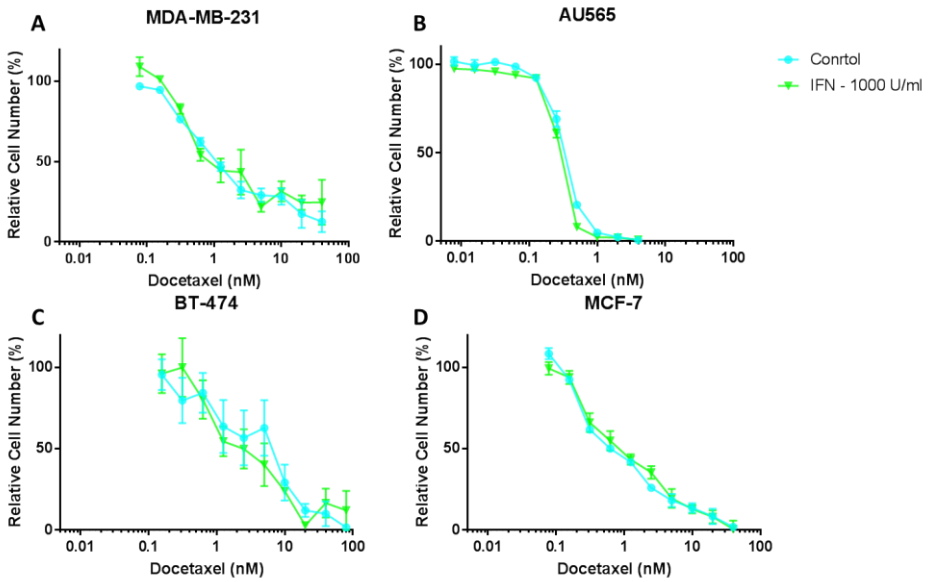

Figure 3.9. Dose response curves of (A) MDA- MB-231, (B) AU565, (C) BT-474 and (D) MCF-7 cells treated with docetaxel and IFNα1 at concentrations of 0 U/ml (Blue line) and 1000 U/ml (green line). Cells were cultured with IFNα1 or vehicle control for 24 h before treatment with chemotherapy and IFNα1 for a further 24 h. cells were then incubation for 72 h in fresh media. Cell viability was then assessed through MTT assay. Absorbance from each dose response was then normalised to vehicle controls treated with the same concentration of IFNα1. Data represents the mean ± SE of 3 biological repeats.

3.3.2 Breast cancer subtypes show differential responses to IFNα1 during chemotherapy treatment in long-term growth assays

To test further for IFN-dependant resistance in breast cancer cell lines, colony forming assays were performed to assess the effects of IFNα1 on chemotherapy-induced long-term cell survival. Previous research has shown a greater increase in chemoresistance in response to IFNα1 in colony forming assays as compared to that observed in MTT assays and therefore this may provide a more sensitive measure of IFN-dependant resistance. Cells were treated with IFNα1 and chemotherapy for 24 h then reseeded at low density and left to grow in the absence of drugs. The replicative potential of cells was then assessed through their ability to form colonies.

As previously established (see section 3.3.1), breast cancer cell lines display very different growth kinetics. As a result, seeding density was optimised for the formation of an appropriate number of colonies in each cell line. The number of colonies produced in the untreated control cells must be low enough to allow that resultant colonies are distinct from each other and therefore that counting is reliable but must also be high enough that the growth inhibitory effects can be accurately measured in the chemotherapy treated cultures. Colony numbers between 100 – 200 per plate were considered suitable.

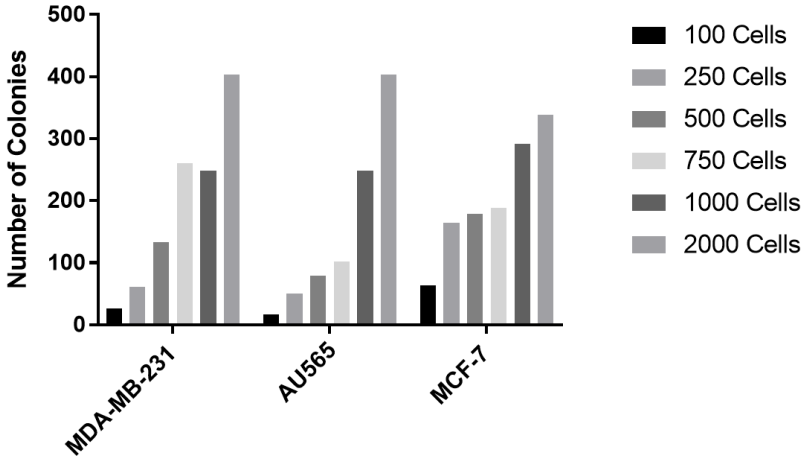

Figure 3.10. Colonies formed by breast cancer cell lines seeded at different densities in 10 cm tissue culture dishes. Cells were incubated for 2 weeks before colonies were fixed, stained and counted. Data represents the mean of 2 technical replicates.

Optimal seeding densities differed between breast cancer cell lines (Figure 3.10). MDA-MB-231, MCF-7 and AU565 cell lines were able to produce approximately 100-200 colonies at seeding densities of 500 and 750 cells per plate, respectively. BT-474 cells required much longer incubation times because of their low growth rate (see Figure 3.1) and data was therefore not produced in time for this thesis.

As well as seeding density, concentrations of chemotherapeutics were optimised due to the differential innate resistance of breast cancer cell lines found during MTT assays. Breast cancer cell lines were treated with a range of epirubicin or docetaxel doses for 24 h before being reseeded for colony forming assays (Figure 3.11); concentrations are necessarily much lower than for MTT assays as this alternative assay is far more sensitive to lower amounts of DNA damage. Concentrations corresponding to approximate IC50 values were selected for

each cell line (for epirubicin treatment: 10 nM for MDA-MB-231 and MCF-7, and 20 nM for AU565; for docetaxel treatment: 1 nM for all cell lines).

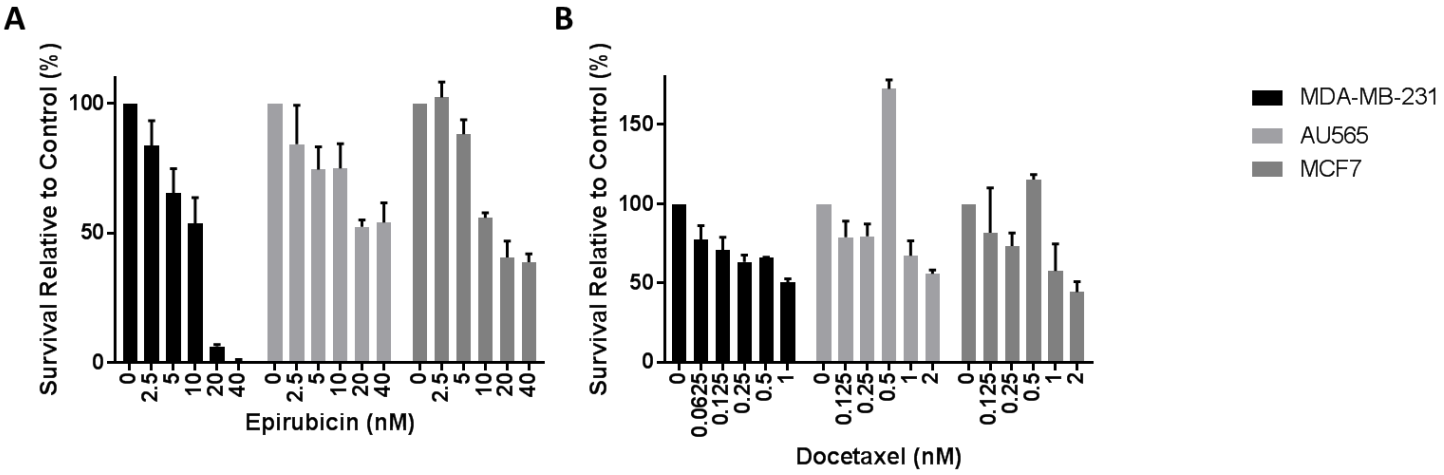

Figure 3.11. Dose-response of breast cancer cell lines to (A) epirubicin and (B) docetaxel in colony forming assays. Cells were treated with chemotherapy for 24 h before being incubated for 2 weeks. Colonies were then fixed, stained, and counted. Data are normalised to vehicle controls and represent the mean ±range of 2 technical replicates.

After the optimisation of chemotherapy concentrations, colony forming assays were repeated at approximate IC50 doses of epirubicin or docetaxel with increasing concentrations of IFNα1 (0, 250, 500, 1000 U/ml). This assay was initially performed with the positive control cell line (MDA-MB-231) and epirubicin.

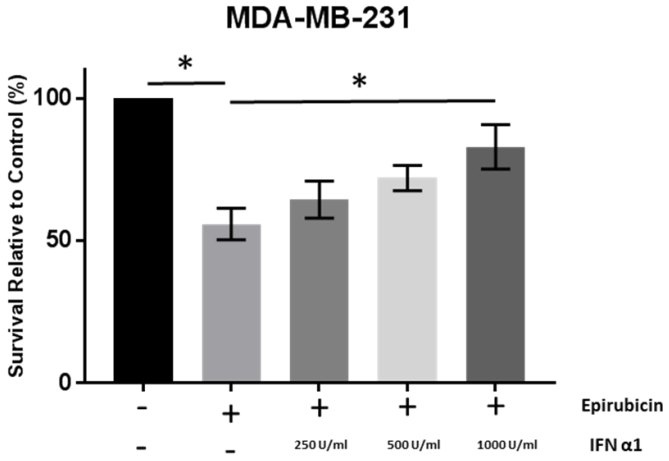

Figure 3.12. The effects of IFNα1 on epirubicin treatment in MDA-MB-231 in colony forming assays. Cells were cultured with IFNα1 for 24 h before treatment with epirubicin (10 nM) and IFNα1 for an additional 24 h. Colonies were then fixed, stained, and counted. Data are normalised to vehicle controls and represent the mean ± SE of 3 biological replicates. Significance was calculated using the Mann Whitney U test (*p = 0.05).

IFNα1 induced epirubicin resistance in a concentration dependant manner in MDA-MB-231 cells. However, this increase was only significant at 1000 U/ml IFN, where relative survival was 27.1 % higher with IFN compared to the epirubicin only treatment (P = 0.05) (Figure 3.12). This dose-dependent protection is consistent with both MTT assay results (section 3.3.1a) and previously published data, showing IFN-dependant resistance in MDA-MB-231 is reproducible both between assays and independent researchers [120]. Colony forming assays were then repeated for both other cell lines with epirubicin (Figure 3.13) or docetaxel (Figure 3.14).

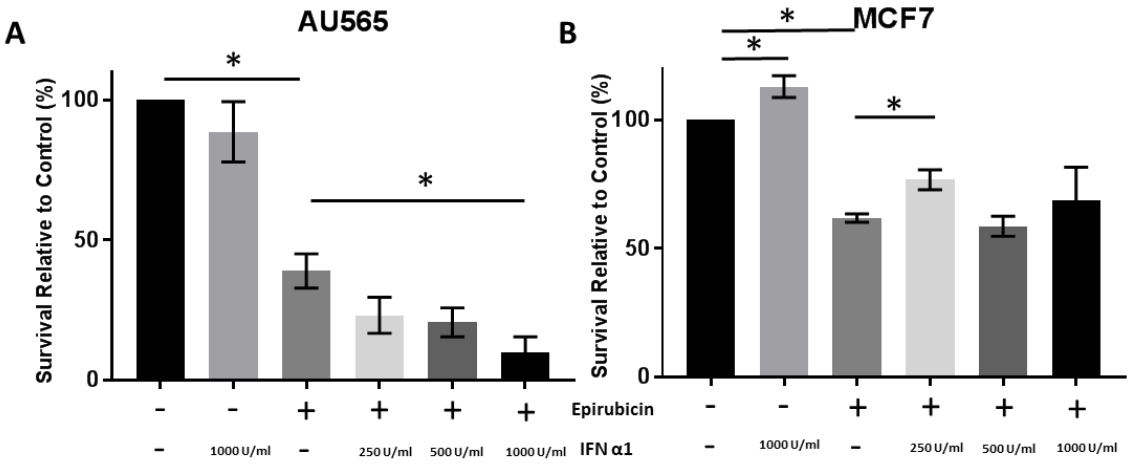

Figure 3.13. The effects of IFNα1 on epirubicin treatment in (A) AU565 and (B) MCF7 cells in colony forming assays. Cells were cultured with IFNα1 for 24 h before treatment with epirubicin (20 nM and 10 nM for AU565 and MCF-7 respectively) and IFNα1 for an additional 24 h. Colonies were then fixed, stained, and counted. Data are normalised to vehicle controls and represent the mean± SE of 3 biological replicates. Significance was calculated using the Mann Whitney U test (*p = 0.05).

Intriguingly, IFNα1 induced a non-significant decrease in AU565 survival of 21.4 % relative to untreated controls (Figure 3.13a). Moreover, in combination with epirubicin, IFNα1 induced a dose-dependent decrease in relative survival. IFNα1 significantly decreased survival relative to epirubicin alone at 1000 U/ml suggesting IFN has a survival/growth inhibitory effect.

In MCF-7 cells, IFNα1 alone induced a significant 13.0 % increase in clonogenicity (Figure 3.13b). Moreover, IFNα1 induced a similar increase in clonogenicity at 250 U/ml relative to epirubicin treated cells (14.9 %). However, this increase did not persist at higher concentrations of IFNα1. I concluded that neither MCF-7 nor AU565 cells showed any evidence of IFN-induced chemoprotection as had been seen with MDA-MB-231 cells.

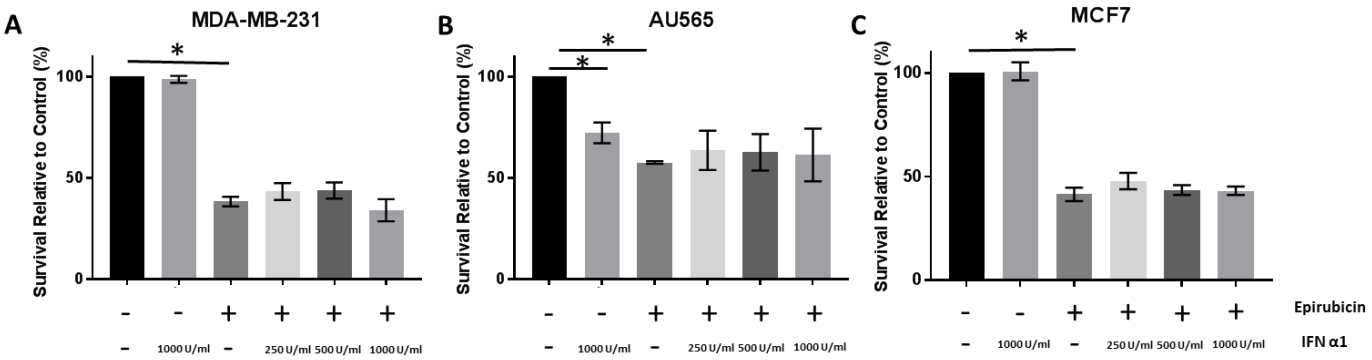

Figure 3.14. The effects of IFNα1 on docetaxel treatment in (A) MDA-MB-231 (B) AU565 and (C) MCF7 cells in colony forming assays. Cells were cultured with IFNα1 for 24 h before treatment with docetaxel (1 nM) and IFNα1 for an additional 24 h. Colonies were then fixed, stained, and counted. Data are normalised to vehicle controls and represent the mean ± SE of 3 biological replicates. Significance was calculated using the Mann Whitney U test (*p = 0.05).

With respect to docetaxel, IFNα1 induced no significant changes in number of colonies formed after docetaxel treatment in any breast cancer cell line tested (Figure 3.14), a finding that is consistent with MTT assays finding of no IFN-induced protection (Figure 3.8). As previously (Figure 3.14a), IFN alone induced a significant decrease in colony formation in AU565 of 27.8 %, although unlike for the epirubicin treated cells, this decrease did not contribute to further reductions in colony formation in combination with docetaxel. I concluded that there was no evidence of IFN-dependant protection from docetaxel in any cell line.

3.3.3 IFNα1 caused reduced DNA double-stranded break formation in MDA-MB-231 treated with epirubicin

As IFNα1 was capable of inducing resistance to epirubicin but not docetaxel in both short- and long-term viability assays, I hypothesised that the mechanism of IFN-dependant resistance may be specific to the mechanism of action of epirubicin. Several studies have implicated

type-I IFN signalling in resistance to DNA-damage inducing therapies such as radiotherapy and anthracycline-based therapy, both of which induce double stranded breaks [172, 173]. I therefore investigated the effects of IFNα1 on DSB formation during epirubicin treatment using a γ-H2Ax immunofluorescence assay. H2Ax is a histone protein which is phosphorylated (once phosphorylated it is referred to as γ-H2Ax) at the site of DNA double stranded breaks and therefore provides a good indication of DSB frequency in cells [174].

