

THERAPY-INDUCED SENESENCE IN TRIPLE-NEGATIVE BREAST CANCER: BIOMARKERS AND REVERSAL STRATEGIES

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ABSTRACT

Therapy induced senescence (TIS) is a state of stable cell cycle arrest, often arising as a consequence of DNA damage, caused by chemotherapy drugs like doxorubicin. TIS is characterised by the senescence associated secretory phenotype (SASP), a heterogeneous collection of pro-inflammatory factors that initially promotes immune surveillance and clearance of damaged cells. However, persistent SASP signalling impairs this response, enabling senescent cell accumulation. Persistent SASP secretion creates a pro-inflammatory and pro-tumorigenic microenvironment, in which SASP components drive angiogenesis, microenvironment remodelling, tumour invasion and metastasis. Breast cancer (BC) is the most common cancer in the UK, of which triple negative BC (TNBC) is a clinically challenging subtype, due to its aggressive behaviour and high relapse rates. TNBC accounts for approximately 15% of BC cases but nearly 40% of BC deaths. As doxorubicin is a first line TNBC treatment and a potent inducer of TIS, it represents a clinically relevant model for investigating TIS. Given that TIS can arise as a consequence of chemotherapy and contribute to tumour progression, inflammation, and treatment response, it is essential to both be able to identify and mitigate its detrimental effects.

One aim of this research was to identify predictive or prognostic biomarkers of TIS in TNBC. TIS models were developed, using doxorubicin, in two TNBC cell lines (MDA-MB-231 and HCC1806) and one non-tumorigenic breast cell line (MCF10A). Senescence was confirmed using multiple hallmarks, including senescence associated β -galactosidase staining, nuclear area changes, γ -H2AX staining, and altered expression of key senescence associated genes. A literature review identified several candidate biomarkers associated with senescence, cancer and the cytoskeleton, whilst a bioinformatics approach assessed their potential predictive and prognostic significance. Candidate biomarkers were evaluated for their presence in TIS TNBC models. Although several genes showed comparable expression patterns in TIS induced non-malignant cells, these trends were often not conserved in TIS TNBC cell lines, highlighting the complexity of defining a universal TIS biomarker. Among the candidates assessed, *Lamin B1* emerged as a reliable marker of TIS and was therefore used in subsequent experiments.

Metformin was assessed for its senotherapeutic potential in TIS models, to mitigate the detrimental consequences of TIS, based on its established tolerability and reported anti-senescence effects, extending beyond SASP attenuation. As existing literature predominantly uses metformin at supra-

pharmacological concentrations, this study compared a clinically relevant concentration (50 μ M) with a supra-pharmacological concentration (500 μ M). Treatment of TIS models with metformin did not result in clear attenuation of senescence hallmarks. To determine whether this was due to insufficient uptake or pathway activation, expression of key metformin transporters were assessed using qPCR and immunostaining, and pathway activation (AMPK and mTOR phosphorylation) was assessed using western blot. Key transporters were present in cell models, and preliminary data indicated pathway activation was consistent, to some extent, with metformin's expected mechanism of action.

To investigate the paracrine effects of TIS and their modulation by metformin, indirect co-culture models were developed using patient derived fibroblasts (a key component of the tumour microenvironment), and TIS HCC1806. Fibroblasts were assessed for markers of senescence (senescence associated β -galactosidase staining and inflammatory gene expression) and fibroblast activation (scratch assays and morphological analysis), both of which contribute to cancer progression. Fibroblasts developed a pro-inflammatory gene expression profile when co-cultured with TNBC cells, which was further enhanced by TIS. These findings indicate that SASP signalling may promote pro-tumorigenic alterations within the tumour microenvironment. Treatment with supra-pharmacological metformin concentrations (500 μ M) reduced the expression of several inflammatory factors which were not attenuated by physiological concentrations (50 μ M), suggesting that clinically relevant metformin concentrations may be insufficient to effectively suppress SASP signalling. Metformin treatment simultaneously increased expression of the inflammatory factor *interleukin 8*, underlining the complexity of metformin's role in senescence attenuation.

In summary, gene expression analysis of candidate biomarkers, identified through bioinformatics analysis, revealed that that TIS associated gene expression is context dependent and emphasised the difficulty of identifying a universal TIS biomarker. SASP signalling was shown to drive an inflammatory gene expression profile in fibroblasts, potentially contributing to pro-tumorigenic changes within the TME. Although metformin did not directly attenuate TIS in the cell models used, it modulated inflammatory signalling at supra-physiological concentrations, indicating that it may have senotherapeutic potential in certain contexts, but is unlikely to be effective at clinically relevant doses.

Keywords: Therapy induced senescence, triple negative breast cancer, biomarker, metformin

ABBREVIATIONS

AF	Alexa-Fluor
AGE	Advanced glycation end products
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
apCAF	Antigen presenting cancer associated fibroblast
α-SMA	Alpha smooth muscle actin
AT	Accession numbers
ATM	ATM serine/threonine kinase
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and RAD3-related
AU	Arbitrary units
AUC	Area under the curve
BARD1	BRCA1-associated RING domain protein 1
BC	Breast cancer
BRCA	Breast invasive carcinoma
CAF	Cancer associated fibroblast
CCL	Chemoattractant protein
CDCA5	Cell division cycle associated 5
CDH1	Cadherin-1
CDK (I)	Cyclin dependent kinase (inhibitor)
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CF	Chromatin fragments
cGAMP	Cyclic guanosine monophosphate-adenosine monophosphate
CI	Confidence interval
CM	Conditioned media
COL4A1	Collagen alpha-1(IV) chain
CPR	Complete pathological response
Ct	Cycle threshold
cGAS	Cyclic GMP–AMP synthase
CHK	Checkpoint kinase
CXC	Chemokine
CXCL1	C-X-C motif chemokine ligand 1
CytoDNA	Endogenous cytoplasmic DNA
DDR	DNA damage repair
DICER-1	Dicer 1, ribonuclease III
DMEM	Dulbecco's Modified Eagle Medium/Nutrient Mixture
DMF	N, N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
ER	Oestrogen receptor
FAP	Fibroblast activation protein
FBS	Foetal bovine serum
GEPIA	Gene expression profiling interactive analysis

GTE_x	Genotype-tissue expression
GTP	Guanosine triphosphate
GPX7	Glutathione peroxidase 7
HBOC	Hereditary Breast and Ovarian Cancer
HCL	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
HMGB2	High mobility group box 2
HNRNP_D	Heterogeneous nuclear ribonucleoprotein D
HR	Hazard ratio
HRP	Horseradish Peroxidase
H3K27me₃	Trimethylation of histone H3 at lysine 27
iCAF	Inflammatory cancer associated fibroblast
IGFBP	Insulin Like Growth Factor Binding Protein
IKK	Kinase and inhibitor of nuclear factor kappa-B kinase
IKKα/β	I κ B kinase α/β
IL	Interleukin
IIS	Insulin/IGF-1 signaling
IRF3	Interferon regulatory factor 3
KDM6A	Lysine demethylase 6A
KIFC1	Kinesin family member C1
KM	Kaplan-Meir
LINE-1	Long interspersed element-1
LMNB1	Lamin B1
MAPK	Mitogen-activated Protein Kinase
MATE1	Multidrug and toxin extrusion protein 1
MBNL1	Muscleblind like splicing regulator 1
MDM2	Murine double minute 2
MEC	Minimum effective concentration
MGI	Mean Grey Intensity
MMPs	Matrix metalloproteinases
miRNA	Mature microRNAs
mRNA	Messenger RNA
MSK1	Mitogen and stress activated protein kinase 1
mtDNA	Mitochondrial DNA
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
myCAF	Myofibroblastic cancer associated fibroblast
NaOH	Sodium Chloride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	Nuclear factor erythroid 2-related factor 2
NUP62	Nuclear pore glycoprotein p62
NST	No special type
OCT1	Organic cationic transporter 1
OCT3	Organic cationic transporter 3
OD	Optical density
OS	Overall survival
OSIS	Oxidative stress induced senescence
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2

PGC1-a	Proliferator-activated receptor-gamma coactivator-1 alpha
PMAT	Plasma membrane monoamine transporter
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
Rag-GTPase	Ras-related GTP-binding protein
REC	Research Ethics Committee
REDD1	DNA damage response 1
RFS	Relapse free survival
Rheb	Ras homolog enriched in brain
RB	Retinoblastoma protein
RNA	Ribonucleic acid
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
RTs	Retrotransposons
SAMD	Senescence-associated mitochondrial dysfunction
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SCAP	Senescent cell anti-apoptotic pathways
SD	Standard deviation
SIRT	Sirtuin
SLS	Scientific Laboratory Supplies
SMC6	Structural maintenance of chromosomes protein 6
STAT3	Signal transducer and activator of transcription 3
STIM1	Stromal interaction molecule 1
STING	Stimulator of interferon genes
STK11	Serine/threonine kinase 11
SYNE	Nesprin
S6K1	Ribosomal protein S6 kinase
TBK1	TANK binding kinase 1
TCGA	The cancer genome atlas
TERF2	Telomeric repeat-binding factor 2
TGF-β	Transforming growth factor beta
TIS	Therapy induced senescence
TNBC	Triple negative breast cancer
TME	Tumour microenvironment
TNM	Tumour, node, metastasis
TPM	Transcripts per million
TTBS	Tris-buffered saline
TP53	Tumour protein 53
TREX2	Three prime repair exonuclease 2
TSC2	Tuberous sclerosis complex 2
TUBG1	γ -tubulin 1
T2DM	Type 2 diabetes mellitus
UALCAN	University of Alabama at Birmingham cancer data analysis portal
UKHSA	UK Health Security Agency
ULK1	Unc-51 like autophagy activating kinase 1
Upar	Urokinase receptor
VEGF	Vascular endothelial growth factor
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1

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Chapter 1

General Introduction

1 GENERAL INTRODUCTION

1.1 HALLMARKS OF AGING

In eukaryotes, aging can be defined as the decline in cellular integrity and function, as a consequence of time passing. This deterioration is the primary risk factor of many pathologies including cancer. Aging occurs as a result of many biological events which have been summarised into 12 hallmarks (Figure 1.1) (López-Otín et al., 2013; (López-Otín et al., 2023)). These can be further categorised into primary hallmarks (cause cellular damage), antagonistic hallmarks (responses which try to re-establish homeostasis) and integrative hallmarks (consequences of primary and antagonistic hallmarks, ultimately leading to cellular decline). These hallmarks are not always independent and are often impacted by one another.

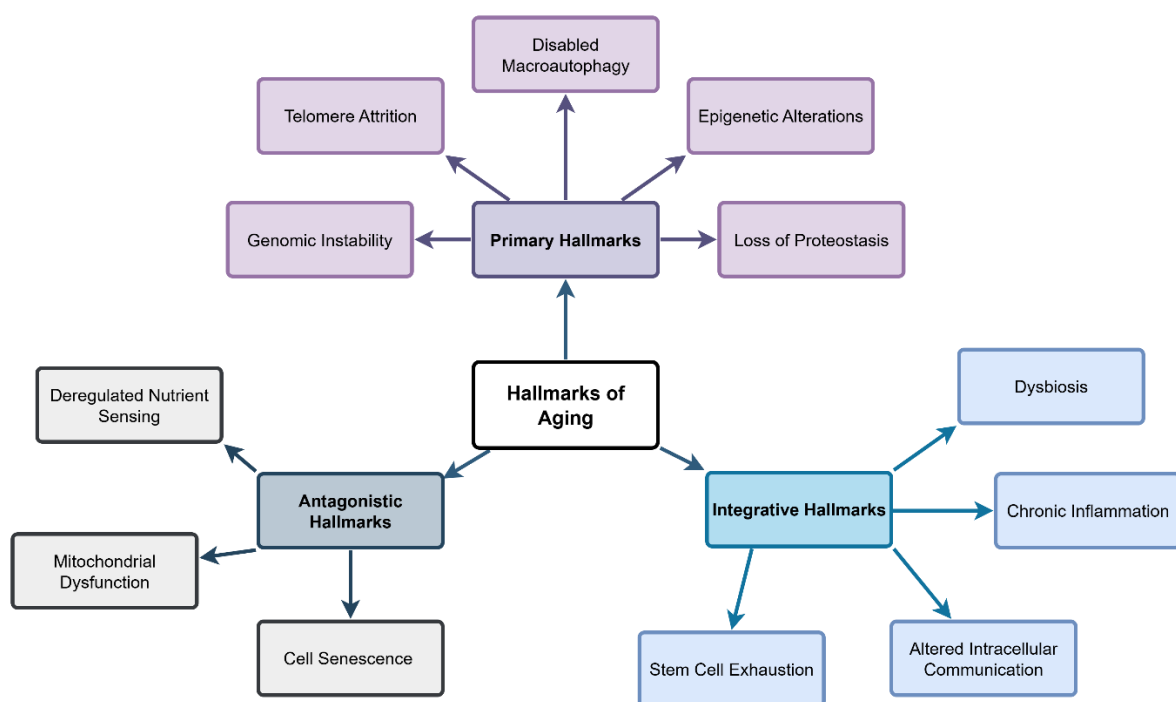


Figure 1.1. Hallmarks of aging. There are 12 hallmarks of aging as classified by López-Otín et al., (2023), each of which can be classified based on its consequences. Primary hallmarks cause cellular damage, resulting in genomic instability, telomere attrition, disabled macroautophagy, epigenetic alterations and loss of proteostasis. Antagonistic hallmarks are responses which occur to re-establish homeostasis, these include deregulated nutrient sensing, mitochondrial dysfunction and senescence. Integrative hallmarks such as dysbiosis, chronic inflammation, stem cell exhaustion and altered intracellular communication, occur as a consequence of both primary and antagonistic hallmarks.

1.2 CELLULAR SENESENCE

Aged cells lose their replicative potential and enter senescence, a state of irreversible cell cycle arrest (Figure 1.2). Senescence can be a beneficial compensatory response to deoxyribonucleic acid (DNA) damage, however, can become damaging if these cells accumulate. As a component of their senescence associated secretory phenotype (SASP), senescent cells secrete proinflammatory cytokines, such as interleukin (IL) 6 and IL8, to facilitate immune clearance (Kumari and Jat, 2021; McHugh and Gill, 2018). However, persistent secretion can diminish the immune response, allowing senescent cells to accumulate. This is termed inflammageing, the low-grade, chronic inflammation associated with aging.

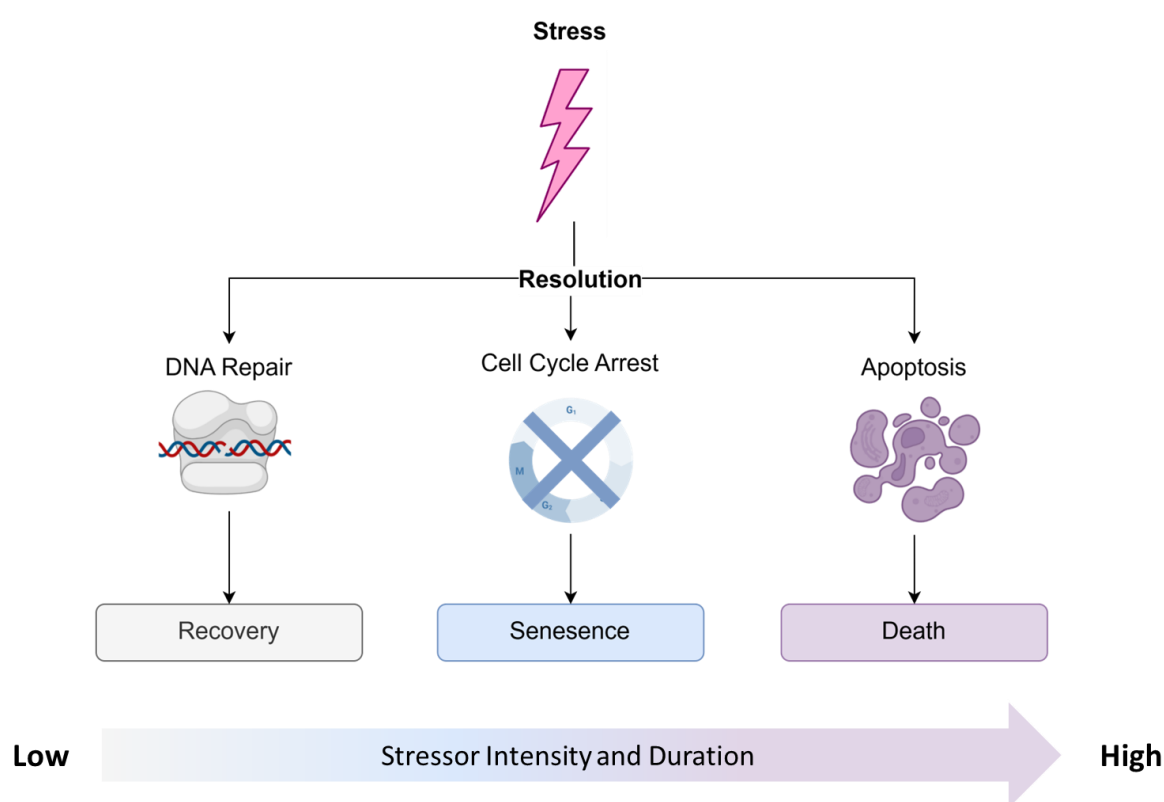


Figure 1.2. Possible fates of cells undergoing stress. The application of cell stress can induce three outcomes: recovery, senescence, or death. These outcomes are dependent on the duration and intensity of the stressor, whereby cells undergoing high levels of stress are more likely to undergo apoptosis, then cells with low level damage which will undergo DNA repair and recover. Cells undergoing moderate damage will likely enter a state of permanent cell cycle arrest, becoming senescent (Childs et al., 2014).

Senescent cells lack a defined phenotype therefore several hallmarks must be used to indicate senescence (Table 1.1) (Hernandez-Segura, Nehme and Demaria, 2018). There are many types of senescence, which share similar hallmarks, but differ in their induction methods. Senescence inducing stimuli can be intrinsic (oxidative stress, hyperproliferation and telomere attrition) or extrinsic

(ultraviolet light and chemotherapy drugs), depending on whether they occur internally or externally (Kumari and Jat, 2021).

Table 1.1. Hallmarks of senescence. Senescence associated secretory phenotype (SASP).

Category	Hallmark	Description
Signalling	DNA damage	DNA damage can be induced by a wide range of stimuli which ultimately promote senescence induction to prevent the proliferation of damaged cells.
	SASP	Senescent cells secrete a plethora of cytokines, chemokines, proteases, and growth factors as part of their highly heterogenous SASP. This not only promotes cell survival but also allows communication with neighbouring cells, influencing their outcome.
	Apoptosis resistance	Senescent cells promote their survival by resisting apoptosis. Commonly implicated proteins include members of the B-cell lymphoma 2 (Bcl-2) family.
	Cell cycle arrest	Senescent cells enter an irreversible cell cycle arrest in the G ₁ phase of the cell cycle.
Morphology	Cell size and shape	Senescent cells often appear larger and flatter than healthy cells.
	Plasma membrane composition	Caveolin-1 is upregulated in senescent cells. Increased levels cause caveolae formation, small invaginations of the plasma membrane.
	Increased lysosomal content	Senescent cells upregulate several lysosomal proteins and have increased lysosomal content, although the mechanism behind this is not fully understood, it can contribute to changes in autophagy resulting in mitochondrial accumulation.
	Mitochondrial accumulation	As age increases, mitochondrial integrity and functionality decrease. An accumulation of large, dysfunctional mitochondria is indicative of senescence.
	Nuclear changes	In senescent cells, a loss of Lamin B1 causes heterochromatin loss and cytoplasmic chromatin fragment formation, resulting in a loss of nuclear envelope integrity and multinucleation.

Signal transduction is essential to the induction and maintenance of senescence. Two particularly important pathways are the p53/p21^{WAF/CIP1} and p16^{INK4A}/Rb pathways which induce senescence by inhibiting cyclin activation (Figure 1.3) (Mijit et al., 2020). Activation of senescence pathways is context dependent and varies depending on the inducer and stage of senescence (Saul et al., 2025). Although p53/p21^{WAF/CIP1} is reported to be induced early in senescence and p16^{INK4A}/Rb associated with its maintenance, opposing patterns have been reported, reflecting the heterogeneity of senescence (Beauséjour et al., 2003; Dolan et al., 2015; Kumari and Jat, 2021; Mijit et al., 2020; Saul et al., 2025).

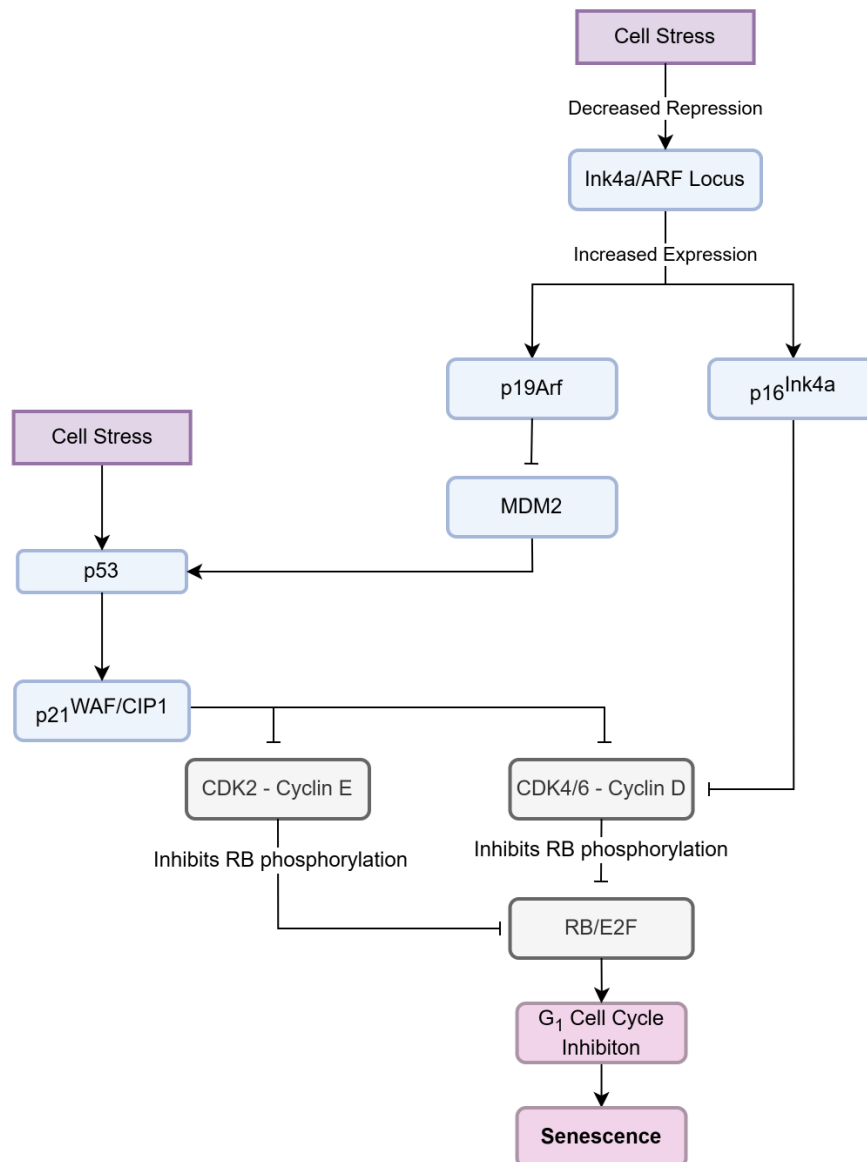


Figure 1.3. Summary of the key pathways involved in senescence induction and maintenance. Senescence can be triggered by different stressors but ultimately results in the activation of p53/p21^{WAF/CIP1} and p16^{INK4A}/Rb pathways, which induce senescence by inhibiting cyclin activation. Cyclin dependent kinase (CDK), murine double minute 2 (MDM2), retinoblastoma protein (RB).

1.2.1 p16^{Ink4a}/Rb

The inhibitors of *cyclin dependent kinase 4 (Ink4)* locus encodes for several proteins, including p16^{Ink4a}, also known as cyclin-dependent kinase inhibitor 2A (CDKN2A) (Sharpless, 2005). P16^{Ink4a} competitively binds to cyclin dependent kinase (CDK)4/6, blocking the formation of cyclin D-CDK4/6 complexes and preventing retinoblastoma protein (RB) phosphorylation (Sharpless, 2005). In its hypophosphorylated state RB forms a complex with E2F transcription factors (Ohtani et al., 2004). RB-E2F complexes bind to the promotor regions of genes essential to cell cycle progression, repressing their transcription and arresting the cell cycle in G₁ (Kumari and Jat, 2021). When stressed or epigenetically modified, cells

overexpress p16^{Ink4a}. The mechanism behind this is not fully understood, although this upregulation may result from a reduction in proteins that normally repress *CDKN2A* expression (Kumari and Jat, 2021; Martin, Beach and Gil, 2014).

1.2.2 p19Arf/p53/p21^{Cip1}

p53 is a tumour suppressor protein, considered to be the ‘guardian of the genome’ and is upregulated in most senescent cells. Its function is dependent on post-translational modifications, however, the primary roles of p53 are to regulate cell cycle arrest, DNA repair, apoptosis, and senescence (Kumari and Jat, 2021). p19^{Arf} is an inhibitor of murine double minute 2 (MDM2), encoded for by the *Arf* gene locus, which overlaps the *Ink4a* gene locus (Sharpless, 2005). MDM2 is a ubiquitin ligase which negatively regulates p53 by directly inhibiting it or degrading it through its related proteases. When DNA is damaged, p19^{Arf} is upregulated, inhibiting MDM2 subsequently activating p53 (Wu and Prives, 2018). Activated p53 then induces the expression of p21^{WAF/CIP1} also known as cyclin-dependent kinase inhibitor 1A (CDKN1A). p21^{WAF/CIP1} is a cyclin dependent kinase inhibitor (CDKI), capable of inhibiting multiple cyclin dependent kinases and preventing cell cycle progression (Chen et al., 1996). It inhibits RB phosphorylation, causing RB-E2F complexes to accumulate, inducing cell cycle arrest (Kumari and Jat, 2021). p21^{WAF/CIP1} expression influences cell cycle progression, where high levels inhibit cyclin D-CDK4/6 complex activity and low levels promote it (LaBaer et al., 1997). In many cases, p21^{WAF/CIP1} levels decrease after senescence is induced, suggesting it primarily functions in senescence induction (Kumari and Jat, 2021; Stein et al., 1999). Although its mechanism is unclear, under stressful conditions, p21^{WAF/CIP1} can directly induce cell cycle arrest, however some cells regulated solely by the p53/p21^{WAF/CIP1} pathway may re-enter the cell cycle after the pathway is turned off (Kumari and Jat, 2021).

1.3 SASP

Most senescent cells secrete a plethora of soluble signalling factors including cytokines, chemokines, and proteases, as part of their highly heterogeneous SASP (Table 1.2). SASP composition varies drastically depending on the inducer and duration of senescence, although some SASP components, such as IL6 and IL8 are widely conserved (Kumari and Jat, 2021). A majority of SASP proteins can be directly secreted, although some, such as TNF- α are secreted as transmembrane proteins and must undergo ectodomain shedding, a form of proteolytic cleavage which facilitates removal of

extracellular domains (Stow and Murray, 2013). Alternatively, some SASP components can be secreted in small extracellular vesicles (Kumari and Jat, 2021; Takasugi et al., 2017).

Table 1.2. Summary of common senescence-associated secretory phenotype (SASP) factors. Table summarised using information from Basisty et al., 2020; Coppé et al., 2010; Kumari and Jat, 2021, Wang et al., 2024a. Interleukin (IL), C-C motif chemokine ligand (CCL), C-X-C motif chemokine ligand (CXCL), Transforming Growth Factor beta (TGF- β), Vascular Endothelial Growth Factor (VEGF), Matrix Metalloproteinase (MMP), Urokinase-type Plasminogen Activator Receptor (upar).

Component	Example	Description
Proinflammatory cytokines	IL1A, IL1B, IL6	Short term secretion promotes immune clearance, but prolonged secretion can drive chronic inflammation, tissue remodelling, NF- κ B activation and senescence induction. IL6 is a highly conserved SASP component.
Chemokines	CCL2, CCL5, CXCL1, CXCL2, CXCL2, IL8	Promotes inflammation, tumorigenesis and immune cell recruitment. IL8 is a highly conserved SASP component.
Growth factors	TGF- β , VEGF	Mediates paracrine signalling, cell proliferation and tissue repair. Can promote paracrine senescence.
Proteases	MMP1, MMP3	Degrades extracellular matrix, remodelling tissue contributing to fibrosis and tumorigenesis.
Soluble factors	Prostaglandin, leukotrienes, upar	Inflammatory mediators, which promote tissue repair and fibrosis.
Extracellular vesicles	Exosomes, microvesicles	Transport various signalling molecules, including proteins and lipids, promoting paracrine senescence.

In young tissue, the SASP contributes to maintaining tissue homeostasis, wound healing, and repair but in aged tissue contributes to chronic inflammation and tumorigenesis (Basisty et al., 2020; Neves et al., 2015; Tominaga, 2015). The SASP can re-enforce senescence through autocrine signalling, and also induce senescence in neighbouring cells, known as paracrine senescence (Lopes-Paciencia et al., 2019; Özcan et al., 2016). The SASP is dynamically regulated at many levels, and although DNA damage is a key driver of SASP, in some incidences it is also inducible via chromatin reorganisation and activation of p38 MAPK and Cyclic GMP-AMP synthase (cGAS)- Stimulator of interferon genes (STING) pathways, as explained below (Figure 1.4) (Coppé et al., 2010; Freund et al., 2010; Hao, Wang and Zhang, 2022; Miller et al., 2021).

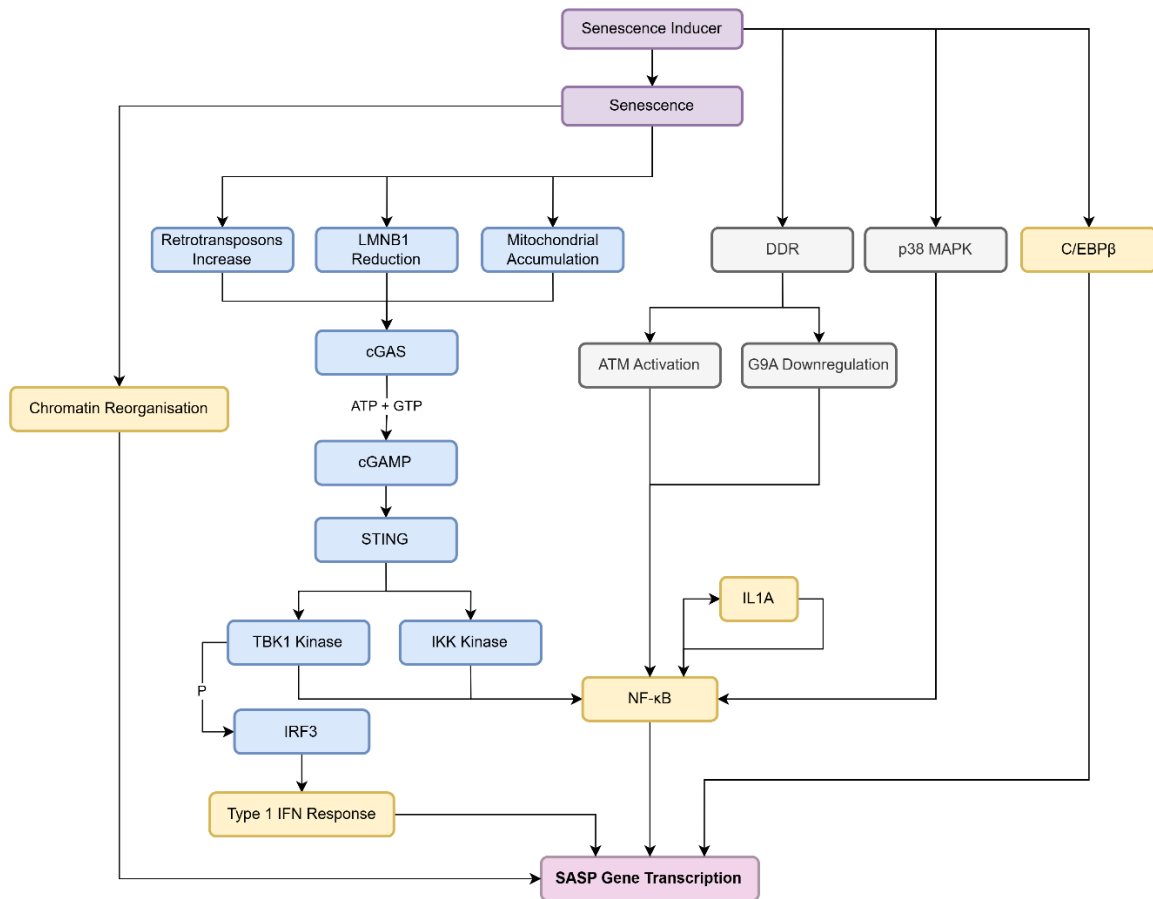


Figure 1.4. Summary of key SASP regulators. The SASP is dynamically regulated at many levels, and although DNA damage is a key driver of SASP in senescence, in some incidences SASP is also inducible via chromatin reorganisation and activation of p38 MAPK and cGAS-STING pathways. DNA damage repair (DDR), senescence associated secretory phenotype (SASP), Lamin B1 (LMNB1), Cyclic GMP-AMP synthase (cGAS), Stimulator of interferon genes (STING), cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), TANK binding kinase 1 (TBK1), inhibitor of nuclear factor kappa-B kinase (IKK), interferon regulatory factor 3 (IRF3), interferon (IFN), interleukin (IL), Ataxia-telangiectasia mutated (ATM), euchromatic histone-lysine N-methyltransferase 2 (G9a) and p38 Mitogen-activated Protein Kinase (p38 MAPK). Factors which directly contribute to SASP gene transcription are highlighted in yellow, whilst the pathways contributing to them are highlighted in blue and grey.

1.3.1 DNA Damage

Persistent DNA damage repair (DDR) signalling is a hallmark of senescence and is likely to induce SASP via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Kumari and Jat, 2021). Sustained ataxia telangiectasia mutated (ATM) signalling, driven by chronic DNA damage responses, regulates NF-κB, promoting expression of SASP factors, including IL6 and IL8 (Rodier et al., 2009). Continuous DNA damage also leads to downregulation of euchromatic histone-lysine N-methyltransferase 2 (G9a), a histone methyltransferase which increases euchromatin in regions encoding for SASP factors (Freund et al., 2010).