I first confirmed that epirubicin was capable of inducing H2Ax phosphorylation in MDA-MB-231. Cells were treated with epirubicin (0, 10, 50 and 100 nM) for 2 h before fixing and fluorescent labelling of γ-H2Ax. Images were then taken using a confocal microscope and relative H2Ax phosphorylation was assessed through number of foci per cell using image J analysis software.

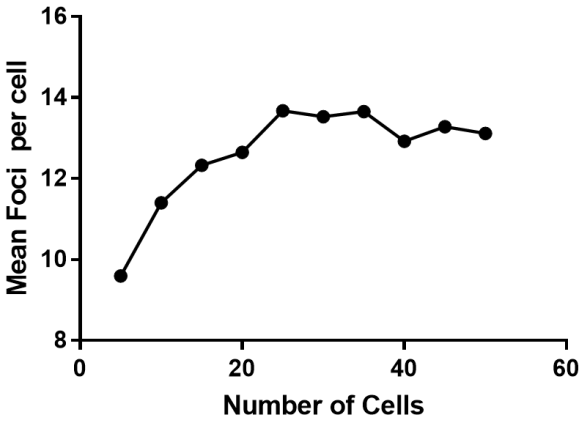

Figure 3.15. Changes in mean γH2Ax Foci per cell as the number of cells measured is increased. Nuclear foci were counted using image J.

To ensure enough data were collected to produce an accurate mean, averages of foci per cell were taken in my 100 nM treatment group with an increasing number of cells. A mean of 30 cells was determined to be sufficient, as mean values using a larger number of cells showed only small fluctuations between 12.9 and 13.7 foci per cell (Figure 3.15). Mean foci per cell values were then calculated for each treatment group. As expected, epirubicin induced a dose-dependent increase in foci number per cell relative to untreated controls using doses ranging from 10 to 100 nM (Figure 3.16).

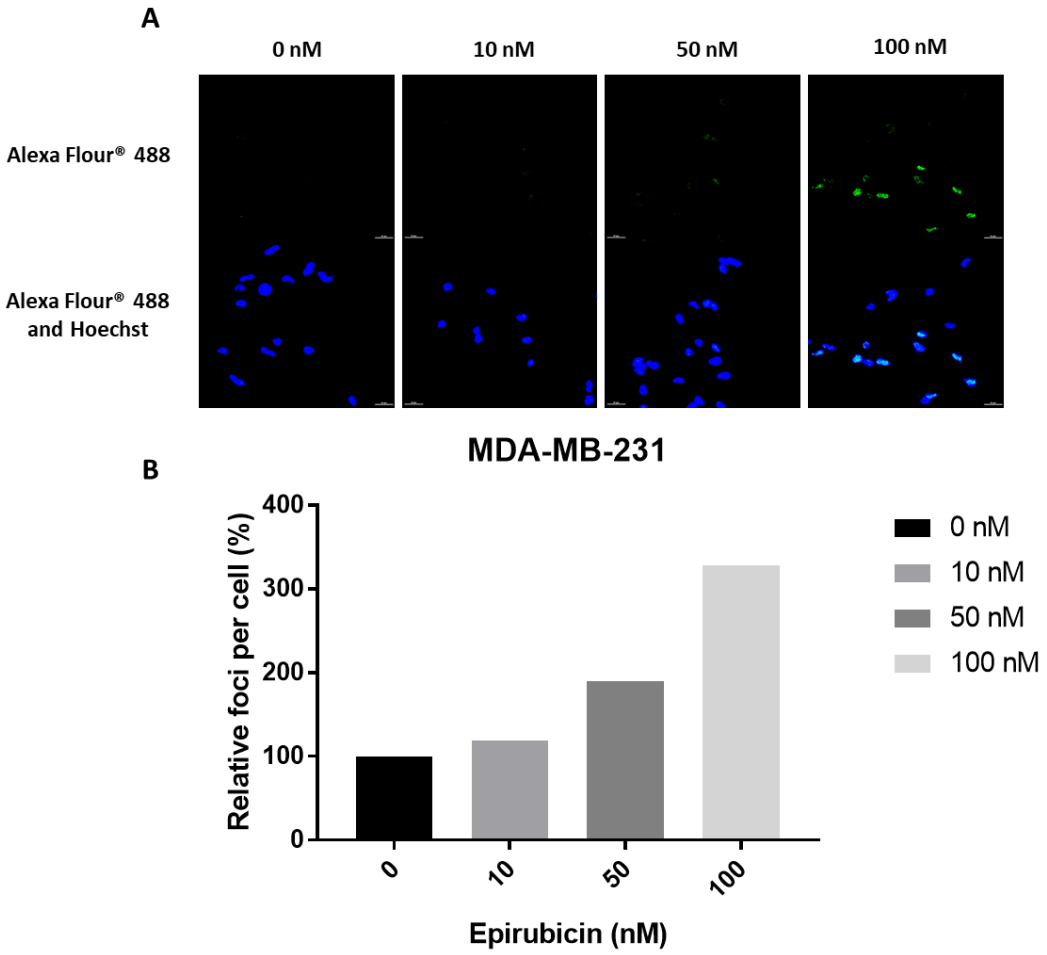

Figure 3.16. The effects of epirubicin on γ-H2Ax expression in MDA-MB-231 cells. Cells were treated with epirubicin for 24 h before being fixed and fluorescently labelling. (A) Imaging was then performed using a confocal microscope and (B) nuclear foci were counted using image J. Foci per cell were then normalised to the vehicle control. Data represent the mean foci per cell of 30 cells per treatment group.

Epirubicin treatment was then repeated in the presence and absence of IFNα1 and γ-H2Ax foci were counted. As in the previous experiment epirubicin induced a significant increase in H2Ax phosphorylation (P = 0.05). However, the introduction of IFNα1 to epirubicin treatment induced a significant 1.8-fold decrease in γ-H2Ax expression (P = 0.05; Figure 3.17). I concluded that IFN did potentially decrease the DNA damage induced by epirubicin, and therefore that this was most likely a component of the IFN-induced resistance.

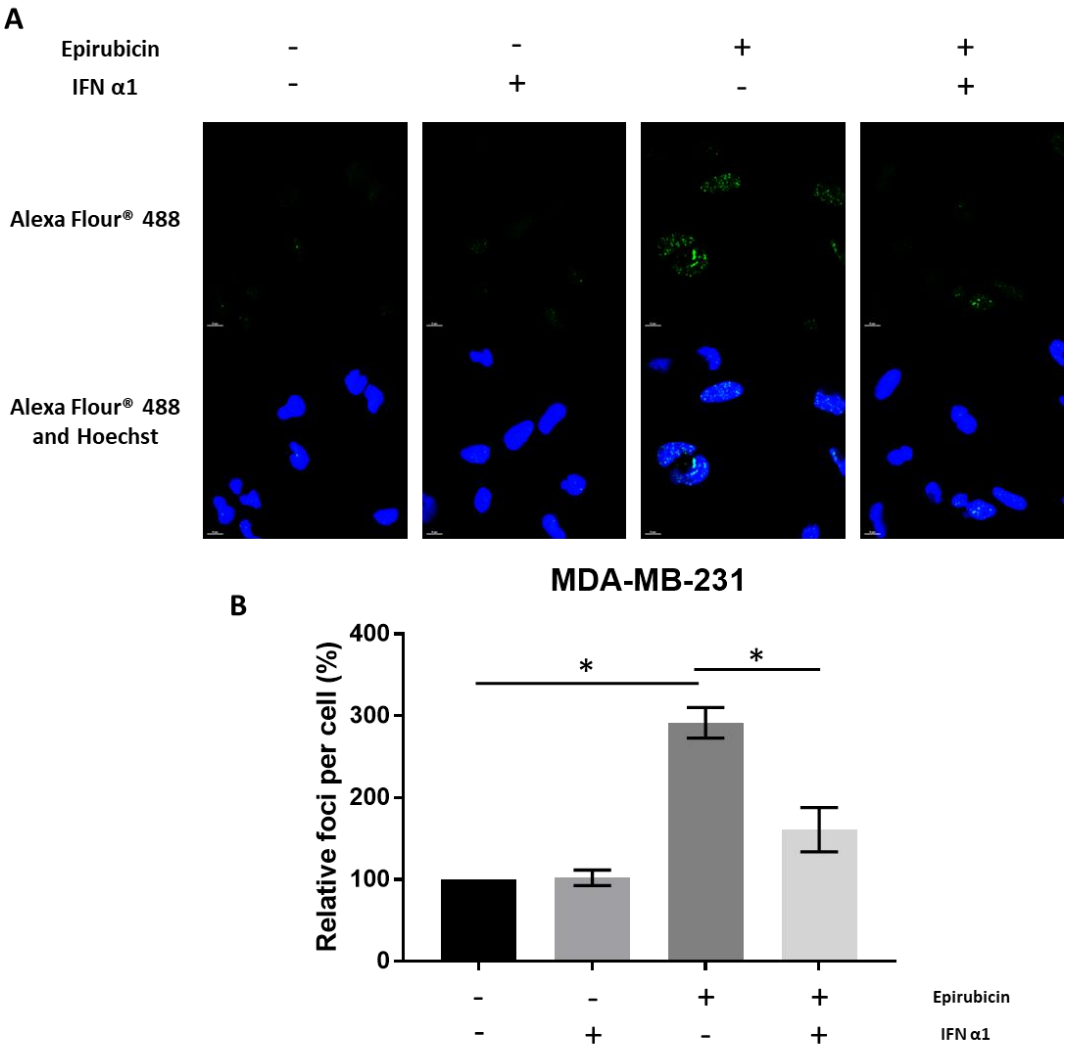

Figure 3.17. The effects of epirubicin and IFNα1 on γ-H2Ax expression in MDA-MB-231 cells. Cells were pre-treated with IFNα1 (1000 U/ml) for 24 h before treated with epirubicin (50 nM) and IFNα1 for an additional 24 h. Cells were then fixed and fluorescently labelled. Imaging was then performed using a confocal microscope and representative images can be found in (A). (B) Nuclear foci were counted using image J and foci per cell were then normalised to the vehicle control. Data represents the mean ± SE of 3 biological repeats. Significance was calculated using the Mann Whitney U test (*p

3.4 Discussion

Resistance to chemotherapy presents a major obstacle in breast cancer treatment and is commonly responsible for local recurrence and metastasis [96]. Therefore, identification of resistance pathways may lead to new strategies to overcome resistance and improve treatment response rates. A recent paper published by the Hughes group has identified paracrine type-I IFN signalling between CAFs and breast cancer cells can induce anthracycline resistance in claudin-low TNBC and may be inhibited to improve chemotherapy response [120]. Although most prevalently used in TNBC, chemotherapy is also commonly used in the treatment of HER2-enriched, luminal B and aggressive luminal A BC subtypes [56]. The role of type-I IFN signalling in these subtypes has not yet been established and may present an opportunity to expand the potential beneficiaries of chemosensitising treatments in the future. This chapter therefore focused on assessing the chemoresistance properties of type-I IFN in cell lines representing these breast cancer subtypes.

The initial aim of this chapter was to establish a viability assay protocol to screen cell lines representing 4 breast cancer subtypes for IFN-dependant resistance to anthracyclines. As IFN-dependant resistance has previously been observed in MDA-MB-231, this was used as my positive control [120].

3.4.1 Type-I IFNs induce epirubicin resistance in claudin-low TNBC

The addition of IFNα1 induced a dose-dependent resistance to epirubicin in MDA-MB-231 cells in both MTT and colony forming assays. Broad *et al.* has also shown an IFN-dependent resistance to epirubicin in two claudin-low TNBC cell lines (MDA-MB-231 and MDA-MB-157) through a CAF paracrine signalling mechanism [120]. In this study IFNα1 alone was capable of inducing a dose-dependent resistance to epirubicin during colony forming assays. Colony forming assays used in this chapter were specifically modelled from those used by Broad *et al.* to allow comparison. At equivalent concentrations of epirubicin and IFNα1 (epirubicin = 10 nM; IFN = 500 U/ml), IFN induced a similar increase in clonogenicity relative to epirubicin alone of between 15 – 20 % [120]. This study also showed inhibition of type-I IFN signalling reverted CAF-induced resistance in both cell lines, suggesting inhibition of this pathway could be used in the sensitisation of claudin-low TNBC to chemotherapy. Boelens *et al.* have also shown CAF-induced upregulation of IFN signalling to increase resistance to radiotherapy and cisplatin treatment in breast cancer cells [162]. Moreover, the cell lines effected by this

resistance pathway, including MDA-MB-231 and MDA-MB-157, were representative of claudin-low TNBC. However, contrary to the previous study, this paper suggested IFN signalling was induced through the release of miRNA containing exosomes which activated STAT1 signalling in breast cancer cells through activation of RIG-1. Although this chapter did not address the role of CAFs in IFN-dependant resistance, it should be noted I have shown exogenous IFNα1 is capable of inducing resistance in concordance with the former study. However, this does not exclude the possibility of resistance induced by other means such as exosomes.

3.4.2 Type-I IFNs reduce epirubicin-dependent DNA-damage in the claudin-low TNBC cell line MDA-MB-231

In MTT assays, MDA-MB-231 showed a biphasic dose-response to epirubicin treatment. Biphasic response is associated with the presence of multiple mechanisms of cytotoxicity in which each curve is associated with a distinct mechanism of action. Evidence of the biphasic action of epirubicin can also be observed in the dose response of HCT-116 and LXFL-529 in the NCI-60 database (https://dtp.cancer.gov/dtpstandard/servlet/dwindex?searchtype=CAS&chemnameboolean=and&outputformat=html&searchlist=CAS+56390-09-1%0D%0A&Submit=Submit).

Epirubicin has been shown to induce cytotoxicity through the inhibition of topoisomerase II resulting in DNA DSBs as well as through the production of ROS [77]. Biphasic cytotoxicity seen in MTT assays could therefore be a result of these two distinct mechanisms of action.

The addition of IFNα1 resulted in the apparent inhibition of the initial mechanism of reduced relative cell number induced by epirubicin in MDA-MB-231 cells. Moreover, immunofluorescence assays showed a significant decrease in DNA damage marker, γ-H2Ax, induced by epirubicin in the presence of IFNα1. Gewirtz *et al.* observed protein-associated DNA strand breaks, such as those associated with topoisomerase II inhibition, occurred at lower anthracycline concentrations whereas free radical induced cytotoxicity was associated with higher concentrations [175]. This suggests the induction of DNA DSBs may be responsible for the initial dose-response curve observed in MTT assays, which is subsequently inhibited by the addition of IFNα1 in MDA-MB-231 cells. In further support of this, IFNα1 had no effect on resistance to docetaxel in MTT and colony forming assays, in which the primary mechanism of cytotoxicity is not related to DNA damage [176].

It should be noted, the mechanism by which IFN α1 reduces γ-H2Ax expression is still unknown. This reduction in H2Ax phosphorylation can occur due to a decrease in double-stranded break formation, suppression of DNA damage repair pathways (reducing H2Ax expression or phosphorylation) or an increase in DNA damage repair. Further study of this may identify targets for inhibition to sensitise resistant tumours to chemotherapy.

The involvement of type-I IFN signalling in DNA-damage resistance has been observed in several studies [158]. Rickardson *et al.* found the expression of STAT1 significantly correlated with topoisomerase II inhibitor resistance across a panel of 10 human cancer cell lines [172]. Moreover, several studies have found an association between expression of a group of ISGs known as the IFN-related DNA damage resistance signature (IRDS) and resistance to DNA damaging therapies [173]. High expression of ISGs, STAT1, MX1 and OAS1 has been observed in breast cancer cell lines resistance to cisplatin and radiotherapy, both of which induce apoptosis through DSB formation. Inhibition of STAT1 restored sensitivity to radiotherapy in these resistance cell lines [162]. Human squamous carcinoma cells selected for radiotherapy resistance have also been shown to have increased expression of an IRDS, which induced resistance to both radiotherapy and doxorubicin treatment. Like in the previous study, inhibition of STAT1 restored doxorubicin sensitivity. Also, overexpression of STAT1 in the SK-BR3 breast cancer cell line has been shown to induce doxorubicin resistance [159]. In the clinical setting, expression of an IRDS has been associated with poor response to adjuvant chemotherapy in a cohort of 295 early breast cancer patients [159].

Both pre-clinical and clinical data suggest type-I IFN signalling is capable of inducing DNA damage resistance in some cancers. In this chapter, I have shown type-I IFNs can reduce epirubicin-induced DSB-formation in the claudin-low TNBC cell line MDA-MB-231, suggesting a possible mechanism of resistance to genotoxicity. Further study of the mechanism utilised by type-I IFN to reduce DNA damage may provide insight into which chemotherapeutics are susceptible to this pathway and how best to inhibit IFN-dependant resistance.

3.4.3 The effects of Type-I IFNs on chemotherapy response are subtype dependent

Viability assays were extended to additional cell lines representing three breast cancer subtypes. BC cell lines showed different innate resistance to epirubicin with MDA-MB-231 and BT-474 showing greater resistance than AU565 and MCF-7 cells. This is consistent with comparisons found in the literature that show MCF7 to be more sensitive to anthracyclines

than BT-474 and MDA-MB-231 [177]. Three forms of dose-response could be observed in breast cancer cell lines treated with epirubicin and IFNα1; (1) biphasic dose response which was sensitive to IFN-dependant chemoresistance (MDA-MB-231); (2) biphasic dose-response which showed little sensitivity to IFN-dependant resistance (MCF-7); (3) monophasic dose-response which showed little or no sensitivity to IFN-dependant resistance (AU565 and BT-474).

Like MDA-MB-231 cells, MCF7s showed a biphasic response to epirubicin, which has also been observed in the literature [178]. Also, like MDA-MB-231, MCF-7 showed a significant increase in $IC50_1$ but no change in $IC50_2$ in the presence of IFNα1, however, this increase was relatively small suggesting IFN has a less pronounce effect on resistance in this cell line. This was further confirmed in colony forming assays where IFN induced a non-dose dependant increase in clonogenicity of MCF-7 in the presence of epirubicin.

Expression of ISGs has been observed in MCF-7 cells selected for radiotherapy resistance. These cells had increased resistance to both tamoxifen and radiotherapy in cell counting viability assays, although significance was calculated from technical and not biological replicates. Contradictory to results found in this chapter (albeit with epirubicin), IFN signalling did not induce resistance to doxorubicin in tamoxifen resistant MCF-7 cells [179].

In colony forming assays, IFNα1 alone induced an increase in the clonogenicity of MCF-7 cells by a similar margin to that seen in combination with epirubicin, suggesting this effect may be a result of increased proliferation and unrelated to epirubicin resistance per se. IFN-β expressing CAFs have been shown to increase proliferation of MCF-7 cells in co-culture models. This increase in proliferation was of a similar level to that seen in this study. Moreover, inhibition of IFN-β using monoclonal antibodies in these co-cultures significantly reduced cancer cell proliferation to levels similar to those of the cancer cell monocultures [163]. High MX1 expression has been associated with the Ki67 marker of proliferation in a cohort of 845 early breast cancer patients suggesting IFN signalling may be responsible for aggressive proliferative phenotypes [161]. Broad *et al.* identified a similar proliferative effect of type-I IFN in MDA-MB-231 cells using colony forming assays [120]. However, this effect was not observed in colony forming assays in this thesis, where IFNα1 had no effect on MDA-MB-231 clonogenicity in the absence of epirubicin.

AU565 showed no sign of IFN-dependant resistance in MTT assays. This absence of resistance was not due to a lack of signalling capability as IFNα1 induced MX1 expression in this cell line. Furthermore, in colony forming assays, IFNα1 induced a decrease in AU565 clonogenicity and further decreased growth potential in combination with epirubicin. The absence of a biphasic response of AU565 to epirubicin in MTT assays suggests either a high sensitivity or resistance to one mechanism of epirubicin cytotoxicity resulting in only one mechanism of action. As shown in MTT assays, IFNα1 may induce resistance to one of multiple mechanisms of epirubicin cytotoxicity through reduced DSB formation. An analysis of gene expression in breast cancer cell lines showed MDA-MB-231 and MCF-7 to express wild type BRCA 1, a protein associated with DNA DSB repair, whereas AU565 showed no measurable expression [180, 181]. Cancer cells deficient in BRCA 1 have been shown to have greater sensitivity to chemotherapeutics which induced double stranded breaks, including doxorubicin [182]. AU565 may therefore have increased sensitivity to epirubicin-induced DSB formation which may overcome the resistance effects of IFNα1. However, further study of the effects of IFNα1 on DSB formation in this cell line are required to define its lack of IFN-dependant resistance.

Intriguingly, during colony forming assays, IFNα1 induced a decrease in clonogenicity of AU565, both alone and in combination with epirubicin. Multiple studies have suggested IFNs can have anti-proliferative and cytotoxic activity in some contexts [183]. In the colon cancer cell line DU145, IFN α was shown to reduce colony formation which was associated with a G_0/G_1 cell cycle arrest [184]. Moreover, growth inhibitory effects of type-I IFNs has been seen in multiple human melanoma cell lines through MTT growth assays [185]. Direct cytotoxic effects of type-I IFNs have also been observed in several cancer cell lines [186]. Melanoma cells treated with high-dose IFNα1 (5000 U/ml) showed an increase in apoptosis relative to controls which was reduced by inhibition of STAT1 [187]. Dedoni *et al.* also found IFNβ to increase cytochrome C release and caspase activation, associated with intrinsic apoptosis in neuroblastoma cells [188]. Although, this chapter has shown type-I IFN to have anti-tumour activity in AU565s, it is unclear whether this has been caused by an anti-proliferative or cytotoxic mechanism, both of which have been observed in the literature.

In this chapter, IFNα1 reduced the clonogenicity of AU565 to a greater extent in combination with epirubicin as compared to alone (28.9 % and 11.4 %, respectively), suggesting it may also have a chemosensitising effect in this cell line. This chemosensitisation was shown to be drug-

specific as IFN did not further decrease the clonogenicity of AU565 cells treated with docetaxel. Type-I IFN has been suggested to increase sensitive of MCA205 fibrosarcoma cells to doxorubicin *in vivo* through a TLR3-IFN-CXCL10 signalling axis. IFN induced a significant increase in sensitivity to doxorubicin in *tlr3-/-* tumour cells implanted in mice, which was inhibited by the addition of anti-IFNAR1 antibodies, suggesting IFN improves chemotherapy response in this context [157]. Due to the anti-tumour effects of IFNα1 seen in this chapter, it is likely HER2-enriched BC may not respond well to inhibition of IFN during chemotherapy.

In this chapter, I have shown the effects of type-I IFNs on chemotherapy response are specific to both breast cancer subtypes and chemotherapeutic drugs. These conflicts may also be found in the literature where type-I IFNs have been shown to induce both chemosensitisation and resistance to anthracyclines in different contexts [120, 157]. The effects of IFN on specific cell types are therefore likely dependant on the relative sensitivity of cells to each of these conflicting effects, defined by their malignant phenotype. Further study into the specific traits associated with IFN-resistance and -sensitising phenotypes is required to better understand when inhibition of this pathway may be relevant in improving chemotherapy response.

It should be noted, the effects of IFN described in this chapter are from *in vitro* experiments performed in monocultures and do not account for the interactions of cancer cells and IFN with the TME. IFN has been associated with influencing chemoresistance and sensitivity through interactions with multiple TME cells including fibroblasts and lymphocytes [120, 189]. Further investigation of IFN-dependant resistance in these cell lines *in vivo* may therefore give a better indication of the susceptibility of BC subtypes in the context of the TME. Additionally, only one cell line was used as a representative of each BC subtype in this study and therefore is likely not representative of the heterogeneity found within these subtypes. Screening of additional cell lines associated with these subtypes would provide a more robust representation of the susceptibility of these subtypes to IFN-dependant resistance. Further analysis of the associations between type-I IFN, ISG expression and chemotherapy response in the clinical setting may also give an indication of which subtypes are affected by this resistance pathway.

4.3.4 Conclusion

In conclusion, this chapter has confirmed type-I IFNs are capable of inducing chemoresistance in claudin-low TNBC *in vitro* and suggests this is likely to occur through a reduction in anthracycline-induced DNA DSB formation. IFN-dependent resistance was also shown to be breast cancer subtype specific as IFN did not induce resistance in cell lines representing HER2-enriched or luminal A breast cancer in viability assays. Moreover, IFN had no effect on resistance to docetaxel in viability assays in any cell line suggesting its mechanism is also chemotherapeutic specific. The results of this chapter therefore suggest that inhibition of IFN signalling may sensitise tumour cells of claudin-low TNBC patients to anthracycline treatments. However, identification of tumours which possess this resistance mechanism will be essential in developing sensitising combination therapies. Future studies should focus on inhibiting this pathway to restore chemotherapeutic efficacy *in vitro* and *in vivo*, with the intention of progressing chemosensitising combination treatments into the clinical setting.

4. Clinical Relevance of IFN-Dependent Chemoresistance in Metastatic Triple-Negative Breast Cancer

4.1 ABSTRACT

Resistance to chemotherapy is a major issue in the treatment of breast cancer, especially in the context of metastasis, where treatment failure is essentially inevitable. As a result, the identification of resistance pathways, with the ultimate goal of inhibiting these to sensitise tumours to chemotherapy, is essential to improving patient outcomes. Recent work by the Hughes group has shown that paracrine CAF-dependant IFN signalling induces resistance to chemotherapy in claudin-low TNBC. In this chapter, I therefore evaluated the clinical relevance of this resistance pathway in metastatic TNBC. The expression of IFNβ1 and MX1 was assessed in the malignant and stromal cells of metastatic tumours from a cohort of 27 TNBC patients using immunohistochemical evaluation. Expression of these IFN signalling biomarkers was correlated between cell types to identify active paracrine or autocrine IFN signalling. Kaplan-Meier survival analysis was also performed to assess the effects of IFN signalling on patient survival. Correlation of IFNβ1 and MX1 expression was further assessed in paired primary tumours of 9 patients from the same cohort. The expression of IFNβ1 in lymphocytes, but not fibroblasts, was significantly associated with MX1 expression in cancer cells of metastatic tumours ($r = 0.376$; $p = 0.049$). No correlation was seen in the expression of IFNβ1 or MX1 between paired primary and metastatic tumours. Neither expression of IFNβ1 or MX1 was associated with survival outcomes in this cohort. This chapter has shown paracrine IFN signalling is active within metastatic TNBC tumours between lymphocytes and cancer cells. However, no association was seen between this signalling axis and patient outcomes probably in part due to the small cohort size of this pilot study. Further studies, using a larger cohort, should be performed to assess the effects of IFN signalling on patient outcomes in the context of metastasis.

4.2 Introduction

As shown in the previous chapter, as well as in independent work by the Hughes group, type-I IFNs are capable of inducing chemoresistance in TNBC cell lines. In addition to this, previous work by the Hughes group has identified an association between IFNβ1 expression in CAFs and MX1 expression in cancer cells in a cohort of 109 primary TNBC patients, suggesting paracrine IFN signalling occurs between these cells in actual tumours. Furthermore, expression of both IFNβ1 in CAFs and MX1 in cancer cells were significantly associated with poor disease-free survival in the same cohort [120]. High expression of MX1 has additionally been associated with poor prognosis in a cohort of 845 primary breast cancer patients [161]. Inhibition of this signalling pathway therefore has the potential to improved disease free survival in breast cancer patients.

Although the role of type-I IFNs has been described in primary breast cancer, little is known about their roles in breast cancer metastases and whether the association with poor outcomes is maintained in this context. Moreover, the primary and metastatic tumour microenvironment show distinct characteristics, including the origins and phenotypes of CAFs and therefore the source of IFN may differ in metastatic breast cancer tumours [128].

Metastasis in breast cancer is not curable using currently available therapies, and treatment in this context is palliative [190]. This is largely due to the high occurrence of chemoresistance in metastatic disease that is often acquired in breast cancer cells during treatment for primary disease [191]. Investigating the relevance of primary breast cancer resistance pathways in metastatic disease may therefore lead to opportunities to overcome chemoresistance in tumours which have already metastasised, improving outcomes for patients.

In this chapter, I therefore investigated the potential of IFNβ1 and MX1 as predictive markers of chemotherapy response in metastatic breast cancer through evaluation of their expression in metastatic tumours in association with survival.

4.3 Results

4.3.1. Assembling a cohort of metastatic TNBC tissues

To assess the relevance of IFN-dependant chemoresistance and identify the relevant cellular source of IFN in metastatic TNBC, a cohort of 31 metastatic triple negative breast cancer samples from 31 patients were collected. The patients from whom these samples were taken all had primary TNBC, were treated with surgery and cytotoxic chemotherapy, and subsequently suffered metastatic recurrences. In 13 cases, it was also possible to collect a paired primary TNBC tumour tissue sample. 3 patients were subsequently excluded from the cohort due to limited available tissue whilst one was excluded because the tumour was a rare form of myoepithelioma, leaving a final cohort of 27 patients with 9 paired primary tissues. A summary of the clinicopathological features and treatment regimens of this final cohort can be seen in Table 4.1.

Table 4.1 Summary of the clinicopathological features and treatment regimens for a cohort of metastatic triple negative breast cancers.

n	27	
Median Age	46 (range: 26 - 77) years	
Median Time to metastasis	31.5 (range: 2 - 497) months	
Primary Tumours		
Median Tumour Size	27.5 (range: 10 - 140) mm	
Tumour Grade	Data not available	2 (7.4 %)
	1	0
	2	5 (18.5 %)
	3	20 (74.1 %)
Lymph node Status	Positive	17 (63 %)
	Negative	10 (37 %)
Primary Treatment	Neoadjuvant	8 (29.6 %)
	Adjuvant	21 (77.8 %)
Primary Chemotherapy Regimen	Anthracyline	3 (11.1 %)
	Taxane	1 (3.7 %)
	Anthracycline and Taxanes	19 (70.4 %)
	Other	4 (14.8 %)
Metastatic Tumours		
Metastasis Chemotherapy Regimen	Platinum Compounds	13 (48.1 %)
	DNA Synthesis Inhibitors	12 (44.4 %)
	Microtubule Inhbitors	16 (59.3 %)
	Other	12 (25.9 %)
Metastasis Site	Liver	4 (14.8 %)
	Brain	5 (18.5 %)
	Bone	2 (7.4 %)
	Lung	4 (14.8 %)
	Lymph Node	3 (11.1 %)
	Skin	3 (11.1 %)
	Other	6 (22.2 %)

The aim of using these samples was to assess evidence for active IFN signalling in the breast cancer cells in both the primary and metastatic setting to determine whether signalling in the primary setting is associated with active signalling in the matched metastatic sample, and whether active signalling in either sample is associated with poor response to chemotherapy in terms of a shorter disease free survival between primary and recurrence and/or a shorter overall survival after diagnosis with metastases. It should be noted that the cohort is not well powered for these analyses, but it comprises all the suitable samples available – therefore this analysis should be regarded as preliminary and hypothesis generating.

As previously, IFN signalling was assessed by determining expression analysis of both IFNβ1 and MX1 [120]. MX1 is an ISG which has been shown to increase in expression during IFN-induced signalling and is therefore used as an indicator of active IFN signalling within the cancer cells in this analysis, whereas IFNβ1 is a driver of IFN signalling previously shown to be released from the cancer associated fibroblast component of the primary breast cancer tissues.

4.3.2. Optimisation of IFNβ1 and MX1 staining and quantification

Optimal dilution of primary antibodies was determined prior to staining of the tissues representing full cohort for both IFNβ1 and MX1 antibodies. This was performed to ensure staining was both antigen specific and sensitive to changes in expression (allowing identification of different levels of antigen expression). The antibodies chosen for this analysis have been used in previous assessment of IFNβ1 and MX1 expression in primary breast cancer and therefore I used the optimised dilutions from this study in my initial staining [120]. To ensure similar staining I also used the same conditions and protocol for antigen retrieval and blocking, which similarly effect staining specificity and intensity.

A cohort of 5 primary breast cancer tissues were used in the optimisation of staining. Staining appeared specific in both IFNβ1 and MX1 stained tissues in that both negative and positive cells could clearly be identified in the same region (Figure 4.1a). IFNβ1 was found to be expressed in the cytoplasm of most identified cell types including cancer cells, fibroblasts, lymphocytes, endothelial cells, and macrophages. Similarly, MX1 was expressed in the cytoplasm of cells and could be expressed by multiple cell types.