1.3.2 p38 MAPK

Although chronic DDR is a key driver of the SASP, it is not entirely responsible for its induction (Freund, Patil and Campisi, 2011; Wang et al., 2024a). p38 Mitogen-activated Protein Kinase (p38 MAPK) is responsible for coordinating cell stress responses to various stimuli, including oncogenic signalling, DNA damage and oxidative stress (Anerillas, Abdelmohsen and Gorospe, 2020; Freund et al., 2010; Xu et al., 2014). Its activation, enhances DNA damage driven NF- κ B signalling, inducing secretion of SASP factors, like IL6 and IL8 through multiple downstream effectors (Freund, Patil and Campisi, 2011). For example, p38 MAPK activates mitogen and stress activated protein kinase 1 (MSK1), which phosphorylates NF- κ B at SASP gene promoters, enhancing chromatin accessibility and promoting transcription of SASP factors (Anerillas, Abdelmohsen and Gorospe, 2020).

1.3.3 cGAS-STING

Endogenous cytoplasmic DNA (cytoDNA) is produced by senescent cells and acts as a ligand for cGAS-STING, activating several cytokine producing pathways (Hong, Tijhuis and Foijer, 2019; Miller et al., 2021; Ou et al., 2021; Wang et al., 2024a). CytoDNA refers to the aberrant presence of any genetic material in the cytoplasm and includes cytoplasmic chromatin fragments (CFs), mitochondrial DNA (mtDNA) and cytoplasmic complimentary DNA (Miller et al., 2021). The mechanism behind this cytoplasmic accumulation is poorly understood, however in senescent cells some DNases, which degrade cytoDNA are downregulated (Ohtani, 2022).

Several processes in senescent cells produce cytoDNA, including Lamin B1 (LMNB1) depletion, which degrades the nuclear membrane and results in the blebbing of chromatin into the cytoplasm, forming CFs (Miller et al., 2021; Ohtani, 2022). Accumulation of dysfunctional mitochondria also result in the release of mtDNA into the cytoplasm (Miller et al., 2021). An increase of retrotransposons (RTs), in senescent cells also contribute to cytoDNA production. RTs are mobile DNA sequences which can move between genomic sites by inserting themselves using a ribonucleic acid (RNA) intermediate. They are usually tightly repressed to prevent genomic instability (Janoušek et al., 2016). In senescent cells the RT, long interspersed element-1 (LINE-1) is no longer repressed, leading to the reverse transcription and accumulation of cytoDNA, which subsequently activates the cGAS-STING pathway (Briggs et al., 2018; Cecco et al., 2019; Ohtani, 2022).

cGAS detects and binds to cytoDNA, triggering cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) production, from adenosine triphosphate (ATP) and guanosine triphosphate (GTP). cGAMP binds to STING, triggering its translocation from the endoplasmic reticulum to golgi apparatus, where it activates TANK binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase (IKK) (Ohtani, 2022; Ou et al., 2021). TBK1 phosphorylates interferon regulatory factor 3 (IRF3), inducing type I interferon responses, which promote SASP signalling by increasing inflammatory cytokine production, enhancing NF- κ B signalling and reinforcing senescence (Frisch and MacFawn, 2020). IKK phosphorylates inhibitor of nuclear factor kappa B alpha (I κ B α) leading to its degradation and translocation of NF- κ B to the nucleus where it drives transcription of SASP genes. (Ohtani, 2022; Ou et al., 2021). The cGAS-STING pathway plays a key role in driving SASP in senescent cells by detecting cytoDNA and activating inflammatory signalling pathways. As the SASP can have both beneficial and detrimental effects, its regulation is a critical component of the senescence response.

1.3.4 Key transcription factors

Many pathways implicated in senescence, converge to activate NF- κ B, a transcriptional regulator modulating many SASP components (Kumari and Jat, 2021; Ohanna et al., 2011; Salminen, Kauppinen and Kaarniranta, 2012). The increased activity of NF- κ B in senescent cells results in the upregulation of several proinflammatory cytokines, chemokines and interleukins which initially can facilitate immune clearance. However, their persistent secretion can diminish the immune response, by generating an immunosuppressive microenvironment, where regulatory T-cells accumulate and inhibit natural killer cells, as a result, senescent cells are less efficiently cleared, allowing them to accumulate (Kumari and Jat, 2021; Wang et al., 2024a). NF- κ B activity is further enhanced by a positive feedback loop generated by IL1A, a cytokine which also regulates *IL6* and *IL8* transcription (Freund et al., 2010). Like NF- κ B, C/EBP β is a transcription factor with increased activity in senescent cells and contributes to SASP secretion (Freund et al., 2010; Salminen, Kauppinen and Kaarniranta, 2012; Wang et al., 2024a). Although its mechanism of induction is unclear, C/EBP β is particularly important to the production of IL6 and IL8, two highly conserved SASP components (Freund et al., 2010; Salminen, Kauppinen and Kaarniranta, 2012). Activation of the cGAS-STING pathways also result in the activation of IRF3, a transcription factor regulating interferon production (Frisch and MacFawn, 2020; Homann et al., 2022). When excessively activated IRF3 promotes a type I interferon response, increasing inflammatory cytokine production, enhancing NF- κ B signalling and reinforcing senescence (Frisch and MacFawn, 2020; Homann et al., 2022).

1.3.5 Chromatin Organisation

Changes in chromatin organisation also regulate SASP composition. Formation of senescence associated heterochromatin foci (SAHF), silences key proliferation genes, by preventing their transcription, but simultaneously allows SASP factors transcription (Hao, Wang and Zhang, 2022). This selective transcriptional regulation is mediated by high mobility group box 2 (HMGB2), which binds to SASP gene loci and prevents them from being incorporated into SAHF. By maintaining an open chromatin state at SASP loci, HMGB2 ensures that SASP factors continue to be highly expressed, when senescent cells are not proliferating (Aird et al., 2016). Alterations in histone modifications also contribute to SASP induction and regulation. Loss of repressive histone modifications, such as H3K9me2 at IL6 and IL8 promoters, increases euchromatin content, allowing their transcription (Lee et al., 2020; Takahashi et al., 2012). Secretion of IL6 and IL8 is further increased by sirtuin 1 (SIRT1) downregulation. SIRT1 is a histone deacetylase which when downregulated results in an increase in histone acetylation, chromatin relaxation and transcription of IL6 and IL8 (Criscione, Teo and Neretti, 2016; Hao, Wang and Zhang, 2022; Hayakawa et al., 2015).

1.4 SENESCENCE DETECTION

Several processes are highly conserved between senescent cells and are therefore considered to be hallmarks of senescence (Table 1.1) (Faragher, 2021; Hernandez-Segura, Nehme and Demaria, 2018). However, as senescent cells do not consistently share a specific phenotype, a combination of detection methods must be used to confirm senescence *in vitro*. It is recommended to identify 3 traits: including morphological changes, cell cycle arrest, and an additional trait, such as the presence of DNA damage or SASP factors (González-Gualda et al., 2021). The methods used in this research are outlined in Figure 1.5. It is important to conduct senescence studies using both senescent and untreated cell lines as a positive control, to compare results and validate each experiment.

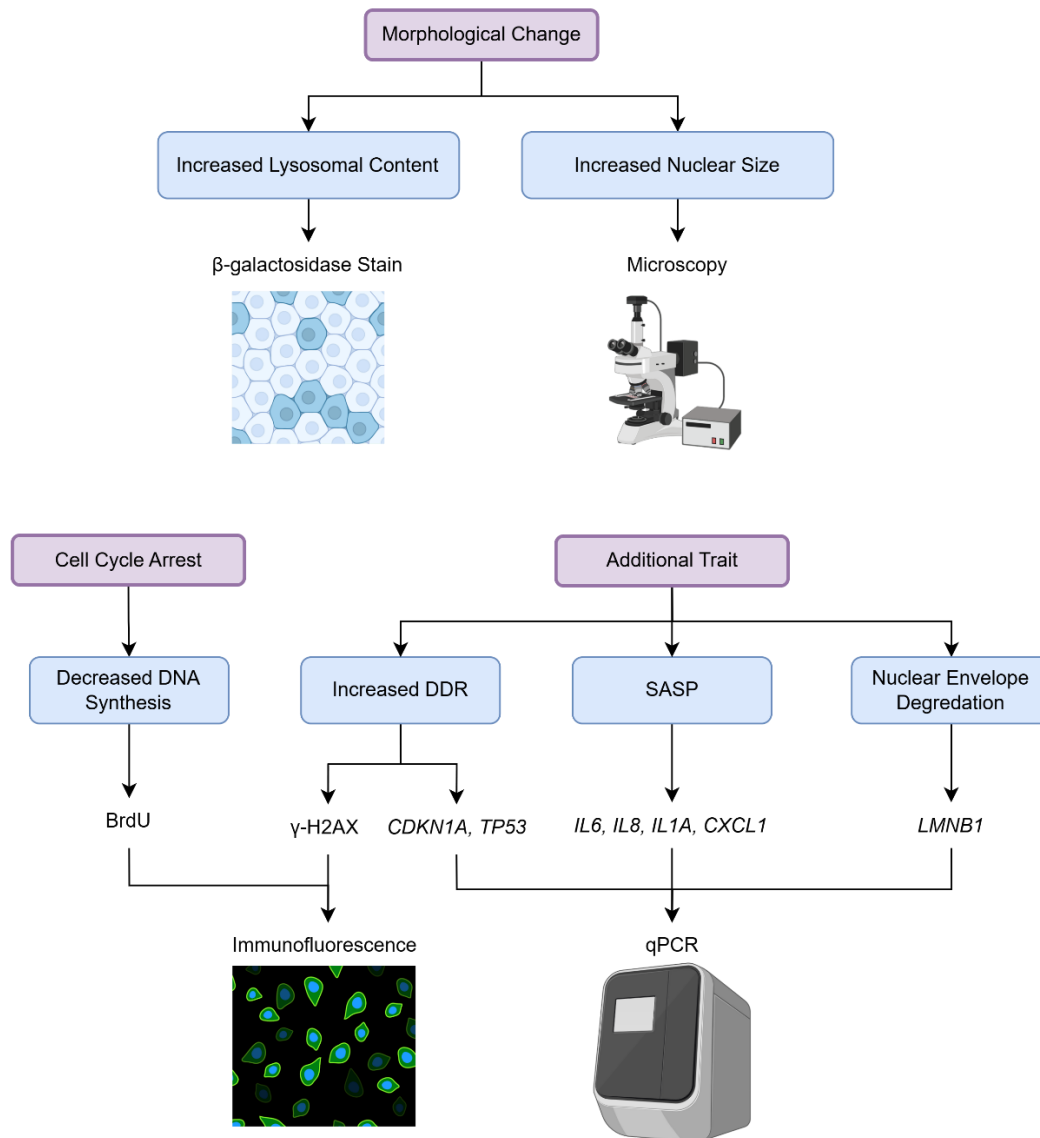


Figure 1.5. Summary of key senescence detection methods. It is recommended to identify 3 traits, highlighted in purple: including morphological changes, cell cycle arrest, and an additional trait. Markers of senescence, used in this research are highlighted in blue. DNA damage repair (DDR), senescence associated secretory phenotype (SASP), bromodeoxyuridine (BrdU), interleukin (IL), *Lamin B1* (*LMNB1*), *tumour protein 53* (*TP53*), chemokine (CXCL).

1.5 BREAST CANCER OVERVIEW

Senescence can contribute to breast cancer (BC) development and can also occur as a result of chemotherapy treatment, which is known as therapy induced senescence (TIS). BC is a heterogenous disease caused by the uncontrolled proliferation of aberrant cells within the breasts (Łukasiewicz et al., 2021). It most commonly originates from the cells lining the breast ducts but can also originate in the breast lobules (Łukasiewicz et al., 2021). Most BC cases are sporadic however 5-10% of BC sufferers are genetically predisposed (Table 1.3). BC is the most common cancer in the UK and is the

fourth most common source of cancer death, causing 11,200 deaths annually (Cancer Research UK, 2022).

Traditionally BC is classified based on its morphology, behaviour, and prognosis (Table 1.4) (Eliyatkin et al., 2015). Although, molecular traits of BC are now being used to make more accurate classifications (Table 1.5) (Eliyatkin et al., 2015). Triple negative BC (TNBC) accounts for 15% of all BC cases and 40% of BC deaths, it is one of the most clinically challenging cancers due to its aggressive metastasis and high relapse rates (Cancer Research UK, 2022). Although, BC survival is improving and has doubled in the last 40 years in the UK, with 85% of women surviving for 10 or more years after their diagnosis, 40-50% of TNBC patients are predicted to relapse within 8 years (Alzate-Granados and Niño, 2025; Cancer Research UK, 2022). Staging determines the severity of BC whereby in the numerical and tumour, node, metastasis (TNM) staging systems, an increasing number denotes increasing BC size, lymph node spread and metastasis (Cancer Research UK, 2025) (Table 1.6). Secondary BC is defined as BC which has spread outside of the breast.

Table 1.3. Gene mutations contributing to breast cancer development. Breast cancer (BC) (Bradbury and Olopade, 2007; Cancer Research UK, 2022; My Cancer Genome, 2017; Wang et al., 2021).

Gene	Syndrome	Description	Prevalence
<i>ATM</i> <i>serine/threonine</i> <i>kinase (ATM)</i>	Ataxia- telangiectasia	<i>ATM</i> encodes for a protein which functions in cell division, regulation, and DNA repair. Most mutations result in a non-functional protein causing abnormal DNA damage repair which leads to mutation accumulation and cancer. Ataxia-telangiectasia is an autosomal recessive condition.	Altered in 3.53% of BC's.
<i>BRCA1-associated</i> <i>RING domain</i> <i>protein 1 (BARD1)</i>		<i>BARD1</i> heterodimerises with <i>BRCA1</i> to maintain its stability. Mutations in <i>BARD1</i> lead to aberrant DNA damage repair, in turn causing mutation accumulation and cancer.	Altered in 1.06% of BC's.
<i>Breast cancer</i> <i>gene (BRCA) 1</i> <i>and BRCA2</i>	Hereditary Breast and Ovarian Cancer (HBOC)	<i>BRCA1</i> and <i>BRCA2</i> encode for tumour suppressor proteins which modulate DNA repair and cell cycle checkpoint activation. These proteins are critical to maintaining genome integrity and when mutated cause genome instability, mutation accumulation and cancer. HBOC is an autosomal dominant disorder.	<i>BRCA1</i> is altered in 3.12% of BC's and <i>BRCA2</i> is altered in 4.48% of BC's.
<i>Cadherin-</i> <i>1 (CDH1)</i>		Cadherins regulate cell adhesion within tissues, loss of function mutations can result in increased cell motility, promoting metastasis.	Altered in 3.13% of all cancers with BC accounting for a majority.
<i>Checkpoint kinase</i> <i>2 (CHEK2)</i>	<i>CHEK2</i> -related breast cancer	<i>CHEK2</i> encodes for checkpoint kinase 2 (CHK2), a cell cycle checkpoint kinase which is activated as part of DNA damage repair mechanisms. Commonly, in breast cancer mutations cause a non-functional protein leading to dysfunctional DNA repair, mutation accumulation and cancer. <i>CHEK2</i> mutations are inherited in an autosomal dominant pattern.	Altered in 1.27% of BC's.

<i>Phosphatase and tensin homolog (PTEN)</i>	Cowden's	<i>PTEN</i> encodes for an enzyme which functions as a tumour suppressor. The enzyme encoded for by <i>PTEN</i> controls cell division and apoptosis. Most mutations of <i>PTEN</i> cause uncontrolled cell division. Cowden's is an autosomal dominant disorder.	Altered in 7.35% of BC's.
<i>Serine/threonine kinase 11 (STK11)</i>	Peutz-Jeghers	<i>STK11</i> encodes for a tumour suppressor enzyme which prevents uncontrolled cell division, promotes appropriate cell polarisation, determines cell energy distribution, and promotes apoptosis. When mutated, many non-cancerous polyps in the gastrointestinal tract develop, spread, and become malignant. Peutz-Jeghers syndrome is an autosomal dominant condition.	Altered in 1.37% of BC's.
<i>Tumour protein 53 (TP53)</i>	Li-Fraumeni	<i>TP53</i> encodes for a transcription factor, which acts as a tumour suppressor, regulating many genes. It plays a major role in determining whether cells should repair damage or undergo apoptosis. Mutations in <i>TP53</i> mean its product does not arrest proliferating cells at cell cycle checkpoints or respond correctly to DNA damage, this causes mutation accumulation. Li-Fraumeni is an autosomal dominant disorder.	Altered in 39.68% of BC's.

Table 1.4. Breast cancer classification by morphology, behaviour, and prognosis. Human epidermal growth factor receptor 2 (HER2).

Breast cancer type	Description
Invasive/No special type (NST)	The cancer has spread from the duct lining to surrounding breast tissue. The cancer does not have any defining features.
Ductal carcinoma <i>in situ</i>	Cells in the duct lining are undergoing tumorigenesis, however, are contained within the breast ducts.
Invasive lobular	The cancer originates in cells lining breast lobules (milk producing glands) and has spread to surrounding breast tissue.
Triple negative	The cancer does not have receptors for the hormone's oestrogen and progesterone or HER2.
Inflammatory	The cancer cells block lymph channels in the breast, reducing lymphatic drainage and causing swelling.
Rare	Encompasses a group of less common breast cancers, which are defined by specific features, including mucinous, tubular, and basal BC.

Table 1.5. Breast cancer classification by molecular traits. Human epidermal growth factor receptor 2 (HER2), breast cancer gene 1 (BRCA1).

Breast cancer type	Description
Luminal A	Oestrogen and progesterone receptor positive but HER2 negative. Expresses low levels of the proliferation protein Ki-67 slowing its growth. Is likely to be a low grade and have a good prognosis.
Luminal B	Oestrogen receptor positive, progesterone and HER2 receptor variable. Expresses high levels of Ki-67.
Luminal B like	Oestrogen receptor positive and HER2 positive. Progesterone receptor variable. Can have any level of Ki-67 and grows slower than Luminal A cancers, however, has a worse prognosis.
HER2 enriched	Often oestrogen and progesterone receptor negative but HER2 positive. Grow faster than luminal BC and have a worse prognosis.
Triple negative	The cancer does not have receptors for oestrogen and progesterone or HER2. <i>BRCA1</i> gene faults are common. Is more aggressive than luminal BC.

Table 1.6. Tumour node metastasis staging system for breast cancer. Staging determines the severity of BC whereby an increasing number denotes increasing BC size, lymph node spread and metastasis (Cancer Research UK, 2025). Clinical node staging is shown.

Tumour (size or area of the cancer)			
T1: ≤2 CM	T2: 2-5 cm	T3 >5 cm	T4
T1MI: ≤ 0.1 CM			T4A: cancer has spread into the chest wall
T1A: 0.1-0.5 CM			T4B: cancer has spread into the skin
T1B: 0.5-1 CM			T4C: cancer has spread to both the skin and the chest wall
T1C: 1-2 CM			T4D: inflammatory carcinoma
Node (cancer spread to lymph nodes)			
N0	N1	N2	N3
NX: not assessable	N1: cancer has spread ≥1 lymph nodes in the lower or middle part of the armpit and are 0.2-2 cm	N2A: cancer cells are in the armpit are stuck together	N3a: cancer cells are in the lymph nodes below the collarbone.
N0: no spread to lymph nodes		N2B: cancer cells are in the lymph nodes behind the breastbone but not in the armpit.	N3b: cancer cells are in the lymph nodes around the armpit and behind the breastbone.
			N3c: cancer cells are in the lymph nodes above the collarbone.
Metastasis (cancer spread)			
M0	M0(i+)	M1	
M0: no spread	M0(i+) cancer cells are present in the blood, lymph nodes or far away from the breast	M1: cancer has spread to another part of the body.	

1.5.1 Treatment

TNBC treatment is dependent on the classification and stage of the cancer, although most patients will often undergo a combination of chemotherapy, surgery, targeted therapy, or radiotherapy (Moo et al., 2018). Chemotherapy uses cytotoxic drugs to kill fast-growing cancer cells and is used as a primary treatment for 34% of BC's (Cancer Research UK, 2022). Commonly, anthracyclines are used as a first line TNBC treatment, although several secondary drugs are also used (Table 1.7) (Moo et al., 2018).

Table 1.7. Chemotherapy drugs used in the treatment of breast cancer. Commonly anthracyclines and taxanes are used in combination with fluorouracil and cyclophosphamide to treat breast cancer (BC). The most common chemotherapy drugs and their mechanisms are shown.

Drug	Drug class	Description
Doxorubicin	Anthracycline	Inhibits topoisomerase II, causing an accumulation of torsional stress, inhibiting DNA replication. Also intercalates with DNA, forming DNA adducts inhibiting DNA synthesis and forms free oxygen radicals causing oxidative stress.
Epirubicin	Anthracycline	Inhibits topoisomerase II, causing an accumulation of torsional stress, inhibiting DNA replication. Also intercalates with DNA, forming DNA adducts inhibiting DNA synthesis.
Docetaxel	Taxane	Stabilises tubulin, preventing microtubule disassembly and causing mitotic arrest. Also binds to anti-apoptotic proteins, preventing their function.
Paclitaxel	Taxane	Stabilises tubulin, preventing microtubule disassembly and causing mitotic arrest.

1.6 THERAPY INDUCED SENESENCE

There are many inducers of senescence, however arguably one of the most important and clinically relevant is DNA damage (Kumari and Jat, 2021). Various stimuli can be responsible for DNA damage, including chemotherapeutics, which cause TIS (Moo et al., 2018). Doxorubicin was chosen for the purpose of this research as it is known to induce senescence by DNA damage and is also a primary treatment for TNBC. DNA damage activates DDR mechanisms to prevent the proliferation of damaged cells. However, if DNA damage is irreparable, cells will either undergo apoptosis or senescence (Kumari and Jat, 2021). The decision between apoptosis and senescence is governed by the magnitude and persistence of cell stress, with moderate DNA damage enforcing stable cell cycle arrest and severe damage inducing apoptosis (Childs et al., 2014; Petrova et al., 2016).

Regardless of the stimuli, DNA damage induced senescence is predominantly stimulated via the p53 pathway (Figure 1.6). The kinases, ATM and ataxia-telangiectasia and RAD3-related (ATR) are recruited to the site of DNA damage by various sensors (Maréchal and Zou, 2013; Yang et al., 2004). ATM is primarily activated by double strand breaks (DSBs) whereas ATR can be activated by a broader range of DNA lesions including DSBs, replication stalls and genotoxic stress (Maréchal and Zou, 2013; Yang et al., 2004). ATM and ATR amplify DDR signals by phosphorylating other mediators which localise DNA repair complexes and cause heterochromatin formation, promoting DNA repair (Ayrappetov et al., 2014; Lukas et al., 2003).

Checkpoint kinase (Chk) Chk1/Chk2 are serine/threonine kinases which are downstream of ATR/ATM and propagate DNA damage signalling by phosphorylating effector substrates (Maréchal and Zou, 2013; Smith et al., 2010). Chk1 initiates DNA repair in S phase and at the G₂/M checkpoint by phosphorylating m-phase inducer phosphatase (CDC25) phosphatase, a cyclin and CDK regulator, inducing G₂ cell cycle arrest in response to DNA damage (Liu et al., 2020a; Patil, Pabla and Dong, 2013). Chk2 promotes DNA repair at the G₁/S phase checkpoint by phosphorylating p53. This reduces MDM2-p53 binding affinity, thereby reducing p53 degradation, increasing its levels (Chehab et al., 1999; Kumari and Jat, 2021). p53 subsequently upregulates p21^{WAF/CIP1}, inhibiting cell cycle progression (Chehab et al., 1999). As chronic DDR increases p53 and p16 expression, levels of protein phosphorylation can be measured to indicate senescence. However, as DDR is implicated in healthy, non-senescent cells, this parameter must be used in conjunction with other tests to confirm senescence (Hernandez-Segura, Nehme and Demaria, 2018).

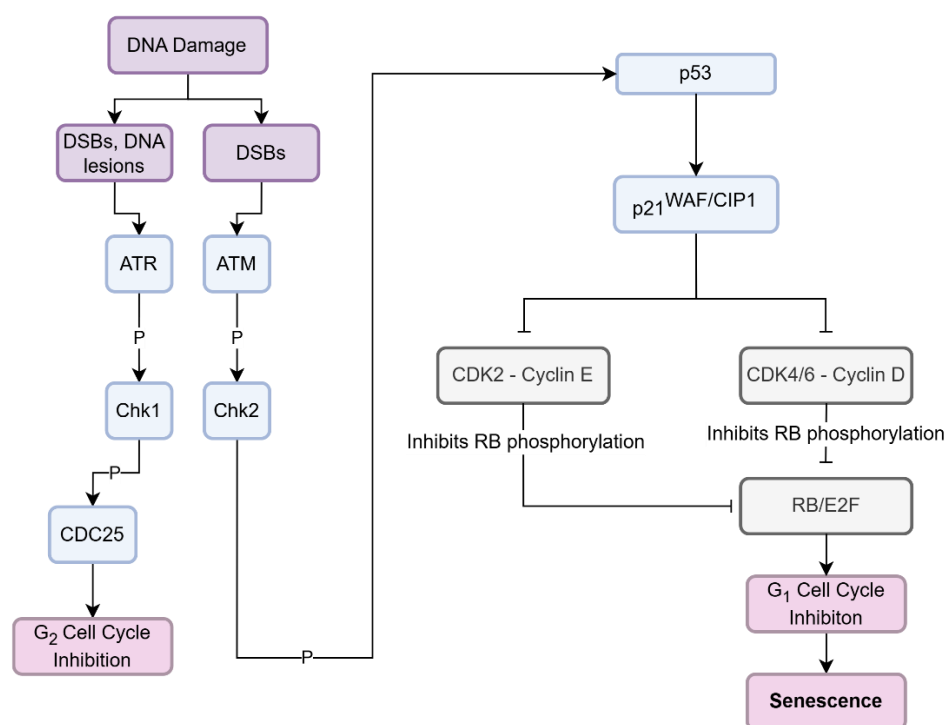


Figure 1.6. Summary of key pathways involved in DNA damaged induced senescence. Double strand breaks (DSBs), DNA damage repair (DDR), phosphorylation (P), ataxia-telangiectasia and RAD3-related (ATR), Ataxia-telangiectasia mutated (ATM), Checkpoint kinase (Chk), m-phase inducer phosphatase (CDC25), cyclin dependent kinase (CDK), retinoblastoma protein (RB).

1.7 THE DUAL ROLE OF TIS IN TNBC

TIS plays a paradoxical role in cancer progression, acting as both a tumour-suppressive and tumour-promoting mechanism. The extent of these effects is treatment dependent but are often a consequence of chemotherapies like doxorubicin, which are administered in short, intensive cycles to limit toxicity (Rawat et al., 2021). DNA-damaging agents induce a more potent senescence response whereas cytoskeletal drugs such as paclitaxel elicit a weaker response (Chang et al., 1999; Luo et al., 2025).

Senescence is generally regarded as a tumour suppressive mechanism, preventing the transformation of pre-malignant cells, or uncontrolled proliferation of cancer cells, in response to damage (Lee and Lee, 2014; Luo et al., 2025). In its early stages, the SASP serves as a protective barrier promoting immune surveillance and clearance of damaged cells. However, chronic SASP secretion impairs this response, enabling immune evasion and senescent cell accumulation (Fitsiou, Soto-Gamez and Demaria, 2022; Lannello et al., 2013; Paula et al., 2023). TIS is inducible in multiple cell types, both

healthy and cancerous which contribute to a pro-tumorigenic microenvironment through SASP signalling. TIS tumour cells can also promote cancer relapse.

Persistent SASP secretion creates a pro-inflammatory and pro-tumorigenic microenvironment where SASP components drive angiogenesis, microenvironment remodelling, tumour invasion and metastasis (Luo et al., 2025). The chronic secretion of SASP factors such as VEGF and IL6, promote tumour progression by enhancing angiogenesis and invasion, respectively (Coppé et al., 2006). Other SASP secretions such as matrix metalloproteinases (MMPs) and chemokines like C-X-C motif chemokine ligand 1 (CXCL1), promote metastasis via tissue vascularisation, remodelling and reducing cell adhesion (Allavena et al., 2008; Fitsiou, Soto-Gamez and Demaria, 2022). IL6 and IL8 induce senescence in MCF7 cells, increasing their migratory capacity. This effect is enhanced by autocrine signalling, which illustrates the tumour promoting potential of the SASP (Ortiz-Montero, Londoño-Vallejo and Vernot, 2017). Persistent SASP reinforces senescence in both an autocrine and paracrine manner, propagating inflammation and promoting tumour progression (Fitsiou, Soto-Gamez and Demaria, 2022; Paula et al., 2023).

Although initially, the SASP of TIS cells promotes immune clearance, prolonged SASP can result in immune evasion (Saleh, Tyutyunyk-Massey and Gewirtz, 2019). SASP components recruit immunosuppressive cells like regulatory T cells, allowing them to evade immune surveillance. Chronic SASP secretions such as TGF- β , IL6, and IL10, promote resistance to immune-mediated killing by inhibiting T cell proliferation and function (Luo et al., 2025; Wang et al., 2020). This effect is further enhanced by the increased metabolic demands of TIS cells, which deprive T cells of nutrients (Chang et al., 2015). Immune cells can also undergo senescence, reducing their ability to clear TIS tumour cells (Luo et al., 2025). By suppressing immune clearance TIS cells create a microenvironment, beneficial for tumour progression.

Although, senescence is generally considered to be irreversible, in tumour cells it is often unstable with some studies showing arrest can be bypassed, allowing senescent tumour cells to re-enter the cell cycle and proliferate (Mastri et al., 2018; (Saleh, 2024; Saleh, Tyutyunyk-Massey and Gewirtz, 2019). This can result in the re-emergence of tumours, weeks after TIS induction, even if a small population of escapes (<1%) (Bajtai et al., 2025; Elmore et al., 2005; Fitsiou, Soto-Gamez and Demaria, 2022). This could be a contributing factor to the >30% relapse rate in BC patients (Chembukavu and

Lindsay, 2024). In some TIS models, including BC, this escape from senescence occurs when TIS cells acquire more stem-like properties (Achuthan et al., 2011; Milanovic et al., 2018; Pacifico et al., 2021; Tatar et al., 2023). In other cancer types, such as head and neck squamous cell carcinoma, TIS escape occurs through p53 or p16 inactivation (Palazzo et al., 2022). It is also important to consider, that whilst studies suggest senescence can be bypassed, without single cell tracking, it isn't possible to determine whether a sub population of non-senescent cells has proliferated. TIS cancer cells can also act as a reservoir for cancer relapse by becoming resistant to apoptosis, which allow TIS cells to accumulate within the tumour microenvironment (TME) (Fitsiou, Soto-Gamez and Demaria, 2022; Prasanna et al., 2021). This prevents them from being targeted by non-specific drugs, reliant on halting DNA replication, such as doxorubicin (Fitsiou, Soto-Gamez and Demaria, 2022). Ultimately the accumulation of TIS cells enhances all the effects described above, through autocrine and paracrine signalling.

The dual role of TIS in TNBC complicates BC treatment options. Therefore, combination approaches, such as the “one-two punch” strategy, which integrates conventional BC treatments with anti-senescence drugs, may be effective in mitigating the pro-tumorigenic effects of TIS (Chembukavu and Lindsay, 2024; Paula et al., 2023).

1.7.1 Clinical relevance of TIS in TNBC

Chemotherapy remains a major component of BC treatment, with ~20-80% of patients receiving chemotherapy as part of their treatment, depending on BC subtype (Saleh et al., 2021). A meta-analysis reported that 81% of chemotherapy is anthracycline based, which results in a higher relapse compared to other treatments (Asselain et al., 2018). As >30% of BC patients experience disease recurrence, it is important to better understand the mechanisms associated with this (Chembukavu and Lindsay, 2024).

TIS has been observed in several cancers including melanoma, lymphoma, lung and breast cancer (Fleury et al., 2019; Luo et al., 2025). In BC specifically, chemotherapy drugs have been shown to induce TIS in tumours. One study found 41% of tumours, from patients receiving chemotherapy, contained β -galactosidase positive cells, compared to only 10% of tumours in untreated patients (Poele et al., 2002). This suggests that treatment increases tumour senescence. More recently, among BC tumours which showed little or no response to chemotherapy 40% showed markers of TIS,

including increased p21 expression and decreased LMNB1 expression (Saleh et al., 2021). Of these, 8% of patients relapsed, potentially indicating that TIS contributes to poor therapeutic response and recurrence. Markers of TIS, including p16, have also been detected systemically, in BC patients receiving anthracycline based chemotherapy (Sanoff et al., 2014). TIS markers increased in T-cells immediately after treatment and remained elevated for 12 months later, potentially indicating TIS cells remained after treatment (Sanoff et al., 2014).

Another study suggested that levels of basal senescence (indicated by elevated β -galactosidase and decreased LMNB1) vary between BC types (Cotarelo et al., 2016). Highest levels of basal senescence were found in Luminal A tumours (75% β -galactosidase positive) whereas the lowest was found in TNBC (0% β -galactosidase positive). This indicates senescence is tumour dependent and potentially affects the extent to which TIS is induced. Ultimately, these findings indicate the clinical presence of senescence in BC. If chemotherapy induces TIS and contributes to relapse, then targeting TIS cells, or their SASP may improve treatment outcomes.

1.8 TNBC BIOMARKERS

TNBC remains one of the most clinically challenging BC subtypes, due to its high recurrence and poor overall survival (Cancer Research UK, 2022). The absence of hormone receptors limits the use of targeted therapies, and the lack of effective early detection methods often results in late stage diagnosis, when treatment is more difficult. However, the emergence of novel prognostic and predictive biomarkers promises to improve patient stratification and treatment personalisation (Veyssière et al., 2022). Biomarkers are measurable biological characteristics, found in blood, body fluids, or tissues, that provide information about biological and pathogenic processes, or responses to therapeutic intervention (Passaro et al., 2024). In oncology, clinicians can use them to aid diagnosis, stratify patients, predict therapeutic response and estimate survival or relapse rates (Banerjee et al., 2024; Califf, 2018; Passaro et al., 2024). Biomarkers encompass a broad range of biological features, including protein, RNA and DNA expression. In TNBC, multiple biomarker types have been reported to be beneficial in determining response to treatment or predicting survival (Table 1.8) (Cocco et al., 2020; Sukumar et al., 2021; Wang et al., 2018).