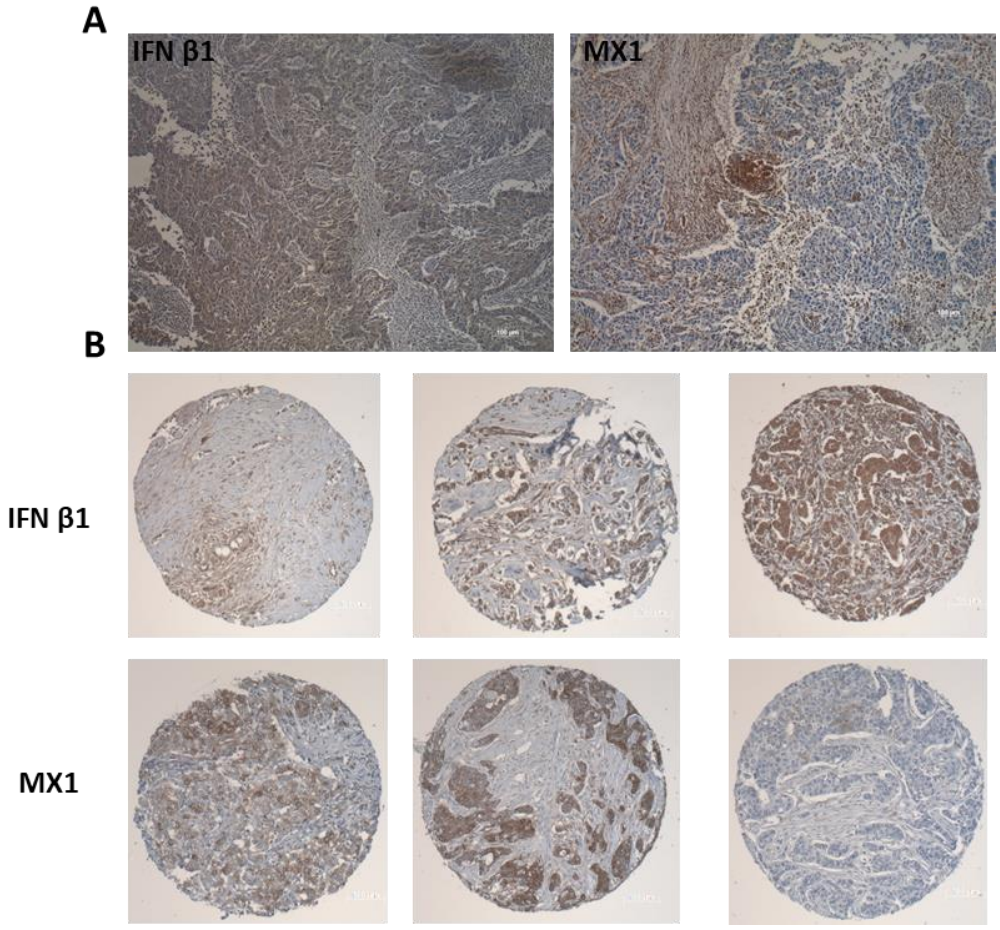

Figure 4.1 Representative images of primary breast cancer tissue stained for IFNβ1 and MX1 for the optimisation of IHC staining protocol in (A) full tissue samples and (B) TMAs.

To further assess the ability of my staining protocol to show variation in staining intensity between tumours, further staining was performed on a tissue microarray of primary breast cancers. Staining varied considerably between tumours both in intensity and proportion of cells stained when stained for either IFNβ1 or MX1 (Figure 4.1b). Tissue was also expertly assessed by Dr Eldo Verghese (Consultant Breast Histopathologist), who confirmed staining appeared specific.

Prior to staining of the full cohort, haematoxylin and eosin staining was performed on all tissues, and samples with no cancer cells present or limited tissue were removed from the cohort. 3 samples showed no cancerous tissue and were therefore removed from the cohort. Another was identified by Dr Eldo Verghese as a rare form of myoepithelioma. This form of breast cancer is extremely rare with distinct and ill-defined prognosis and was therefore

removed from the cohort. This resulted in a cohort of 27 metastatic breast cancer tissues and 9 paired primary breast cancer tissues. The full cohort of tissues was stained for both IFNβ1 and MX1 expression.

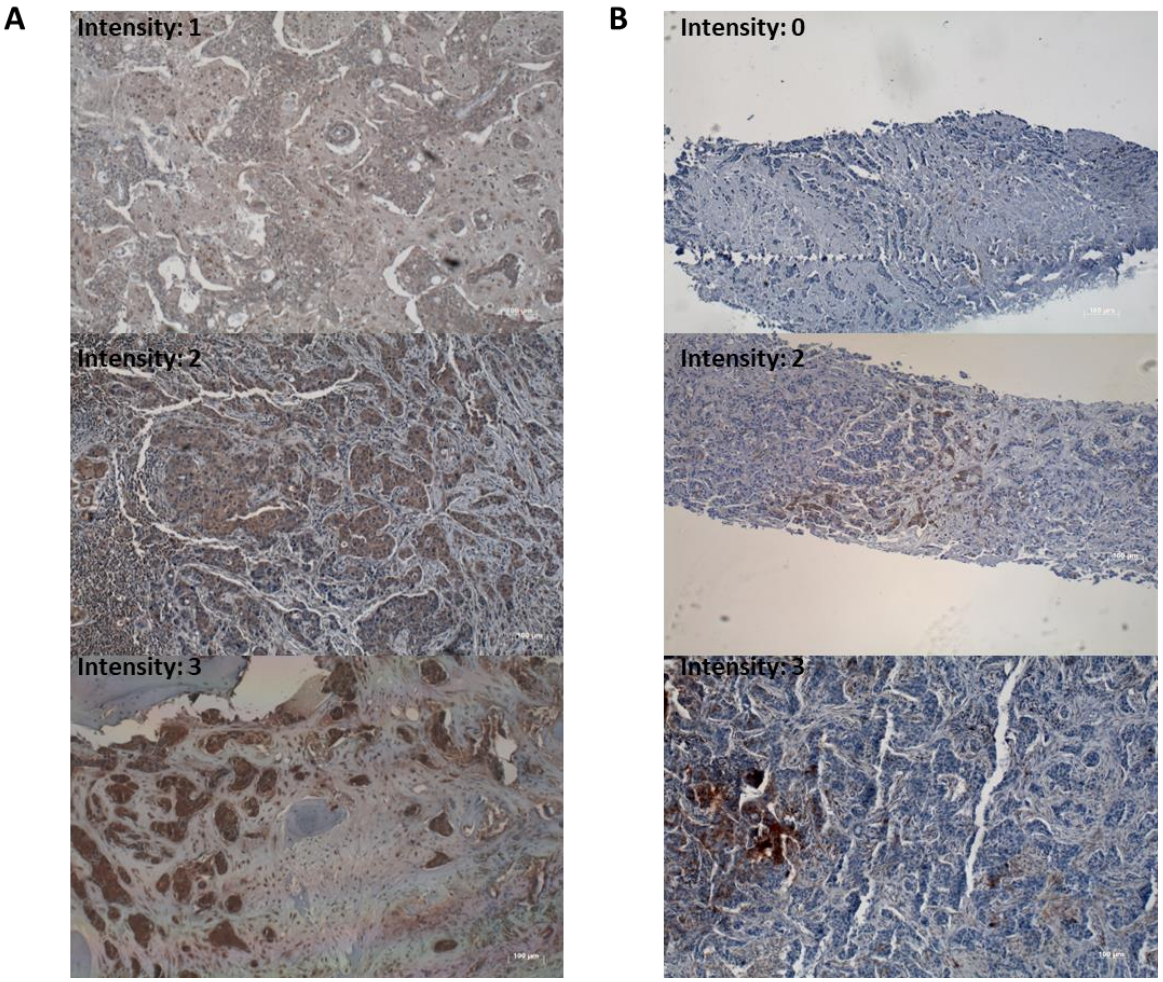

Figure 4.2. Images of immunohistochemical staining in metastatic breast cancer tissue for (A) IFNβ1 and (B) MX1, representative of tissues typical of each intensity scoring. MX1 intensity = 1 is not represented as no examples are found within the cohort.

Parameters were developed to score expression of IFNβ1 and MX1 semi-quantitatively in consultation with Dr Eldo Verghese. For IFNβ1, cancer cells were scored for both intensity and proportion of cancer cells staining positively, as both factors appeared to vary between tissues. Intensity was measured on a scale of 1-3 (1 = weak, 2 = intermediate, 3 = strong) (Figure 4.2a). The proportion of cells stained was also measured on a scale of 1-3 with defined percentage cut offs for each score (1 = 1 - 50 %, 2 = 51 - 75 %, 3 = >75 %). Scores for intensity and proportion were then added together to produce a score of 2 to 6. Similarly, MX1 was

scored for both intensity and portion of cells stained, however scoring for intensity was on a scale of 0-3 as some tumours showed no MX1 expression (0 = no staining, 1 = weak, 2 = intermediate, 3 = strong) (Figure 4.2b). Cut offs for staining coverage also differed from IFNβ1 scoring as MX1 was typically expressed at lower levels (0 = no staining, 1 = <15 %, 2 = 16 – 30 %, 3 = > 30 %). Scores for MX1 were also combined to produce a score of 0 to 6. Expression of IFNβ1 and MX1 also showed variability in cancer-associated fibroblasts and lymphocytes. Scoring was performed individually for these two cell types for proportion of cells stained on a scale of 0-2 (0 = no staining, 1 = < 50 %, 2 = > 50 %) (Figure 4.3). Having outlined a scoring strategy, the full cohort was scored by eye using a light microscope.

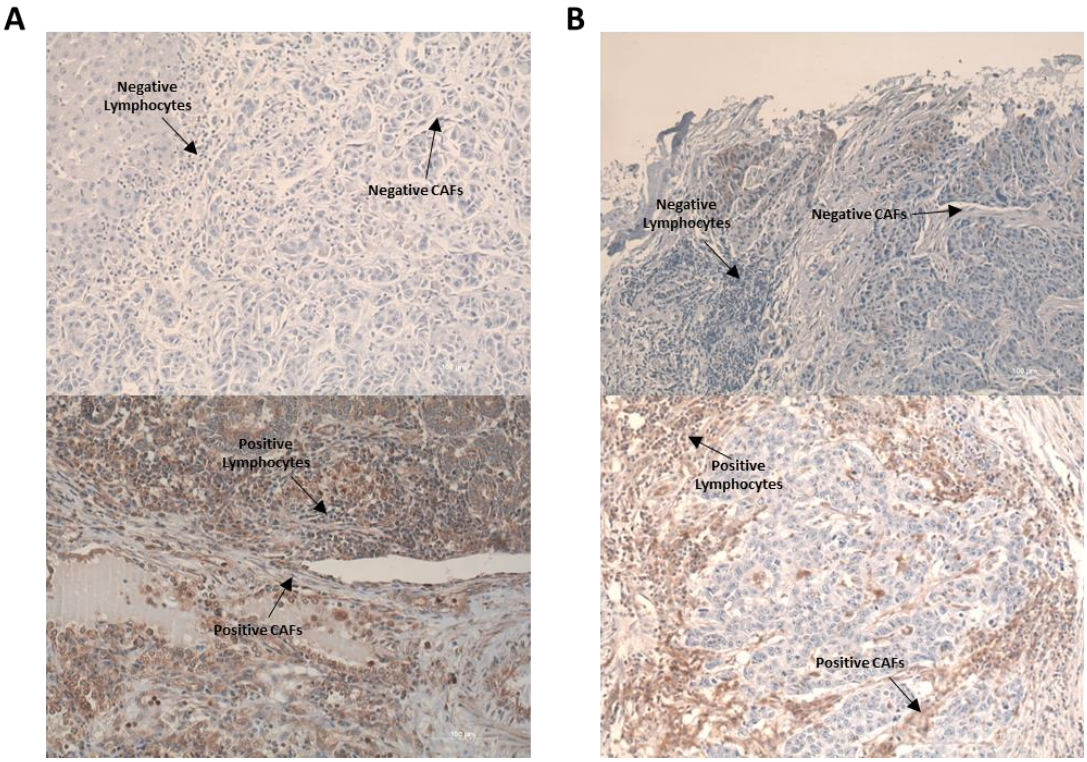

Figure 4.3. Representative Images of lymphocytes and fibroblasts which are expressing or not expressing (A) IFNβ1 and (B) MX1.

Once scored, expression scores were validated by assessing the agreement between two independent scorers (myself and Dr Eldo Verghese). 24 % of the tissues were independently scored by Dr Eldo Verghese for expression of both IFNβ1 and MX1 and inter-scorer concordance was assessed through the Cohen's Kappa statistical method, which considers both relative observer agreement and the probability of chance agreement. For IFNβ1 expression, Cohen's Kappa statistics showed substantial agreement for both intensity and

coverage (0.71 and 0.66, respectively). Similarly, MX1 expression analysis showed a substantial agreement in inter-scorer concordance in both intensity and coverage (0.81 and 0.66 respectively).

Both IFNβ1 and MX1 were expressed at a wide range of levels in both cancer and stromal cells. Distribution of scores for metastatic and primary tumours are found in Figures 4.4 and 4.5 respectively.

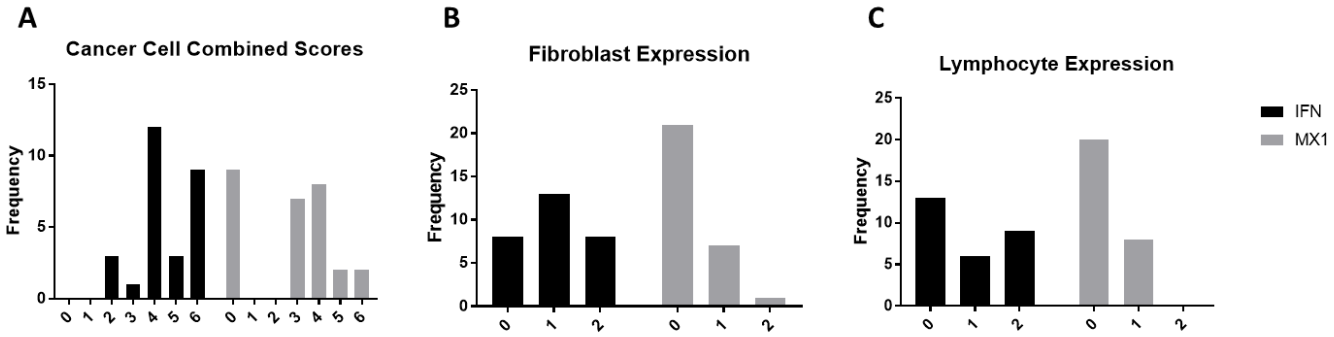

Figure 4.4. Score distributions of IFN β1 (black) and MX1 (grey) in the metastatic TNBC cohort, in (A) cancer cell, (B) fibroblasts and (C) lymphocytes

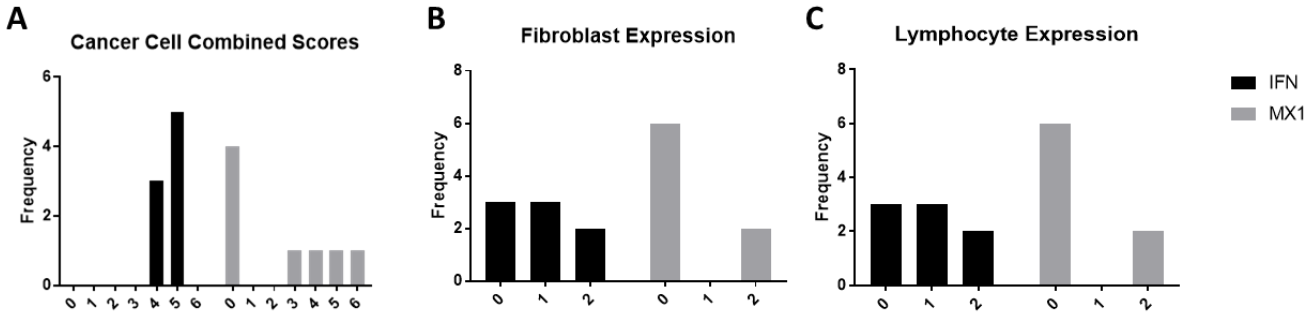

Figure 4.5. Score distributions of IFN β1 (black) and MX1 (grey) in paired primary breast cancer tissues of the metastatic TNBC cohort in (A) cancer cells, (B) fibroblasts and (C) lymphocytes

4.3.3. Expression of INFβ1 in lymphocytes correlates with MX1 expression in metastatic breast cancer cells

To determine the source of IFNβ1 that induces IFN signalling in metastatic breast cancer cells, IFNβ1 expression in lymphocytes, fibroblasts and cancer cells was correlated with MX1 expression in cancer cells using Spearman's rho. IFNβ1 expression in cancer cells showed no correlation with MX1 expression in cancer cells ($r = -0.015$; $p = 0.939$). Similarly, no significant

correlation was observed between IFNβ1 expression in fibroblasts and MX1 expression in cancer cells (r = 0.247; p = 0.206). However, analysis in stromal cells showed a significant positive correlation between IFNβ1 expression in lymphocytes and MX1 expression in metastatic cancer cells (r= 0.376; p = 0.049).

This analysis was then reperformed in primary breast cancer tissues. No correlation was observed between lymphocyte or cancer cell IFNβ1 expression and MX1 expression in cancer cells (r = 0.315; p = 0.443 and r = 0.00; p = 0.771, for lymphocytes and cancer cells respectively). However, a correlation was seen between fibroblast IFNβ1 and cancer cell MX1 expression which was close to significance (r = 0.685; p = 0.086).

To determine whether IFN signalling in cancer or stromal cells from primary tumours persisted in the metastatic tumours, Spearman's Rho correlations were performed for expression of IFNβ1 or MX1 between cells of the same type in paired primary and metastatic tumours. No significant correlation was observed in IFNβ1 or MX1 expression between primary cells of any tested cell type with paired metastatic cells of the same type (Table 4.2).

Table 4.2. Spearman's Rho correlations of the expression of IFNβ1 or MX1 in paired primary and metastatic tumour cells of the same cell type.

		r	p
IFNβ1	**Cancer cells**	-0.062	0.940
	Fibroblasts	-0.412	0.381
	Lymphocytes	0.338	0.452
MX1	**Cancer cells**	0.150	0.735
	Fibroblasts	-0.189	1.000
	Lymphoctes	0.661	0.222

4.3.4 Lymphocyte IFNβ1 and cancer cell MX1 expression does not correlate with survival in a metastatic TNBC cohort

As a significant correlation was observed between IFN β1 in lymphocytes and MX1 expression in metastatic breast cancer cells, Kaplan-Meier survival analysis was performed to identify any association between expression and patient survival. For this analysis, the cohort was

separated into high and low expression of lymphocyte IFNβ1 and cancer cell MX1. Tissues with an IFNβ1 expression score of 0 in lymphocytes were considered low-expressing whereas those with expression of 1-2 were considered high-expressing. MX1 expression ≤ 3 in metastatic cancer cells was considered low whilst expression > 3 was considered high. Kaplan-Meier curves were then produced for overall survival (OS), progression-free survival (PFS), and post-metastasis survival (PMS). No significant difference in OS, PFS or PMS was observed between high and low IFNβ1 or MX1 expressing groups (Figure 4.6).

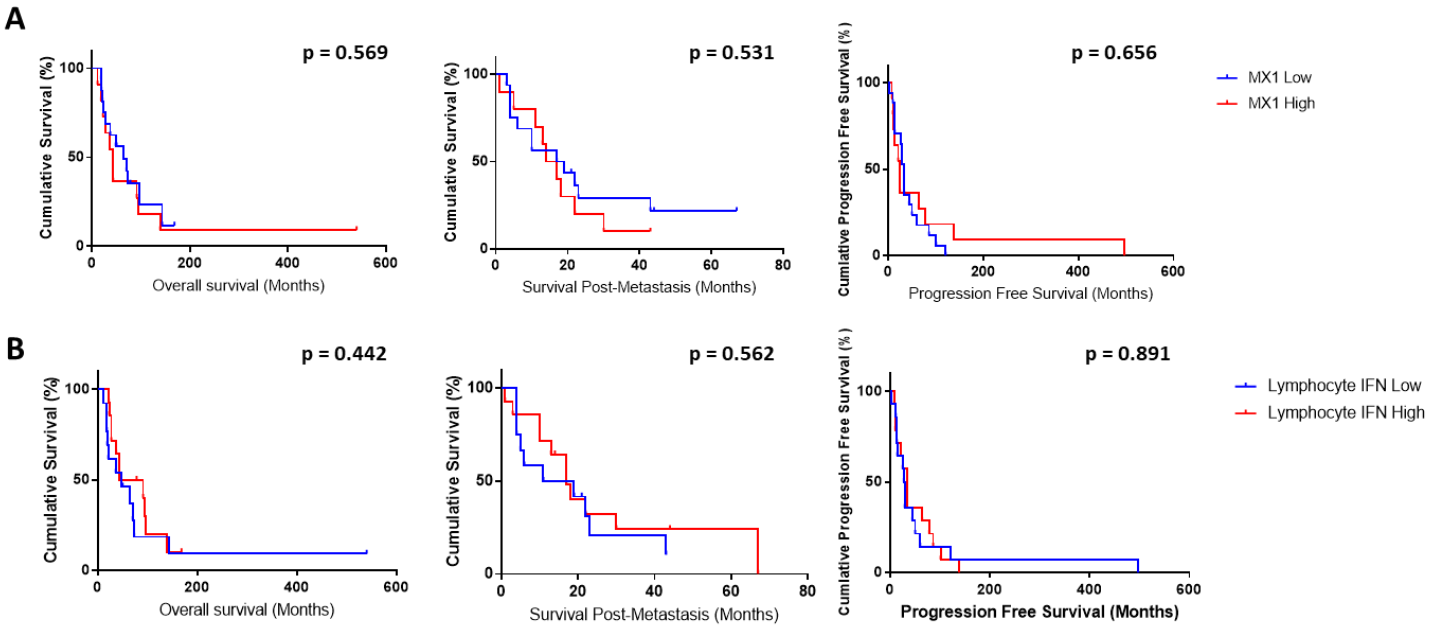

Figure 4.6. Kaplan-Meier analysis of breast cancer survival outcomes (overall survival, survival post-metastasis and progression-free survival) in metastatic TNBC cancer patients (n= 27). Patients were separated into groups defined by (A) expression of MX1 in metastatic cancer cells or (B) expression of IFNβ1 in tumour infiltrating lymphocytes as determined by immunohistochemically analysis.

As seen in chapter three, IFN-dependant resistance is apparently caused by a reduction in chemotherapy-induced DNA damage. I therefore reperformed Kaplan-Meier analysis including only patients treated with DNA-damaging agents during treatment of metastatic tumours. Like the full cohort, no significant change in PMS was observed between groups for IFN β1 ($p = 0.77$) or MX1 ($p = 0.31$) (Figure 4.7).

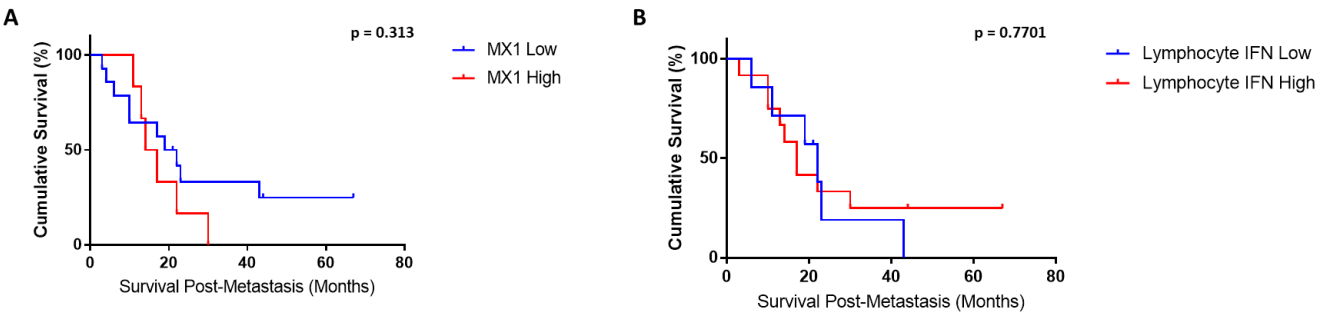

Figure 4.7. Kaplan-Meier analysis of survival post-metastasis in metastatic TNBC cancer patients treated with DNA-damaging agents as part their treatment of metastatic breast cancer (n = 20). Patients were separated into groups defined by (A) expression of MX1 in metastatic cancer cells or (B) expression of IFNβ1 in tumour infiltrating lymphocytes as determined by immunohistochemically analysis.

As no correlation was observed between expression of IFNβ1 or MX1 and patient survival, I investigated the ability of tumour grade, a well-established prognostic marker in breast cancer, to predict overall survival in this cohort through Kaplan-Meier survival analysis (Figure 4.8). Although patients with grade 3 tumours appeared to have worse overall survival (note the steep decrease in survival in this group relative to the grade 2 tumour group in Figure 4.8) no significant difference in OS was identified (p = 0.667).

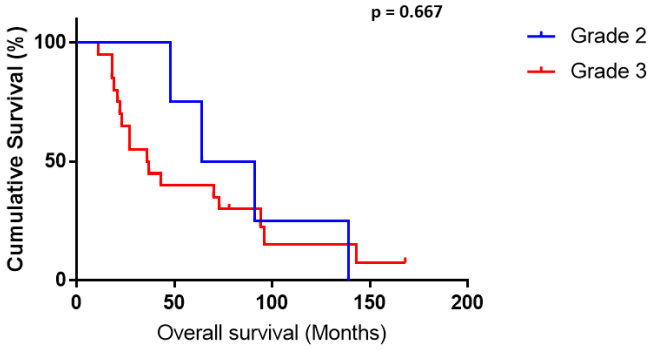

Figure 4.8. Kaplan-Meier analysis of breast cancer overall survival in metastatic TNBC cancer patients (n= 25). Patients were separated into groups defined by the grade of the primary tumour.

4.3.5 CAF IFNβ1 and cancer cell MX1 expression do not correlate with survival in primary breast cancer tissue

In paired primary TNBC tumours IFN β1 expression in CAFs showed a correlation with MX1 expression in cancer cells which was close to significance. Moreover, significant correlation between CAF IFNβ1 expression and cancer cell MX1 expression has been observed in a previous study using a larger cohort [120]. Association between expression of these two markers and survival was therefore analysed using Kaplan-Meier survival analysis (expression cut offs for MX1 are the same as those in section 4.3.4; cut-offs for IFNβ1 in CAFs are the same as those in lymphocytes in section 4.3.4). No significant difference in OS or PFS was observed between high and low expressing IFN β1 or MX1 groups (Figure 4.9).

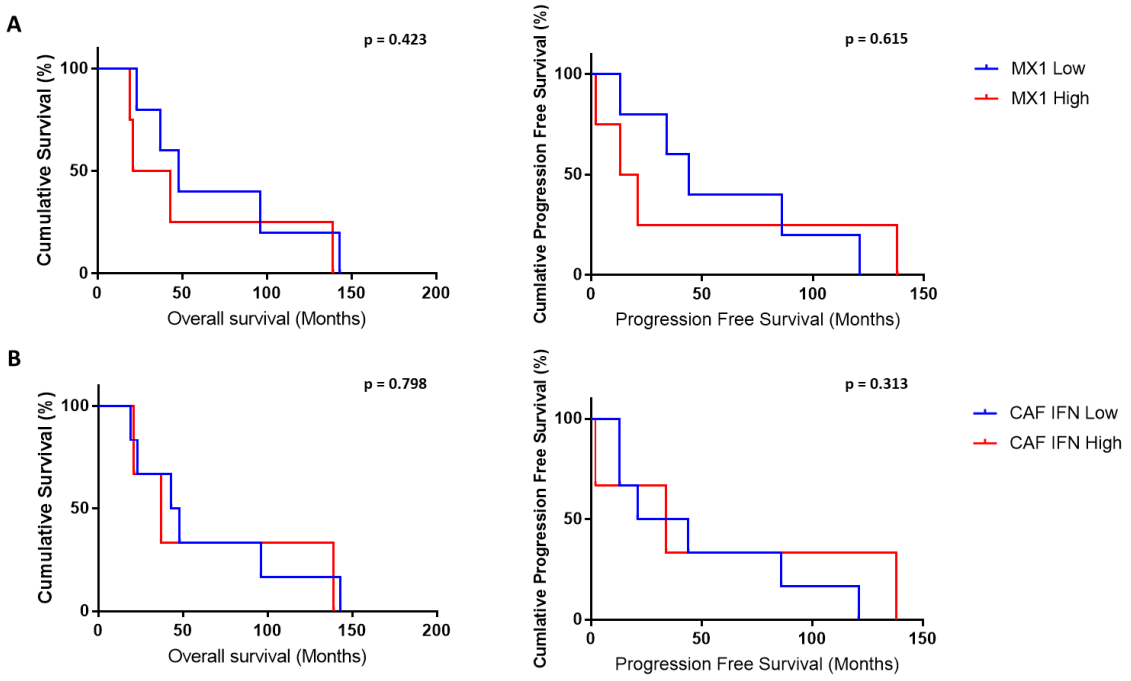

Figure 4.9. Kaplan-Meier analysis of breast cancer overall survival and progression-free survival in primary TNBC cancer patients (n = 9). Patients were separated into groups defined by (A) expression of MX1 in metastatic cancer cells or (B) expression of IFNβ1 in tumour infiltrating lymphocytes as determined by immunohistochemically analysis.

4.4 Discussion

Identification of prognostic markers in metastatic breast cancer may lead to improved treatment options and longer overall survival in this group. Active paracrine type-I IFN signalling has been previously identified in a cohort of 109 primary TNBC patients and was significantly associated with poor disease-free survival [120]. In this chapter, I therefore assessed the presence of active IFN signalling in metastatic breast cancer and evaluated its potential as a prognostic marker in this group, through a preliminary, hypothesis generating study.

4.4.1 Active type-I IFN signaling occurs between lymphocytes and cancer cells in metastatic TNBC

Previous work by the Hughes group has shown a significant correlation between IFNβ1 expression in CAFs and MX1 expression in primary TNBC cells, suggesting active paracrine signalling between these cell types [120]. In this chapter, a similar correlation was also observed in primary TNBC tumours, however this correlation was found to be not significant by a small margin ($r = 0.685$; $p = 0.086$). The lack of significance in this study may have been due to the substantially smaller cohort of primary tissues ($n = 9$). Considering this, the correlation between IFNβ1 expression in CAFs and MX1 expression in primary breast cancer cells is in agreement with that of previous research but may require a larger cohort for statistical conformation.

In the context of metastasis, this chapter showed no association between CAF IFNβ1 expression and metastatic cancer cell MX1 expression, suggesting this paracrine signalling axis may not persist in metastatic breast cancer. Evaluation of metastatic CAF expression of type-I IFNs is scarce within the literature. However, transcriptomic analysis of CAFs originating from primary and metastatic tumours, showed IFN signalling was the most prominently up-regulated pathway in metastatic CAFs relative to primary CAFs. Moreover, metastatic CAFs cocultured with breast cancer cell lines induce a greater increase in resistance to doxorubicin in cancer cells relative to coculture with primary CAFs [129]. However, no direct association was made between CAF-dependant paracrine IFN signalling and chemoresistance suggesting further research into the implications of this signalling axis in metastatic chemoresistance is required.

Intriguingly, the IFNβ1 expression of lymphocytes in the TME was significantly correlated with MX1 expression in metastatic breast cancer cells. This suggests the source of IFN may shift from CAFs to lymphocytes in the metastatic TME. In primary breast cancer, lymphocyte infiltration of tumours is associated with improved prognosis, particularly in TNBC [192]. However, the role of lymphocytes in the metastatic setting is less clear. A study of 85 metastatic breast cancer tumours showed a significant association between increased tumour infiltrating lymphocyte abundance and improved prognosis [193, 194]. Contradictory to this, no association was found between T-lymphocyte abundance and prognosis in a cohort of 34 metastatic patients, however, the abundance of regulatory T-lymphocytes (Tregs) was significantly associated with worse post-recurrence survival [134]. This suggest specific lymphocyte subtypes contribute to a pro-tumorigenic state of the TME however, as in this study, the cohort size for this analysis was limited.

In this chapter, neither lymphocyte IFNβ1 expression or cancer cell MX1 expression correlated with OS, PFS or PMS in this cohort. Although this could suggest IFN signalling has no effect on the progression of metastatic breast cancer, this study was limited in statistical power due to the low number of patients in this cohort, which may reduce the likelihood of identifying significant associations. The statistical power of OS and PMS associations were further reduced as 5 patients were alive and were therefore censored during analysis. The low power in this study is exemplified by the lack of significant differences in OS between high- and low-grade tumours (Figure 4.8), which is a well-established prognostic marker in breast cancer [195].

Broad *et al.* showed paracrine IFN signalling was associated with poor disease-free survival in claudin-low but not claudin-high TNBC [120]. In this chapter, patients were not separated based on the expression of claudin-3, as in the latter study, and therefore the cohort likely consisted of a mix of claudin-low and claudin-high TNBCs. This may have further reduced the strength of associations between IFN signalling and survival, and separation of these groups may be required in future studies.