Table 1.8. Predictive and prognostic TNBC biomarkers. Epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), Human epidermal growth factor receptor 2 (HER2), Breast cancer gene (BRCA), circulating tumour cells (CTC) and circulating tumour DNA (ctDNA). Table summarised using information from Cocco et al., 2020; Sukumar et al., 2021 and Wang et al., 2018.

Type	Biomarker	Description	Summary
Protein	EGFR	Promotes cell survival.	High expression is associated with poor survival.
	VEGF	Promotes angiogenesis.	High expression is associated with metastasis.
	HER2	Promotes cell proliferation.	Predictor of response to tyrosine kinase inhibitors.
RNA	MicroRNAs	Non-coding RNAs regulating transcription.	Several signatures are predictors of poor survival and chemoresistance.
	RNA	Predicts survival.	Differential expression in tissues predicts overall and disease-free survival
DNA	<i>BRCA 1/2</i>	Tumour suppressor genes.	High expression is associated with a positive response to platinum treatments and PARP inhibitors.
Circulating tumour cells	CTC	Cancer cells within the bloodstream.	Increased levels are associated with poor survival.
	ctDNA	Fragments of cancer cell DNA within plasma.	Increased levels are associated with poor survival.

Several biomarkers have been used routinely in BC to stratify patients by risk, guide treatment decisions and facilitate sub-typing (Banerjee et al., 2024). Germline *BRCA1/BRCA2* mutations are used to assess the risk of developing BC and have more recently been used as predictive biomarkers to guide targeted therapy. The PARP inhibitor talazoparib (Talzenna) has been approved for the treatment of *BRCA* mutated BCs and has improved progression free survival in affected patients (Litton et al., 2018). Similarly, programmed death ligand 1 (PDL1) expression $\geq 1\%$ identifies TNBC patients eligible for treatment with atezolizumab and nab paclitaxel, improving survival by ~2.5 months (Schmid et al., 2018). Molecular classification based on receptor status is commonly used to define BC subtypes and ultimately guide therapeutic decisions, where the absence of oestrogen receptor (ER), progesterone receptor (PR), and HER2 characterises TNBC (Eliyatkin et al., 2015).

Despite many biomarkers having *in vitro* success, relatively few have been approved for routine clinical use (Cocco et al., 2020; Füžéry et al., 2013). The limited clinical translation of biomarkers can in part be attributed to the heterogeneity of TNBC. Variability in biomarker expression between patients, temporal changes in TNBC, and expression of markers in healthy tissues complicates the identification of universal biomarkers and prevents reliable expression thresholds from being defined (Banerjee et

al., 2024; Sukumar et al., 2021). As TIS involves extensive transcriptional reprogramming, gene expression changes were explored in this study as potential biomarkers of TIS within TNBC.

1.9 SENOTHERAPY OVERVIEW

Senotherapies target senescent cells to mitigate their detrimental effects on tissue function and overall health. They are broadly categorised into senolytic and senomorphic drugs. Senolytic agents selectively eliminate senescent cells by targeting their survival pathways, whereas senomorphic drugs suppress the SASP without inducing cell death (Zhang et al., 2023). Although, senolytic drugs seem promising, their lack of specificity, off target effects and associated side effects limit their clinical utility. In addition, the heterogeneity of senescent cells, complicates the development of specific senolytics. For this reason, senomorphic drugs have gained increasing attention, as they dampen the SASP, one of the most detrimental consequences of senescence, without causing significant side effects (Saliev and Singh, 2025; Zhang et al., 2023). Several drugs have been investigated for their senomorphic properties, of which rapamycin and metformin are widely researched, and have been proven to inhibit SASP (Saliev and Singh, 2025; Zhang et al., 2023). However, rapamycin is a potent immunosuppressant, with numerous side effects, limiting its clinical utility (NICE, 2025). In contrast, metformin is better tolerated, and is already being used in large scale studies, such as The Targeting Aging with Metformin (TAME) to evaluate its anti-aging properties (Barzilai et al., 2016).

1.9.1 Metformin

Metformin (1,1-dimethylbiguanide hydrochloride) is a well-established, antihyperglycemic drug, which is used as a first line treatment in >200 million patients with type 2 diabetes mellitus (T2DM) (Foretz, Guigas and Viollet, 2023). It is administered orally at a standard dose of 0.5-2.5 g daily and has a plasma concentration of ~4-40 μ M in T2DM patients (Foretz, Guigas and Viollet, 2023; Graham et al., 2011; LaMoia and Shulman, 2021). Although, systemic levels have been measured up to ~70 μ M (He and Wondisford, 2015). Metformin primarily lowers blood glucose levels by suppressing hepatic gluconeogenesis, a metabolic process that synthesises glucose from non-carbohydrate precursors, like amino acids, lactate and glycerol (Foretz, Guigas and Viollet, 2023). It is a hydrophilic compound, which at a physiological pH is charged positively, therefore primarily enters and leaves cells through organic cationic transporter 1 (OCT1) (Kulkarni, Gubbi and Barzilai, 2020). Mechanistically, metformin inhibits mitochondrial complex I, reducing oxidative phosphorylation (the formation of ATP from oxygen reduction) (Foretz, Guigas and Viollet, 2023). Under normal conditions, ATP is rapidly

regenerated from adenosine diphosphate (ADP) by oxidative phosphorylation. However, inhibition of oxidative phosphorylation reduces ATP availability, leading to ADP accumulation (Foretz, Guigas and Viollet, 2023). This drives the adenylate kinase reaction, which converts ADP into ATP and adenosine monophosphate (AMP) (Foretz, Guigas and Viollet, 2023). The rise in AMP, indicates a low energy state, activating AMP-activated protein kinase (AMPK), a key energy sensor, and inhibiting key gluconeogenic enzymes, like adenylate cyclase and fructose-1,6-bisphosphatase-1, reducing hepatic glucose production (Hardie, Ross and Hawley, 2012).

However, metformin's mechanisms are not fully understood and studies have shown that it exerts unintended senotherapeutic effects, such as reducing inflammatory cytokine production (SASP components), reactive oxygen species (ROS) levels, and mitochondrial biogenesis, making it a compelling candidate for the investigation of senescence attenuation and reversal (Barzilai et al., 2016; Nehlin, 2023; Zhang et al., 2023). Mechanistically, metformin's senotherapeutic effects can be broadly categorised into AMPK dependent (Figure 1.7) and independent (Figure 1.8) pathways. It is important to note that these effects are context and concentration dependent as, high metformin concentrations (1-20 mM) have been shown to induce cell cycle arrest, apoptosis, impair proliferation and colony formation in some BC models (Alimova et al., 2009; Liu et al., 2009; Williams et al., 2013).

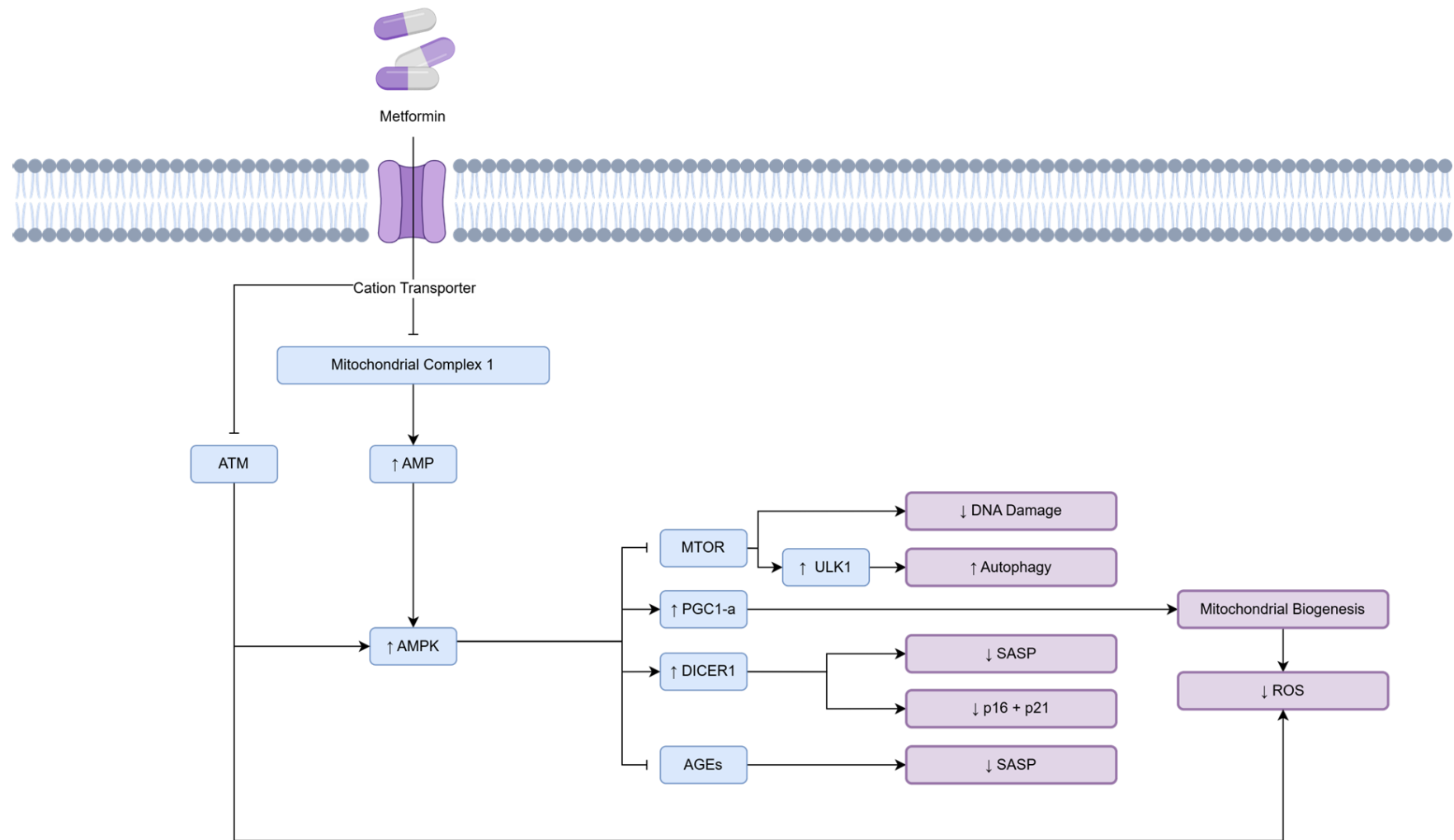


Figure 1.7. AMPK dependent mechanisms of metformin on senescence attenuation. Ataxia-telangiectasia mutated (ATM), adenosine monophosphate (AMP), AMP-activated protein kinase (AMPK), mechanistic target of rapamycin (MTOR), proliferator-activated receptor-gamma coactivator-1 alpha (PGC1A), dicer 1 ribonuclease III (DICER1), advanced glycation end products (AGE), senescence associated secretory phenotype (SASP) and reactive oxygen species (ROS).

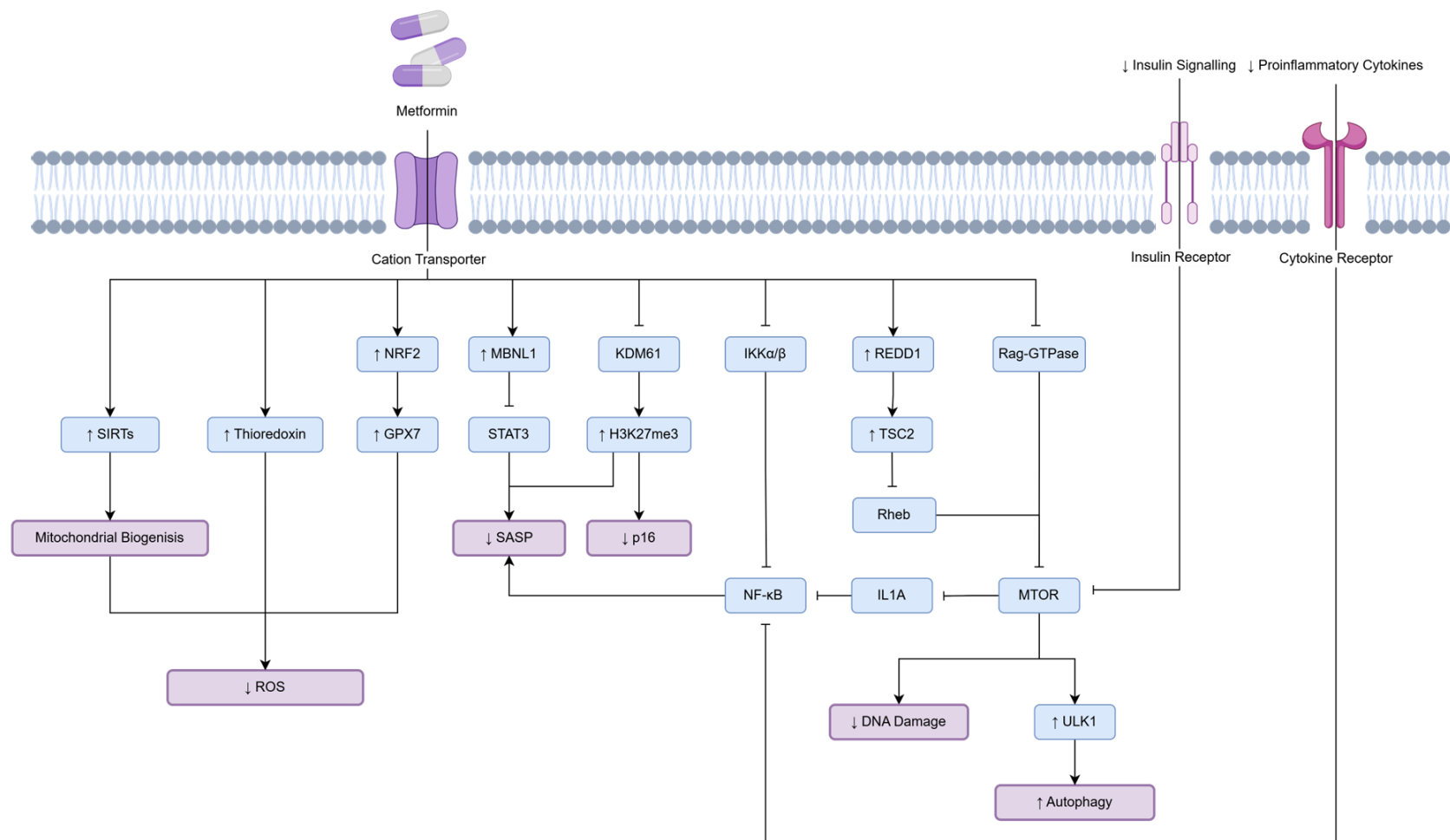


Figure 1.8. AMPK independent mechanisms of metformin on senescence attenuation. Sirtuin (SIRT), nuclear factor erythroid 2-related factor 2 (NRF2), glutathione peroxidase 7 (GPX7), muscle blind like splicing regulator 1 (MBNL1), signal transducer and activator of transcription 3 (STAT3), senescence associated secretory phenotype (SASP), lysine demethylase 6A (KDM6), kinase and inhibitor of nuclear factor kappa-B kinase (IKK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), DNA damage response 1 (REDD1), tuberous sclerosis complex 2 (TSC2), interleukin (IL), mechanistic target of rapamycin (MTOR), Unc-51 like autophagy activating kinase 1 (ULK1), ras homolog enriched in brain (Rheb), ras-related GTP-binding protein (Rag-GTPase), trimethylation of histone H3 at lysine 27 (H3K27me3) and reactive oxygen species (ROS).

1.9.2 AMPK dependent mechanisms

The inhibition of mitochondrial complex 1, ultimately leads to the activation of AMPK and several downstream effects, which could attenuate senescence by reducing SASP, mitochondrial dysfunction, and DNA damage, as well as by promoting autophagy.

1.9.2.1 SASP reduction

By attenuating SASP components, metformin could reduce the inflammatory environment, which promotes paracrine senescence induction. The activation of AMPK by metformin inhibits the production of advanced glycation end products (AGEs), glycated proteins or lipids resulting from sugar exposure. AGEs trigger inflammation by interacting with RAGE, Receptors for AGEs, which ultimately promote NF- κ B activation (Yamagishi, 2011). Therefore, inhibition of AGEs by metformin, results in reduced inflammatory cytokine production and therefore may also act to attenuate the SASP (Chung et al., 2017).

Dicer 1, ribonuclease III (DICER1) is also activated by AMPK and is an enzyme that processes precursor microRNAs (pre-miRNAs) into mature microRNAs (miRNAs). Heterogeneous nuclear ribonucleoprotein D (HNRNPD) is a nucleic acid binding protein, responsible for messenger (m)-RNA pre-processing. In human fibroblasts, HNRNPD stabilises DICER1 mRNA, allowing it to accumulate. This ultimately results in reduced p16 and p21 protein levels (key regulators of senescence induction and maintenance), as well as decreased *IL6*, *IL8*, *CXCL1* and *CXCL2* (SASP components) gene expression (Hooten et al., 2016).

1.9.2.2 Improving mitochondrial function

Senescence associated mitochondrial dysfunction (SAMD) is a hallmark of senescence, known to increase ROS production and active mechanistic target of rapamycin (mTOR), reinforcing and stabilising senescence (Miwa et al., 2022; Nacarelli, Azar and Sell, 2015; Von-Zglinicki, 2024). Persistent DNA damage not only triggers cell cycle arrest but also contributes to mitochondrial dysfunction, amplifying ROS production. This, in turn, exacerbates DNA damage and promotes the secretion of SASP factors (Correia-Melo et al., 2016; Passos et al., 2010; Von-Zglinicki, 2024). Metformin has been shown to alleviate SAMD. Proliferator-activated receptor-gamma coactivator-1 alpha (PGC1- α) is a key regulator of mitochondrial biogenesis (Handschin, 2009). Its expression is increased by metformin and

reduces oxidative stress. This has been demonstrated in both mouse and human hepatocytes, as well as rat skeletal muscle (Aatsinki et al., 2014; Masataka, 2006).

1.9.2.3 Autophagy activation

Autophagy removes damaged organelles, misfolded proteins, and pathogens, by sequestering them into autophagosomes (specialised vesicles), which fuse with lysosomes, to degrade and recycle their contents (Khandia et al., 2019). AMPK promotes autophagy by inhibiting mTOR and activating unc-51 like autophagy activating kinase 1 (ULK1), a kinase required for autophagy initiation. ULK1 plays a critical role in recruiting other autophagy related proteins to the autophagosome formation site (a region in the cytoplasm where proteins assemble to form double membraned autophagosomes). This facilitates the removal of damaged cellular components, via fusion with lysosomes (Khandia et al., 2019). Although autophagy is commonly upregulated in senescent cells, it is often inefficient, resulting in the accumulation of dysfunctional components (Cayo et al., 2021). Metformin, through the activation of AMPK, promotes more efficient autophagy in senescent cells, resulting in the clearance of dysfunctional organelles and misfolded proteins. In oxidative stress induced senescence (OSIS) intervertebral disc cells, metformin promoted autophagy and reduced markers of senescence, such as β -galactosidase staining (Chen et al., 2016). A similar effect was observed in a study by Kuang et al., (2020) who demonstrated that senescence attenuation by metformin was dependent on autophagy. Metformin reduced several hallmarks of senescence in OSIS human ligament cells including DNA damage, β -galactosidase activity, senescence pathway activation (p16, p21, p38, p53), and ROS accumulation (Kuang et al., 2020). In contrast, Luo et al., (2011) reported that autophagy inhibition in human fibroblasts reduced OSIS. The discrepancies between these findings may be attributed to differences in the concentrations of hydrogen peroxide used to induce OSIS, as varying levels of oxidative stress could differentially activate autophagy pathways. It is possible that low doses of hydrogen peroxide promote beneficial and protective autophagy, whereas higher doses overwhelm these mechanisms and lead to senescence.

1.9.2.4 DNA Damage

Several studies suggest that metformin activates ATM, triggering a DDR response (Sacco et al., 2016; Storozhuk et al., 2013; Vazquez-Martin et al., 2011; Zhang et al., 2023). This activation may contribute to the increased DNA damage observed in TIS. However, ATM also functions upstream of AMPK, and mutations near ATM have been associated with reduced AMPK activation (Ambrose and Gatti, 2013; Harries et al., 2011). Therefore, an increase in ATM activation may beneficially enhance AMPK

dependent senotherapeutic effects. In contrast, Halicka et al., (2011) showed that low metformin doses could reduce ATM signalling and lower markers of DNA damage, such as γ -H2AX, potentially by decreasing oxidative stress. Despite these conflicting findings, it is clear that metformin plays a role in ATM activation. Given that this is a key driver of DNA damage induced TIS, it is important to understand how metformin may regulate this.

1.9.3 AMPK Independent Mechanisms

Metformin can also act through AMPK independent mechanisms to alleviate senescence by attenuating SASP and reducing ROS production.

1.9.3.1 SASP Reduction

Metformin also acts as a senotherapeutic, independently of AMPK activation. In fibroblasts, it inhibits the expression of genes encoding for multiple SASP components, including *IL6*, *IL8*, *IL1A* and *CXCL2* (Moiseeva et al., 2013). Metformin inhibits the NF- κ B pathway, a key regulator of inflammation and cell stress responses, by interfering with upstream signalling components. NF- κ B is normally bound to its inhibitor of NF- κ B (I κ B), in the cytoplasm, where it remains inactive. Once I κ B is phosphorylated, by IKK α / β (I κ B kinase α / β), it degrades, allowing NF- κ B to translocate to the nucleus and activate target genes. Metformin prevents NF- κ B activation, by inhibiting I κ B phosphorylation and degradation (Cameron et al., 2016). This reduces the ability of NF- κ B to transcribe pro-inflammatory SASP components (Cameron et al., 2016; Moiseeva et al., 2013).

In renal epithelial cells, metformin reduces senescence by upregulating the expression of the RNA-binding protein muscleblind-like splicing regulator 1 (MBNL1). MBNL1 binds to multiple RNAs, regulating their stability and function, and in BC interacts with tumour suppressor RNAs, preventing metastasis (Fish et al., 2016; Jiang et al., 2020). In metformin treated cells, MBNL1 negatively regulates the transcription factor signal transducer and activator of transcription 3 (STAT3) (Jiang et al., 2020). As STAT3 inhibition has been associated with reduced pro-inflammatory cytokine production, in murine lung cancer, it is possible metformin prevents inflammation by indirectly repressing STAT3 (Mikyskova et al., 2023).

1.9.3.2 Oxidative Stress Reduction

ROS can be generated by endogenous factors, like mitochondrial activity ROS or exogenous factors, like chemotherapy drugs. Metformin reduces genomic instability by mitigating oxidative stress and DNA damage, although the precise mechanisms behind this remain unclear (Najafi et al., 2018). Research indicates that metformin lowers ROS induced by various stressors, thereby protecting cells from genomic instability (Najafi et al., 2018). For example, Śmieszek et al., (2017) showed that metformin could reduce β -galactosidase staining and ROS, when mouse olfactory cells were treated with metformin. Metformin can achieve this by acting as an antioxidant, neutralising free radicals through the donation of hydrogen atoms from its chemical groups. It also enhances the cell's natural antioxidant defences by upregulating thioredoxin, a protein that removes ROS and repairs oxidative damage (Hou et al., 2010; Najafi et al., 2018).

Prolonged metformin treatments extend the lifespan of human fibroblasts and stem cells, by upregulating endoplasmic reticulum-localised glutathione peroxidase 7 (GPx7), which is a peroxidase (uses hydrogen peroxide or organic peroxide to oxidize other molecules), predicted to function in oxidative stress responses. GPx7 expression levels are decreased in senescent cells, contributing to increased ROS and misfolded protein accumulation (Fang et al., 2018). Metformin increases nuclear accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that protects cells from oxidative stress, by activating antioxidant and detoxification genes. Nrf2 binds to the antioxidant response elements in the *GPX7* gene promoter inducing its expression, resulting in decreased β -galactosidase staining and likely reduced ROS (Fang et al., 2018).

Metformin has also been shown to influence sirtuins (SIRTs), a family of proteins critical for metabolic sensing and mitochondrial function (Cuyàs et al., 2018a). Metformin directly activates SIRT1, a protein that supports mitochondrial function and metabolic regulation (Gomes et al., 2013). It also indirectly activates SIRT3, enhancing mitochondrial function in endothelial cells (Karnewar et al., 2018). Therefore, metformin may act on sirtuins to reduce ROS and improve mitochondrial health in senescent cells (Lee et al., 2019).

1.9.3.3 Epigenetic modification

A hallmark of senescence is the formation of SAHF's, which repress key proliferation genes, contributing to the maintenance of cell cycle arrest (Olan, Handa and Narita, 2023). Metformin can

regulate transcriptional activity through histone modifications (Bridgeman et al., 2018; Sabit et al., 2018). In senescence, loss of trimethylation of histone H3 at lysine 27 (H3K27me3, a repressive histone mark, preventing gene transcription), at the *CDKN2A* locus, increases p16 and SASP transcription (Cai et al., 2021; Ito et al., 2018). Histone demethylase lysine demethylase 6A (KDM6A), is a histone demethylase which removes the repressive H3K27me3 mark, leading to chromatin opening and increased gene expression. Since metformin inhibits KDM6A, H3K27me3 is maintained (Cai et al., 2021; Cuyàs et al., 2018b; Kulkarni, Gubbi and Barzilai, 2020). It is therefore possible this prevents p16 and SASP transcription in senescent cells (Cai et al., 2021; Cuyàs et al., 2018b; Kulkarni, Gubbi and Barzilai, 2020).

1.9.4 Inhibition of mTOR

Metformin has also been shown to reduce mTOR activity through both AMPK dependent and independent mechanisms (Figure 1.7 and Figure 1.8) (Dey et al., 2023). mTOR is a serine/threonine kinase involved in the integration of multiple metabolic and growth promoting signals (Cayo et al., 2021; Weichhart, 2018). Metformin has been shown to decrease insulin and insulin-like growth factor 1 levels and signalling in cancer cells (Kulkarni, Gubbi and Barzilai, 2020; Sarfstein, 2013). As insulin signalling strongly activates mTOR, its reduction reduces mTOR activity. Accumulating evidence indicates that mTORC1 (an mTOR complex) activity is required for the orchestration of senescence (Cayo et al., 2021; Papadopoli et al., 2019). Metformin prevents mTORC1 signalling, which integrates nutrient and growth factor signalling, to regulate cell growth and metabolism. mTORC1 activation depends on its localisation within the lysosome, which is regulated by ras-related GTP-binding protein (Rag-GTPases). In the presence of amino acids, Rag-GTPases potentially activate mTORC1, facilitating its localisation, and signalling, at the lysosomal membrane (Lama-Sherpa et al., 2023). Rag-GTPases ensure that mTORC1 is activated in nutrient rich conditions (Lama-Sherpa et al., 2023). As metformin inhibits Rag-GTPases, this prevents mTORC1 from localising at the lysosome surface, blocking downstream signalling, associated with senescence (Cayo et al., 2021; Kalender, 2010; Papadopoli et al., 2019).

Metformin also indirectly inhibits mTOR by upregulating regulated in development and DNA damage response 1 (REDD1). REDD1 upregulates tuberous sclerosis complex 2 (TSC2) activity which subsequently inhibits ras homolog enriched in brain (Rheb), a small GTPase that activates mTORC1 (Sahra et al., 2011). By enhancing TSC2 activity, REDD1 indirectly suppresses mTORC1. AMPK activation also phosphorylates TSC2, further enhancing its ability to inhibit Rheb and suppress

mTORC1. In addition, AMPK can also phosphorylate ribosomal protein S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), downstream effectors of mTORC1, which promote cell growth (Kalender, 2010; Kulkarni, Gubbi and Barzilai). AMPK phosphorylates these proteins at different sites from those targeted by mTORC1, inhibiting their translation. This results in reduced protein synthesis, decreased cell growth, and attenuation of mTORC1 signalling (Kalender, 2010; Kulkarni, Gubbi and Barzilai). Interestingly, mTOR inhibition prevents IL1A expression, dampening SASP signalling. Low IL1A levels, limit the activation of IKK complexes, reducing NF- κ B activation (Laberge et al., 2015). It has also been reported that rapamycin mediated inhibition of mTOR could attenuate senescence induced by doxorubicin or hydrogen peroxide in mesenchymal stem cells, by reducing DNA damage (Liu et al., 2020b).

1.9.4.1 Metformin in BC

Metformin has a well-established, protective role in various age-associated diseases, including obesity, cardiovascular disease, and cancer (Espinoza et al., 2023; Salvatore et al., 2020). As insulin resistance and hyperinsulinemia are independent risk factors for cancer development, metformin is a particularly interesting candidate for its prevention and treatment (Cejuela et al., 2022).

There is conflicting evidence for metformin's role in BC (Cejuela et al., 2022). Some studies suggest that metformin may reduce the risk of developing BC (Chlebowski et al., 2012; Col et al., 2012; Min et al., 2020) whilst others indicate it has no significant effect on BC prevention (Lega et al., 2017; Tang et al., 2018) or treatment outcome (Bayraktar et al., 2012). Interestingly, Anisimov et al., (2010) showed metformin delays the onset of cancer and extends lifespan by 8% in a BC model.

Despite this, several studies have suggested that in patients with diabetes, metformin is associated with improved BC mortality (Hua et al., 2023; Tang et al., 2018). Given that ~15% of BC patients have T2DM, repurposing metformin for cancer therapy holds significant potential (Min et al., 2020). It is therefore beneficial to identify whether metformin's protective properties are linked to TIS reduction or SASP attenuation in TNBC.

1.10 METFORMIN REPURPOSING

Metformin is taken by >200 T2DM million patients, demonstrating its widespread tolerability (Foretz, Guigas and Viollet, 2023). Research is now focusing on exploiting the beneficial effects of metformin, beyond glycaemic control. Since 2014, the number of clinical trials repurposing metformin has increased significantly, of these trials 34% focus on metformin and cancer, 17% of these specifically on BC (Zhou and Xue, 2025). Most of these studies aim to enhance the efficiency of chemotherapy treatments or delaying tumour progression with metformin, highlighting its potential role as a future combination therapy (Zhou and Xue, 2025). This study explores the senotherapeutic potential of metformin, and aims to reduce the detrimental consequences of TIS, which negatively influence patient prognosis and treatment response. Given metformin's clinical repurposing potential, these results may provide a rationale for its use alongside cancer therapies to counteract the harmful effects of TIS.

The TAME study is a large-scale trial, investigating the anti-aging effects of metformin, in >3000 participants, over 6 years (Barzilai et al., 2016). As part of the geroscience approach, the TAME study aims to modulate aging as a whole, rather than treat the specific diseases associated with it. As senescence is a hallmark of aging, modulating it may contribute to beneficial physiological effects. It is therefore important to establish the effects of metformin on TIS, at a cellular level, to understand any effects seen at a patient level.

1.11 RESEARCH RATIONALE

BC is the most commonly diagnosed form of cancer in females, and as the subtype accounting for 40% of its deaths, TNBC was selected as the focus of this study (Banerjee et al., 2024; Cancer Research UK, 2022). To investigate TIS in TNBC, *in vitro* models were developed using doxorubicin, a standard chemotherapy treatment for TNBC, that is also a known TIS inducer. TIS was confirmed using multiple methods to capture the diversity of the senescence phenotype.

Initially, this study aimed to identify predictive or prognostic biomarkers of TIS in TNBC (Figure 1.9), which are currently lacking. Given that TIS is characterised by extensive transcriptional reprogramming, it was hypothesised that changes in senescence associated gene expression may indicate TIS within tumours and correlate with poor treatment response or reduced patient survival.

Cytoskeletal components were of particular interest, as they are implicated in TME remodelling, SASP maintenance, and changes in migratory behaviour, processes commonly associated with aggression and therapeutic resistance in TNBC (Ghosh et al., 2020; Malla et al., 2025; Molina et al., 2025). Candidate genes associated with senescence, the cytoskeleton and cancer, were selected for further investigation when changes in their expression were associated with poor treatment response and reduced patient survival. The intention of this was to identify patients affected by TIS, who may benefit from the incorporation of anti-senescence drugs into their treatment regimens. These biomarkers were evaluated *in vitro* to determine whether their expression was indicative of TIS in TNBC.

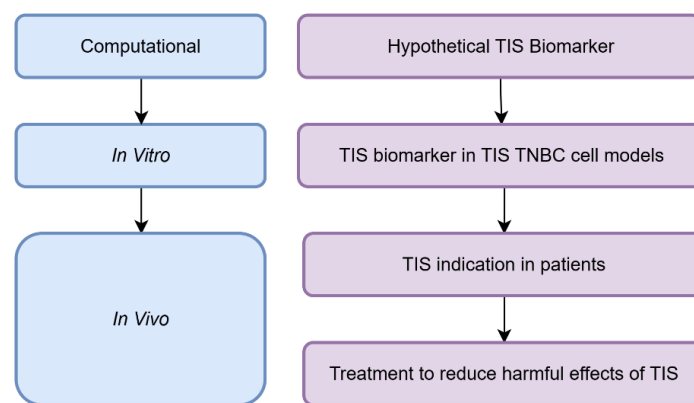


Figure 1.9. The role of biomarker discovery in improving patient response to treatment.

The second part of this research focused on evaluating TIS attenuation strategies in TNBC. Metformin was selected as a candidate senomorphic drug due to its tolerability, potential for repurposing and broad range of senotherapeutic effects, extending beyond SASP attenuation. The overarching goal was to determine whether metformin could mitigate the pro-tumorigenic effects of SASP or attenuate TIS, making cells susceptible to chemotherapy (Figure 1.10). Ultimately, this strategy aims to improve chemotherapy efficacy by reducing the risk of TIS associated TNBC progression and re-emergence.

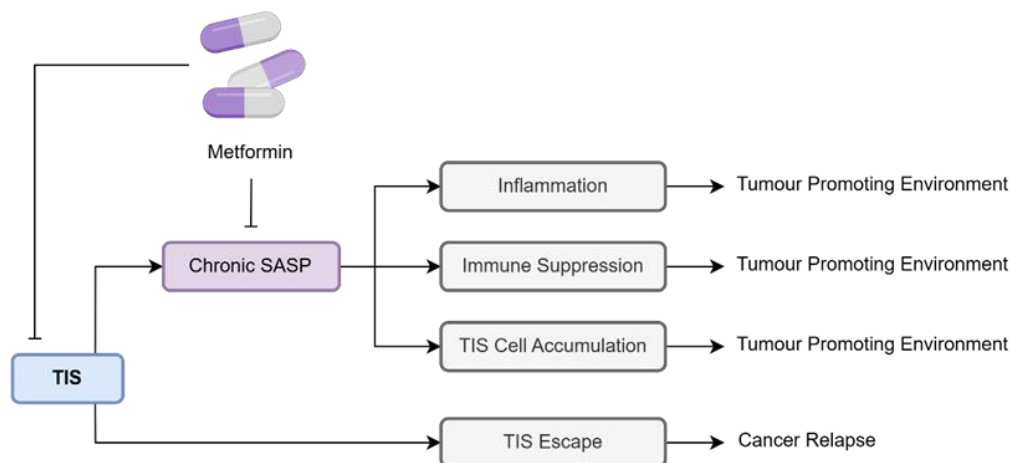


Figure 1.10. The role of metformin in attenuating TIS and preventing tumour progression and relapse.

The final part of this research aimed to identify the paracrine effects of TIS and investigate whether these could be mitigated. Specifically, the goal of this chapter was to determine whether TIS TNBC adversely affected fibroblasts, the primary TME component, and if the paracrine SASP signalling driving these changes, could be prevented using metformin. This approach aims to suppress the inflammatory signalling which promotes tumour progression, via paracrine senescence and fibroblast activation.

1.12 RESEARCH IMPORTANCE

BC is one of the most prevalent cancers in the UK, with TNBC being among the most clinically challenging subtypes, due to its aggressive nature and high mortality rates. TIS plays a complex role in TNBC. Although in its early stages TIS prevents cell transformation and uncontrolled proliferation, chronic TIS has many pro-tumorigenic effects. These arise primarily through the SASP generating a pro-inflammatory microenvironment, beneficial for tumour progression. However, TIS cancer cells have also been shown to escape cell cycle arrest, and drive cancer relapse. As TIS has been observed in breast tumours, it is essential to be able to both identify it and mitigate its detrimental effects.

Doxorubicin, a first-line chemotherapy drug for TNBC, is a well-established inducer of TIS, making it a clinically relevant model of senescence. Since chemotherapy drugs, such as doxorubicin, induce TIS via DNA damage, senomorphic drugs which modulate senescence may enhance treatment efficacy by attenuating SASP or reversing TIS.

As TIS has been detected clinically and is associated with poor treatment response and prognosis, the ability to reliably identify TIS cells within tumours could improve treatment personalisation. TIS biomarkers could help identify patients who may benefit from the incorporation of senotherapeutic drugs, alongside conventional therapies, to mitigate the detrimental consequences of TIS. Cytoskeletal biomarkers are of particular interest, as cytoskeletal remodelling not only drives phenotypic alterations in TIS cells but also contributes to tumour aggression and therapeutic resistance in TNBC.

Given metformin's known senomorphic effects, understanding its role in TIS and TNBC is crucial for evaluating its therapeutic potential. Ultimately, if metformin can attenuate senescence, it may help counteract TIS driven inflammation and cancer relapse, improving treatment outcomes. Whilst metformin's influence on TIS has been investigated at supra-pharmacological concentrations, its effects at clinically relevant doses remain largely unknown. As a well-tolerated drug with the potential to modulate TIS, metformin represents a promising candidate for further study and repurposing. Given the therapeutic challenges of TNBC, advancing our understanding of metformin's effects on TIS could contribute to the development of more effective treatment or prevention strategies.

It is also essential to investigate the paracrine effects of TIS to understand its broader role in TNBC. Assessing how SASP influences fibroblasts, the primary TME component, provides insight into the indirect consequences of TIS on surrounding breast tissue. Given that senotherapies, like metformin, may be more effective at mitigating paracrine SASP signalling than directly reversing senescence, it is important to evaluate how it modulates TIS driven TME alterations. Ultimately, if metformin can reduce TIS driven changes *in vitro*, it could be used clinically to prevent TIS driven inflammation.

1.13 AIMS AND OBJECTIVES

The overall aim of this project was to improve understanding of TIS in TNBC by identifying its biomarkers and potential strategies for its attenuation (Figure 1.11). This was achieved by, first, identifying biomarkers associated with TIS in TNBC; second, assessing whether metformin could attenuate TIS in TNBC models; third, investigating why metformin didn't attenuate TIS in TNBC and fourth, identifying the paracrine effects of TIS TNBC on healthy cells and investigating their modulation by metformin. A summary of each chapter's specific aims and objectives are provided below.

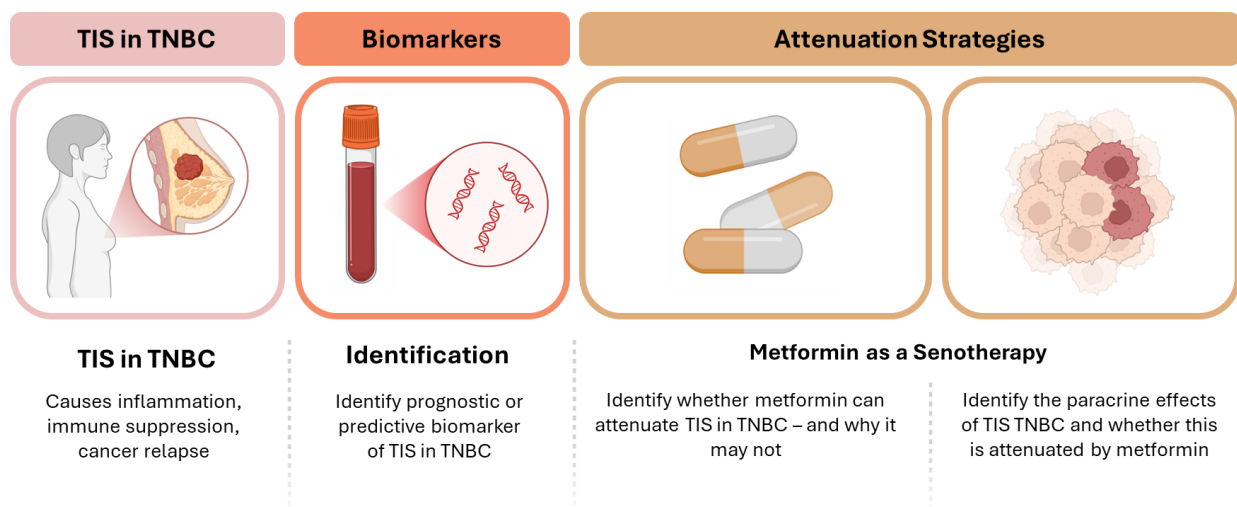


Figure 1.11. Overview of research aims. The key aims of this project, were to identify novel biomarkers of therapy induced senescence (TIS) in triple negative breast cancer (TNBC) and investigate the senotherapeutic properties of metformin. Metformin's was investigated for its role in attenuating TIS in TNBC models and also in mitigating paracrine inflammation.

Chapter 3: Biomarkers of TIS in TNBC

- **3.1:** Identify candidate genes, associated with TIS induced cytoskeletal changes, which may be biomarkers of TIS in TNBC
 - **3.1A:** Conduct a literature review to identify key genes implicated in senescence and the cytoskeleton.
 - **3.1B:** Conduct a bioinformatics analysis to identify candidates with predictive or prognostic significance specifically by using:
 - ROC Plotter to evaluate whether candidates are associated with treatment response.
 - KM Plotter to assess whether candidates are associated with patient survival.
 - GEPIA to determine whether candidates differ between cancerous and healthy tissue.
 - UALCAN to examine whether candidates are differentially expressed in BC stages or subtypes.
- **3.2:** Identify whether candidate genes are differentially expressed in TIS TNBC models.
 - **3.2A:** Develop robust TIS models in immortalised cell lines MDA-MB-231, HCC1806 and MCF10A through characterisation of β -galactosidase staining, nuclear area, γ -H2AX staining and gene expression of key TIS genes, including *IL1A*, *IL6*, *CXCL1*, *IL8*, *CDKN1A*, *TP53* and *LMNB1*.

- **3.2B:** assess whether changes in the expression of candidate genes with potential prognostic or predictive significance are associated with TIS in TNBC, using qPCR.

Chapter 4: Metformin as a senotherapy in TNBC

- **4.1:** Determine whether metformin reverses TIS in TNBC models.
 - **4.1A:** Establish effective working concentrations of metformin by performing MTT assays to assess cell viability, and apoptosis assays to determine the proportion of live cells within the population.
 - **4.1B:** Determine whether metformin attenuates TIS in cell models (MCF10A, HCC1806 and MDA-MB-231) by assessing various markers of senescence including:
 - Gene expression changes in SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*), markers of senescence pathway activation (*CDKN1A* and *TP53*) and nuclear integrity (*LMNB1*).
 - β -galactosidase staining as a marker of increased lysosomal content.
 - Nuclear area as a marker of morphological changes.
 - BrdU staining as a measure of cell proliferation.
 - γ -H2AX staining as a marker of DNA damage.

Chapter 5: Mechanisms limiting metformin efficacy in TNBC models

- **5.1:** Investigate the potential reasons for limited metformin efficacy in TIS models.
 - **5.1A:** Confirm the functional effects of metformin by using MTT assays to assess cell viability and BrdU staining to assess replicative alterations in MCF7 cells, as a positive control for metformin function.
 - **5.1B:** Assess whether key downstream targets of metformin are regulated in accordance with its expected mechanism of action by performing western blots to evaluate activation of key pathway components such as AMPK and mTOR.
 - **5.1C:** Determine whether cell models express the cation transporters required for metformin uptake using qPCR to assess gene expression and immunofluorescence to examine protein expression. Specifically, this objective aimed to:
 - Assess whether the transporters necessary for metformin uptake are present in cell models.

- Determine whether transporter expression is altered in TIS samples, potentially affecting metformin uptake.
- **5.2:** Assess the relevance of the pre-clinical cell models to patient gene expression profiles.
 - **5.2A:** Perform bioinformatics analysis, using UALCAN and GEPIA to identify relevance of cell models to patient expression profiles.

Chapter 6: Paracrine effects of TIS TNBC and modulation by metformin

- **6.1:** Determine whether secretions from TIS TNBC cells induce senescence or functional changes in healthy fibroblasts
 - **6.1A:** Establish a co-culture system using TIS TNBC cells and patient derived fibroblasts by:
 - Assessing changes in fibroblast and TNBC viability, using MTT assays.
 - Confirming senescence in HCC1806 maintained in fibroblast media, using β -galactosidase staining and gene expression analysis of SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).
 - **6.1B:** Determine whether TIS secretions induce cancer associated fibroblast formation in fibroblasts by using:
 - Scratch assays to assess migratory changes in fibroblasts co-cultured with TNBC and TIS TNBC.
 - Aspect ratio measurements to assess morphological changes in fibroblasts co-cultured with TNBC and TIS TNBC.
 - **6.1C:** Determine whether TIS secretions induce senescence in fibroblasts by assessing:
 - β -galactosidase staining as a marker of increased lysosomal content.
 - Gene expression changes in SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).
- **6.2:** Assess whether metformin can mitigate TIS induced inflammation in a co-culture system.
 - **6.2A:** Determine working metformin concentrations in fibroblasts, using MTT assays to assess cell viability.
 - **6.2B:** Determine whether metformin attenuates inflammation in fibroblasts co-cultured with TIS TNBC by analysing changes in gene expression of SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).

Chapter 2

Universal Materials and Methods

2 UNIVERSAL MATERIALS AND METHODS

2.1 CELL CULTURE OF IMMORTALISED CELL LINES

Experiments were conducted using three immortalised, adherent, cell lines; MCF10A, HCC1806, and MDA-MB-231 (Table 2.1), donated by staff at the University of Hertfordshire and Anglia Ruskin University. MCF10A was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Fisher Scientific, Leicestershire, UK) supplemented with, 5% horse serum (MERCK, Dorset, UK), 1% penicillin/streptomycin (Fisher Scientific, Leicestershire, UK), 10 µg/mL insulin (MERCK, Dorset, UK), 20 ng/mL epidermal growth factor (EGF) (Scientific Laboratory Supplies (SLS), Nottinghamshire, UK), 100 ng/mL cholera toxin (MERCK, Dorset, UK) and 0.5 µg/mL hydrocortisone (SLS, Nottinghamshire, UK). To ensure their sterility, media components which were not pre-prepared, were filter sterilised using 0.2 µm polyethersulfone filtropur filters (Sarstedt, Leicestershire, UK). MDA-MB-231 and HCC1806 were cultured in High Glucose (GlutaMAX) DMEM (Fisher Scientific, Leicestershire, UK) supplemented with 10% foetal bovine serum (FBS) (SLS, Nottinghamshire, UK).

Table 2.1. Summary of cell lines used. Each cell line was isolated from a different patient tissue; therefore, vary in their type, growth characteristics and conditions.

Cell line	Tissue	Type	Mutations
MCF10A	Non-tumorigenic, epithelial cells, isolated from the mammary gland of a female with fibrocystic breasts. Immortalised spontaneously.	Healthy control.	<i>CDKN2A (p16/p14)</i> and <i>CDKN2B (p15)</i> deletion
MDA-MB-231	Epithelial cells isolated from the breast tissue of a female adenocarcinoma patient.	Triple negative.	<i>CDKN2A (p16/p14)</i> and <i>CDKN2B (p15)</i> deletion, <i>BRAF</i> , <i>KRAS</i> , <i>TERT</i> , <i>TP53</i> mutation
HCC1806	Epithelial cells isolated from the mammary gland of a female acantholytic squamous cell carcinoma patient.	Triple negative.	<i>TP53</i> mutation and <i>FHIT</i> deletion

Cell lines were maintained using aseptic technique and subcultured at 80% confluence, as determined by microscopic inspection. To subculture cells, medium was removed, cells were washed once with phosphate buffered saline (PBS) (Fisher Scientific, Leicestershire, UK) and detached from the flask using Gibco™ TrypLE™ Express Enzyme (Fisher Scientific, Leicestershire, UK). Cells were incubated at

37 °C with 5% CO₂, until detachment was confirmed by microscopy. Once detached, cells were resuspended in fresh medium and centrifuged at 150 x g for 5 minutes, at room temperature. The resulting cell pellet was resuspended in medium and seeded as required. All cell lines were cultured in an incubator maintained at 37 °C with 5% CO₂. Unless otherwise stated, all cell culture media and reagents were pre-warmed to 37 °C before use.

2.1.1 Cell line authentication

Cell lines were authenticated by the UK Health Security Agency (UKHSA), using short tandem repeat profiling of 16 loci. STRs are sequences of repeated, hypervariable DNA which differ between individuals, which when amplified by polymerase chain reaction (PCR), can be used to generate an STR profile (Culture Collections, 2025). Cell lines with STR profiles >80% are considered to be generated from the same source.

2.2 PHASE CONTRAST MICROSCOPY

An Olympus CKX41 phase-contrast inverted microscope, with 4X, 10X, 20X and 40X objective lenses was used to view cells during routine cell culture. Cell images were captured using a GX camera, linked to GX Capture software.

2.3 CALCULATING CELL SEEDING DENSITY

Cell density was calculated using a Neubaur haemocytometer, provided by the university. To prepare cells for counting, confluent cells were trypsinised, resuspended in PBS and centrifuged at 150 x g for 5 minutes. The cell pellet was then resuspended in 4 mL medium, 10 µL of which was diluted with 10 µL Trypan Blue, supplied by the university. 10 µL of this solution was pipetted between the coverslip and haemocytometer. Viable, unstained cells were counted in each of the 4 corner squares using an inverted microscope at 10X magnification. Equation 1 was used to calculate cell density/mL. The depth of the Neubaur haemocytometer was accounted for by multiplying the output by $\times 10^4$. Cells were seeded as required, using values obtained from Equation 2, where concentration (C) refers to concentration of cells/mL. Adjustments were made to cell seeding densities to account for the requirements of each experiment.

Equation 1

$$Cells/mL = \frac{2 \text{ (Average cell count per square)}}{\text{Number of squares counted}} \times 10^4$$

Equation 2

$$C_1V_1 = C_2V_2$$

C_1 : initial concentration, V_1 : initial volume, C_2 : final concentration and V_2 : final volume

2.4 DOXORUBICIN SOLUTION PREPARATION

Doxorubicin (MERCK, Dorset, UK) was supplied in a powder and diluted to 3 mM in distilled water, as determined from Equation 3. This stock was filtered sterilised using 0.2 µm polyethersulfone filtropur filters (Sarstedt, Leicestershire, UK) and further diluted in media, using equation 2, to generate a 30 µM working stock. Doxorubicin powder and solution was stored at 3-5 °C. Non-treated controls were also prepared, using distilled water and media. In experiments where cells were treated with doxorubicin, a corresponding untreated control was included, to ensure that any changes observed were due to the treatment itself rather than experimental conditions.

Equation 3

$$Mass = Concentration \times Volume \times Molecular Weight$$

2.5 METFORMIN SOLUTION PREPARATION

Metformin (MERCK, Dorset, UK) was supplied in a powder and diluted to 60.38 mM in distilled water (Equation 3). This stock was filter sterilised using 0.2 µm polyethersulfone filtropur filters (Sarstedt, Leicestershire, UK) and further diluted in media, to generate a 500 µM working stock (Equation 2). Metformin powder was stored and room temperature and solution was stored at -20 °C. Non-treated controls were also prepared, using distilled water and media. In experiments where cells were treated with metformin, a corresponding untreated control was included, to ensure that any changes observed were due to the treatment itself rather than experimental conditions. In chapter 5 (Mechanisms Limiting Metformin Efficacy in TIS Models) MCF7 cells were included as a positive control for metformin function.

2.6 MTT ASSAY

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays were conducted to assess cell metabolic activity, as an indicator of cell viability.

2.6.1 MTT Assay for immortalised cells

Unless otherwise stated, cells were seeded in triplicate (technical replicates $n=3$), on a 96-well plate at 40,000 cells/100 μL well for 24-hour assays and 10,000 cells/100 μL well for 96-hour assays. Three medium only wells were added to each plate and used to eliminate background noise during data processing. Non-treated controls were also included to ensure any observed changes were due to treatments, rather than experimental conditions.

To assess doxorubicin cytotoxicity, cells were seeded and allowed to attach overnight. Medium was then removed and replaced with serial log dilutions of doxorubicin from 10 nM to 100 μM . Control wells (medium only and non-treated) received fresh medium. Plates were incubated for 24 hours to allow the drug to exert its effects. To assess metformin cytotoxicity, cells were seeded directly in serial log dilutions of metformin ranging from 5 nM to 50 mM, for either 24 or 96 hours

After drug incubations, 25 μL of MTT reagent (5 mg/mL Tetrazolium Bromide Thiozyl Blue MTT prepared in PBS) (MERCK, Dorset, UK) was added to all wells, including medium only and non-treated controls, then incubated for 2 hours at 37 °C, allowing surviving cells to metabolise MTT to a purple formazan. Plates were covered due to the light sensitivity of the reagent. Medium was carefully removed by inversion, so to leave the formazan in place, and 100 μL DMSO was added to solubilise the crystals. Plates were placed on a shaker, at room temperature, for 30 minutes to maximise solubilisation. Formazan concentration was determined using a Multiskan II plate reader, measuring optical density (OD) at 540 nm. Values were interpreted by CLARIOstar Plus plate reader (BMG Lab Tech, Buckinghamshire, UK) and MARS data analysis software (BMG Lab Tech, Buckinghamshire, UK), then exported to Microsoft Excel, where data was processed. MTT experiments were repeated at least 3 times, independently to determine drug response (biological replicates $n=3$).

2.6.2 Data processing

Data was processed using Microsoft Excel and plotted on a graph using GraphPad Prism. Initially, data was normalised using medium only wells as a reference to remove background noise, from all wells. Cell viability (%) was calculated using Equation 4 which measures the proportion of live, metabolising cells within a population, by normalising them to the untreated control. Bar graphs were plotted in GraphPad Prism, to visualise the viability of each cell line and drug treatment. The standard deviation (SD) for each datapoint was plotted to illustrate sampling distribution.

Equation 4

$$\% Viability = \frac{Average OD_{Treated}}{Average OD_{Untreated}} \times 100$$

2.7 SENESCENCE INDUCTION

Senescence was induced over 7 days, using doxorubicin (Figure 2.1). On day 1, cells were seeded in appropriate volumes (2 mL for 6-well plates and 1 mL for 12-well plates) and incubated overnight, allowing cells to adhere. Untreated and treated samples were seeded at different densities to account for the replication arrest observed in treated cells (Table 2.2). Control samples were seeded to achieve approximately 80% confluence by Day 7. On day 2, cell medium was removed, and each well was washed once with PBS. Appropriate doxorubicin treatments (Table 2.3), or fresh medium for controls, was added to each well. Plates were incubated for 24 hours, after which media was removed, and the cells were washed twice with PBS (day 3). Fresh medium was then added, and the plates were incubated for 96 hours, to allow the cells to enter senescence. Tests to confirm senescence were performed at day 7.

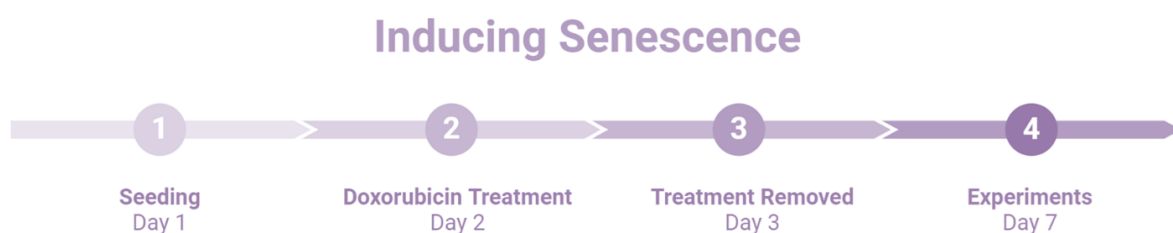


Figure 2.1. Summary of senescence induction protocol. Senescence was induced over 7 days. On day 1, cells were seeded, on day 2 cells were treated with doxorubicin for 24 hours, on day 3 doxorubicin treatments were removed. Cells were allowed to recover for 96 hours and were tested for senescence on day 7.

Table 2.2. Cell seeding densities for senescence induction. Seeding densities for senescence induction were calculated in cells/cm² to account for the varying surface areas of the plates used. The surface area of a standard 6-well plate is 9.6 cm², whilst that of a 12-well plate is 3.5 cm².

Cell Line	Cells/Cm ²	
	Control	Treated
MCF10A	1667	20833
MDA-MB-231	2083	10417
HCC1806	5833	20833

Table 2.3. Senescence inducing doxorubicin concentrations. As shown below, differing concentrations of doxorubicin were needed to induce senescence in each cell line used.

Cell Line	Senescence inducing doxorubicin concentration
MCF10A	100 nM
MDA-MB-231	175 nM
HCC1806	25 nM

2.8 METFORMIN TREATMENT

Immortalised cells were treated with either 50 µM (physiological concentration) or 500 µM (supra-physiological concentration) metformin to evaluate its potential role in reversing senescence (Foretz, Guigas and Viollet, 2023; Graham et al., 2011; He and Wondisford, 2015; LaMoia and Shulman, 2021). Metformin treatments were added 24 hours after cells were seeded and incubated with cells for the duration of the experiment (Figure 2.2).

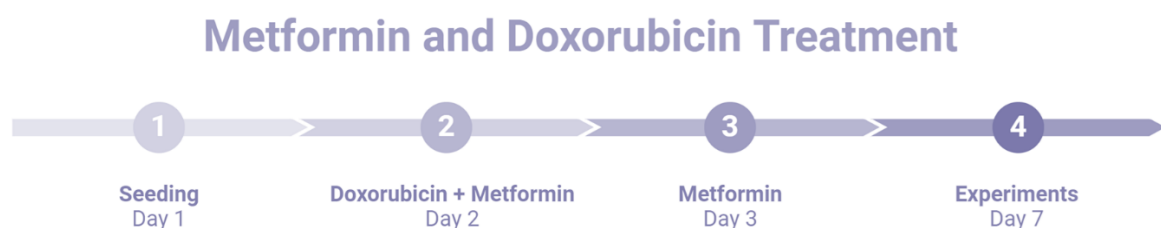


Figure 2.2. Summary of metformin treatments during senescence induction. On day 1, cells were seeded, on day 2 cells were treated with doxorubicin and metformin for 24 hours, on day 3 doxorubicin treatments were removed and metformin was replaced. Cells were incubated with metformin for 96 hours and were tested on day 7.

2.9 BETA GALACTOSIDASE STAINING

Increased lysosomal content was detected using senescence β -galactosidase Staining Kit (#9860, Cell Signalling Technology, London, UK). Reagents were prepared according to manufacturer's instructions, with the amendment that X-Gal was dissolved in Dimethyl sulfoxide (DMSO) (Fisher Scientific, Leicestershire, UK) rather than N, N-Dimethylformamide (DMF) as this caused significant crystal formation and debris. The pH of the β -galactosidase staining solution was adjusted using hydrochloric acid (HCl) or sodium hydroxide (NaOH) to fall within the optimal range of pH 5.9-6.1, as deviations from this range could lead to false positives (low pH) or false negatives (high pH).

Cells were washed once with PBS and fixed with the 1X fixative solution, supplied as part of the kit, for 13 minutes, at room temperature. After fixation, cells were washed twice with PBS, and sufficient β -galactosidase staining solution was added to cover each well. Plates were wrapped in parafilm and incubated at 37 °C, in a dry incubator, overnight, to prevent crystal formation due to pH change. The following day cells were microscopically examined and imaged using GX Capture software. For the extended storage of plates, staining solution was removed, wells were washed once with PBS and covered in 70% glycerol. Plates were stored in the fridge at 4 °C.

2.9.1 Stain interpretation

To quantify levels of β -galactosidase staining, a total of 150 cells were counted, and then % positive stained cells were determined (Equation 5). Cells were classified as positively stained, if lysosomes, present in the cytoplasm were stained blue. If there was a marked increase in staining compared to controls (>50% cells positively stained), cells were considered to be senescent. Bar graphs were plotted in GraphPad Prism to visualise the percentage of cells that were positively stained for β -galactosidase. The SD for each datapoint was plotted to illustrate sampling distribution.

Equation 5

$$\% \text{ Positively Stained Cells} = \left(\frac{\text{Number of positively stained cells}}{\text{Total number of cells}} \right) \times 100$$

2.10 FLUORESCENT STAINING

Coverslips were sterilised in a 1:10 HCl solution, for 1 hour at room temperature, washed extensively with distilled and deionised water, rinsed with IMS, air dried, and autoclaved prior to use. Sterile glass coverslips (size 16 mm, thickness 0.13 to 0.16 mm) were added to plates, prior to cell seeding. Antibody specific information is detailed below, however unless otherwise stated, cells were fixed and permeabilised directly in culture plates, before being blocked and incubated with antibodies in. Coverslips were kept moist throughout the process by placing them on dampened filter paper, in petri dishes and adding approximately 30 μ L of reagent to ensure they did not dry out. Unless otherwise stated, incubations were carried out at room temperature. Antibodies were prepared in antibody dilution buffer (1% BSA and 0.3% Triton X-100 in PBS). DyLight™ 488 Goat Anti-Mouse IgG (product code: 35502, Fisher Scientific, Leicestershire, UK), conjugated to Alexa-Fluor 488 (AF-488), was supplied at 1 mg/mL, diluted to 500 μ g/mL in glycerol, and further diluted to a final working concentration of 1 μ g/mL in antibody dilution buffer. In addition to non-treated controls, secondary antibody-only controls were used to confirm the specificity of primary antibody binding. Coverslips were mounted onto SuperFrost microscope slides (kindly provided by staff at Brunel University) using ProLong™ Diamond Antifade Mountant with DAPI (product code: 15382192, Fisher Scientific, Leicestershire, UK), which was equilibrated to room temperature for 1 hour before use, to prevent solidification. Coverslips were placed cell-side down and allowed to cure in the dark at room temperature for 24 hours before visualisation.

2.10.1 Phospho-H2AX Staining

γ -H2AX (ser 139) was used to detect DNA damage in samples. Cells were washed once with PBS and fixed with 4% paraformaldehyde (diluted in PBS) for 15 minutes, followed by three 5 minute PBS washes. Cells were permeabilised using 0.5% Triton X-100 in PBS for 10 minutes. After removing the buffer, cells were washed three more times with PBS and blocked in 10% BSA in PBS for 1 hour to prevent nonspecific binding. Coverslips were washed nine times with PBS after blocking.

The γ -H2AX (ser 139) primary antibody (product code: 05-636-25UG, MERCK, Dorset, UK) was diluted 1:500 (final concentration 2 μ g/mL) in antibody dilution buffer and incubated on coverslips for 1 hour. After incubation, the antibody was removed, and the coverslips were washed nine times with PBS. The secondary antibody, DyLight™ 488 Goat Anti-Mouse IgG, was diluted 1:500 (final concentration 1 μ g/mL) in antibody dilution buffer and incubated for 1 hour, in the dark, due to its light sensitivity.

Excess antibody was blotted off, and the coverslips were washed nine times, with the final wash in deionised water to prevent crystal formation. Coverslips were mounted onto slides using DAPI mountant (Fisher Scientific, Leicestershire, UK) as described in section 2.10.

2.10.2 Bromodeoxyuridine staining

Bromodeoxyuridine (BrdU) staining was used to detect DNA synthesis in proliferating cells. BrdU powder (product code: ab142567, Abcam, Cambridge, UK) was equilibrated to room temperature for 1 hour and diluted to 10 mg/mL (32.6 mM) in water. Doxifluridine (FrdU) (product code: F8791, MERCK, Dorset, UK) was prepared to 1 mg/mL (4.06 mM) in water and further diluted for a working stock of 0.1 µg/mL (406.17 nM). BrdU and FrdU stocks were aliquoted, stored at -20 °C, and thawed as needed. BrdU was added to cell cultures at a final concentration of 30 µg/mL (97.7 µM). FrdU was added at a final concentration of 0.156 ng/mL (1.22 nM) to inhibit thymidine synthesis, forcing cells to incorporate exogenous BrdU as the primary thymidine analog. Immortalised cells were incubated for 2 hours, at 37 °C with 5% CO₂, with BrdU and FrdU, to allow DNA incorporation.

Cells were washed once with PBS and fixed with 70% ice-cold ethanol (diluted in water) for 15 minutes, at room temperature, to dehydrate and preserve the samples. After fixation, cells were washed three times with PBS, permeabilised with 0.5% Triton X-100 in PBS for 10 minutes, and washed three times with PBS. DNA was denatured into single strands by treating samples with 2 M HCl (prepared by diluting 5 M stock in water) for 30 minutes, at room temperature. This enabled antibodies to bind to BrdU incorporated into cell DNA. Cells were blocked in 10% BSA in PBS for 1 hour to minimise nonspecific binding and washed nine times with PBS. BrdU primary antibody (product code: 66241-1-Ig, ProteinTech, Manchester, UK) was diluted 1:150 (final concentration 6.7 µg/mL) in antibody dilution buffer and incubated for 1 hour. Coverslips were washed nine times with PBS after incubation.

The secondary antibody, DyLight™ 488 Goat Anti-Mouse IgG, was diluted 1:500 (final concentration 1 µg/mL) and incubated for 1 hour in the dark. Excess antibody was removed, and the coverslips were washed nine times, with a final wash in deionised water. Coverslips were mounted onto slides using DAPI mountant (Fisher Scientific, Leicestershire, UK).

2.10.3 Stain visualisation and interpretation

After 24 hours slides were microscopically examined and imaged using a Zeiss Axioimager M2 microscope (fitted with an Axiocam 503 imaging device), paired with Zen Blue software (Zeiss, Cambridge, UK). Table 2.4 provides a summary of fluorescently labelled antibodies used and their excitation wavelengths. To quantify staining and determine the percentage of positively stained cells (Equation 5), 150 cells per condition were counted. For BrdU staining, cells were categorised as negative (unstained) or positive (stained) (Figure 2.3). Initially, in Chapter 3, γ -H2AX staining was classified as negative (unstained) or positive (stained), however, to capture differences in staining patterns in subsequent chapters (Chapter 4) γ -H2AX staining was further classified as unstained, weakly positive (less than half the nucleus stained), or strongly positive (more than half the nucleus stained). Cells were also classified as weakly stained if their staining was diffuse, and fewer foci were observed. In contrast, cells were classified as strongly positive when many discrete foci were observed across the nucleus. Representative images of each staining pattern are shown in chapter specific methods (3.3 and 4.3). Bar graphs were plotted in GraphPad Prism to visualise the percentage of cells that were positively stained. The SD for each datapoint was plotted to illustrate sampling distribution.

Table 2.4. Summary of fluorescently labelled antibodies.