As well as the size of the cohort, the distinct characteristics of different metastatic sites may affect the contribution of IFN signalling to cancer progression and thus reduce the strength of associations between markers and patient survival. For example, during gene expression profiling, CAFs taken from breast cancer metastases of the skin and lungs formed a distinct

cluster from those taken from metastasis of the liver, suggesting they may behave differently within the TME [129]. Moreover, the ratio of CD8+ and $FOXP_3$+ lymphocytes (Tregs) was found to differ between sites of breast cancer metastasis, with skin metastasis showing significantly higher abundance of $FOXP_3$+ lymphocytes than other sites [196].

It should also be noted, other cells of the TME were not considered in this study including tumour associated macrophages (TAMs) and tumour infiltrating neutrophils (TINs). This was partially due to the difficulty of accurately identifying these cell types in tissues without additional cell-specific immunohistochemical markers. In breast cancer, a greater abundance of M2-like macrophages was identified in metastatic tumours relative to paired primary tumours in a cohort of 49 patients [133]. M2-like macrophages have been shown to have pro-tumorigenic properties and their abundance in the TME has been associated with poor survival in multiple breast cancer studies [197]. Additionally, TAMs have been shown to increase breast cancer resistance to taxols *in vivo* as well as increased resistance to doxorubicin in co-culture models [198]. Similarly, TINs have been associated with tumour progression and increased risk of metastasis [199]. In breast cancer high abundance of TINs, post neo-adjuvant therapy, was significantly associated with poor prognosis suggesting they may also play a role in therapy resistance [200]. As both cell types have been associated with poor prognosis and treatment resistance in breast cancer, these cell types should be considered in future investigations of IFN-dependant resistance in metastatic tumours.

The expression of IFN signalling markers was also not considered in metastasis-site specific cells due to the limited number of tumours from each metastatic site in this cohort. However, multiple site-specific cells have been implicated in metastatic breast cancer progression [127]. Cancer cells in bone metastasis have been shown to increase osteoclast activity and bone resorption leading to release of TGFβ which has pro-tumorigenic affects [201]. Moreover, Kupffer cells have also been shown to secrete growth factors and inflammatory cytokines promoting the establishment of metastasis in the liver [202]. Consideration of these cells in future studies may identify further sources of IFN paracrine signalling in breast cancer metastasis.

4.4.2 Conclusions

This chapter has shown IFN signalling occurs within the TME of metastatic TNBC breast cancer, although was unable to identify any associations between IFN signalling and survival

in this context. As a hypothesis generating study, this was expected due to the small cohort and therefore low power of statistical analysis. However, correlations between expression of type-I IFNs in lymphocytes and MX1 expression in metastatic cancer cells suggests this pathway may still influence cancer progression within metastatic TNBC breast cancer. Further studies, into the influence of this paracrine signalling pathway on metastatic breast cancer outcomes should therefore be performed using larger cohorts. In future studies, the expression of claudin-3 should also be considered as previous studies have associated IFN-dependant resistance with claudin-low TNBCs specifically. Additionally, the role of metastasis-site specific cells as well as further immune cells of the TME should be considered in future studies due to their role in chemoresistance found in the literature. Further investigation of IFN signalling in metastatic breast cancer patients may lead to new treatment strategies and improved response to chemotherapy in this group.

5 Discussion

Despite the development of targeted therapies that have improved survival in several breast cancer subtypes, a third of patients still rely on systemic chemotherapy as a component of their treatment for breast cancer [28]. The development of resistance to chemotherapy is the most common cause of relapse and metastasis, which is not considered curable using current treatments; therefore, resistance presents a major obstacle in breast cancer treatment [96]. Although, several new targeted therapies are currently being introduced, such as PD-L1 inhibitors and CDK 4/6 inhibitors, these are not applicable in all cases and developing new target treatments can take a long time [203, 204]. An alternative solution is to inhibit resistance pathways to sensitise cells to chemotherapy and improve treatment response using drugs that are already approved for clinical use, thus reducing the time taken in clinical testing. To achieve this, it is therefore essential to identify chemoresistance pathways which can be inhibited to improve treatment response.

5.1 Type-I IFN-dependant resistance in breast cancer subtypes and metastasis

This thesis showed IFN-dependant resistance, identified in claudin-low TNBC, is subtype dependant and may not be induced in other subtypes. For example, IFN-induced resistance was not evident in the luminal A breast cancer cell line, MCF-7. Additionally, in a cell line that is representative of the HER2-enriched subtype, AU565, type-I IFN induced dose dependant chemosensitisation, as opposed to resistance. Due to the heterogeneity in response of different breast cancer subtypes, biomarkers to identify IFN-dependant resistant tumours will be essential in identifying patients who will benefit from additional sensitisation therapies (see section 5.2 below).

Additionally, clinical evaluation showed paracrine type-I IFN signalling is active within the metastatic TME of TNBC but failed to show an association between this signalling and survival. It should be noted, clinical evaluation of this resistance pathway in metastasis was only performed in TNBC. Although IFN-dependant resistance was not found *in vitro* in luminal A and HER2-enriched breast cancer cell lines, this resistance pathway may still occur within metastasis of these subtypes. Genetic and expression analysis of paired primary and metastatic breast cancer cells have shown them to be distinct, which may influence their response to treatment [205-207]. Moreover, cells of the TME are distinct within primary and metastatic tumours both in abundance and expression profiles [129, 133, 208]. Transcriptome

analysis of CAFs from paired primary and metastatic tumours showed IFN signalling to be the most upregulated pathway in metastatic verses primary CAFs, suggesting this signalling pathway can be increased in the metastatic TME [129]. As a result, evaluation of this signalling pathway in metastasis should be expanded to other breast cancer subtypes.

5.2 Targeting Type-I IFN signalling during chemotherapy

In this thesis I have shown type-I IFNs are capable of inducing anthracycline resistance in claudin-low TNBC breast cancer cell line, MDA-MB-231 through *in vitro* viability assays. This therefore presents an opportunity for intervention, through inhibition of IFN signalling in order to sensitise claudin-low TNBC cells to chemotherapy.

Several inhibitors of JAK/STAT signalling have already been developed, such as ruxolitinib which is currently used in the treatment of myelofibrosis and graft vs host disease [209]. As ruxolitinib has already been approved for clinical use with established pharmacokinetics and pharmacodynamics, combination treatments may be developed relatively quickly to improve treatment response in these patients [210].

Additionally, several clinical trials have previously investigated the use of ruxolitinib in breast cancer and have shown it is well tolerated in this context [211]. Ruxolitinib as a monotherapy was well tolerated in patients with STAT3-positive metastatic TNBC and showed on-target activity assessed through expression of JAK-STAT target genes in a non-randomised phase II clinical trial. However, this study was discontinued as it failed to show clinical response [212]. In the context of combination therapies, a randomised phase II clinical trial assessing ruxolitinib in combination with capecitabine, in patients with HER2-negative breast cancer, showed that ruxolitinib was generally tolerable, however showed no improvement in OS or PFS relative to the capecitabine plus placebo arm [213]. Similarly, ruxolitinib in combination with weekly paclitaxel was well tolerated and showed evidence of clinical activity in a phase I clinical trial [214]. Several additional clinical trials are also either recruiting or active in the investigation of ruxolitinib in breast cancer treatment (NCT03012230 and MCT02876302).

Although clinical trials have shown little evidence of clinical activity of ruxolitinib in chemosensitisation, based on the results of this thesis, this may be improved by targeting these treatments to those with claudin-low TNBC. Additionally, this thesis has shown IFN-dependant resistance to epirubicin but not docetaxel suggesting combination of JAK

inhibitors with anthracyclines but not taxanes may be beneficial in these patients. In future studies, evaluation of the ability of ruxolitinib and other JAK inhibitors to inhibit IFN-dependant resistance *in vitro* and *in vivo* should be assessed in the context of claudin-low TNBC, with the intention of progressing combination treatments to clinical trials. Furthermore, as this thesis has shown paracrine IFN signalling to be active in metastatic TNBC (specifically between lymphocytes and cancer cells), clinical studies, through immunohistochemical evaluation of IFN signalling markers, should be performed with a larger cohort to assess the relevance of this pathway in metastatic TNBC.

5.3 Conclusion

In conclusion, type-I IFNs are capable of inducing resistance to chemotherapy *in vitro* in a subtype and chemotherapeutic dependent manner. The results of this thesis suggests targeting IFN inhibiting therapies to claudin-low TNBC patients in the context of anthracycline treatment may sensitise patients to chemotherapy and improve therapy response. Moreover, paracrine type-I IFN signalling was shown to persist in metastatic TNBC, although the origin of type-I IFNs may differ between primary and metastatic tumours. Further investigation of this pathway using a larger cohort is required to determine the effects of this signalling axis on survival in metastatic breast cancer.

6. References

1. Sung, H., *et al.*, *Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* Ca-a Cancer Journal for Clinicians, 2021. **71**(3): p. 209-249.
2. O'Driscoll, L. and M. Clynes, *Biomarkers and multiple drug resistance in breast cancer.* Current Cancer Drug Targets, 2006. **6**(5): p. 365-384.
3. DeSantis, C.E., *et al.*, *Breast cancer statistics, 2019.* Ca-a Cancer Journal for Clinicians, 2019. **69**(6): p. 438-451.
4. Min, N.N., *et al.*, *Advancement of prognostic models in breast cancer: a narrative review.* Gland Surgery, 2021. **10**(9): p. 2815-2831.
5. Ronchi, A., *et al.*, *Current and potential immunohistochemical biomarkers for prognosis and therapeutic stratification of breast carcinoma.* Seminars in Cancer Biology, 2021. **72**: p. 114-122.
6. Rakha, E.A., *et al.*, *Breast cancer prognostic classification in the molecular era: the role of histological grade.* Breast Cancer Research, 2010. **12**(4).
7. O'Donnell, M., *et al.*, *Non-epithelial malignancies and metastatic tumours of the breast.* Ulster Med J., 2009. **78**: p. 2.
8. Makki, J., *Diversity of Breast Carcinoma: Histological Subtypes and Clinical Relevance.* Clinical Medicine Insights- Pathology, 2015. **8**.
9. Sinn, H.P. and H. Kreipe, *A Brief Overview of the WHO Classification of Breast Tumors, 4th Edition, Focusing on Issues and Updates from the 3rd Edition.* Breast Care, 2013. **8**(2): p. 149-154.
10. Seijen, M., *et al.*, *Ductal carcinoma in situ: to treat or not to treat, that is the question.* British Journal of Cancer, 2019. **121**.
11. van der Borden, C.L., *et al.*, *Avoiding Overtreatment of Ductal Carcinoma in situ.* Trends in Cancer, 2019. **5**(7): p. 391-393.
12. Wallis, M.G., *Addressing the overtreatment of DCIS: the LORIS trial.* Breast Cancer Research and Treatment, 2020. **180**(2): p. 590-590.
13. Decker, T., D. Hungermann, and W. Bocker, *Prognostic and predictive factors of invasive breast cancer.* Pathologe, 2009. **30**(1): p. 49-55.
14. Haybittle, J.L., *et al.*, *A Prognostic Index in Primary Breast Cancer.* British Journal of Cancer, 1982. **45**(3): p. 361-366.

15. Rakha, E.A., *et al.*, *Prognostic significance of Nottingham histologic grade in invasive breast carcinoma.* Journal of Clinical Oncology, 2008. **26**(19): p. 3153-3158.
16. van Dooijeweert, C., P.J. van Diest, and I.O. Ellis, *Grading of invasive breast carcinoma: the way forward.* Virchows Archiv, 2022. **480**(1): p. 33-43.
17. van Dooijeweert, C., *et al.*, *Significant inter- and intra-laboratory variation in grading of invasive breast cancer: A nationwide study of 33,043 patients in the Netherlands.* International Journal of Cancer, 2020. **146**(3): p. 769-780.
18. Plichta, J.K., *Breast cancer prognostic staging and internal mammary lymph node metastases: a brief overview.* Chinese Clinical Oncology, 2019. **8**.
19. Narod, S.A., *Tumour size predicts long-term survival among women with lymph node-positive breast cancer.* Current Oncology, 2012. **19**(5): p. 249-+.
20. Rosen, P.P., *et al.*, *Factors Influencing Prognosis in Node-Negative Breast Carcinoma - Analysis of 767 T1N0m0/T2N0M0 Patients with Long-Term Follow-up.* Journal of Clinical Oncology, 1993. **11**(11): p. 2090-2100.
21. Carter, C.L., C. Allen, and D.E. Henson, *Relation of Tumor Size, Lymph-Node Status, and Survival in 24.740 Breast Cancer Cases.* Cancer, 1989. **63**(1): p. 181-187.
22. Cianfrocca, M. and L.J. Goldstein, *Prognostic and predictive factors in early-stage breast cancer.* Oncologist, 2004. **9**(6): p. 606-616.
23. Solak, M., *et al.*, *The lymph node ratio as an independent prognostic factor for non-metastatic node-positive breast cancer recurrence and mortality.* Journal of Buon, 2015. **20**(3): p. 737-745.
24. Hetterich, M., *et al.*, *Adjuvant chemotherapy for breast cancer patients with axillary lymph node micrometastases.* Breast Cancer Research and Treatment, 2021. **187**(3): p. 715-727.
25. Park, M., *et al.*, *Breast Cancer Metastasis: Mechanisms and Therapeutic Implications.* International Journal of Molecular Sciences, 2022. **23**(12).
26. Wu, Q., *et al.*, *Breast cancer subtypes predict the preferential site of distant metastases: a SEER based study.* Oncotarget, 2017. **8**(17): p. 27990-27996.
27. Gote, V., *et al.*, *Drug Resistance in Metastatic Breast Cancer: Tumor Targeted Nanomedicine to the Rescue.* International Journal of Molecular Sciences, 2021. **22**(9).
28. Rivera, E. and H. Gomez, *Chemotherapy resistance in metastatic breast cancer: the evolving role of ixabepilone.* Breast Cancer Research, 2010. **12**.

29. Bertucci, F., *et al.*, *Genomic characterization of metastatic breast cancers.* Nature, 2019. **569**(7757): p. 560-+.

30. Goldhirsch, A., *et al.*, *Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013.* Annals of Oncology, 2013. **24**(9): p. 2206-2223.

31. Perou, C.M., *et al.*, *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-752.

32. Sorlie, T., *et al.*, *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(19): p. 10869-10874.

33. Sorlie, T., *et al.*, *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(14): p. 8418-8423.

34. Koboldt, D.C., *et al.*, *Comprehensive molecular portraits of human breast tumours.* Nature, 2012. **490**(7418): p. 61-70.

35. Puppe, J., *et al.*, *Genomic Signatures in Luminal Breast Cancer.* Breast Care, 2020. **15**(4): p. 355-365.

36. Liu, M.C., *et al.*, *PAM50 gene signatures and breast cancer prognosis with adjuvant anthracycline- and taxane-based chemotherapy: correlative analysis of C9741 (Alliance).* Npj Breast Cancer, 2016. **2**.

37. Prat, A., *et al.*, *Clinical implications of the intrinsic molecular subtypes of breast cancer.* Breast, 2015. **24**: p. S26-S35.

38. Dowsett, M., *et al.*, *Assessment of Ki67 in Breast Cancer: Recommendations from the International Ki67 in Breast Cancer Working Group.* Jnci-Journal of the National Cancer Institute, 2011. **103**(22): p. 1656-1664.

39. Johnson, K.S., E.F. Conant, and M.S. Soo, *Molecular Subtypes of Breast Cancer: A Review for Breast Radiologists.* Journal of Breast Imaging, 2021. **3**(1): p. 12-24.

40. Cheang, M.C.U., *et al.*, *Responsiveness of Intrinsic Subtypes to Adjuvant Anthracycline Substitution in the NCIC.CTG MA.5 Randomized Trial.* Clinical Cancer Research, 2012. **18**(8): p. 2402-2412.

41. Metzger, 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.* Journal of Clinical Oncology, 2013. **31**(25): p. 3083-+.

42. Mohammed, A.A., *Prognostic parameter differences in breast cancer patients between luminal A and luminal B types after application of the new classification according to Ki67 score.* International Journal of Surgery Open, 2021. **34**.

43. Yersal, O. and S. Barutca, *Biological Subtypes of Breast Cancer: Prognostic and Therapeutic Implications.* World Journal of Clinical Oncology, 2014. **5**(3).

44. Sestak, I., *et al.*, *Factors Predicting Late Recurrence for Estrogen Receptor-Positive Breast Cancer.* Jnci-Journal of the National Cancer Institute, 2013. **105**(19): p. 1504-1511.

45. Garcia-Alvarez, A., A. Papakonstantinou, and M. Oliveira, *Brain Metastases in HER2-Positive Breast Cancer: Current and Novel Treatment Strategies.* Cancers, 2021. **13**(12).

46. Brosnan, E.M. and C.K. Anders, *Understanding patterns of brain metastasis in breast cancer and designing rational therapeutic strategies.* Annals of Translational Medicine, 2018. **6**(9).

47. Perez, E.A., *et al.*, *Trastuzumab Plus Adjuvant Chemotherapy for Human Epidermal Growth Factor Receptor 2-Positive Breast Cancer: Planned Joint Analysis of Overall Survival From NSABP B-31 and NCCTG N9831.* Journal of Clinical Oncology, 2014. **32**(33): p. 3744-+.

48. Derakhshani, A., *et al.*, *Overcoming trastuzumab resistance in HER2-positive breast cancer using combination therapy.* Journal of Cellular Physiology, 2020. **235**(4): p. 3142-3156.

49. Nedeljkovic, M. and A. Damjanovic, *Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge.* Cells, 2019. **8**(9).

50. Abad, M.N., et al., *Update on systemic treatment in early triple negative breast cancer.* Therapeutic Advances in Medical Oncology, 2021. **13**.

51. Dent, R., *et al.*, *Triple-negative breast cancer: Clinical features and patterns of recurrence.* Clinical Cancer Research, 2007. **13**(15): p. 4429-4434.

52. Bianchini, G., *et al.*, *Treatment landscape of triple-negative breast cancer - expanded options, evolving needs.* Nature Reviews Clinical Oncology, 2022. **19**(2): p. 91-113.

53. Hennessy, B.T., *et al.*, *Characterization of a Naturally Occurring Breast Cancer Subset Enriched in Epithelial-to-Mesenchymal Transition and Stem Cell Characteristics.* Cancer Research, 2009. **69**(10): p. 4116-4124.

54. Prat, A., *et al.*, *Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer.* Breast Cancer Research, 2010. **12**(5).

55. Tang, Y.H., *et al.*, *Neuropilin-1 is over-expressed in claudin-low breast cancer and promotes tumor progression through acquisition of stem cell characteristics and RAS/MAPK pathway activation.* Breast Cancer Research, 2022. **24**(1).

56. Waks, A.G. and E.P. Winer, *Breast Cancer Treatment A Review.* Jama-Journal of the American Medical Association, 2019. **321**(3): p. 288-300.

57. Li, W.H., *et al.*, *Breast-conserving therapy versus mastectomy for breast cancer: a ten-year follow-up single-center real-world study.* Gland Surgery, 2022. **11**(7): p. 1148-1165.

58. Zumsteg, Z.S., *et al.*, *Breast-Conserving Therapy Achieves Locoregional Outcomes Comparable to Mastectomy in Women with T1-2N0 Triple-Negative Breast Cancer.* Annals of Surgical Oncology, 2013. **20**(11): p. 3469-3476.

59. Keelan, S., M. Flanagan, and A.D.K. Hill, *Evolving Trends in Surgical Management of Breast Cancer: An Analysis of 30 Years of Practice Changing Papers.* Frontiers in Oncology, 2021. **11**.

60. Liu, Y.P., *et al.*, *Molecular mechanisms of chemo- and radiotherapy resistance and the potential implications for cancer treatment.* Medcomm, 2021. **2**(3): p. 315-340.

61. Hortobagyi, G., *Adjuvant therapy for breast cancer.* Annual Review of Medicine, 2000. **51**: p. 377-392.

62. Matuschek, C., *et al.*, *The benefit of adjuvant radiotherapy after breast conserving surgery in older patients with low risk breast cancer- a meta- analysis of randomized trials.* Radiation Oncology, 2017. **12**.

63. Van de Steene, J., G. Soete, and G. Storme, *Adjuvant radiotherapy for breast cancer significantly improves overall survival: the missing link.* Radiotherapy and Oncology, 2000. **55**(3): p. 263-272.

64. Ahmed, M., F. Jozsa, and M. Douek, *A systematic review of neo-adjuvant radiotherapy in the treatment of breast cancer.* Ecancermedicalscience, 2021. **15**.

65. Anampa, J., D. Makower, and J.A. Sparano, *Progress in adjuvant chemotherapy for breast cancer: an overview.* Bmc Medicine, 2015. **13**.

66. Hassan, M.S.U., *et al.*, *Chemotherapy for breast cancer (Review).* Oncology Reports, 2010. **24**(5): p. 1121-1131.

67. Houssami, N., *et al.*, *Meta-analysis of the association of breast cancer subtype and pathologic complete response to neoadjuvant chemotherapy.* European Journal of Cancer, 2012. **48**(18): p. 3342-3354.

68. Mieog, J.S.D., J.A. van der Hage, and C.J.H. van de Velde, *Preoperative chemotherapy for women with operable breast cancer.* Cochrane Database of Systematic Reviews, 2007(2).

69. Alberro, J.A., *et al.*, *Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials.* Lancet Oncology, 2018. **19**(1): p. 27-39.

70. Zarotti, C., *et al.*, *Biomarker dynamics and prognosis in breast cancer after neoadjuvant chemotherapy.* Scientific Reports, 2022. **12**(1).

71. Abe, O., *et al.*, *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet, 2005. **365**(9472): p. 1687-1717.

72. Andreopoulou, E. and J. Sparano, *Chemotherapy in Patients with Anthracycline- and Taxane-Pretreated Metastatic Breast Cancer: An Overview.* Curr Breast Cnacer Rep, 2014. **5**(1).

73. Crown, J., M. O'Leary, and W.S. Ooi, *Docetaxel and paclitaxel in the treatment of breast cancer: A review of clinical experience.* Oncologist, 2004. **9**: p. 24-32.

74. Nabholtz, J.-M., *et al.*, *Evidence for the use of chemotherapy in breast cancer.* 10.1007/s101470200037, 2002. **7**(4).

75. Carrick, S., *et al.*, *Single agent versus combination chemotherapy for metastatic breast cancer.* Cochrane Database of Systematic Reviews, 2009(2).

76. Martins-Teixeira, M.B. and I. Carvalho, *Antitumour Anthracyclines: Progress and Perspectives.* Chemmedchem, 2020. **15**(11): p. 933-948.

77. Beretta, G.L. and F. Zunino, *Molecular mechanisms of anthracycline activity.* Anthracycline Chemistry and Biology Ii: Mode of Action, Clinical Aspects and New Drugs, 2008. **283**: p. 1-19.

78. McClendon, A.K. and N. Osheroff, *DNA topoisomerase II, genotoxicity, and cancer.* Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 2007. **623**(1-2): p. 83-97.

79. Shah, A.N. and W.J. Gradishar, *Adjuvant Anthracyclines in Breast Cancer: What Is Their Role?* Oncologist, 2018. **23**(10): p. 1153-1161.

80. Huober, J., *et al.*, *Effect of neoadjuvant anthracycline-taxane-based chemotherapy in different biological breast cancer phenotypes: overall results from the GeparTrio study.* Breast Cancer Research and Treatment, 2010. **124**(1): p. 133-140.

81. Albain, K., *et al.*, *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**(9814): p. 432-444.

82. Cardinale, D., F. Iacopo, and C.M. Cipolla, *Cardiotoxicity of Anthracyclines.* Frontiers in Cardiovascular Medicine, 2020. **7**.

83. Cai, F.F., *et al.*, *Anthracycline-induced cardiotoxicity in the chemotherapy treatment of breast cancer: Preventive strategies and treatment.* Molecular and Clinical Oncology, 2019. **11**(1): p. 15-23.

84. Maloney, S.M., *et al.*, *Mechanisms of Taxane Resistance.* Cancers, 2020. **12**(11).

85. Field, J.J., A. Kanakkanthara, and J.H. Miller, *Microtubule-targeting agents are clinically successful due to both mitotic and interphase impairment of microtubule function.* Bioorganic & Medicinal Chemistry, 2014. **22**(18): p. 5050-5059.

86. Choi, Y.H. and Y.H. Yoo, *Taxol-induced growth arrest and apoptosis is associated with the upregulation of the Cdk inhibitor, p21(WAF1/CIP1) in human breast cancer cells.* Oncology Reports, 2012. **28**(6): p. 2163-2169.

87. Willson, M.L., *et al.*, *Taxanes for adjuvant treatment of early breast cancer.* Cochrane Database of Systematic Reviews, 2019(9).

88. Gradishar, W.J., *Taxanes for the Treatment of Metastatic Breast Cancer.* Breast Cancer (Auckl), 2012. **6**.

89. Sivasanker, M., *et al.*, *Clinical and pathologic response following taxane based neoadjuvant chemotherapy in locally advanced breast cancer patients in a tertiary care centre in India.* Indian Journal of Cancer, 2016. **53**(2): p. 220-225.

90. Bayraktar, S., *et al.*, *Clinical outcome and toxicity from taxanes in breast cancer patients with BRCA1 and BRCA2 pathogenic germline mutations.* Breast Journal, 2020. **26**(8): p. 1572-1582.

91. Varayathu, H., *et al.*, *Taxane induced pulmonary toxicity in breast cancer.* Journal of Clinical Oncology, 2020. **38**(15).

92. Schneider, B.P., *et al.*, *Neuropathy Is Not Associated With Clinical Outcomes in Patients Receiving Adjuvant Taxane-Containing Therapy for Operable Breast Cancer.* Journal of Clinical Oncology, 2012. **30**(25): p. 3051-3057.

93. O'Shaughnessy, J., *Extending survival with chemotherapy in metastatic breast cancer.* Oncologist, 2005. **10**: p. 20-29.

94. Wang, X., H.Y. Zhang, and X.Z. Chen, *Drug resistance and combating drug resistance in cancer.* Cancer Drug Resistance, 2019. **2**(2): p. 141-160.

95. Mansoori, B., *et al.*, *The Different Mechanisms of Cancer Drug Resistance: A Brief Review.* Advanced Pharmaceutical Bulletin, 2017. **7**(3): p. 339-348.

96. Lainetti, P.D., *et al.*, *Mechanisms of Resistance to Chemotherapy in Breast Cancer and Possible Targets in Drug Delivery Systems.* Pharmaceutics, 2020. **12**(12).

97. Martin, H., L. Smith, and D. Tomlinson, *Multidrug-Resistant Breast Cancer: Current Perspectives.* Breast Cancer (Dove Med Press), 2014. **6**.

98. Boichuk, S., *et al.*, *Establishment and characterization of a triple negative basal-like breast cancer cell line with multi-drug resistance.* Oncology Letters, 2017. **14**(4): p. 5039-5045.

99. Trock, B.J., F. Leonessa, and R. Clarke, *Multidrug resistance in breast cancer: A meta-analysis of MDR1/gp170 expression and its possible functional significance.* Journal of the National Cancer Institute, 1997. **89**(13): p. 917-931.

100. Luo, B., *et al.*, *Cytochrome P450: Implications for human breast cancer.* Oncology Letters, 2021. **22**(1).

101. McFadyen, M.C.E., *et al.*, *Cytochrome P450CYP1B1 protein expression: a novel mechanism of anticancer drug resistance.* Biochemical Pharmacology, 2001. **62**(2): p. 207-212.

102. Marsh, S., *et al.*, *Pharmacogenetic analysis of paclitaxel transport and metabolism genes in breast cancer.* Pharmacogenomics Journal, 2007. **7**(5): p. 362-365.

103. Neophytou, C.M., *et al.*, *Apoptosis Deregulation and the Development of Cancer Multi-Drug Resistance.* Cancers, 2021. **13**(17).

104. Lucantoni, F., *et al.*, *BCL(X)L and BCL2 increase the metabolic fitness of breast cancer cells: a single-cell imaging study.* Cell Death and Differentiation, 2021. **28**(5): p. 1512-1531.

105. Qin, M., *et al.*, *Let-7i Reduces Chemotherapy Resistance in Breast Cancer Cells Through Down-Regulation of K-Ras and Bcl2 Expression.* Chinese journal of cellular and molecular immunology, 2019. **35**(11).

106. Abdel-Fatah, T.M.A., *et al.*, *Bcl2 is an independent prognostic marker of triple negative breast cancer (TNBC) and predicts response to anthracycline combination (ATC) chemotherapy (CT) in adjuvant and neoadjuvant settings.* Annals of Oncology, 2013. **24**(11): p. 2801-2807.

107. Hientz, K., *et al.*, *The role of p53 in cancer drug resistance and targeted chemotherapy.* Oncotarget, 2017. **8**(5): p. 8921-8946.

108. Ungerleider, N.A., *et al.*, *Breast cancer survival predicted by TP53 mutation status differs markedly depending on treatment.* Breast Cancer Research, 2018. **20**.

109. Yang, M.C., *et al.*, *Bim directly antagonizes Bcl-xl in doxorubicin-induced prostate cancer cell apoptosis independently of p53.* Cell Cycle, 2016. **15**(3): p. 394-402.

110. Tiek, D. and S.Y. Cheng, *DNA damage and metabolic mechanisms of cancer drug resistance.* Cancer Drug Resistance, 2022. **5**(2): p. 368-379.

111. Karimi-Busheri, F., *et al.*, *Senescence evasion by MCF-7 human breast tumor-initiating cells.* Breast Cancer Research, 2010. **12**(3).

112. Asakawa, H., *et al.*, *Prediction of breast cancer sensitivity to neoadjuvant chemotherapy based on status of DNA damage repair proteins.* Breast Cancer Research, 2010. **12**(2).

113. Depowski, P.L., *et al.*, *Topoisomerase II alpha expression in breast cancer: Correlation with outcome variables.* Modern Pathology, 2000. **13**(5): p. 542-547.

114. Antberg, L., *et al.*, *Pathway-Centric Analysis of the DNA Damage Response to Chemotherapeutic Agents in Two Breast Cell Lines.* EuPA Open Proteomics, 2015. **8**: p. 128-136.

115. Annaratone, L., *et al.*, *The Multifaceted Nature of Tumor Microenvironment in Breast Carcinomas.* Pathobiology, 2020. **87**(2): p. 125-142.

116. Zhou, J., *et al.*, *Cancer-Associated Fibroblasts Correlate with Tumor-Associated Macrophages Infiltration and Lymphatic Metastasis in Triple Negative Breast Cancer Patients.* Journal of Cancer, 2018. **9**(24): p. 4635-4641.

117. Hu, G.M., *et al.*, *Activated Tumor-infiltrating Fibroblasts Predict Worse Prognosis in Breast Cancer Patients.* Journal of Cancer, 2018. **9**(20): p. 3736-3742.

118. Jena, B.C., *et al.*, *Cancer associated fibroblast mediated chemoresistance: A paradigm shift in understanding the mechanism of tumor progression.* Biochimica Et Biophysica Acta-Reviews on Cancer, 2020. **1874**(2).

119. Su, S.C., *et al.*, *CD10(+) GPR77(+) Cancer-Associated Fibroblasts Promote Cancer Formation and Chemoresistance by Sustaining Cancer Stemness.* Cell, 2018. **172**(4): p. 841-+.

120. Broad, R.V., *et al.*, *Inhibition of interferon-signalling halts cancer-associated fibroblast-dependent protection of breast cancer cells from chemotherapy.* British Journal of Cancer, 2021. **124**(6): p. 1110-1120.

121. Stanton, S.E., S. Adams, and M.L. Disis, *Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes A Systematic Review.* Jama Oncology, 2016. **2**(10): p. 1354-1360.

122. Denkert, C., *et al.*, *Tumor-Associated Lymphocytes As an Independent Predictor of Response to Neoadjuvant Chemotherapy in Breast Cancer.* Journal of Clinical Oncology, 2010. **28**(1): p. 105-113.

123. Salemme, V., *et al.*, *The Crosstalk Between Tumor Cells and the Immune Microenvironment in Breast Cancer: Implications for Immunotherapy.* Frontiers in Oncology, 2021. **11**.

124. Xiao, M.Y., *et al.*, *Tumor-Associated Macrophages: Critical Players in Drug Resistance of Breast Cancer.* Frontiers in Immunology, 2021. **12**.

125. Dong, X.L., *et al.*, *Glutathione S-transferases P1-mediated interleukin-6 in tumor-associated macrophages augments drug-resistance in MCF-7 breast cancer.* Biochemical Pharmacology, 2020. **182**.

126. Yang, C.X., *et al.*, *Increased drug resistance in breast cancer by tumor-associated macrophages through IL-10/STAT3/bcl-2 signaling pathway.* Medical Oncology, 2015. **32**(2).

127. Zarrilli, G., *et al.*, *The Tumor Microenvironment of Primitive and Metastatic Breast Cancer: Implications for Novel Therapeutic Strategies.* International Journal of Molecular Sciences, 2020. **21**(21).