Antibody	Colour	Excitation Wavelength (nm)	Emission Wavelength (nm)
DAPI	Blue	360	460
AF488	Green	492	519

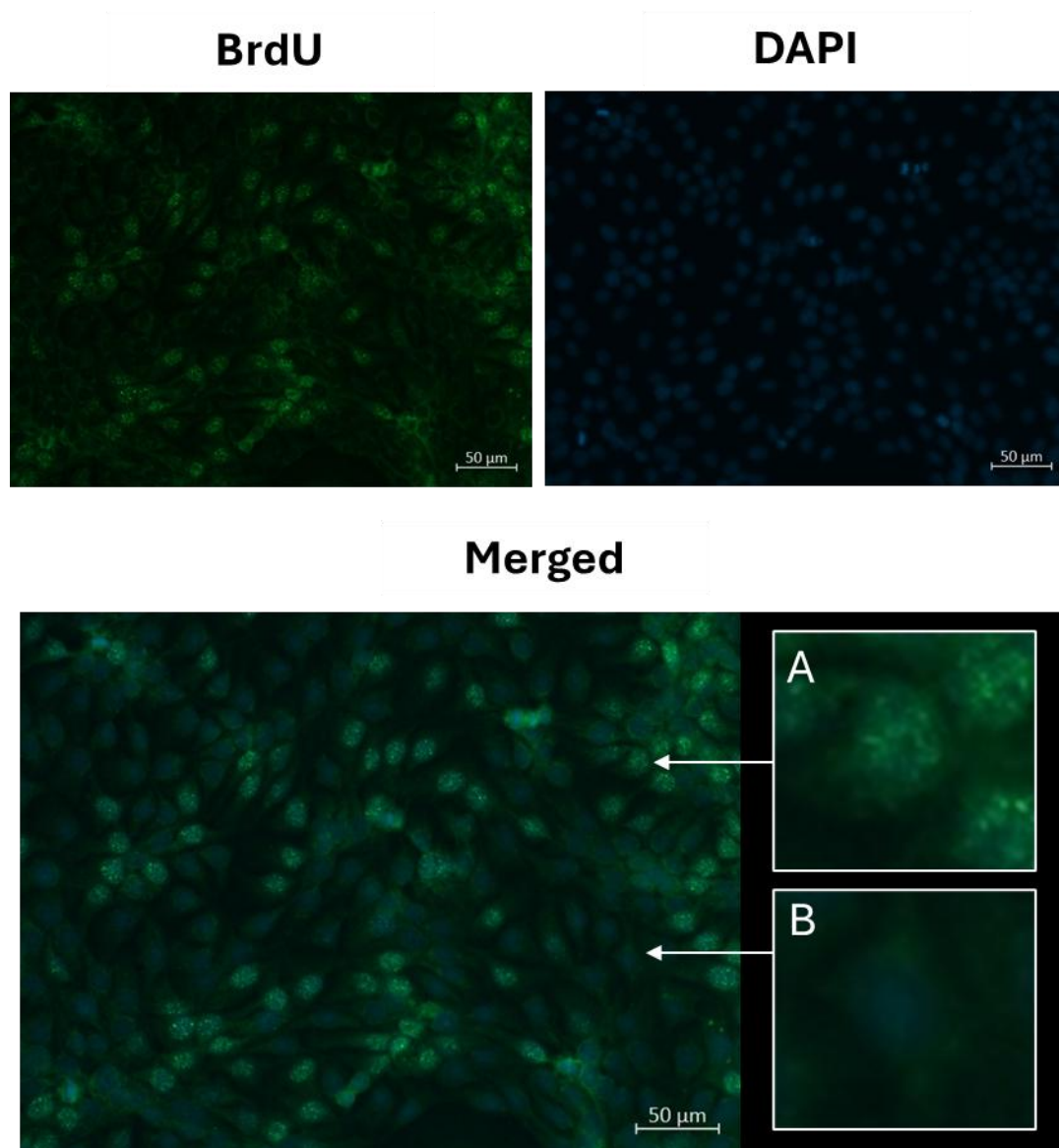


Figure 2.3. Representative fluorescence microscopy images illustrating BrdU classification in HCC1806 cells. Cells were untreated and stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 20× magnification and have a scale bar of 50 μm. BrdU fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm). Individual fluorescence channel images are shown alongside the merged images. The merged images combine both fluorescence channels, where A) represents a BrdU positive cell and B) a negative cell.

2.10.4 Nuclear Area

As alterations in cell morphology are a hallmark of senescence, nuclear area was measured to assess nuclear hypertrophy (Beck, Horikawa and Harris, 2020; Hernandez-Segura, Nehme, and Demaria, 2018; Herranz and Gil, 2018). Nuclear area (μm^2) was measured, using Fiji (ImageJ) (Schindelin et al., 2012) software, across 150 cells per treatment group from DAPI stained images. The scale was set, images was converted to greyscale (16-bit), and a threshold was adjusted to segment nuclei. After the threshold was applied, the nuclei were measured using the analyse particle's function. Bar graphs

were plotted in GraphPad Prism to quantify alterations in nuclear area. The SD for each datapoint was plotted to illustrate sampling distribution.

2.11 APOPTOSIS STAIN

Cell viability was assed using an apoptosis assay kit (product code: ab176749, Abcam, Cambridge, UK). This assay distinguished live, apoptotic, and necrotic cells, requiring time-sensitive handling to maintain cell viability. Reagents were prepared and the protocol was followed according to manufacturer's instructions, with an additional wash step after the 45 minute dye incubation to remove debris. Imaging was performed using a Zeiss Axioimager M2 microscope, equipped with an Axiocam 503 imaging device, and Zen Blue software (Zeiss, Cambridge, UK). A summary of the dyes used is provided in Table 2.5.

Table 2.5. Summary of fluorescently labelled dyes used for viability staining. Ex: Excitation wavelength max, Em: Emission wavelength max.

Dye	Colour	Ex (nm)	Em (nm)	Detects
Apoxin Green	Green	490	525	Apoptosis. Binds to Phosphatidylserine, which upon apoptosis is transferred to the outer plasma membrane.
7-AAD	Red	550	650	Necrosis. Membrane impermeable dye which will only enter cell during necrosis or late apoptosis. Detects loss of plasma integrity by labelling nucleus.
Cytocalcein Violet 450	Blue	405	450	Live. Sequestered in the cytoplasm of live cells. Fluorescent when stain is hydrolysed.

2.11.1 Stain interpretation

A total of 150 cells were counted, to determine the percentage of positively stained cells (apoptotic, necrotic, or live) using Equation 5. To positively identify cells, phase contrast images were overlayed onto fluorescent images. Dye colour was indicative of cell fate, where blue staining indicated a live cell, green an apoptotic cell and red a necrotic cell (Figure 2.4). Although dye localisation was sometimes difficult to determine, cells that were stained blue (absent of green and red dyes) were classified as live. Cells stained green or both green and red, were considered apoptotic, as 7-AAD can also stain cells in late apoptosis. Cells that were only stained red, were classified as necrotic. Structures were classified as debris if they lacked a visible nucleus and appeared irregular or fragmented. Debris

was observed for both Cytocalcein Violet and Apoxin Green staining. Although these structures likely contained some cellular material, any artifact that could not be confidently identified as an individual cell was excluded from the analysis. Bar graphs were created in GraphPad Prism to visualise data, where the percentage of positively stained cells were plotted, with SD included to represent sampling distribution.

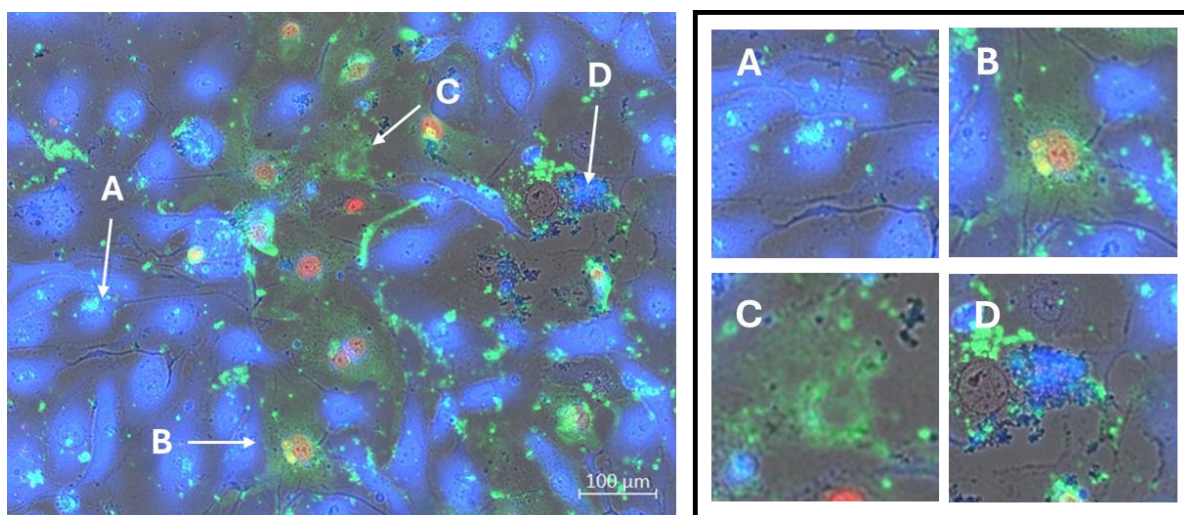


Figure 2.4. Representative fluorescence microscopy images illustrating cell fate classification in doxorubicin treated MDA-MB-231 cells. Cells were stained using a fluorescence-based apoptosis assay. Images were captured at 10× magnification with a 100 μm scale bar. Cells were treated with 175 nM doxorubicin. Live cells were stained with Cytocalcein Violet, visualised on the CeTCV channel. Apoptotic cells were stained with Apoxin Green, visualised on the FITC channel. Necrotic cells were stained with 7-AAD, visualised on the Texas Red channel. Merged images combining all fluorescence channels are shown, where A) represents a live cell, B) an apoptotic cell, C) a necrotic cell, and D) debris.

2.12 RNA EXTRACTION AND QUALITY ASSESSMENT

Cells were cultured as required, after which media was aspirated, and cells were washed twice with PBS. Total RNA was extracted using Aurum™ Total RNA Mini Kit (Biorad 7326820, Hertfordshire, UK), as per the manufacturer's instructions for 'Mammalian Cell Spin Protocol'. Genomic contamination was removed using DNase I, included within the kit. The extracted RNA was eluted in 60 μL RNA elution solution for 6-well plates, and was stored at -80 °C.

The quantity and quality of extracted RNA was assessed using the CLARIOstar Plus plate reader (BMG Lab Tech, Buckinghamshire, UK) and MARS data analysis software (BMG Lab Tech, Buckinghamshire, UK). 2 μL of RNA was loaded to a LVis Plate (BMG Lab Tech, Buckinghamshire, UK) and measured at wavelengths 260 nm (absorption peak for nucleic acids) and 280 nm (absorption peak of proteins).

RNA samples with an A260/A280 ratio of ~2 were considered to be high quality, pure extracts. RNA quality was confirmed by agarose gel electrophoresis (1.1% Agarose Gel, run at 70 V for 90 minutes), where clear 18s (2kb) and 28s (5kb) bands were observed.

2.13 cDNA SYNTHESIS

cDNA was synthesised from RNA using a High-Fidelity cDNA synthesis kit (ROCHE 05 081 955 00, Hertfordshire, UK) using the random hexamer primer protocol, as per the manufacturer's instructions for 'first-strand cDNA synthesis for a two-step RT-PCR'. The protocol was carried out at room temperature, keeping RNA and reagents on ice where possible to prevent their degradation. cDNA was synthesised using an Eppendorf mastercycler nexus gradient PCR thermal cycler. Various concentrations of RNA were used (in general 1 µg per reaction), ensuring levels remained within the kit's sensitivity range (1 pg to 1 µg per reaction). The resulting cDNA was diluted in RNase free water, as required for subsequent experiments and was stored at -80 °C. A negative control without a template and without an enzyme was included.

2.14 PRIMER DESIGN

Primers were designed to target multiple splice variants of each gene, ensuring gene expression across isoforms was detected in subsequent experiments. Transcript variant accession numbers were identified using the NCBI nucleotide database and were used in NCBI Primer BLAST to design primers common to each transcript. Default primer BLAST parameters were applied to generate primers with a length of 18-25 bp, GC content 40-60%, and melting temperature (T_m) 55-65 °C with a maximum difference of ≤2 °C between primer pairs. Primer pairs were designed to generate PCR amplicons of <200 bp. Primer specificity was confirmed using BLAST alignment, and potential secondary structures, including likelihood of primer dimers, were assessed using MERCK OligoEvaluator. Primers were sourced through the university, using Invitrogen. Primers were supplied in lyophilised form and resuspended in RNase-free water.

2.15 PCR FOR PRIMER VERIFICATION

To test primers and confirm product sizes for quantitative PCR (qPCR), PCR was performed using REDTaq® DNA Polymerase (MERCK, Dorset, UK). REDTaq® DNA Polymerase is a Taq DNA polymerase

combined with an inert red dye, which enables the direct loading of PCR product onto an agarose gel, without the need for additional loading dye. A PCR master mix was prepared, and 25 ng of cDNA was added per reaction. Product sizes were confirmed by agarose gel electrophoresis, where 10 μ L of the PCR product was loaded into each well each well. A 1.1% agarose gel was prepared by dissolving agarose in Tris-borate-EDTA (TBE) buffer, provided by the university, and heating for 1 minute until fully dissolved. After cooling slightly, 3 μ L of GelRed (used to visualise cDNA) was added before the solution was poured into a gel cassette and allowed to set. Gels were run in a standard Bio-Rad horizontal midi-gel electrophoresis tank, with a Bio-Rad PowerPac electrophoresis power supply, using TBE running buffer at 70 V for 90 minutes. Gels were viewed using the iBright™ FL1500 Imaging System (Fisher Scientific, Leicestershire, UK). A negative control (no template) was included in all reactions to ensure specificity and identify contamination. Components of the PCR reaction are detailed in Table 2.6, and the cycling conditions used on the Eppendorf mastercycler nexus gradient PCR thermal cycler are provided in Table 2.7. The primers used for this study are listed in Table 2.8 and their functions are shown in Table 2.9.

Table 2.6. PCR Reaction components.

Component	Volumes for 1X 25 μL Reaction Mix (μL)
REDTaq® DNA Polymerase Ready Mix	12.5
5 μ M Forward Primer	1.25
5 μ M Reverse Primer	1.25
cDNA (25 ng/ μ L)	1
Water	9
Total Volume	25

Table 2.7. PCR cycling conditions for primer verification.

Step	Action			
Initial denaturation	94 °C for 2 minutes			
PCR profile	Stage	Temp	Time	Cycles
	Denaturation	94 °C	1 minute	30
	Annealing	60 °C	1 minute	
	Elongation	72 °C	1 minute	
	Final elongation	72 °C	5 minutes	1
	Cooling	4 °C		

Table 2.8. Primers for senescence studies. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *pumilio RNA binding family member 1 (PUM1)*, *C-X-C motif chemokine ligand (CXCL1)*, *interleukin (IL)*, *cyclin-dependent kinase inhibitor 1A (CDKN1A)*, *tumour protein p53 (TP53)* and *Lamin B1 (LMNB1)*.

Gene	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Product Size (bp)
<i>GAPDH</i>	AGC CAC ATC GCT CAG ACA C	GCC CAA TAC GAC CAA ATC C	66
<i>PUM1</i>	CGG TCG TCC TGA GGA TAA AA	CGT ACG TGA GGC GTG AGT AA	121
<i>CXCL1</i>	TTT CTG AGG AGC CTG CAA CA	ACA TTC CCC TGC CTT CAC AA	94
<i>IL1A</i>	GAC CAA CCA GTG CTG CTG AA	TTA GTG CCG TGA GTT TCC CA	98
<i>IL6</i>	CCG GGA ACG AAA GAG AAG CT	GCG CTT GTG GAG AAG GAG TT	68
<i>IL8</i>	AAA CCA CCG GAA GGA ACC AT	AAT CAG GAA GGC TGC CAA GA	81
<i>CDKN1A</i> (p21)	CCT AAT CCG CCC ACA GGA AG	GGC AGA AGA TGT AGA GCG GG	73
<i>TP53</i> (p53)	TGG GAC GGA ACA GCT TTG AG	ATT CTC TTC CTC TGT GCG CC	71
<i>LMNB1</i>	TAG AGG TGG ATT CTG GGC GT	TAC AGC CTC ACT TGG GCA TC	97

Table 2.9. Summary of primers used and their functions. Senescence associated secretory pathway (SASP), *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *pumilio RNA binding family member 1 (PUM1)*, *C-X-C motif chemokine ligand (CXCL1)*, *interleukin (IL)*, *cyclin-dependent kinase inhibitor 1A (CDKN1A)*, *tumour protein p53 (TP53)* and *Lamin B1 (LMNB1)*.

Gene	Function
<i>GAPDH</i>	Housekeeping gene
<i>PUM1</i>	Housekeeping gene
<i>CXCL1</i>	SASP Component
<i>IL1A</i>	SASP Component
<i>IL6</i>	SASP Component
<i>IL8</i>	SASP Component
<i>CDKN1A</i> (p21)	Marker of senescence pathway activation
<i>TP53</i> (p53)	Marker of senescence pathway activation
<i>LMNB1</i>	Marker of nuclear integrity

2.16 QUANTITATIVE PCR (qPCR)

Quantitative PCR (qPCR) was used to determine cycle threshold (Ct) values, the number of amplification cycles required for the fluorescent signal to surpass a defined threshold. This threshold was set automatically based on the point at which the fluorescence signal was distinguishable from background noise. These Ct values were subsequently used to assess changes in gene expression across various target genes.

qPCR was performed using SYBR® Green Master Mix (Biorad, Hertfordshire, UK). Each 10 µL reaction contained 5 µL of 2× SYBR® Green master mix (including polymerase, dNTPs, and fluorescent dye), 3 µL of primer mix (1 µM forward and 1 µM reverse), and 2 µL of cDNA diluted to 5 ng/µL in nuclease-free water (10 ng cDNA per reaction).

Initially, a master mix of SYBR® Green and primers was prepared for each primer pair and 8 µL was added in triplicate, to a 96-well plate (technical replicates n=3). The plate was centrifuged briefly, to collect the master mix at the bottom of each well. After which, 2 µL of diluted cDNA was added to the respective wells. A negative control (no template) was included for each primer, to ensure primer specificity, identify contamination and highlight background noise. Melt curve analysis was routinely performed to verify amplification specificity and showed the presence of a single peak, indicative of a single product.

The plate was sealed with microseal 'B' PCR Film (product code: #MSB1001B, Biorad, Hertfordshire, UK), centrifuged briefly, and loaded into a Bio-Rad CFX Opus 96 qPCR machine (Biorad, Hertfordshire, UK). Cycling conditions are provided in Table 2.10. Data was exported in Excel file format, via the Bio-Rad Maestro software. All reactions were performed under dark conditions to protect the light-sensitive SYBR® Green dye. qPCRs were repeated at least 3 times, independently to determine average Ct values (biological replicates n=3).

Table 2.10. qPCR Cycling Conditions.

Stage	Action			
Initial denaturation	95 °C for 10 minutes			
PCR profile	Setup	Temp	Time	Cycles
	Denaturation	95 °C	15 Seconds	40
	Annealing/Extending/Luminescence	60 °C	1 minute	
Standard Curve		95 °C	1 minute	1
		60 °C	30 Seconds	1
		95 °C	30 Seconds	1

2.16.1 Data Processing

qPCR was also used to identify gene expression in both untreated cell lines and TIS models. The housekeeping genes *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and *pumilio RNA binding family member 1 (PUM1)* were selected based on their stability across BC's, and their mean relative expression was used as a single normalisation factor to calculate fold changes in gene expression (Gorji-Bahri, Moradtabrizi and Hashemi, 2021; Kılıç, Çelebiler and Sakızlı, 2014). The relative quantification method ($2^{-\Delta\Delta Ct}$), shown in Equation 6, was used to assess gene expression levels of various senescence markers, including SASP components and markers of key senescence pathways. Fold changes in gene expression were calculated relative to their respective controls, where, unless otherwise stated, treated cells were compared to untreated cells of the same cell line. The control sample is represented as a fold change of 1 as this was the baseline after normalisation. Fold change values >1 indicate higher expression relative to the control, whereas values <1 indicate lower expression. Data was processed using Microsoft Excel and plotted on a graph using GraphPad Prism. Bar graphs were plotted to visualise the fold change in gene expression. The SD for each datapoint was plotted, using the fold change from each replicate (n=3), to illustrate sampling distribution.

Equation 6

$$Fold\ Change = 2^{-[(Ct_{GOI} - Ct_{HKG})_{Experimental} - (Ct_{GOI} - Ct_{HKG})_{Control}]}$$

GOI: Gene of interest, HKG: Housekeeping gene

2.17 STATISTICAL TESTING

Statistical testing was performed and plotted in GraphPad Prism. For comparative quantitative data with <5 samples, normality was first assessed using the Shapiro-Wilk normality test. If the data followed a normal distribution and was parametric, a paired t-test was used for comparisons between two groups, whilst an ANOVA was performed for comparisons of >2 groups. For unpaired parametric data, an unpaired t-test was used for two group comparisons, and ANOVA was applied for comparisons involving >2 groups. For unpaired non-parametric data, the Mann-Whitney U test was applied to two group comparisons, whilst the Kruskal-Wallis test was used for comparisons involving more than >2 groups. In instances where significant differences were observed, post hoc tests were used to determine which treatment group differed from the control. The post-hoc test was selected based on the type of ANOVA performed: Dunnett's post hoc test was used for comparisons of multiple samples to a single control, whereas Tukey's test was used for comparisons between all groups. Statistical tests were selected based on the comparisons being made and the number of samples analysed. In chapter 3, t-tests were often used to compare untreated and treated samples, whereas in chapter 4, ANOVA was used to compare multiple treatment groups, including untreated and treated samples, as well as different metformin treatments. Graphs are presented as mean $\pm$ SD with statistical significance indicated as follows: Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

2.18 FIGURES

Unless otherwise stated, figures were generated solely by the author, using a combination of Microsoft Powerpoint, Biorender (Biorender, 2025) and draw.io (Draw IO, 2025).

Chapter 3

Biomarkers of TIS in TNBC

3 BIOMARKERS OF TIS IN TNBC

3.1 INTRODUCTION

3.1.1 Senescence Biomarkers

Biomarkers of TNBC are lacking, despite their importance in guiding therapeutic decisions and predicting patient outcomes (Passaro et al., 2024). The use of senescence associated cancer biomarkers is a novel area of research which for many years has been unexplored. However, advances in the field have highlighted the importance of TIS in cancer progression and relapse, as well as its contribution to chemotherapy side effects, including frailty, cardiac dysfunction, and inflammation (Domen et al., 2022; Ewald et al., 2010; Schmitt, Wang and Demaria, 2022; Wang, Kohli and Demaria, 2020). Therefore, identifying senescent cells, within tumours, using predictive or prognostic biomarkers could be beneficial in identifying the likelihood of a therapeutic intervention being successful or predicting survival rates (Califf, 2018).

Although several studies have attempted to define biomarkers of senescence, the heterogeneity in both cancer and senescence has made the identification of a universal marker challenging (Hernandez-Segura, Nehme and Demaria, 2018; Łukasiewicz et al., 2021). Using biomarkers to predict the prognostic significance of senescence have had varied and sometimes contradictory outcomes, whilst some studies suggest that higher senescence signatures are associated with poor prognosis, others indicate that senescence may contribute to improved outcomes (Althubiti et al., 2014; Santos-Sousa, Da Rosa and Filippi-Chiela, 2025; Ungvari et al., 2025). Regardless, the identification of a universal biomarker is required to identify TIS within TNBC so that treatments can be personalised.

Although several markers of senescence have been identified, limitations in their specificity have prevented them from reliably detecting TIS. For example, p21 upregulation is frequently associated with senescence and has been detected in tumours, however, its diagnostic potential is limited due to its overexpression in other cell states, such as transient cell cycle arrest and stress responses (Niro et al., 2025). Similarly, p16 overexpression has been linked to poor prognosis in basal-like BC and is often associated with senescence, however its overexpression is insufficient to identify senescent cells, as it is also expressed in non-senescent states (O’Sullivan et al., 2024). Beyond single gene markers, senescence associated gene signatures (a combination of genes used together), including cell cycle

regulators and DNA repair genes, have been shown to predict disease progression and patient outcome in prostate and pancreatic cancers, however markers of TIS in TNBC are lacking (Kellers et al., 2022; Lin et al., 2025a).

As TIS is characterised by extensive transcriptional reprogramming, this study investigated gene expression changes as potential biomarkers of TIS in TNBC. Cytoskeletal components were of particular interest, given their established roles in TME remodelling, SASP maintenance, and changes in migratory behaviour, processes commonly associated with aggression and therapeutic resistance in TNBC (Ghosh et al., 2020; Malla et al., 2025; Molina et al., 2025). Ultimately, the identification of universal TIS biomarkers may improve the detection and stratification of tumours, as well as improving prediction of patient prognosis and treatment response (Passaro et al., 2024). Improved TIS biomarkers may also enable more accurate detection of TIS within tumours and therefore provide a rationale for incorporating senotherapeutic drugs into TNBC treatments, to mitigate the detrimental effects of TIS.

3.1.2 Cytoskeletal changes in senescence

Senescence is not only characterised by widespread transcriptional reprogramming but also by morphological alterations. Senescent cells typically have an increased size, flattened morphology and enlarged nuclei, all of which are driven by cytoskeletal changes. These changes can often be attributed to alterations in actin dynamics, microtubules, the nucleoskeleton, stiffness or focal adhesions (complexes that anchor the cytoskeleton to the extracellular matrix (ECM) outside the cell) (Ghosh et al., 2020; Molina et al., 2025; Moujaber et al., 2019; Mu et al., 2020). Cytoskeletal remodelling in senescent cells also contributes to functional alterations in cellular mechanics and behaviour. In senescent cells, alterations in actin organisation and adhesion dynamics, increase production of ECM components and matrix modifying enzymes, altering the TME and facilitating cancer progression, whilst preventing senescent cell migration and proliferation (Ghosh et al., 2020; Molina et al., 2025; Moujaber et al., 2019).

In the context of TNBC, where cytoskeletal plasticity is closely associated with tumour aggression, invasiveness, and resistance to therapy, identifying cytoskeletal genes linked to TIS may provide a way of identifying this behaviour *in vivo* (Malla et al., 2025). If cytoskeletal alterations are consistently associated with TIS and correlate with invasive potential or treatment resistance, such changes may

serve as predictive or prognostic biomarkers. The identification of cytoskeletal markers, specific to TIS, may also be potential therapeutic targets. Given that drugs targeting the cytoskeleton such as taxanes (microtubule inhibitors) are used clinically, this approach may be beneficial in targeting TIS cells within tumours (Huang, Mei and Han, 2022).

3.1.3 Bioinformatics approaches for biomarker analysis

Given the clinical challenges that TNBC poses, it is essential to develop reliable biomarkers with predictive or prognostic significance. With the increasing availability of large-scale transcriptomic datasets, such as the Cancer Genome Atlas (TCGA), bioinformatics approaches have become a powerful tool for the identification of candidate biomarkers (Clark and Lillard, 2024). Bioinformatics analysis offers a way of identifying candidate biomarkers without the need for initial high-throughput experiments, which are often costly and time-consuming, streamlining biomarker discovery pipelines (Figure 3.1) (Hristova and Chan, 2019; Sheng et al., 2020). Countless web-based platforms have been developed to interpret public transcriptomic databases, such as TCGA, Gene Expression Omnibus and Human Protein Atlas (Bravo-Merodio et al., 2019; Das et al., 2020). These resources are commonly used in publications to identify predictive and prognostic biomarkers by correlating gene expression patterns with clinical outcomes (Cai et al., 2019; Fekete and Györfy, 2019; Houghton-Gisby et al., 2022; Hu et al., 2022c; Phan et al., 2018; Qin et al., 2022; Zheng et al., 2020).



Figure 3.1. Summary of generic biomarker discovery pipeline. In preclinical stages biomarkers are usually identified and analysed using bioinformatics software or high-throughput methods. Cell and animal models will be used to determine the biomarkers performance and safety before being tested in clinical trials. If successful, biomarkers will be reviewed by regulatory bodies and subsequently used in a clinical setting.

3.1.4 Cell Models

TIS is a consequence of chemotherapy, which results in cells entering a stable cell cycle arrest (González-Gualda et al., 2021). TNBC is an aggressive BC subtype with limited treatment options and accounts for 15% of all BC cases but 40% of BC deaths (Cancer Research UK, 2022). Doxorubicin is a primary TNBC treatment and was selected as a clinically relevant TIS inducer. As TIS is increasingly recognised as a complex contributor to cancer progression and therapeutic response, modelling TIS in TNBC provides an opportunity to identify predictive TIS markers and explore potential therapeutic

strategies. This is particularly relevant as senescent cells have been detected clinically in patient tumours, including in BC (Fleury et al., 2019; Luo et al., 2025; Saleh et al., 2021).

Two TNBC cell lines, MDA-MB-231 and HCC1806 were studied to identify variability within TIS responses, and a non-tumorigenic breast cell line, MCF10A was included as a healthy like control. Including both malignant and non-malignant cell lines was beneficial to discriminate between general TIS responses, and TNBC specific TIS responses. Although doxorubicin induced TIS in MDA-MB-231 is frequently described in literature there is considerable variation in treatment duration (24-72 hours) and post-treatment recovery (3-11 days), highlighting the absence of a standardised TIS model (Bojko et al., 2019; Chang et al., 1999; Soto-Gamez et al., 2022). Fewer, if any, reports exist for other cell lines, including HCC1806 and MCF10A. Therefore, for this project TIS models were fully characterised to ensure their reproducibility. Senescence is heterogenous and cannot be defined by a single marker therefore multiple detection methods were used to confirm its successful induction, including β -galactosidase staining as an indicator of increased lysosomal content, γ -H2AX as a marker of DNA damage, nuclear area as an indicator of hypertrophy and gene expression analysis of senescence associated genes. These *in vitro* TIS models provide a way in which to investigate potential biomarkers and treatments of TIS.

3.2 AIMS AND OBJECTIVES

The primary aims of this chapter were to (3.1) identify candidate genes, associated with TIS induced cytoskeletal changes, which may be biomarkers of TIS in TNBC and (3.2) identify whether candidate genes are differentially expressed in TIS TNBC models.

To fulfil aim (3.1) the following objectives will be completed:

- Objective 3.1A: Conduct a literature review to identify key genes implicated in senescence and the cytoskeleton.
- Objective 3.1B: Conduct a bioinformatics analysis to identify candidates with predictive or prognostic significance specifically by using:
 - ROC Plotter to evaluate whether candidates are associated with treatment response.
 - KM Plotter to assess whether candidates are associated with patient survival.
 - GEPIA to determine whether candidates differ between cancerous and healthy tissue.

- UALCAN to examine whether candidates are differentially expressed in BC stages or subtypes.

To fulfil aim (3.2) the following objectives will be completed:

- Objective 3.2A: Develop robust TIS models in immortalised cell lines MDA-MB-231, HCC1806 and MCF10A through characterisation of β -galactosidase staining, nuclear area, γ -H2AX staining and gene expression of key TIS genes, including *IL1A*, *IL6*, *CXCL1*, *IL8*, *CDKN1A*, *TP53* and *LMNB1*.
- Objective 3.2B: assess whether changes in the expression of candidate genes with potential prognostic or predictive significance are associated with TIS in TNBC, using qPCR.

3.3 MATERIALS AND METHODS

Materials and methods specific to this chapter are described below. Unless otherwise stated, protocols used were those outlined in Chapter 2 (Universal Materials and Methods). This includes methods of TIS induction and detection, as well as statistical analysis.

3.3.1 Biomarker Pipeline

As the aim of this research was to identify biomarkers of TIS in TNBC, that were associated with clinical outcome and treatment response, candidate genes were selected based on bioinformatic analysis showing correlations between their expression and poor treatment response and reduced patient survival. This approach allowed for the large scale screening of candidate biomarkers, which were subsequently evaluated for their presence in TNBC TIS models (Figure 3.2).

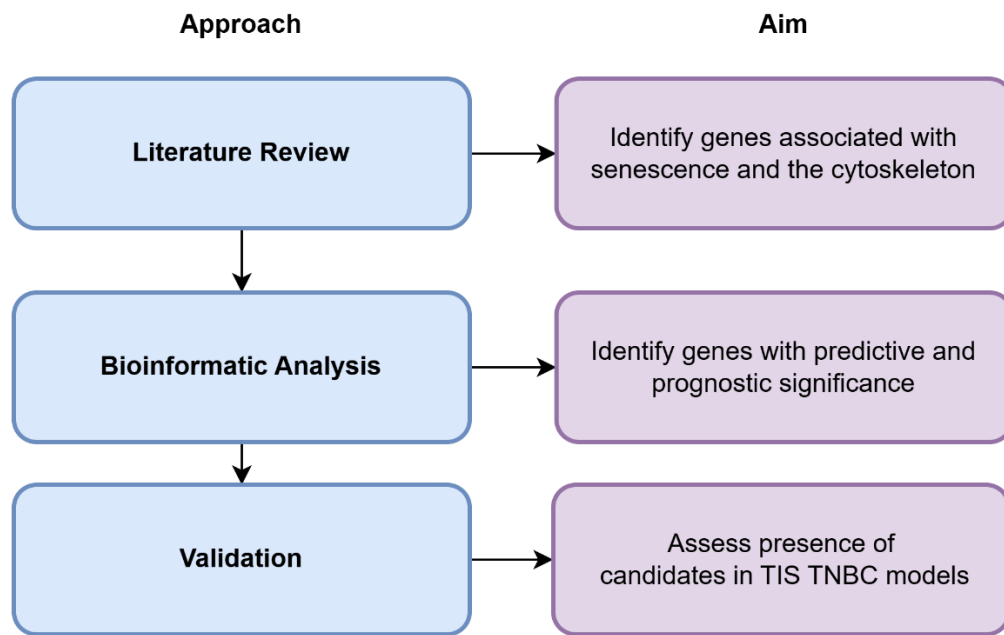


Figure 3.2. Summary of biomarker identification pipeline. Biomarkers associated with senescence and the cytoskeleton were initially identified through a literature review. Bioinformatic screening was then used to select genes with predictive and prognostic significance. Candidate genes were then validated in TNBC TIS models to assess their presence.

3.3.2 γ -H2AX Staining classification

γ -H2AX staining was performed as described in materials and methods section (2.10.1). In this chapter, any cell which fluoresced with γ -H2AX (Ser139) staining was classified as positively stained (Figure 3.3). However, as this did not account for more subtle differences in staining patterns, an alternative classification approach was used in subsequent chapters (4.3.1) where cells were classified as unstained, weakly positive (diffuse staining or <50% of the nucleus with foci) and strongly positive (numerous discrete foci across >50% of the nucleus).

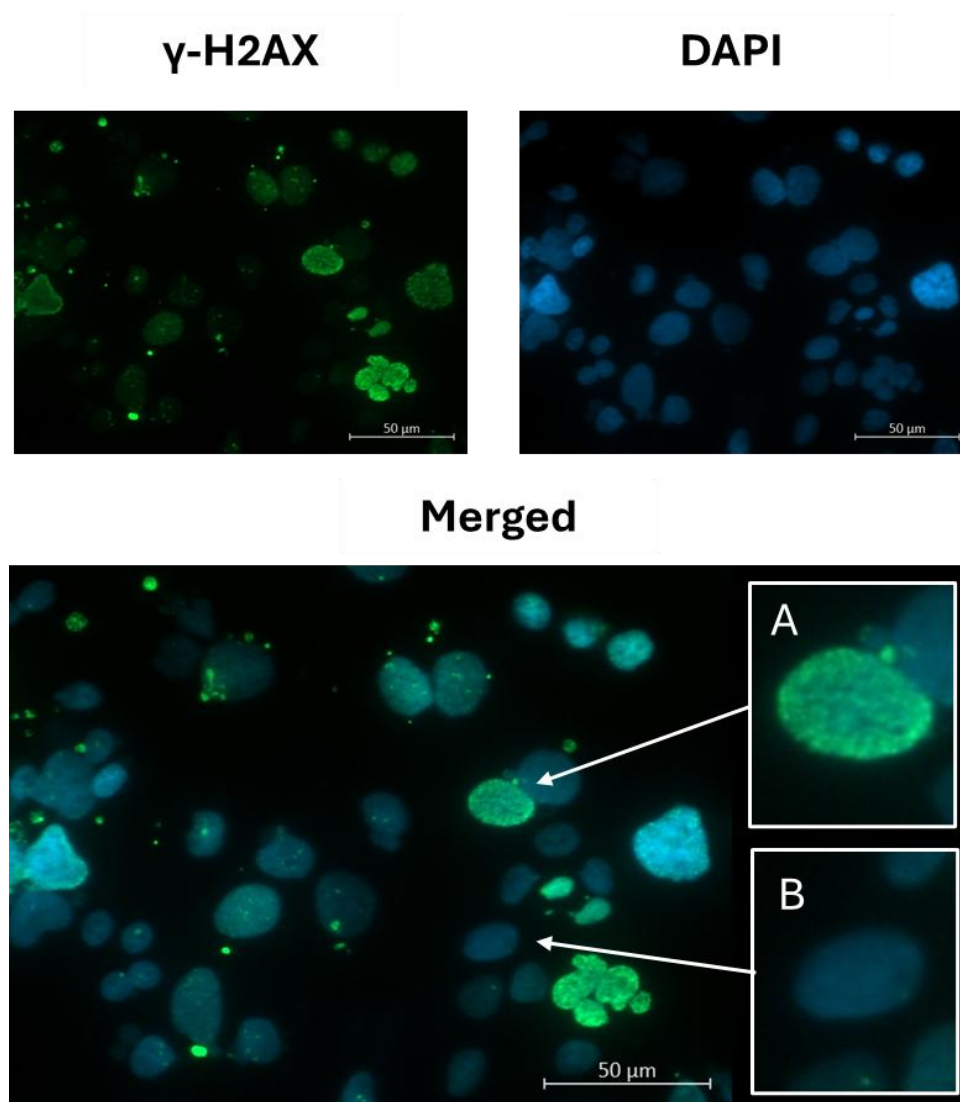


Figure 3.3. Chapter 3 γ -H2AX Staining classification. Representative staining is captured in HCC1806 cells, treated with 25 nM doxorubicin. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. γ -H2AX fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 380ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX positive cell and B) a negative cell.

3.3.3 Literature review

A literature review was conducted using primary and review articles, accessed through bibliographic databases including PubMed and Google Scholar to identify candidate genes. Key phrases for this search included 'senescence', 'cytoskeleton', and 'cancer' (Figure 3.4). Only articles written in English were included. Articles included primary studies and review articles. Further genes of interest were identified using Ami-GO 2 (<https://amigo.geneontology.org/amigo/landing>), an online catalogue which searches the gene ontology database, using key terms. This search was refined by organism (*Homo sapiens*) and function (cytoskeleton and senescence).

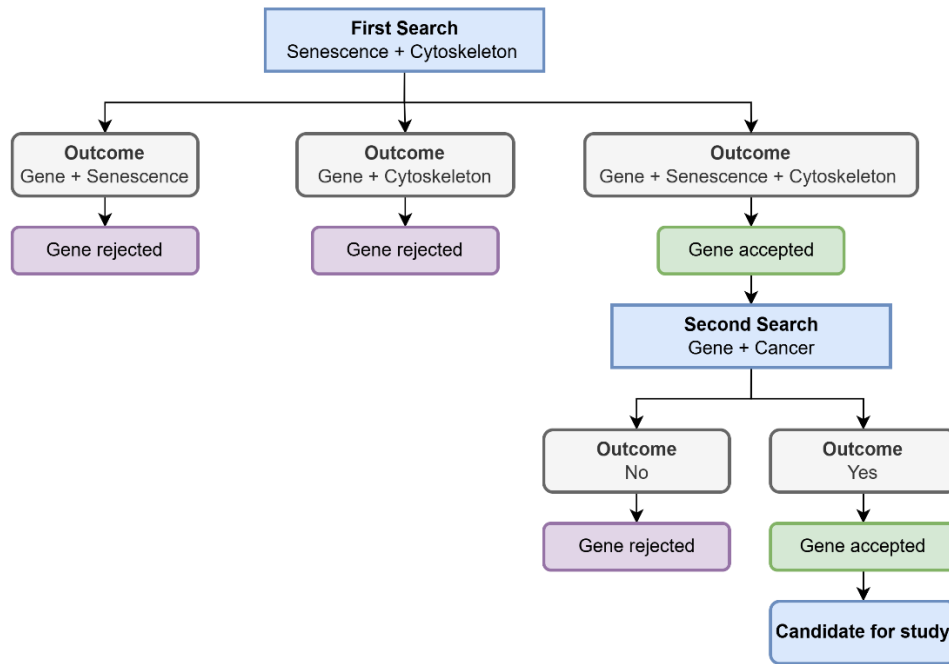


Figure 3.4. Flowchart of literature review search criteria. Initially searches were made for genes functioning in both senescence and the cytoskeleton, if both search terms were associated with a gene, it was queried in a second search. If the gene was involved in cancer it was chosen as a candidate for study.

3.3.4 ROC Plotter

The receiver operating characteristic curve (ROC) plotter (<https://rocplot.com/>) was used to determine whether the expression of the genes identified from the literature review and Ami-GO 2 search impacted therapeutic outcomes of BC patients. The ROC plotter correlates gene expression and patient response using transcriptomic data of BC's acquired from curated public datasets (Fekete and Györfy, 2019). Gene expression values are normalised by ROC plotter to make datasets comparable and represent relative levels of gene expression, in arbitrary units (AU). All searches were refined to molecular subtype: TNBC (cohort n=658) and patients treated with anthracycline (cohort n=348). Searches were further refined by therapy response, the clinical endpoint of the study. Complete pathological response (cohort n=250) (CPR) compares the pathological complete response (absence of cancer) vs residual disease (cancer presence) after therapy, whilst relapse free survival (RFS) at 5 years (cohort n=98) compares relapse free survival at 5 years vs samples relapsed before 5 years. The CPR cohort was initially used to refine candidate genes by their clinical significance, as it had a larger sample size than the RFS cohort (n=250>n=98), making the dataset more robust, and giving it more statistical power. Cohort sizes and associated dataset values were accurate as of the analysis performed in October 2022. Accession numbers (AT) are recorded to identify genes.

ROC plotter compares gene expression levels between two patient groups: responders and non-responders. For each gene, ROC plotter tests every observed expression level as a potential threshold capable of distinguishing between responders and non-responders. For each threshold, it predicts which patients would be classified as responders or non-responders, based on their expression levels, and then compares these predictions to the actual clinical outcomes. From this, ROC plotter calculates the specificity and sensitivity of each threshold. The specificity is defined as the proportion of true non-responders correctly identified at that threshold and the sensitivity as the proportion of true responders correctly identified at each threshold. These values are plotted to generate a ROC curve, which illustrates the gene's ability to discriminate between responders and non-responders at every threshold.

The overall predictive performance of each gene is summarised by the area under the curve (AUC), which shows how well the gene discriminates between responders and non-responders across all thresholds. An AUC of 0.5 indicates the gene has little predictive ability and any associations between its expression and response are likely due to chance. Higher AUC values indicate a stronger association between gene expression and patient response. Generally, genes with an $AUC < 0.6$ are considered to have potential predictive utility. Statistical significance was assessed using the p-value calculated by ROC Plotter, with $p < 0.05$ indicating that the observed discrimination was unlikely to have occurred by chance. In this study, genes were highlighted as candidates if they had an $AUC \geq 0.58$, and $p < 0.05$ so not to exclude candidates with near discriminatory ability, in this exploratory phase.

3.3.5 Kaplan-Meir Plotter

The Kaplan-Meir (KM) plotter (<https://kmplot.com/analysis/>) was used to determine whether candidate genes, identified from ROC plotter, were associated with BC patient survival. KM plotter integrates transcriptomic and proteomic data from curated public databases, including gene expression omnibus, European genome-phenome Archive, the cancer genome atlas (TCGA) and PubMed. Its primary purpose is to identify potential prognostic biomarkers in cancers. The clinical endpoint is reported as overall survival (OS) and is defined as the time (in months) from diagnosis, or the start of treatment, to death (Györfy, 2021). Gene and protein expression values are normalised by KM plotter to make datasets comparable; therefore, results represent relative levels of expression (AU). For each patient, a normalised expression value is linked to an OS time (Györfy, 2021).

Searches were restricted to BC datasets and refined by molecular subtype, specifically TNBC (ER-, PR-, and HER2-). Patients were split into high and low expression cohorts using median expression level as a cut off. Patients with expression levels above the median were assigned to the high expression cohort, whilst those with expression at or below the median were assigned to the low expression cohort. Median splitting divides the cohort into two equal groups and ensures both contain sufficient numbers for statistical comparison.

Initially, the breast protein database was used to assess whether alterations in candidate protein levels were associated with OS. Protein analysis was prioritised as mRNA abundance does not necessarily correlate with protein expression. However, as this feature was still under development, only a limited number of proteins were available, and the cohort size was small ($n = 126$). Therefore, a second analysis was performed using the mRNA-seq breast database, which contained a substantially larger cohort and data for all candidate genes ($n = 7,462$). This made the dataset more robust and gave it more statistical power. Cohort sizes and associated dataset values were accurate as of the analysis performed in October 2022.

KM survival curves were plotted for the high and low expression cohorts (Györfy, 2021). Survival probability (0-1) was plotted against time (months). Survival probability is the estimated chance that a patient is still alive at a given time point. At the start of follow-up, the survival probability is 1 (100%). However, each time a patient dies, the probability is reduced according to the proportion of patients alive at that time point (Györfy, 2021). The KM curve therefore forms a series of downward steps each time a patient dies. At early time points, when many patients are still alive, a death causes a small drop in survival probability. However, at later time points, when fewer patients remain, each death produces a larger drop. Flat sections of the curve represent periods when no deaths occur. KM plotter estimates survival probability rather than direct survival of patients as it accounts for patients who were not monitored until their death (Györfy, 2021). These patients are removed from the cohort at the last time their survival was known and do not cause a drop in the KM curve. KM plotter assumes that these patients have the same survival probability as those still being followed. Therefore, the KM curve provides an estimated survival probability for the entire cohort, even though some individual outcomes are unknown.

KM Plotter also provides three statistical outputs: hazard ratio (HR), 95% confidence interval (CI), and logrank p-value. HR quantifies the differences in death between the high and low expression cohorts, at each time point. These differences are averaged using a Cox proportional hazards model, within KM plotter, to provide an overall measure of relative risk, where HR = 1: no survival difference in low or high expression cohorts, HR > 1: high expression is associated with worse survival and HR < 1: high expression is associated with improved survival. The 95% CI indicates the precision of the HR, by calculating a range of error that it is likely to fall within. A narrow CI indicates the HR is a precise estimation whilst a wide CI indicates it is not. If the 95% CI includes 1, the association between the gene and survival may not be reliable, as a HR of 1 indicates no difference in survival between the high and low expression cohorts. The logrank p-value indicates whether survival differences in cohorts are due to chance. At each time point, it compares the number of observed deaths to the number of expected deaths if both cohorts had identical survival. The log-rank test then combines these differences across all time points to calculate a logrank p-value. A p-value >0.05 indicates the result is likely due to chance, whereas a p-value <0.05 indicates results are significantly different.

3.3.6 GEPIA

Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/>) was used to compare gene expression in healthy tissues and breast invasive carcinoma (BRCA) to determine whether the candidate genes could distinguish between cancerous and healthy tissue. GEPIA integrates expression data from 9,736 tumour samples in the TCGA database, and 8,587 normal tissue samples in the genotype-tissue expression (GTEx) project database (Tang et al., 2017).

GEPIA reports gene expression using transcripts per million (TPM). GEPIA normalises both gene length and sequencing depth, using an internal algorithm (RNA-Seq by expectation maximisation), so that gene expression (TPM) can be compared across samples and conditions, they represent the median of each group (Tang et al., 2017). For each candidate, gene expression was compared in tumour and normal samples. Differential gene expression was assessed using GEPIA's internal one-way ANOVA analysis, which determined whether expression statistically differed between tumour and normal samples (Tang et al., 2017). Significance was reported as q-values, which are p-values adjusted to account for the increased likelihood of false positive results in large datasets. A default significance threshold of q-value < 0.01 was applied by GEPIA, which minimised the detection of false positive results (Tang et al., 2017). Fold change in expression was also calculated by internal algorithms to quantify differences in gene expression between healthy and cancerous samples. A $|\text{Log}_2\text{FC}|$ (fold

change) ≥ 1 was set as a default threshold by GEPIA, to remove any genes from the analysis which were unlikely to be biologically meaningful (Tang et al., 2017). GEPIA reported Log_2FC , which was converted to fold change ($\text{FC} = 2^{\text{Log}_2\text{FC}}$) for ease of interpretation. GEPIA calculates Log_2FC based on normalised expression data across all samples, not directly from simple TPM averages. Cohort sizes and associated dataset values were accurate as of the analysis performed in October 2022.

3.3.7 UALCAN

The University of Alabama at Birmingham Cancer data analysis Portal (UALCAN) (<https://ualcan.path.uab.edu/>), is an online resource for analysing cancer OMICS data (Chandrashekar et al., 2017). It was used to determine whether the expression of candidate biomarkers could stratify BC's by stage or subtype. Analysis was refined to the BRCA TCGA, PanCancer Atlas dataset, which was selected as it is one of the most comprehensive studies integrating both clinical and genomic data. UALCAN categorised gene expression of candidates according to the pathological stages and molecular subtypes, defined by the TCGA dataset. For stage comparisons, the cohort included $n = 1,179$ samples: normal ($n = 114$), stage I ($n = 183$), stage II ($n = 615$), stage III ($n = 247$), and stage IV ($n = 20$). For subtype comparisons, the cohort included $n = 833$ samples: normal ($n = 114$), luminal ($n = 566$) (ER+, PR+/-, HER2 +/-), HER2 positive ($n = 37$) (HER2+, ER-, PR-), and TNBC ($n = 116$) (HER2-, ER-, PR-). Cohort sizes and associated dataset values were accurate as of the analysis performed in October 2022.

UALCAN reports gene expression as TPM (Chandrashekar et al., 2017). It normalises both gene length and sequencing depth, using an internal algorithm (RNA-Seq by expectation maximisation), so that gene expression (TPM) can be compared across samples and conditions (Chandrashekar et al., 2022). For each BC stage and subtype, boxplots were generated to visualise the distribution of gene expression. Boxplots show the interquartile range (IQR), the median (middle value), and the minimum and maximum dataset values. The IQR (coloured box) contains the central 50% of values, where Q1 is the lower (25th) percentile and Q3 is the upper (75th) percentile. The line inside the box represents the median, and the error bars indicate the minimum and maximum values in the dataset. Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and presented below the graphs, with p-values indicating significance: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

3.4 RESULTS

3.4.1 Identification of candidate biomarkers

3.4.1.1 ROC Plotter

ROC plotter was used to identify candidate biomarkers with potential utility for predicting therapeutic outcomes. A total of 108 genes were identified through a literature review and Ami-GO 2 search and analysed using ROC Plotter (Table 3.1). When using CPR to classify patients, 16 genes were identified with an AUC ≥ 0.58 and $p < 0.05$, indicating they may be a useful marker of therapeutic response and that this is unlikely due to chance (Table 3.2). Of these 16 candidates, 8 met the same criteria when classified by RFS (Table 3.3). AUC and p-values were not used to identify candidates in the RFS cohort, as its smaller sample size resulted in it having reduced statistical power, compared with the CPR cohort. Therefore, candidates were selected for further investigation based on whether gene expression trends in both datasets were consistent. Expression patterns in both cohorts corroborated for 12 genes which showed either increased or decreased expression in both cohorts. The genes selected for further investigation were *cell division cycle associated 5 (CDCA5)*, *collagen alpha-1(IV) chain (COL4A1)*, *kinesin family member C1 (KIFC1)*, *lamin B1 (LMNB1)*, *nuclear pore glycoprotein p62 (NUP62)*, *structural maintenance of chromosomes protein 6 (SMC6)*, *nesprin-1 (SYNE1)*, *nesprin-2 (SYNE2)*, *γ -tubulin 1 (TUBG1)*, *stromal interaction molecule 1 (STIM1)*, *telomeric repeat-binding factor 2 (TERF2)* and *three prime repair exonuclease 2 (TREX2)* (Table 3.4).

Table 3.1. ROC Plotter analysis of genes associated with senescence, the cytoskeleton and cancer. Genes were identified from a literature review and Ami-Go 2 search. The query was refined to complete pathological response and anthracycline treated, triple negative breast cancer patients (cohort $n = 250$). The area under the curve (AUC) reflects the predictive performance of each gene; genes with AUC ≥ 0.58 and $p < 0.05$ were considered to have meaningful predictive utility, which was unlikely due to chance. Genes meeting these criteria are highlighted. The summary column shows the mean gene expression of non-responders compared with responders. $\downarrow$ indicates expression is lower in non-responders than responders, $\uparrow$ indicates expression is higher in non-responders than responders, $\rightarrow$ indicates both responders and non-responders had the same expression. Hyphens (-) indicate data was not available. Accession numbers (AT) are recorded to identify genes.

Gene	Gene symbol	AT	AUC	p-value	Summary
A disintegrin and metalloproteinase	<i>ADAM17</i>	205745	0.545	4.7e-02	$\uparrow$
A disintegrin and metalloproteinase with thrombospondin motifs	<i>ADAMTSS5</i>	229357	0.529	0.31	$\uparrow$
Aurora kinase B	<i>AURKB</i>	209464	0.528	0.15	$\uparrow$
Bifunctional 3'-5' exonuclease/ATP-dependent helicase WRN	<i>WRN</i>	205667	0.52	0.24	$\rightarrow$
Breast cancer type 2 susceptibility protein	<i>BRCA2</i>	214727	0.547	4e-02	$\uparrow$
Calreticulin	<i>CALR</i>	214315	0.567	5.8e-03	$\uparrow$
Cell Division Cycle 42	<i>CDC42</i>	214230	0.548	3.9e-02	$\uparrow$
Cell Division Cycle Associated 5	<i>CDCA5</i>	224753	0.633	1.2e-02	$\downarrow$
Cell division cycle associated 8	<i>CDCA8</i>	221520	0.55	3.1e-02	$\downarrow$

Cellular Communication Network Factor 1	<i>CCN1(CYR61)</i>	201289	0.516	0.27	↓
Cellular Communication Network Factor 2	<i>CCN2 (CTGF)</i>	209101	0.515	0.29	↓
Cellular tumor antigen p53	<i>TP53</i>	201746	0.579	1.8e-03	↑
Centrosomal protein 192	<i>Cep192</i>	218827	0.575	2.3e-03	↓
Collagen alpha-1(IV) chain	<i>COL4A1</i>	211980	0.583	1.2e-03	↓
Collagen Type I Alpha 1 Chain	<i>COL1A1</i>	202311	0.554	2.5e-02	↓
Collagen Type I Alpha 2 Chain	<i>COL1A2</i>	202404	0.563	1.1e-02	↓
Collagen Type III Alpha 1 Chain	<i>COL3A1</i>	211161	0.538	0.085	↓
Collagen Type IV Alpha 2 Chain	<i>COL4A2</i>	211964	0.57	4.4e-03	↓
Collagen Type V Alpha 1 Chain	<i>COL5A1</i>	212488	0.513	0.32	↓
Collagen Type XVI Alpha 1 Chain	<i>COL16A1</i>	213428	0.553	2.5e-02	↓
c-Src	<i>SRC</i>	213324	0.508	0.38	↓
Cyclin-dependent kinase 2	<i>CDK2</i>	204252	0.56	1.2e-02	↓
Cyclin-dependent kinase inhibitor 1B	<i>CDKN1B</i>	209112	0.554	2e-02	↓
Desmin	<i>DES</i>	214027	0.515	0.29	↑
DnaJ homolog subfamily A member 3	<i>DNAJA3</i>	205963	0.579	1.7e-03	↑
Dual specificity mitogen-activated protein kinase kinase 1	<i>MAP2K1</i>	202670	0.52	0.23	↑
Dual specificity mitogen-activated protein kinase kinase 6	<i>MAP2K6</i>	205698	0.534	0.11	↑
Dynamin-like 120 kDa protein	<i>OPA1</i>	214306	0.504	0.44	↑
Elastin	<i>ELN</i>	212670	0.564	9.1e-03	↑
Fibronectin	<i>FN1</i>	212464	0.559	1.7e-02	↓
G2 And S-Phase Expressed 1	<i>GTSE1</i>	204317	0.523	0.2	↑
Gamma-Tubulin Ring Complex Targeting Factor	<i>NEDD1</i>	234984	0.554	0.21	↑
GTPase HRas	<i>HRAS</i>	212983	0.523	0.2	↓
Histone acetyltransferase KAT5	<i>KAT5</i>	206689	0.556	2e-02	↑
Histone H2AX	<i>H2AFX</i>	205436	0.524	0.19	↓
Histone H3 Associated Protein Kinase	<i>GSG2</i>	223759	0.501	0.49	↑
Interferon Regulatory Factor 3	<i>IRF3</i>	202621	0.602	8.2e-05	↑
Keratin 14	<i>KRT14</i>	209351	0.518	0.25	↑
Keratin 5	<i>KRT5</i>	201820	0.563	8.1e-03	↑
Keratin 7	<i>KRT7</i>	209016	0.521	0.22	↓
Keratin 8	<i>KRT8</i>	209008	0.558	1.5e-02	↓
Kinesin Family Member 22	<i>KIF22</i>	202183	0.505	0.43	↑
Kinesin family member 23	<i>KIF23</i>	204709	0.542	0.06	↓
Kinesin family member C1	<i>KIFC1</i>	209680	0.587	5.5e-04	↓
Kinetochore associated 1	<i>KNTC1</i>	206316	0.562	9.6e-03	↓
Lamin B1	<i>LMNB1</i>	203276	0.698	2.2e-16	↓
Lamina-associated polypeptide 2 α	<i>LAP2α</i>	-	-	-	-
Laminin A1	<i>LAMA1</i>	227048	0.514	0.4	↑
Laminin A2	<i>LAMA2</i>	216840	0.529	0.14	↓
Laminin A3	<i>LAMA3</i>	203726	0.517	0.26	↑
Laminin A4	<i>LAMA4</i>	202202	0.524	0.2	↓
Laminin A5	<i>LAMA5</i>	210150	0.509	0.36	↑
Laminin B1	<i>LAMB1</i>	201505	0.533	0.12	↓

Laminin B2	<i>LAMB2</i>	216264	0.53	0.13	↓
Laminin B3	<i>LAMB3</i>	209270	0.545	4.9e-02	↑
Laminin B4	<i>LAMB4</i>	215516	0.57	3.9e-03	↑
Laminin C1	<i>LAMC1</i>	200771	0.533	0.11	↓
Laminin C2	<i>LAMC2</i>	202267	0.572	3.2e-03	↑
Laminin C3	<i>LAMC3</i>	219407	0.577	1.5e-03	↑
MAP kinase-activated protein kinase 5	<i>MAPKAPK5</i>	212871	0.533	0.11	↓
Matrix metalloproteinase 1	<i>MMP1</i>	204475	0.534	0.11	↓
Matrix metalloproteinase 2	<i>MMP2</i>	201069	0.523	0.2	↑
Matrix metalloproteinase 3	<i>MMP3</i>	205828	0.518	0.26	↓
Mitogen-activated protein kinase 14	<i>MAPK14</i>	202530	0.521	0.21	↑
Nesprin-1	<i>SYNE1</i>	209447	0.603	5e-05	↓
Nesprin-2	<i>SYNE2</i>	202761	0.64	5.3e-08	↓
Nesprin-3	<i>SYNE3</i>	-	-	-	-
Nuclear Mitotic Apparatus	<i>NuMA1</i>	200747	0.577	1.7e-03	↓
Nuclear pore glycoprotein p62	<i>NUP62</i>	202153	0.59	3.3e-04	↓
Nucleophosmin	<i>NPM1</i>	221923	0.521	0.22	↑
Ostenonectin	<i>SPARC</i>	212667	0.549	3.7e-02	↓
Paxillin	<i>PXN</i>	201087	0.514	0.29	↑
Pericentrin	<i>PCNT</i>	203660	0.553	2.2e-02	↓
Pericentriolar material 1	<i>PCM1</i>	228905	0.622	1.9e-02	↓
Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha	<i>PIK3CA</i>	204369	0.504	0.44	↑
Polo-like kinase 4	<i>PLK4</i>	204886	0.579	1.4e-03	↓
Prelamin-A/C	<i>LMNA</i>	212089	0.551	3e-02	↑
Protein kinase C delta type	<i>PRKCD</i>	202545	0.558	1.3e-02	↑
Rac Family Small GTPase 1	<i>RAC1</i>	208640	0.514	0.31	↓
Ras Homolog Family Member A	<i>RHOA</i>	200059	0.538	0.078	↓
Ras Homolog Family Member D	<i>RHOD</i>	209885	0.529	0.14	↑
Rho associated coiled coil containing protein kinase 1	<i>ROCK1</i>	235854	0.588	0.088	↓
Rho Associated Coiled-Coil Containing Protein Kinase 2	<i>ROCK2</i>	202762	0.544	0.051	↓
Serine-protein kinase ATM	<i>ATM</i>	212672	0.549	3.3e-02	↓
Serum response factor	<i>SRF</i>	202401	0.533	0.11	↑
Stromal Interaction Molecule 1	<i>STIM1</i>	202764	0.594	1.8e-04	↑
Structural maintenance of chromosomes protein 6	<i>SMC6</i>	236535	0.658	3e-03	↓
SUN domain-containing protein 1	<i>SUN1</i>	212074	0.503	0.46	↓
SUN domain-containing protein 2	<i>SUN2</i>	212144	0.511	0.33	↑
TANK Binding Kinase 1	<i>TBK1</i>	218520	0.569	5.1e-03	↓
Telomeric repeat-binding factor 2	<i>TERF2</i>	203611	0.592	3.2e-04	↑
Three prime repair exonuclease 1	<i>TREX1</i>	34689	0.508	0.39	↓
Three prime repair exonuclease 2	<i>TREX2</i>	211788	0.591	2.3e-04	↑
Thrombospondin 1	<i>THBS1</i>	201110	0.574	3e-03	↓
Tissue inhibitors of metalloproteinase 1	<i>TIMP1</i>	201666	0.515	0.29	↑

Tissue inhibitors of metalloproteinase 2	<i>TIMP2</i>	231579	0.685	9.8e-04	↓
Tissue inhibitors of metalloproteinase 3	<i>TIMP3</i>	201150	0.529	0.14	↓
Tissue inhibitors of metalloproteinase 4	<i>TIMP4</i>	206243	0.509	0.37	↑
Transforming Acidic Coiled-Coil Containing Protein 3	<i>TACC3</i>	218308	0.522	0.2	↑
tubulin β -3	<i>TUBB3</i>	202154	0.573	3e-03	↓
Tyrosine-protein kinase ABL1	<i>ABL1</i>	202123	0.517	0.26	↓
Vimentin	<i>VIM</i>	201426	0.512	0.33	↓
ZW10 Interacting Kinetochore Protein	<i>ZWINT</i>	204026	0.56	1.3e-02	↓
α -tubulin 1	<i>TUBA1A</i>	209118	0.528	0.15	↓
β 3-integrin	<i>ITGB3</i>	204627	0.516	0.28	↑
β -Actin	<i>ACTB</i>	213867	0.514	0.29	↑
γ -Actin	<i>ACTG1</i>	211970	0.638	3.8e-08	↓
γ -tubulin 1	<i>TUBG1</i>	201714	0.596	1.2e-04	↓

Table 3.2. Summary of candidate genes, identified in ROC plotter analysis, refined by complete pathological response. The query was refined to complete pathological response and anthracycline treated, triple negative breast cancer patients (cohort n = 250). The area under the curve (AUC) reflects the predictive performance of each gene; genes with AUC $\geq$ 0.58 and p < 0.05 were considered to have meaningful predictive utility, which was unlikely due to chance. The summary column shows the mean gene expression of non-responders compared with responders. ↓ indicates expression is lower in non-responders than responders, ↑ indicates expression is higher in non-responders than responders. Accession numbers (AT) are recorded to identify genes. The mean gene expression is reported in arbitrary units, as normalised by ROC plotter.

Gene symbol	AT	AUC	p-value	Mean responder gene expression	Mean non-responder gene expression	Summary
<i>ACTG1</i>	211970	0.638	3.8e-08	32588	28103	↓
<i>CDCA5</i>	224753	0.633	1.2e-02	741	526	↓
<i>COL4A1</i>	211980	0.583	1.2e-03	5579	3984	↓
<i>IRF3</i>	202621	0.602	8.2e-05	595	705	↑
<i>KIFC1</i>	209680	0.587	5.5e-04	563	469	↓
<i>LMNB1</i>	203276	0.698	2.2e-16	980	585	↓
<i>NUP62</i>	202153	0.590	3.3e-04	1161	1023	↓
<i>PCM1</i>	228905	0.622	1.9e-02	619	311	↓
<i>SMC6</i>	236535	0.658	3e-03	337	216	↓
<i>STIM1</i>	202764	0.594	1.8e-04	247	299	↑
<i>SYNE1</i>	209447	0.603	5e-05	394	285	↓
<i>SYNE2</i>	202761	0.640	5.3e-08	1043	766	↓
<i>TERF2</i>	203611	0.592	3.2e-04	612	700	↑
<i>TIMP2</i>	231579	0.685	9.8e-04	10794	7151	↓
<i>TREX2</i>	211788	0.591	2.3e-04	7	11	↑
<i>TUBG1</i>	201714	0.596	1.2e-04	754	619	↓

Table 3.3. Candidate genes, identified in ROC plotter analysis, refined by relapse free survival. The query was refined to relapse free survival and anthracycline treated, triple negative breast cancer patients (cohort n = 98). The area under the curve (AUC) reflects the predictive performance of each gene; genes with AUC ≥ 0.58 and $p < 0.05$ were considered to have meaningful predictive utility, which was unlikely due to chance. Genes meeting these criteria are highlighted. The summary column shows the mean gene expression of non-responders compared with responders. ↓ indicates expression is lower in non-responders than responders, ↑ indicates expression is higher in non-responders than responders. Accession numbers (AT) are recorded to identify genes. The mean gene expression is reported in arbitrary units, as normalised by ROC plotter.

Gene symbol	AT	AUC	p-value	Mean responder gene expression	Mean non-responder gene expression	Summary
<i>ACTG1</i>	211970	0.623	5.5e-03	27521	30554	↑
<i>CDCA5</i>	224753	0.672	7.6e-03	566	425	↓
<i>COL4A1</i>	211980	0.555	0.14	3870	3564	↓
<i>IRF3</i>	202621	0.558	0.12	955	825	↓
<i>KIFC1</i>	209680	0.528	0.29	541	509	↓
<i>LMNB1</i>	203276	0.539	0.22	536	522	↓
<i>NUP62</i>	202153	0.642	1.6e-03	1314	1053	↓
<i>PCM1</i>	228905	0.509	0.45	114	115	↑
<i>SMC6</i>	236535	0.554	0.24	143	132	↓
<i>STIM1</i>	202764	0.653	6.1e-04	268	343	↑
<i>SYNE1</i>	209447	0.52	0.35	238	222	↓
<i>SYNE2</i>	202761	0.611	1.1e-02	839	685	↓
<i>TERF2</i>	203611	0.614	9.1e-03	711	822	↑
<i>TIMP2</i>	231579	0.644	2.3e-02	4857	5981	↑
<i>TREX2</i>	211788	0.59	3.5e-02	8	9	↑
<i>TUBG1</i>	201714	0.539	0.22	868	838	↓

Table 3.4. Summary of candidate genes, identified in ROC plotter analysis. The summary column shows the mean gene expression of non-responders compared with responders. ↓ indicates expression is lower in non-responders than responders, ↑ indicates expression is higher in non-responders than responders. Accession numbers (AT) are recorded to identify genes.

Gene symbol	AT	Expression Summary
<i>CDCA5</i>	224753	↓
<i>COL4A1</i>	211980	↓
<i>KIFC1</i>	209680	↓
<i>LMNB1</i>	203276	↓
<i>NUP62</i>	202153	↓
<i>SMC6</i>	236535	↓
<i>STIM1</i>	202764	↑
<i>SYNE1</i>	209447	↓
<i>SYNE2</i>	202761	↓
<i>TERF2</i>	203611	↑
<i>TREX2</i>	211788	↑
<i>TUBG1</i>	201714	↓

3.4.1.2 KM Plotter

The 12 genes identified from ROC plotter analysis were queried in KM plotter, to identify candidate biomarkers with potential utility for predicting patient survival. Initial results were obtained using BC protein analysis (Table 3.5). However, as the dataset was still in development, the analysis was limited by cohort size ($n = 126$) and number of proteins available. Therefore, a second search using mRNA Seq analysis was conducted, which had a larger cohort size ($n = 7,462$) and data for all candidates (Table 3.6). The survival of cohorts (months) was the average survival of low and high expression cohorts. Candidates for further investigation were selected based on the consistency of their expression patterns with both therapeutic response in ROC Plotter and survival in the mRNA KM plotter database. Specifically, candidate genes were selected when the expression pattern associated with poor treatment response in ROC plotter (high or low) was associated with poor OS in KM plotter. For example, a gene whose high expression predicted poor treatment response in ROC Plotter and poor survival in KM Plotter was selected. This approach ensured that the chosen genes had both predictive (treatment response) and prognostic (survival) potential. This refined the search from 12 candidates to 6 (*CDCA5*, *KIFC1*, *LMNB1*, *SMC6*, *STIM1* and *TUBG1*) (Table 3.7) The low expression cohorts for *CDCA5*, *KIFC1*, *LMNB1*, *SMC6*, and *TUBG1*, had a lower average OS than high expression cohorts, whereas the high expression cohort for *STIM1* had a lower average OS than high expression cohorts (Table 3.6).