128. Wang, Z.M., *et al.*, *Metastasis-associated fibroblasts: an emerging target for metastatic cancer.* Biomarker Research, 2021. **9**(1).

129. Gui, Y.R., *et al.*, *Metastatic Breast Carcinoma-Associated Fibroblasts Have Enhanced Protumorigenic Properties Related to Increased IGF2 Expression.* Clinical Cancer Research, 2019. **25**(23): p. 7229-7242.

130. Sansone, P., *et al.*, *Evolution of Cancer Stem-like Cells in Endocrine-Resistant Metastatic Breast Cancers Is Mediated by Stromal Microvesicles.* Cancer Research, 2017. **77**(8): p. 1927-1941.

131. Lee, H., K.J. Na, and H. Choi, *Differences in Tumor Immune Microenvironment in Metastatic Sites of Breast Cancer.* Frontiers in Oncology, 2021. **11**.

132. Ogiya, R., *et al.*, *Comparison of tumor-infiltrating lymphocytes between primary and metastatic tumors in breast cancer patients.* Journal of Clinical Oncology, 2015. **33**(15).

133. Zhu, L., *et al.*, *Metastatic breast cancers have reduced immune cell recruitment but harbor increased macrophages relative to their matched primary tumors.* Journal for Immunotherapy of Cancer, 2019. **7**(1).

134. Stenstrom, J., I. Hedenfalk, and C. Hagerling, *Regulatory T lymphocyte infiltration in metastatic breast cancer-an independent prognostic factor that changes with tumor progression.* Breast Cancer Research, 2021. **23**(1).

135. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nature Reviews Immunology, 2005. **5**(5): p. 375-386.

136. Fish, E.N. and L.C. Platanias, *Interferon Receptor Signaling in Malignancy: A Network of Cellular Pathways Defining Biological Outcomes.* Molecular Cancer Research, 2014. **12**(12): p. 1691-1703.

137. Ali, S., *et al.*, *Sources of Type I Interferons in Infectious Immunity: Plasmacytoid Dendritic Cells Not Always in the Driver's Seat.* Frontiers in Immunology, 2019. **10**.

138. Lee, A.J. and A.A. Ashkar, *The Dual Nature of Type I and Type II Interferons.* Frontiers in Immunology, 2018. **9**.

139. McNab, F., *et al.*, *Type I interferons in infectious disease.* Nature Reviews Immunology, 2015. **15**(2): p. 87-103.

140. Fu, X.Y., C. De Angelis, and R. Schiff, *Interferon Signaling in Estrogen Receptor-positive Breast Cancer: A Revitalized Topic.* Endocrinology, 2022. **163**(1).

141. Borden, E.C., *Interferons alpha and beta in cancer: therapeutic opportunities from new insights.* Nature Reviews Drug Discovery, 2019. **18**(3): p. 219-234.

142. Schindler, C., *et al.*, *Interferon-Dependent Tyrosine Phosphorylation of a Latent Cytoplasmic Transcription Factor.* Science, 1992. **257**(5071): p. 809-813.

143. Provance, O.K. and J. Lewis-Wambi, *Deciphering the role of interferon alpha signaling and microenvironment crosstalk in inflammatory breast cancer.* Breast Cancer Research, 2019. **21**.

144. Michalska, A., *et al.*, *A Positive Feedback Amplifier Circuit That Regulates Interferon (IFN)-Stimulated Gene Expression and Controls Type I and type II IFN Responses.* Frontiers in Immunology, 2018. **9**.

145. van Boxel-Dezaire, A.H.H., *et al.*, *Major Differences in the Responses of Primary Human Leukocyte Subsets to IFN-beta.* Journal of Immunology, 2010. **185**(10): p. 5888-5899.

146. Stark, G.R., H. Cheon, and Y.X. Wang, *Responses to Cytokines and Interferons that Depend upon JAKs and STATs.* Cold Spring Harbor Perspectives in Biology, 2018. **10**(1).

147. Mazewski, C., *et al.*, *Type I Interferon (IFN)-Regulated Activation of Canonical and Non-Canonical Signaling Pathways.* Frontiers in Immunology, 2020. **11**.

148. Verma, A., *et al.*, *Cutting edge: Activation of the p38 mitogen-activated protein kinase signaling pathway mediates cytokine-induced hemopoietic suppression in aplastic anemia.* Journal of Immunology, 2002. **168**(12): p. 5984-5988.

149. Uddin, S., *et al.*, *Activation of the p38 mitogen-activated protein kinase by type I interferons.* Journal of Biological Chemistry, 1999. **274**(42): p. 30127-30131.

150. Mayer, I.A., *et al.*, *The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells.* Journal of Biological Chemistry, 2001. **276**(30): p. 28570-28577.

151. Verma, A., *et al.*, *Activation of the p38 mitogen-activated protein kinase mediates the suppressive effects of type I Interferons and transforming growth factor-beta on normal hematopoiesis.* Journal of Biological Chemistry, 2002. **277**(10): p. 7726-7735.

152. Kaur, S., *et al.*, *Dual regulatory roles of the phosphatidylinositol 3-kinase in interferon signaling.* Cytokine, 2008. **43**(3): p. 237-237.

153. Yeh, Y.H., *et al.*, *Inflammatory interferon activates HIF-1 alpha-mediated epithelial-to-mesenchymal transition via PI3K/AKT/mTOR pathway.* Journal of Experimental & Clinical Cancer Research, 2018. **37**.

154. Musella, M., *et al.*, *Type-I-interferons in infection and cancer: Unanticipated dynamics with therapeutic implications.* Oncoimmunology, 2017. **6**(5).

155. Gresser, I. and C. Bourali, *Exogenous Interferon and Inducers of Interferon in Treatment of BALB/C Mice Inoculated with RC19 Tumour Cells.* Nature, 1969. **223**(5208): p. 844-&.

156. Borden, E.C., *et al.*, *Interferons at age 50: past, current and future impact on biomedicine.* Nature Reviews Drug Discovery, 2007. **6**(12): p. 975-990.

157. Sistigu, A., *et al.*, *Cancer cell-autonomous contribution of type I interferon signaling to the efficacy of chemotherapy.* Nature Medicine, 2014. **20**(11): p. 1301-1309.

158. Padariya, M., *et al.*, *Functional Interfaces, Biological Pathways, and Regulations of Interferon-Related DNA Damage Resistance Signature (IRDS) Genes.* Biomolecules, 2021. **11**(5).

159. Weichselbaum, R.R., *et al.*, *An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(47): p. 18490-18495.

160. Khodarev, N.N., *et al.*, *STAT1 Pathway Mediates Amplification of Metastatic Potential and Resistance to Therapy.* Plos One, 2009. **4**(6).

161. Aljohani, A.I., *et al.*, *Myxovirus resistance 1 (MX1) is an independent predictor of poor outcome in invasive breast cancer.* Breast Cancer Research and Treatment, 2020. **181**(3): p. 541-551.

162. Boelens, M.C., *et al.*, *Exosome Transfer from Stromal to Breast Cancer Cells Regulates Therapy Resistance Pathways.* Cell, 2014. **159**(3): p. 499-513.

163. Hosein, A.N., *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**.

164. Ambjorn, M., *et al.*, *IFNB1/interferon-beta-induced autophagy in MCF-7 breast cancer cells counteracts its proapoptotic function.* Autophagy, 2013. **9**(3): p. 287-302.

165. Heiser, L.M., *et al.*, *Subtype and pathway specific responses to anticancer compounds in breast cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(8): p. 2724-2729.

166. Ganapathi, R.N. and M.K. Ganapathi, *Mechanisms regulating resistance to inhibitors of topoisomerase II.* Frontiers in Pharmacology, 2013. **4**.

167. Orr, G.A., *et al.*, *Mechanisms of Taxol resistance related to microtubules.* Oncogene, 2003. **22**(47): p. 7280-7295.

168. Dai, X.F., *et al.*, *Breast Cancer Cell Line Classification and Its Relevance with Breast Tumor Subtyping.* Journal of Cancer, 2017. **8**(16): p. 3131-3141.

169. Gomez, D. and N.C. Reich, *Stimulation of primary human endothelial cell proliferation by IFN.* Journal of Immunology, 2003. **170**(11): p. 5373-5381.

170. Holzinger, D., *et al.*, *Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling.* Journal of Virology, 2007. **81**(14): p. 7776-7785.

171. Legrier, M.E., *et al.*, *Activation of IFN/STAT1 signalling predicts response to chemotherapy in oestrogen receptor-negative breast cancer.* British Journal of Cancer, 2016. **114**(2): p. 177-187.

172. Rickardson, L., *et al.*, *Identification of molecular mechanisms for cellular drug resistance by combining drug activity and gene expression profiles.* British Journal of Cancer, 2005. **93**(4): p. 483-492.

173. Khodarev, N.N., B. Roizman, and R.R. Weichselbaum, *Molecular Pathways: Interferon/Stat1 Pathway: Role in the Tumor Resistance to Genotoxic Stress and Aggressive Growth.* Clinical Cancer Research, 2012. **18**(11): p. 3015-3021.

174. Mah, L.J., A. El-Osta, and T.C. Karagiannis, *gamma H2AX: a sensitive molecular marker of DNA damage and repair.* Leukemia, 2010. **24**(4): p. 679-686.

175. Gewirtz, D.A., *A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics Adriamycin and daunorubicin.* Biochemical Pharmacology, 1999. **57**(7): p. 727-741.

176. Abal, M., J.M. Andreu, and I. Barasoain, *Taxanes: Microtubule and Centrosome Targets, and Cell Cycle Dependent Mechanisms of Action.* Current Cancer Drug Targets, 2003. **3**(3): p. 193-203.

177. Wen, S.H., *et al.*, *Sulbactam-enhanced cytotoxicity of doxorubicin in breast cancer cells.* Cancer Cell International, 2018. **18**.

178. Khongkow, M., *et al.*, *SIRT6 modulates paclitaxel and epirubicin resistance and survival in breast cancer.* Carcinogenesis, 2013. **34**(7): p. 1476-1486.

179. Post, A.E.M., *et al.*, *Interferon-Stimulated Genes Are Involved in Cross-resistance to Radiotherapy in Tamoxifen-Resistant Breast Cancer.* Clinical Cancer Research, 2018. **24**(14): p. 3397-3408.

180. Wu, J.X., L.Y. Lu, and X.C. Yu, *The role of BRCA1 in DNA damage response.* Protein & Cell, 2010. **1**(2): p. 117-123.

181. Smith, S.E., *et al.*, *Molecular characterization of breast cancer cell lines through multiple omic approaches.* Breast Cancer Research, 2017. **19**.

182. Foulkes, W.D., *BRCA1 and BRCA2: chemosensitivity, treatment outcomes and prognosis.* Familial Cancer, 2006. **5**(2): p. 135-142.

183. Arico, E., *et al.*, *Type I Interferons and Cancer: An Evolving Story Demanding Novel Clinical Applications.* Cancers, 2019. **11**(12).

184. Hobeika, A.C., P.S. Subramaniam, and H.M. Johnson, *IFN alpha induces the expression of the cyclin-dependent kinase inhibitor p21 in human prostate cancer cells.* Oncogene, 1997. **14**(10): p. 1165-1170.

185. Sarkar, D., E.S. Park, and P.B. Fisher, *Defining the mechanism by which IFN-beta dowregulates c-myc expression in human melanoma cells: pivotal role for human polynucleotide phosphorylase (hPNPase(old-35)).* Cell Death and Differentiation, 2006. **13**(9): p. 1541-1553.

186. Kotredes, K.P. and A.M. Gamero, *Interferons as Inducers of Apoptosis in Malignant Cells.* Journal of Interferon and Cytokine Research, 2013. **33**(4): p. 162-170.

187. Arulampalam, V., *et al.*, *Activation of STAT1 is required for interferon-alpha-mediated cell death.* Experimental Cell Research, 2011. **317**(1): p. 9-19.

188. Dedoni, S., M.C. Olianas, and P. Onali, *Interferon-beta induces apoptosis in human SH-SY5Y neuroblastoma cells through activation of JAK-STAT signaling and down-regulation of PI3K/Akt pathway.* Journal of Neurochemistry, 2010. **115**(6): p. 1421-1433.

189. Minn, A.J., *Interferons and the Immunogenic Effects of Cancer Therapy.* Trends in Immunology, 2015. **36**(11): p. 725-737.

190. Cao, J., *et al.*, *Chemoresistance and Metastasis in Breast Cancer Molecular Mechanisms and Novel Clinical Strategies.* Frontiers in Oncology, 2021. **11**.

191. Yardley, D., *Drug Resistance and the Role of Combination Chemotherapy in Improving Patient Outcomes.* International Journal of Breast Cancer, 2013.

192. Gao, Z.H., *et al.*, *Predictive and prognostic role of tumour-infiltrating lymphocytes in breast cancer patients with different molecular subtypes: a meta-analysis.* Bmc Cancer, 2020. **20**(1).

193. azaki, S., *et al.*, *Impact of Adjuvant Chemotherapy and Radiotherapy on Tumour-Infiltrating Lymphocytes and PD-L1 Expression in Metastatic Breast Cancer.* British Journal of Cancer, 2022.

194. Yazaki, S., *et al.*, *Impact of Adjuvant Chemotherapy and Radiotherapy on Tumour-Infiltrating Lymphocytes and PD-L1 Expression in Metastatic Breast Cancer.* British Journal of Cancer, 2022.

195. Esteva, F.J. and G.N. Hortobagyi, *Prognostic molecular markers in early breast cancer.* Breast Cancer Research, 2004. **6**(3): p. 109-118.

196. Dieci, M.V., *et al.*, *Immune characterization of breast cancer metastases: prognostic implications.* Respiratory Research, 2018. **20**.

197. Larionova, I., *et al.*, *Interaction of tumor-associated macrophages and cancer chemotherapy.* Oncoimmunology, 2019. **8**(7).

198. Shree, T., *et al.*, *Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer.* Genes & Development, 2011. **25**(23): p. 2465-2479.

199. Wu, M.Y., *et al.*, *Neutrophil: A New Player in Metastatic Cancers.* Frontiers in Immunology, 2020. **11**.

200. Geng, S.K., *et al.*, *Tumor infiltrating neutrophil might play a major role in predicting the clinical outcome of breast cancer patients treated with neoadjuvant chemotherapy.* Bmc Cancer, 2021. **21**(1).

201. Ottewell, P.D., *The role of osteoblasts in bone metastasis.* Journal of Bone Oncology, 2016. **5**(3): p. 124-127.

202. Van den Eynden, G.G., *et al.*, *The Multifaceted Role of the Microenvironment in Liver Metastasis: Biology and Clinical Implications.* Cancer Research, 2013. **73**(7): p. 2031-2043.

203. Piezzo, M., *et al.*, *Targeting Cell Cycle in Breast Cancer: CDK4/6 Inhibitors.* International Journal of Molecular Sciences, 2020. **21**(18).

204. Planes-Laine, G., *et al.*, *PD-1/PD-L1 Targeting in Breast Cancer: The First Clinical Evidences are Emerging-A Literature Review.* Cancers, 2019. **11**(7).

205. Iwamoto, T., *et al.*, *Distinct gene expression profiles between primary breast cancers and brain metastases from pair-matched samples.* Scientific Reports, 2019. **9**.

206. Ongaro, E., *et al.*, *Comparison of primary breast cancer and paired metastases: biomarkers discordance influence on outcome and therapy.* Future Oncology, 2018. **14**(9): p. 849-859.

207. Poplawski, A.B., *et al.*, *Frequent genetic differences between matched primary and metastatic breast cancer provide an approach to identification of biomarkers for disease progression.* European Journal of Human Genetics, 2010. **18**(5): p. 560-568.

208. Liu, T., *et al.*, *Single cell profiling of primary and paired metastatic lymph node tumors in breast cancer patients.* Nature Communications, 2022. **13**(1).

209. Schwartz, D.M., *et al.*, *JAK inhibition as a therapeutic strategy for immune and inflammatory diseases (vol 16, pg 843, 2017).* Nature Reviews Drug Discovery, 2018. **17**(1).

210. Spinelli, F.R., *et al.*, *JAK inhibitors: Ten years after.* European Journal of Immunology, 2021. **51**(7): p. 1615-1627.

211. Qureshy, Z., D. Johnson, and J. Grandis, *Targeting the JAK/STAT Pathway in Solid Tumors.* Journal of Cancer Metastasis and Treatment, 2020.

212. Stover, D.G., *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**.

213. 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 Research and Treatment, 2018. **170**(3): p. 547-557.

214. 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 Chemotherapy and Pharmacology, 2021. **87**(5): p. 673-679.