Figure 3.5 summarises the KM mRNA plots of candidates and shows their associated HR's, CI's, and logrank p-values. Until ~125 months both low and high expression cohorts have similar OS rates, after which expression differences result in altered survival times. The HRs for *CDCA5*, *KIFC1*, *LMNB1*, *SMC6*, and *STIM1* were slightly above 1, indicating that higher expression is associated with poorer survival, whilst *TUBG1* had an HR just below 1, suggesting higher expression is linked to improved survival. This contradicts with the average OS calculated by KM Plotter (Table 3.6). However, as the CI for each gene includes 1, the associations between gene expression and survival may not be reliable in either case. This is further supported by logrank p-values > 0.05 for all candidates, indicating that any observed differences in OS are not significant and are likely due to chance.

Table 3.5. Analysis of overall patient survival in candidate proteins. The query was refined to triple negative breast cancer patients (ER-, PR-, and HER2-) The cohort size of the breast protein database was n = 126. Patients were split into high and low expression cohorts using the median expression level as a cut off. Where data is not available a hyphen (–) is shown. The survival of cohorts (months) was the average survival of low and high expression cohorts.

Protein	Survival of low expression cohort (months)	Survival of high expression cohort (months)
<i>CDCA5</i>	-	-
<i>COL4A1</i>	-	-
<i>KIFC1</i>	-	-
<i>LMNB1</i>	65	47
<i>NUP62</i>	-	-
<i>SMC6</i>	-	-
<i>STIM1</i>	35	57
<i>SYNE1</i>	46	127
<i>SYNE2</i>	152	46
<i>TERF2</i>	-	-
<i>TREX2</i>	-	-
<i>TUBG1</i>	-	-

Table 3.6. Analysis of overall patient survival in candidate mRNAs. The query was refined to triple negative breast cancer patients (ER-, PR-, and HER2-) The cohort size of the breast mRNA database was n = 7462. Patients were split into high and low expression cohorts using the median expression level as a cut off. The survival of cohorts (months) was the average survival of low and high expression cohorts.

mRNA	Survival of low expression cohort (months)	Survival of high expression cohort (months)
<i>CDCA5</i>	124.53	131.5
<i>COL4A1</i>	131.97	116.4
<i>KIFC1</i>	122.3	131.37
<i>LMNB1</i>	129.1	131.37
<i>NUP62</i>	215.2	129.1
<i>SMC6</i>	131.37	131.97
<i>STIM1</i>	215.2	129.1
<i>SYNE1</i>	131.37	122.3
<i>SYNE2</i>	215.2	122.3
<i>TERF2</i>	129.1	142.23
<i>TREX2</i>	124.53	148.53
<i>TUBG1</i>	129.1	131.37

Table 3.7. Summary of candidate genes, identified in KM plotter analysis. The summary column shows the mean gene expression of non-responders compared with responders. ↓ indicates expression is lower in non-responders and patients with poorer overall survival than responders and patients with prolonged survival, ↑ indicates expression is higher in non-responders and patients with poorer overall survival than responders and patients with prolonged survival. Accession numbers (AT) are recorded to identify genes.

Gene symbol	AT	Expression Summary
<i>CDCA5</i>	224753	↓
<i>KIFC1</i>	209680	↓
<i>LMNB1</i>	203276	↓
<i>SMC6</i>	236535	↓
<i>STIM1</i>	202764	↑
<i>TUBG1</i>	201714	↓

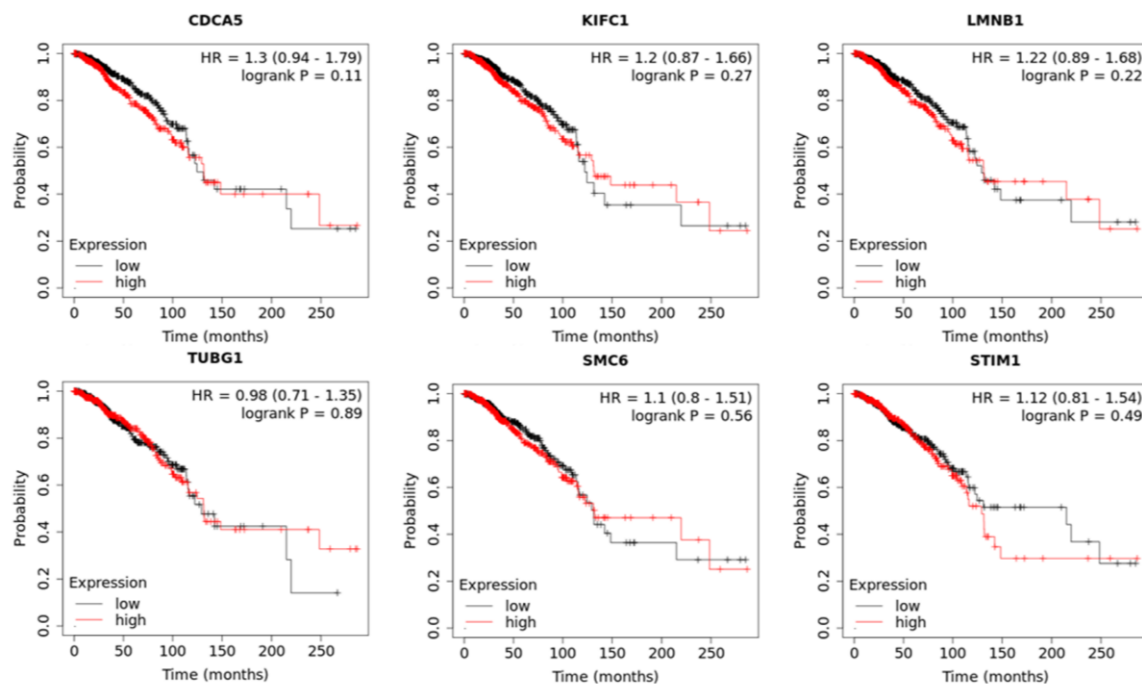


Figure 3.5. KM survival curves for high and low gene expression cohorts. Survival probability (0-1) is plotted against time (months) and represents the estimated likelihood that a patient remains alive at each time point. Each step down in the curve corresponds to a patient death, with larger drops occurring at later time points when fewer patients remain. Flat sections indicate periods with no deaths. KM estimates account for patients who are not monitored by assuming their survival probability is the same as patients still being monitored. Hazard ratio (HR), 95% confidence interval (CI), and log-rank p-value are also presented. The HR quantifies differences in survival between high and low expression cohorts (HR = 1: no difference, HR > 1: high expression associated with worse survival, HR < 1: high expression associated with improved survival). The 95% CI indicates HR precision and provides a range in which the HR is likely to fall. If the CI includes 1, this suggests the association may be unreliable. The logrank p-value indicates whether survival differences are due to chance, with $p < 0.05$ indicating statistical significance. The query was refined to triple negative breast cancer patients (ER-, PR-, and HER2-). The cohort size of the breast mRNA database was $n = 7462$. Patients were split into high and low expression cohorts using the median expression level as a cut off.

3.4.1.3 GEPIA

GEPIA was used to compare gene expression in healthy tissues and BRCA, to determine whether candidate expression could differentiate between cancerous and healthy tissue (Table 3.8). For most candidates (*CDCA5*, *KIFC1*, *LMNB1*, *SMC6*, and *TUBG1*), expression was higher in BRCA compared to healthy tissue, whereas *STIM1* expression was lower in BRCA compared to healthy tissue. Differential expression statistics were not available for *SMC6* or *STIM1*, however, for all other genes, the difference in expression was statistically significant, as indicated by q-values < 0.01.

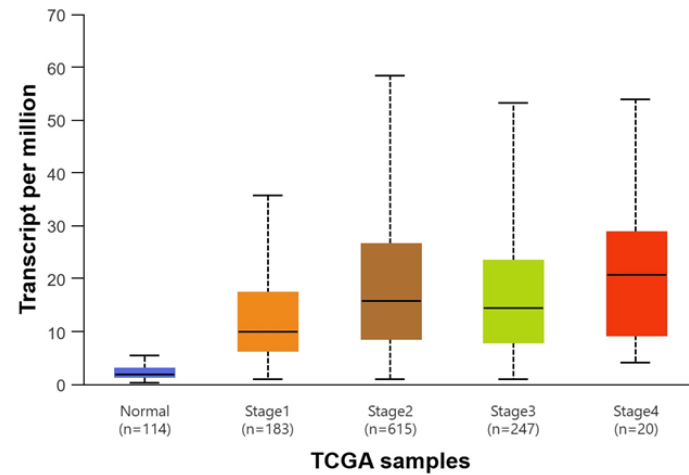
Table 3.8. GEPIA expression analysis of candidates across healthy tissue and breast invasive carcinoma. The analysis was restricted to breast invasive carcinoma (BRCA) patients. GEPIA integrates RNA-seq expression data from tumour samples (n = 9,736) and normal tissue samples (n = 8,587). Gene expression was normalised by GEPIA and reported as transcripts per million (TPM). Statistical differences in gene expression were assessed using GEPIA's internal one-way ANOVA. A default q-value threshold of < 0.01 was applied to define statistical significance and minimise the likelihood of false positives. Fold change (FC) in expression was calculated using GEPIA's internal pipeline, which quantified differences in gene expression between tumour and normal tissue. A $|\text{Log}_2\text{FC}| \geq 1$ was applied as a default threshold to filter out genes with expression differences unlikely to be biologically meaningful. GEPIA calculates Log_2FC based on normalised expression data across all samples, not directly from TPM averages. GEPIA reported Log_2FC , which was converted to fold change ($\text{FC} = 2^{\text{Log}_2\text{FC}}$) for ease of interpretation. Differential expression statistics were not available for all genes, where data was unavailable, a hyphen (-) is shown.

Gene symbol	TPM in BRCA	TPM in normal	LOG ₂ FC	Fold Change	q-value
<i>CDCA5</i>	11.36	0.92	2.69	6.45	1.33e-166
<i>KIFC1</i>	15.24	1.42	2.75	6.73	3.59e-170
<i>LMNB1</i>	30.44	3.37	2.85	7.21	1.54e-170
<i>SMC6</i>	12.1	8.59	-		-
<i>STIM1</i>	25.89	39.48	-		-
<i>TUBG1</i>	42.34	19.69	1.07	2.1	7.14e-91

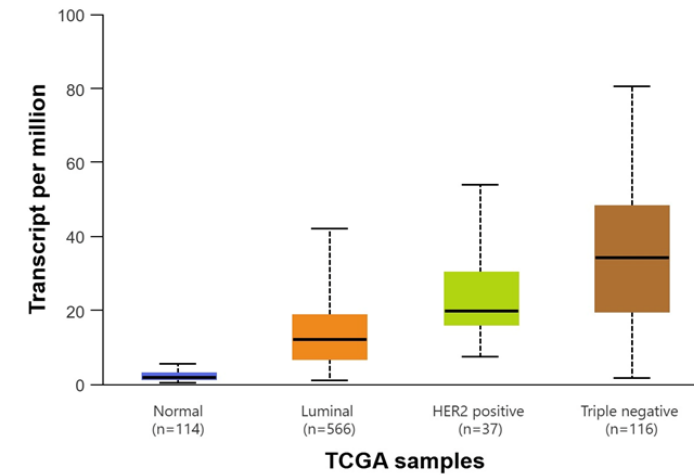
3.4.1.4 UALCAN

UALCAN was used to determine whether the expression of candidate biomarkers could be used to stratify BC by stage or subtype (Figure 3.6 - Figure 3.11). For a majority of candidates (*CDCA5*, *KIFC1*, *LMNB1*, *SMC6* and *TUBG1*), gene expression was highest in TNBC compared with other BRCA subtypes. *CDCA5* and *KIFC1* expression (Figure 3.6 and Figure 3.7) was significantly different in all pairwise comparisons, whereas *TUBG1* and *SMC6* (Figure 3.11 and Figure 3.9) were significantly different between tumour and normal tissue, but not between cancer subtypes. *LMNB1* expression was significantly different in all pairwise comparisons except luminal and HER2 positive BC (Figure 3.8). *STIM1* expression was lower in TNBC compared with other healthy tissue, although this difference was not statistically significant (Figure 3.10). For all candidates, expression was similar across BRCA stages.

Expression of CDCA5 in BRCA based on individual cancer stages



Expression of CDCA5 in BRCA based on breast cancer subclasses

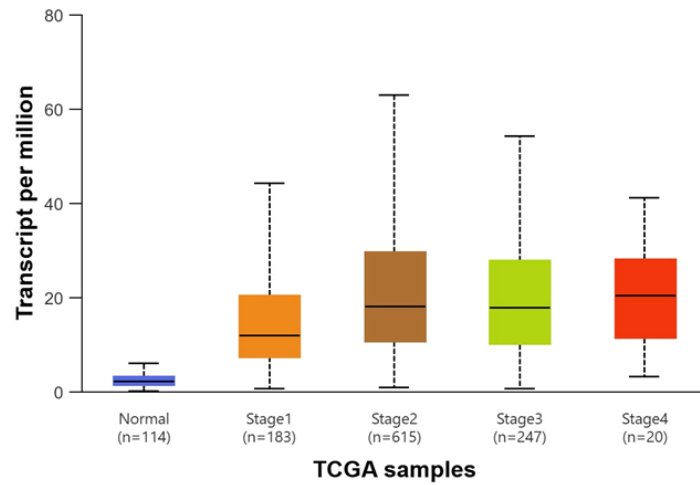


Comparison	Statistical significance	Symbol
Normal-vs-Stage1	1.62E-12	****
Normal-vs-Stage2	1.62E-12	****
Normal-vs-Stage3	1.62E-12	****
Normal-vs-Stage4	1.76E-05	****
Stage1-vs-Stage2	3.88E-05	****
Stage1-vs-Stage3	2.39E-02	*
Stage1-vs-Stage4	9.02E-02	
Stage2-vs-Stage3	8.52E-02	
Stage2-vs-Stage4	9.71E-01	
Stage3-vs-Stage4	5.10E-01	

Comparison	Statistical significance	Symbol
Normal-vs-Luminal	1.62E-12	****
Normal-vs-HER2 Positive	4.17E-11	****
Normal-vs-TNBC	1.62E-12	****
Luminal-vs-HER2 Positive	5.56E-03	**
Luminal-vs-TNBC	1.62E-12	****
HER2 Positive-vs-TNBC	9.23E-04	***

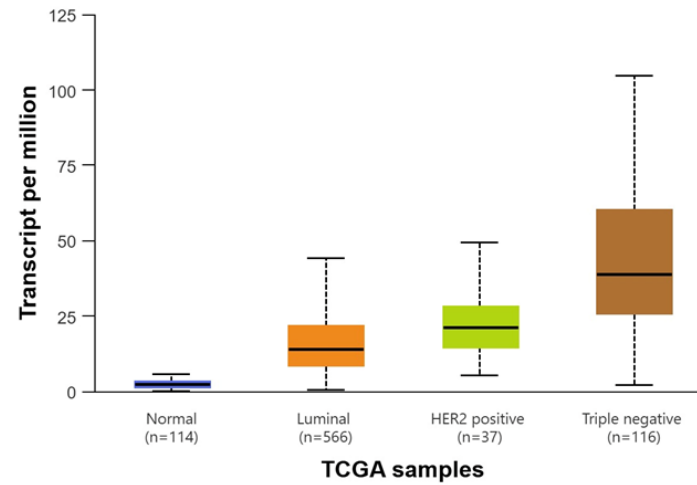
Figure 3.6. UALCAN Analysis of CDCA5 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was highest in TNBC compared with other BRCA subtypes and was significantly different in all pairwise comparisons. CDCA5 expression was similar across BRCA stages.

Expression of KIFC1 in BRCA based on individual cancer stages



Comparison	Statistical significance	Symbol
Normal-vs-Stage1	1.62E-12	****
Normal-vs-Stage2	<1E-12	****
Normal-vs-Stage3	<1E-12	****
Normal-vs-Stage4	4.99E-06	****
Stage1-vs-Stage2	6.41E-05	****
Stage1-vs-Stage3	1.33E-01	
Stage1-vs-Stage4	4.82E-01	
Stage2-vs-Stage3	3.86E-03	**
Stage2-vs-Stage4	3.29E-01	
Stage3-vs-Stage4	9.05E-01	

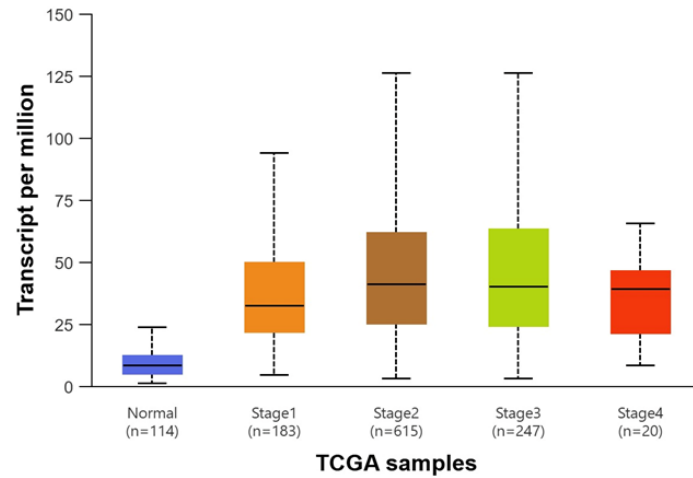
Expression of KIFC1 in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	1.29E-11	****
Normal-vs-TNBC	1.62E-12	****
Luminal-vs-HER2 Positive	3.72E-03	**
Luminal-vs-TNBC	<1E-12	****
HER2 Positive-vs-TNBC	3.04E-08	****

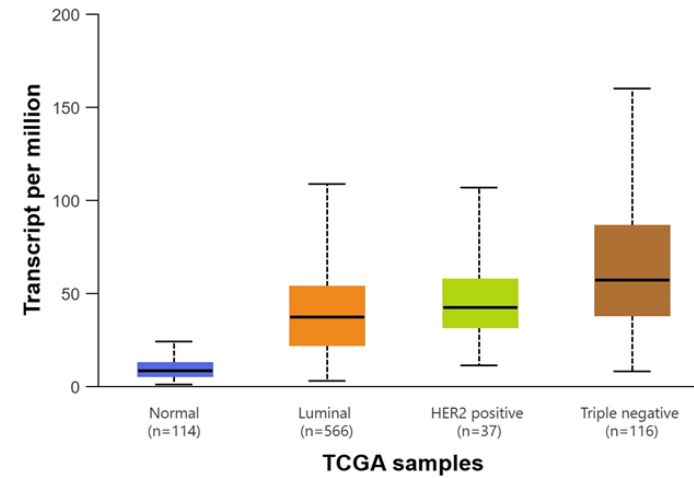
Figure 3.7. UALCAN Analysis of KIFC1 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was highest in TNBC compared with other BRCA subtypes and was significantly different in all pairwise comparisons. KIFC1 expression was similar across BRCA stages.

Expression of LMNB1 in BRCA based on individual cancer stages



Comparison	Statistical significance	Symbol
Normal-vs-Stage1	1.62E-12	****
Normal-vs-Stage2	1.62E-12	****
Normal-vs-Stage3	<1E-12	****
Normal-vs-Stage4	6.03E-05	****
Stage1-vs-Stage2	1.89E-02	*
Stage1-vs-Stage3	1.10E-01	
Stage1-vs-Stage4	9.55E-01	
Stage2-vs-Stage3	6.22E-01	
Stage2-vs-Stage4	3.47E-01	
Stage3-vs-Stage4	4.64E-01	

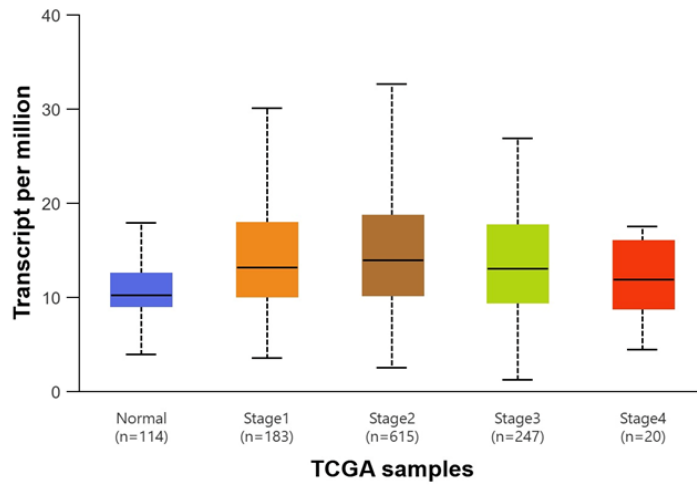
Expression of LMNB1 in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	4.80E-11	****
Normal-vs-TNBC	<1E-12	****
Luminal-vs-HER2 Positive	1.68E-01	
Luminal-vs-TNBC	8.66E-08	****
HER2 Positive-vs-TNBC	8.36E-03	**

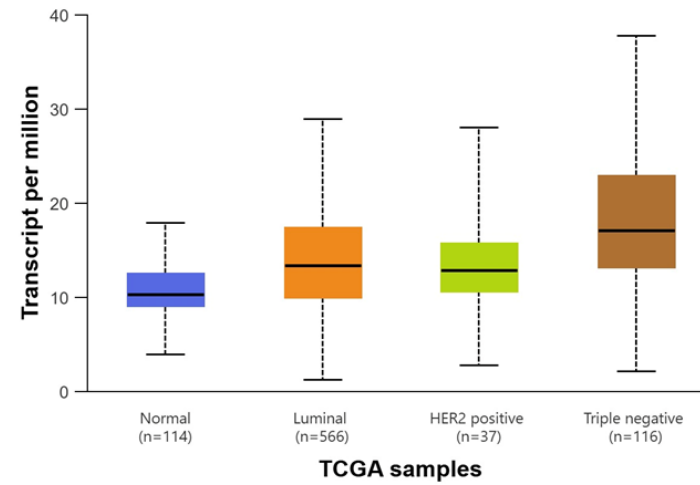
Figure 3.8. UALCAN Analysis of LMNB1 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was highest in TNBC compared with other BRCA subtypes and was significantly different in all pairwise comparisons, except Luminal and HER2 Positive. LMNB1 expression was similar across BRCA stages.

Expression of SMC6 in BRCA based on individual cancer stages



Comparison	Statistical significance	Symbol
Normal-vs-Stage1	7.45E-11	****
Normal-vs-Stage2	<1E-12	****
Normal-vs-Stage3	1.38E-11	****
Normal-vs-Stage4	1.51E-01	
Stage1-vs-Stage2	2.03E-01	
Stage1-vs-Stage3	4.35E-01	
Stage1-vs-Stage4	9.38E-03	**
Stage2-vs-Stage3	1.09E-02	*
Stage2-vs-Stage4	7.77E-04	***
Stage3-vs-Stage4	2.46E-02	*

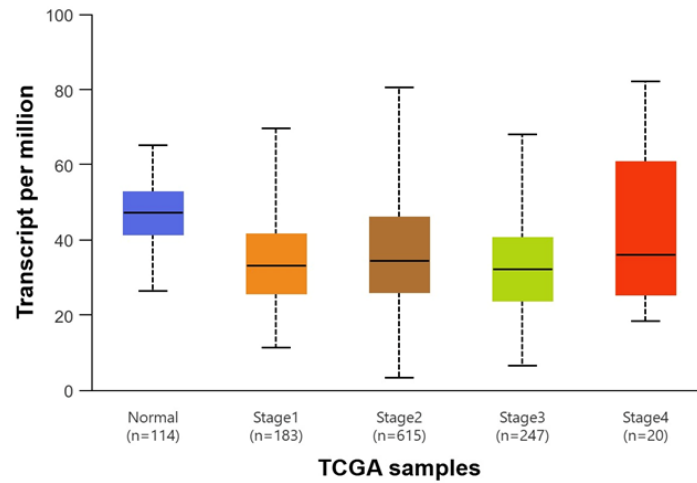
Expression of SMC6 in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	1.12E-03	**
Normal-vs-TNBC	4.44E-16	****
Luminal-vs-HER2 Positive	1.94E-01	
Luminal-vs-TNBC	9.84E-08	****
HER2 Positive-vs-TNBC	9.20E-02	

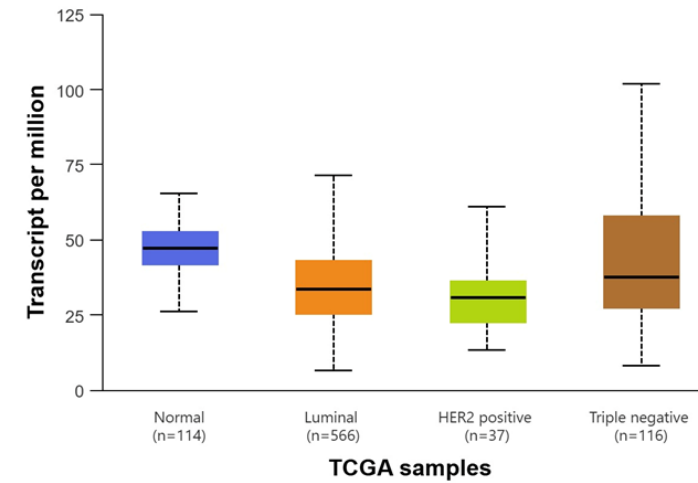
Figure 3.9. UALCAN Analysis of SMC6 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was highest in TNBC compared with other BRCA subtypes but was significantly different in all pairwise comparisons. SMC6 expression was similar across BRCA stages.

Expression of STIM1 in BRCA based on individual cancer stages



Comparison	Statistical significance	Symbol
Normal-vs-Stage1	5.60E-07	****
Normal-vs-Stage2	1.46E-10	****
Normal-vs-Stage3	1.62E-12	****
Normal-vs-Stage4	3.50E-01	
Stage1-vs-Stage2	5.05E-01	
Stage1-vs-Stage3	7.37E-02	
Stage1-vs-Stage4	4.06E-01	
Stage2-vs-Stage3	8.41E-04	***
Stage2-vs-Stage4	5.27E-01	
Stage3-vs-Stage4	6.12E-02	

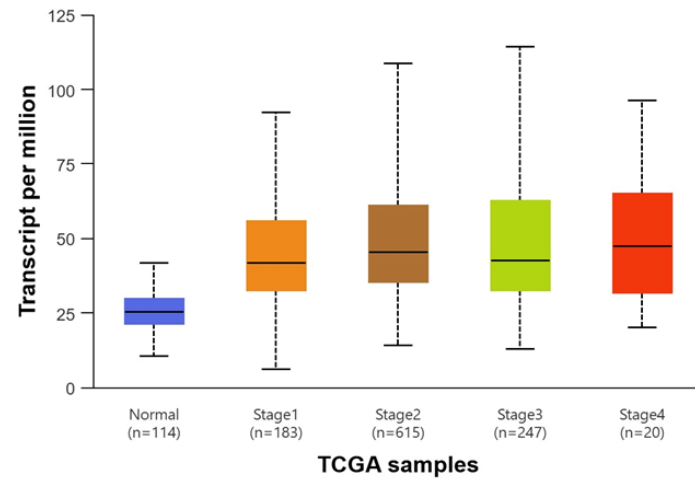
Expression of STIM1 in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	1.61E-05	****
Normal-vs-TNBC	9.29E-01	
Luminal-vs-HER2 Positive	1.77E-01	
Luminal-vs-TNBC	6.97E-04	***
HER2 Positive-vs-TNBC	4.60E-04	***

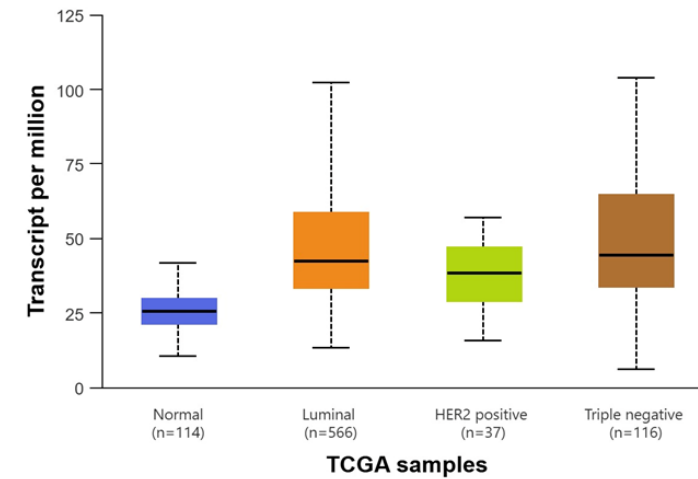
Figure 3.10. UALCAN Analysis of STIM1 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. STIM1 expression was lower in TNBC compared with other normal tissue, although this difference was not statistically significant. STIM1 expression was similar across BRCA stages.

Expression of TUBG1 in BRCA based on individual cancer stages



Comparison	Statistical significance	Symbol
Normal-vs-Stage1	1.62E-12	****
Normal-vs-Stage2	<1E-12	****
Normal-vs-Stage3	<1E-12	****
Normal-vs-Stage4	2.24E-04	***
Stage1-vs-Stage2	1.06E-01	
Stage1-vs-Stage3	4.98E-01	
Stage1-vs-Stage4	4.19E-01	
Stage2-vs-Stage3	3.49E-01	
Stage2-vs-Stage4	8.44E-01	
Stage3-vs-Stage4	6.19E-01	

Expression of TUBG1 in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	2.94E-06	****
Normal-vs-TNBC	1.62E-12	****
Luminal-vs-HER2 Positive	8.40E-02	
Luminal-vs-TNBC	3.70E-01	
HER2 Positive-vs-TNBC	2.71E-02	*

Figure 3.11. UALCAN Analysis of TUBG1 gene expression in breast invasive carcinoma stages and subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to pathological stage and molecular subtype, as defined in the TCGA clinical metadata. For stage comparisons, the cohort included n = 1,179 samples: normal (n = 114), stage I (n = 183), stage II (n = 615), stage III (n = 247), and stage IV (n = 20). For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was highest in TNBC compared with other BRCA subtypes but was not significantly different between all pairwise comparisons. TUBG1 expression was similar across BRCA stages.

3.4.2 Developing TIS models

Given the diverse phenotype of senescent cells, no single marker is sufficient to characterise them, therefore TIS was confirmed using multiple assays. Several hallmarks of senescence were assessed to confirm TIS induction in each cell line, including β -galactosidase staining, γ -H2AX staining, nuclear area analysis, and qPCR analysis of senescence associated genes.

3.4.2.1 Identification of non-cytotoxic TIS inducing doxorubicin concentrations

MTT and apoptosis assays confirmed that the selected doxorubicin concentrations induced a cytostatic response, rather than cytotoxic effects. MTT assays were conducted under the same conditions as TIS experiments, using the same seeding densities, 24 hour doxorubicin treatment, and 96 hour post treatment incubation time, corresponding to the TIS assay endpoint (2.7). For each cell line, metabolic activity was normalised and compared with the untreated control. No cell line showed a significant reduction in viability indicating the selected drug concentrations did not induce extreme cytotoxicity (Figure 3.12). Apoptosis assays were used to confirm that cells were predominantly live, following treatment. This was particularly important as MTT assays assess metabolic activity rather than cell fate. For all cell lines, the majority of cells in both untreated and treated groups were live, with no statistically significant differences observed between treatment groups (Figure 3.16-Figure 3.18). A small proportion of cells undergoing apoptosis and necrosis was detected in each cell line which is to be expected in healthy cell populations. These results indicated that the selected doxorubicin concentrations were appropriate for inducing TIS, rather than cell death. Staining patterns were as expected, where cytochrome c stained the cytoplasm of live cells, annexin green stained the outer membrane of apoptotic cells and 7-AAD stained the nucleus of necrotic cells. Representative images of each cell fate are shown in Figure 3.13- Figure 3.15.

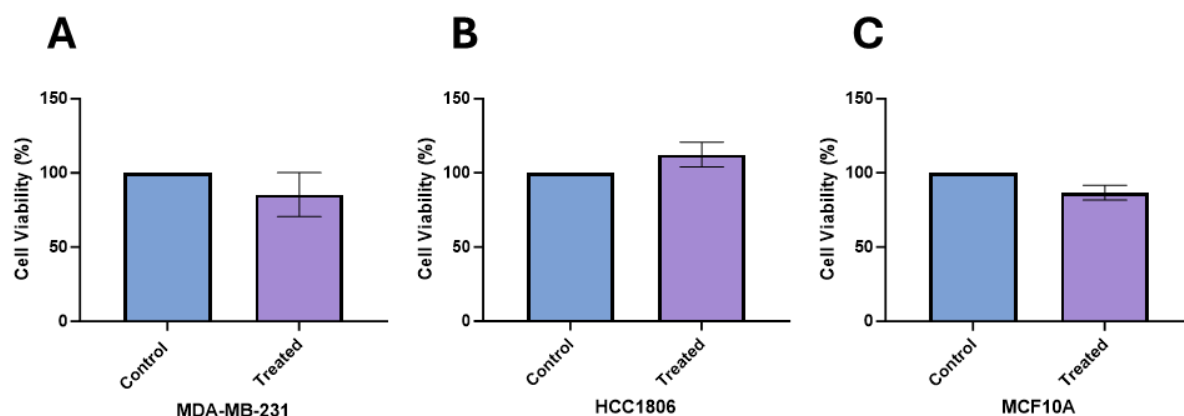


Figure 3.12. Effect of TIS inducing doxorubicin concentrations on cell viability, assessed using MTT assays. Cell viability following a 24 hour doxorubicin treatment and 96 hour post treatment recovery in (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A cells. Viability (%) is expressed as a proportion of the untreated control (100%). Cells were treated at equivalent levels to TIS experiments (MDA-MB-231 175 nM doxorubicin, HCC1806 25 nM doxorubicin and MCF10A 100 nM doxorubicin). No statistically significant reductions in viability were observed. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests.

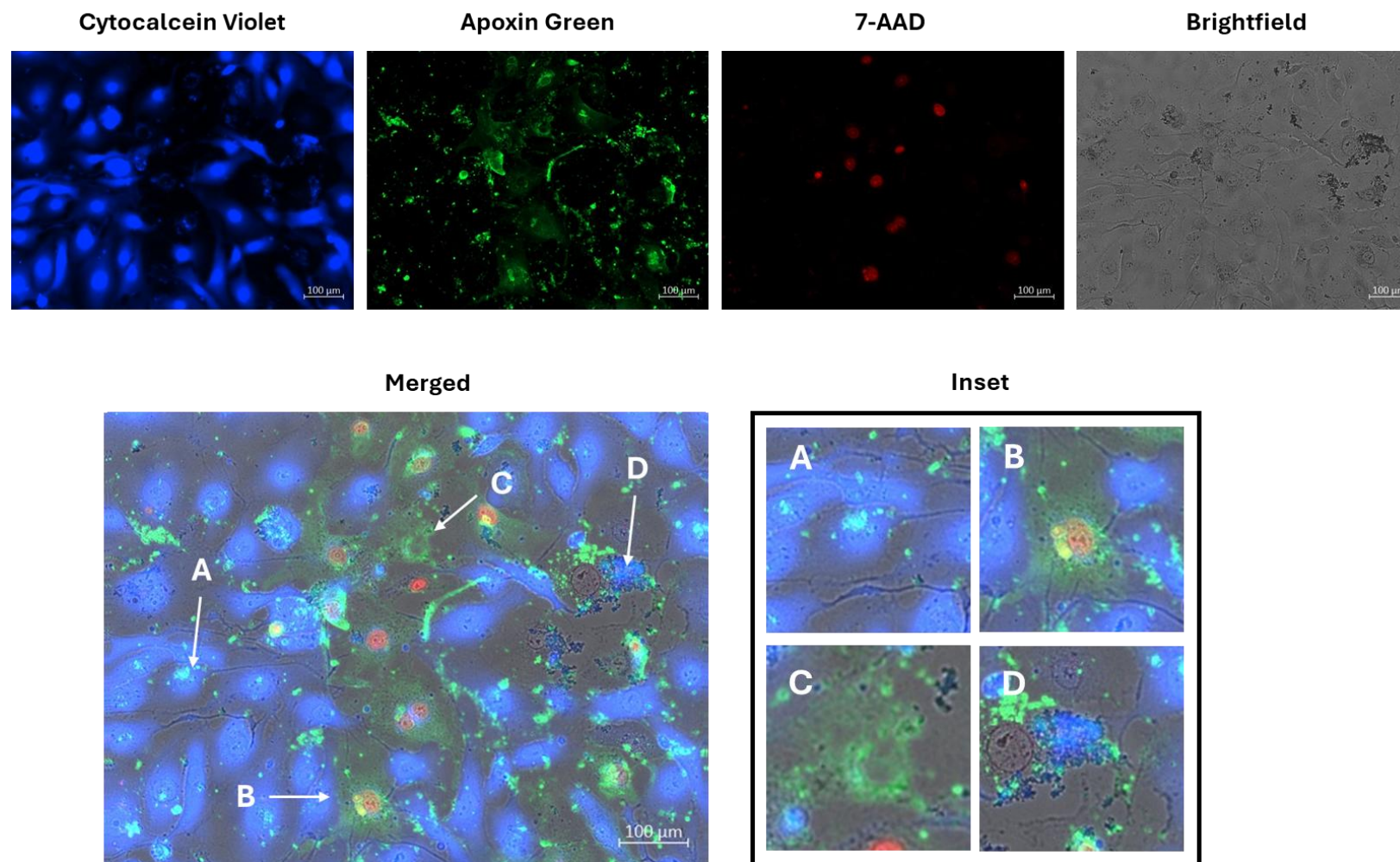


Figure 3.13. Representative fluorescence microscopy images illustrating distinct cell fates in MDA-MB-231. Cells were stained using a fluorescence-based apoptosis assay. Images were captured at 10× magnification. Cells were treated with 175nM doxorubicin. Live cells were stained with Cytocalcein Violet, visualised on the CeTCV channel. Apoptotic cells were stained with Apoxin Green, visualised on the FITC channel. Necrotic cells were stained with 7-AAD, visualised on the Texas Red channel. Merged image combining all fluorescence channels, where A) represents a live cell, B) an apoptotic cell, C) a necrotic cell, and D) debris.

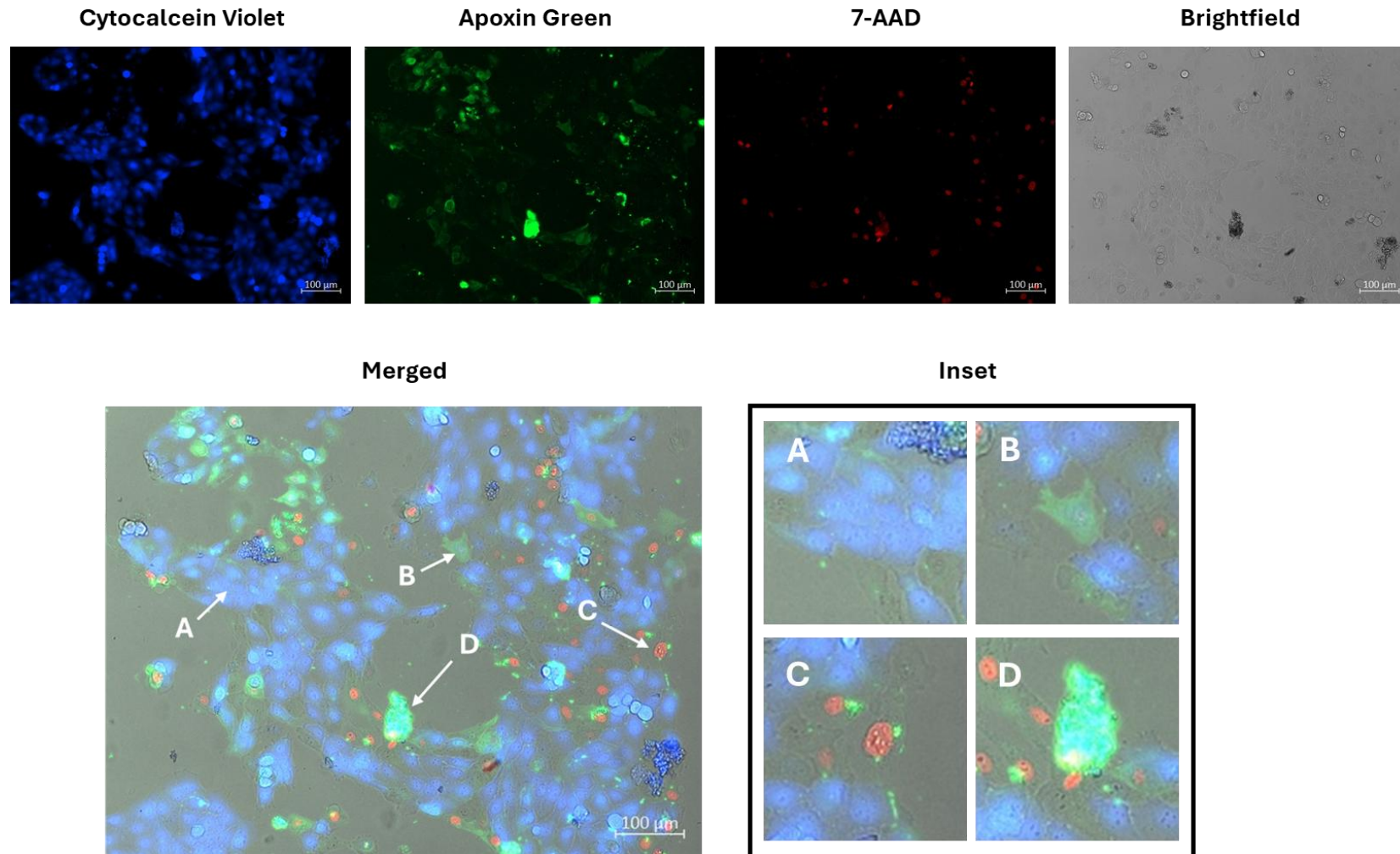


Figure 3.14. Representative fluorescence microscopy images illustrating distinct cell fates in HCC1806. Cells were stained using a fluorescence-based apoptosis assay. Images were captured at 10× magnification. Cells were treated with 25nM doxorubicin. Live cells were stained with Cytocalcein Violet, visualised on the CeTCV channel. Apoptotic cells were stained with Apoxin Green, visualised on the FITC channel. Necrotic cells were stained with 7-AAD, visualised on the Texas Red channel. Merged image combining all fluorescence channels, where A) represents a live cell, B) an apoptotic cell, C) a necrotic cell, and D) debris.

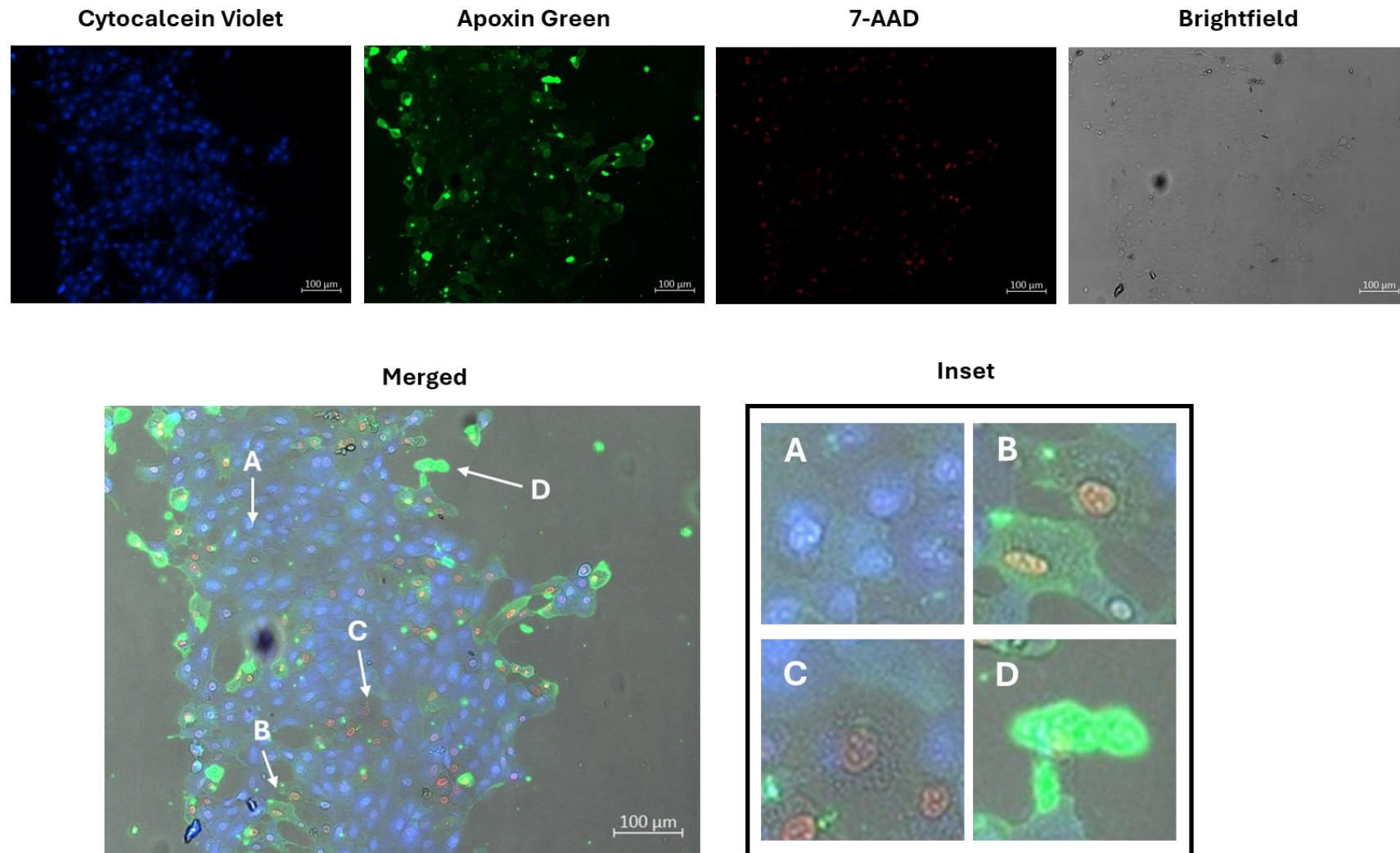


Figure 3.15. Representative fluorescence microscopy images illustrating distinct cell fates in MCF10A. Cells were stained using a fluorescence-based apoptosis assay. Images were captured at 10× magnification. Cells were treated with 100nM doxorubicin. Live cells were stained with Cytocalcein Violet, visualised on the CeTCV channel. Apoptotic cells were stained with Apoxin Green, visualised on the FITC channel. Necrotic cells were stained with 7-AAD, visualised on the Texas Red channel. Merged image combining all fluorescence channels, where A) represents a live cell, B) an apoptotic cell, C) a necrotic cell, and D) debris.

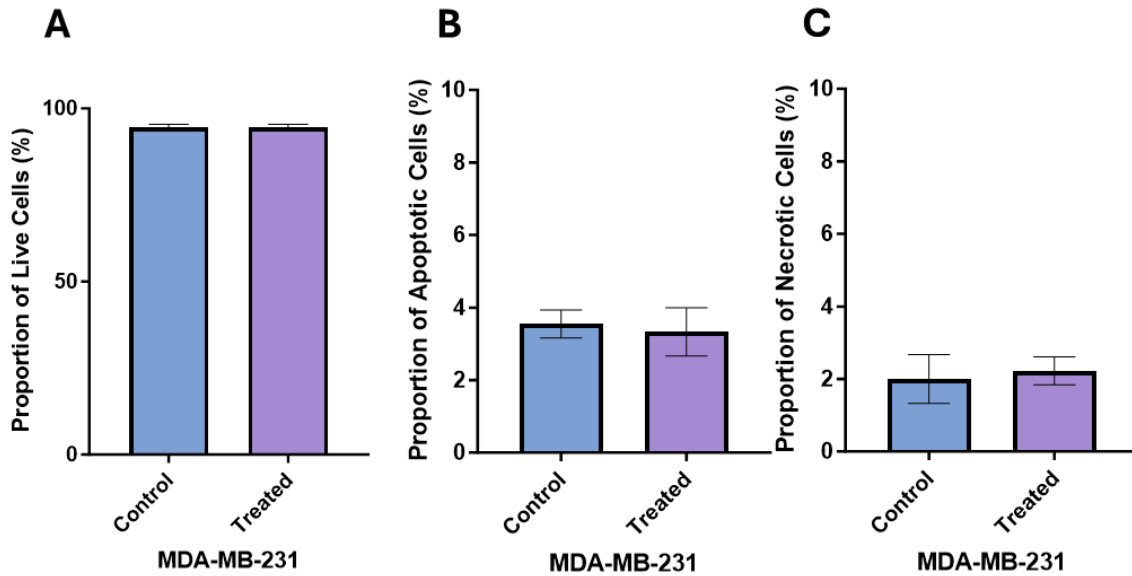


Figure 3.16. Effect of TIS inducing doxorubicin concentrations on MDA-MB-231 cell fate. Cells were treated for 24 hours with 175nM doxorubicin and allowed to recover for 96 hours post treatment. Cells were seeded at equivalent levels to TIS experiments. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

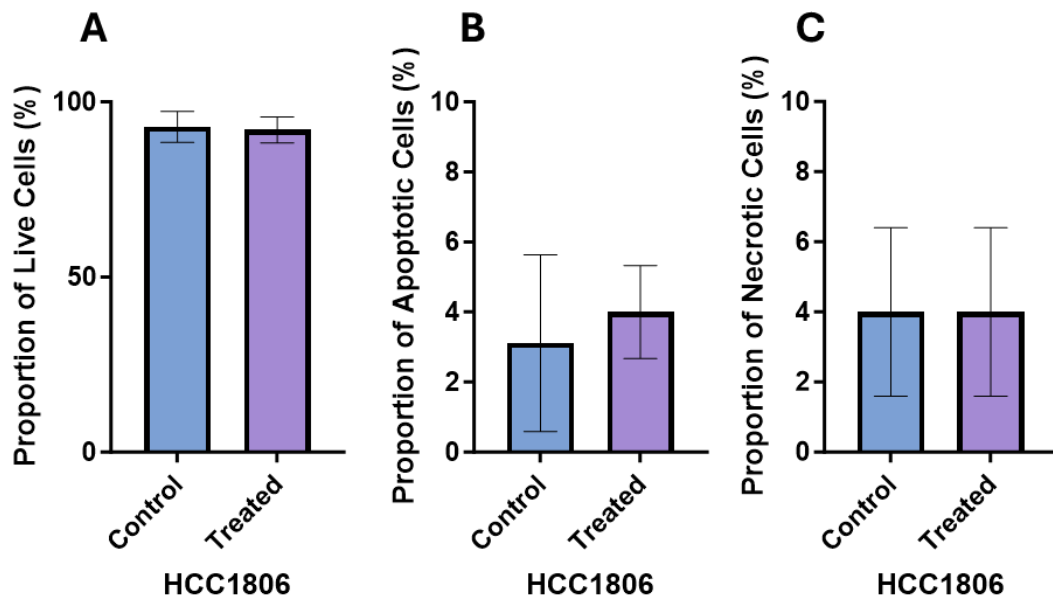


Figure 3.17. Effect of TIS inducing doxorubicin concentrations on HCC1806 cell fate. Cells were treated for 24 hours with 25nM doxorubicin and allowed to recover for 96 hours post treatment. Cells were seeded at equivalent levels to TIS experiments. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

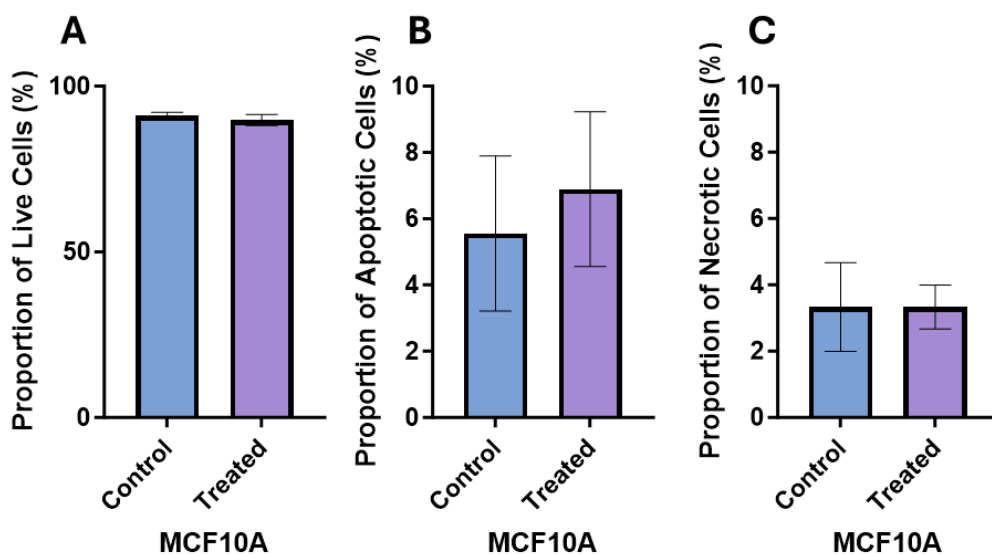


Figure 3.18. Effect of TIS inducing doxorubicin concentrations on MCF10A cell fate. Cells were treated for 24 hours with 100nM doxorubicin and allowed to recover for 96 hours post treatment. Cells were seeded at equivalent levels to TIS experiments. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

3.4.2.2 β -galactosidase staining

β -galactosidase staining is a widely used method for detecting senescence. Senescent cells have increased lysosomal content and upregulate several lysosomal proteins, leading to characteristic blue cytoplasmic staining at pH 5.9-6.1, indicative of TIS (Hernandez-Segura, Nehme and Demaria, 2018; Park et al., 2018). The % of β -galactosidase positive cells, for each cell type and treatment is presented in Figure 3.19. Comparable levels of staining were observed between cell lines. All doxorubicin treated cells showed a significant increase in β -galactosidase staining compared to untreated controls, indicating successful TIS induction. Representative images of β -galactosidase positive (B) and negative (A) cells are shown in Figure 3.20. Lysosomal staining was frequently observed surrounding the nuclei of cells.

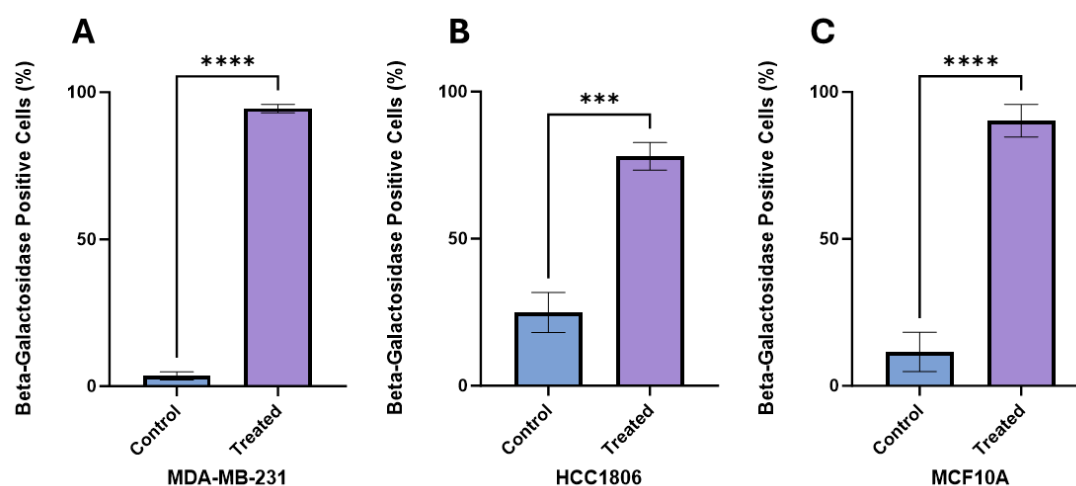


Figure 3.19. β -galactosidase staining following TIS induction. Cells were stained using a β -galactosidase staining kit, and a total of 150 cells were counted to determine the percentage of β -galactosidase positive (A) MDA-MB-231, (B) HCC1806, and (C) MCF10A cells. The proportion of positive cells (%) was calculated relative to the total cell population. A statistically significant increase in staining was observed in all doxorubicin treated (TIS) samples compared to untreated controls. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

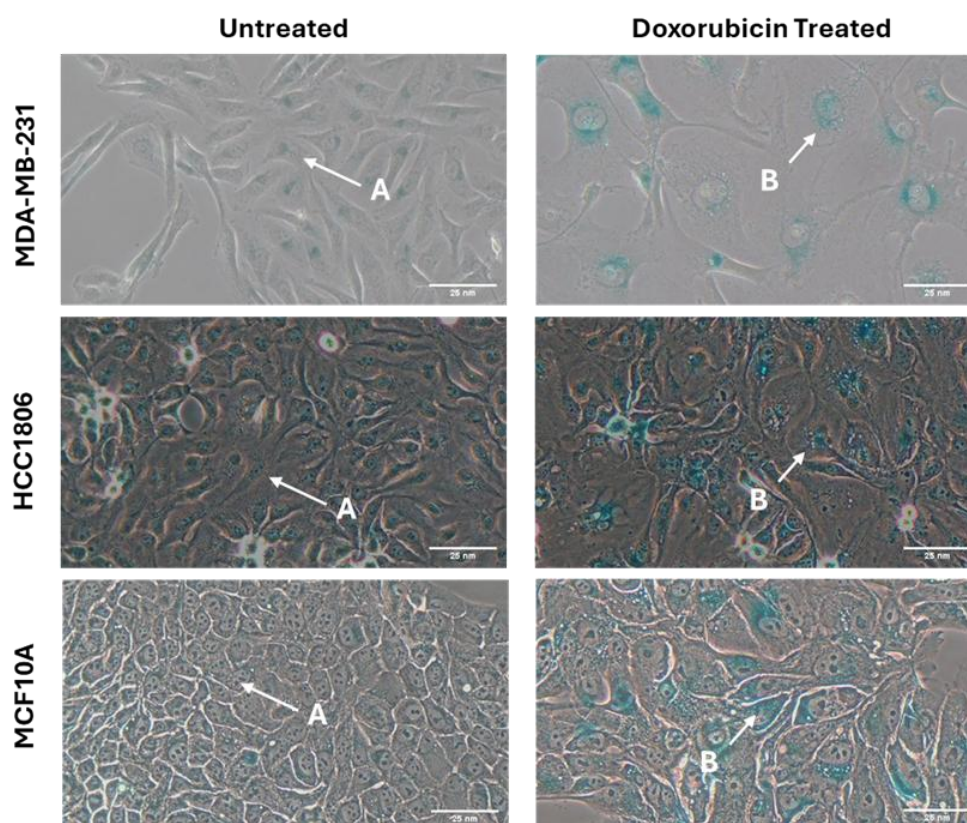


Figure 3.20. Representative β -galactosidase staining images of immortalised cells. Cells were stained using a β -galactosidase staining kit, to determine the percentage of β -galactosidase positive MDA-MB-231, HCC1806, and MCF10A cells. Images show untreated controls and doxorubicin treated cells. Images were captured at 20 $\times$ magnification, using a phase contrast microscope. A) Example of a β -galactosidase negative cell and B) Example of a β -galactosidase positive cell, indicative of senescence.

3.4.2.3 γ -H2AX staining

Chronic DDR is a characteristic of TIS cells, commonly induced by DNA damaging drugs such as doxorubicin (Hernandez-Segura, Nehme and Demaria, 2018). γ -H2AX (Ser139) the phosphorylated form of the histone variant H2AX, is a well-established marker of DSBs, and when increased indicates DNA damage, as expected in cells undergoing TIS. As expected for a histone modification, γ -H2AX staining was confined to the nucleus (Figure 3.21 - Figure 3.23). A combination of punctate and pan γ -H2AX staining was observed, reflecting heterogeneity in the DDR among individual cells. In some cells, large, distinct punctate nuclear foci were observed, whereas in others, smaller and less defined foci with more diffuse staining were present. Any cell which clearly fluoresced was interpreted as positively stained. Overall, stronger nuclear staining was detected in doxorubicin treated samples compared to controls. An increase in γ -H2AX staining, indicative of increased DDR and TIS, was observed in each cell line, statistically for MDA-MB-231 and MCF10A. The largest increase in damage was observed in MDA-MB-231, followed by MCF10A and HCC1806 (Figure 3.24).

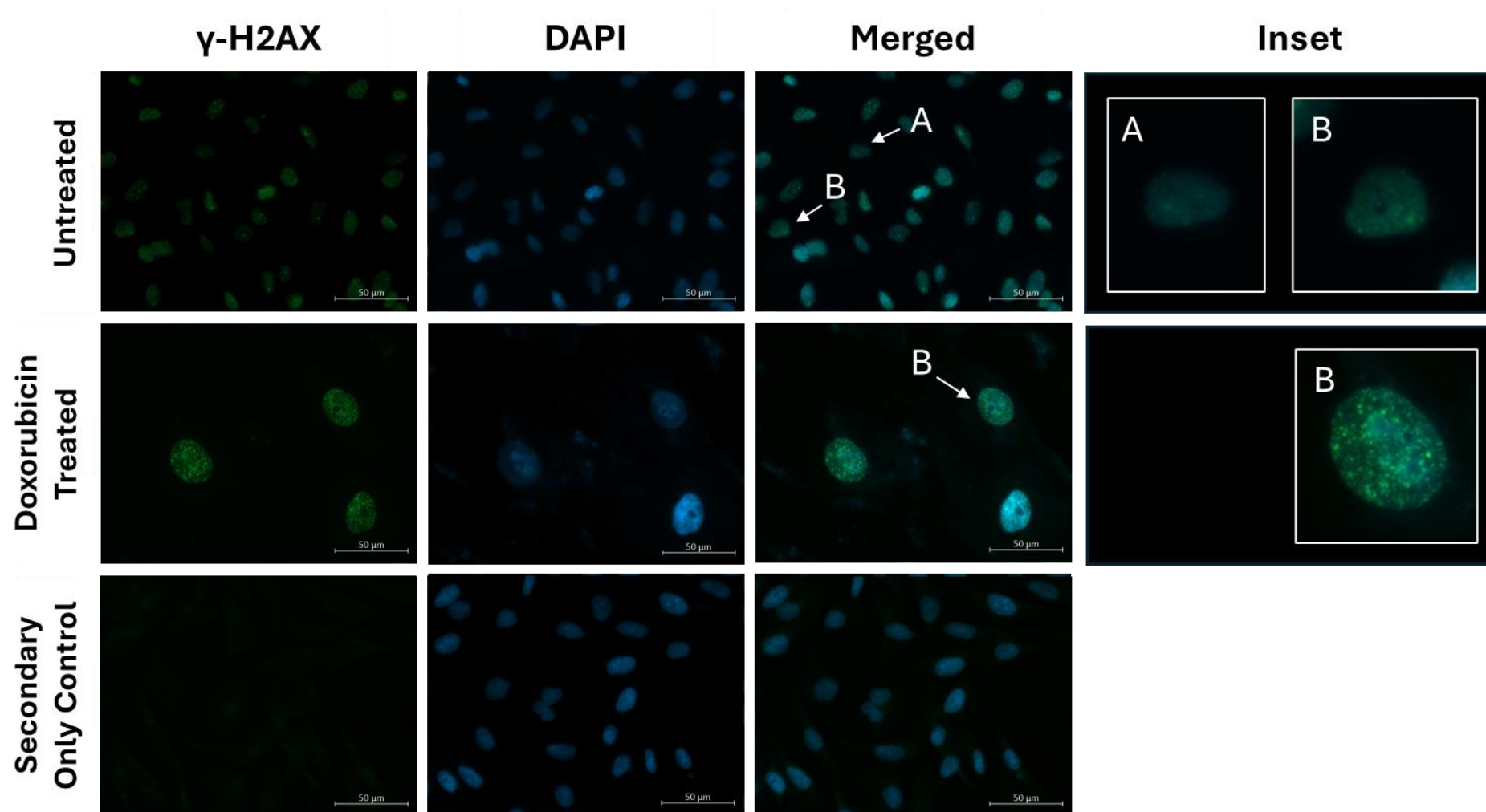


Figure 3.21. Representative fluorescence microscopy images illustrating γ -H2AX staining in MDA-MB-231 cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: untreated cells, cells treated with 175nM doxorubicin, and a secondary antibody only control. γ -H2AX fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm). Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell and B) a positive cell.

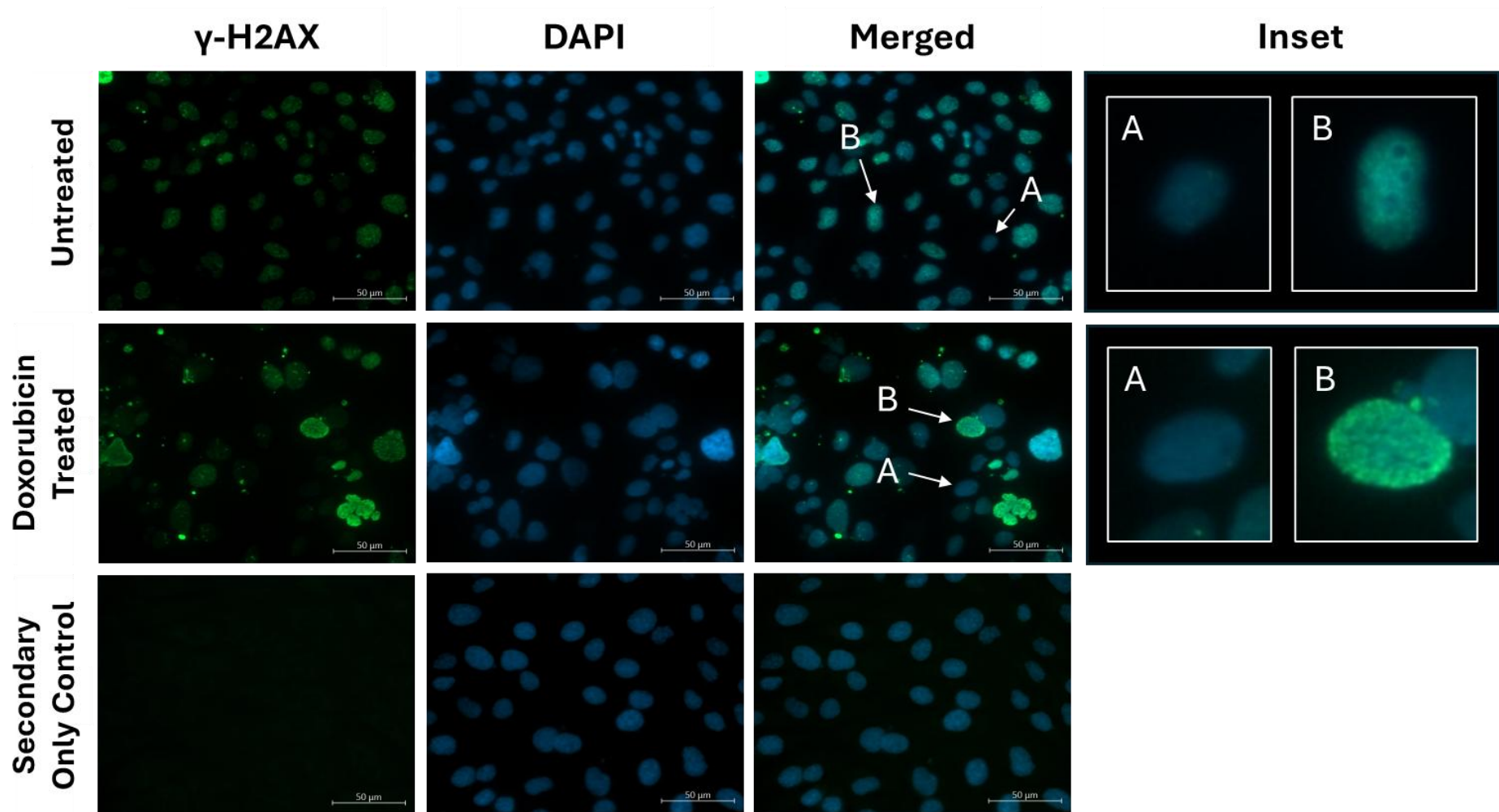


Figure 3.22. Representative fluorescence microscopy images illustrating γ -H2AX staining in HCC1806 cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: untreated cells, cells treated with 25nM doxorubicin, and a secondary antibody only control. γ -H2AX fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm). Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell and B) a positive cell.

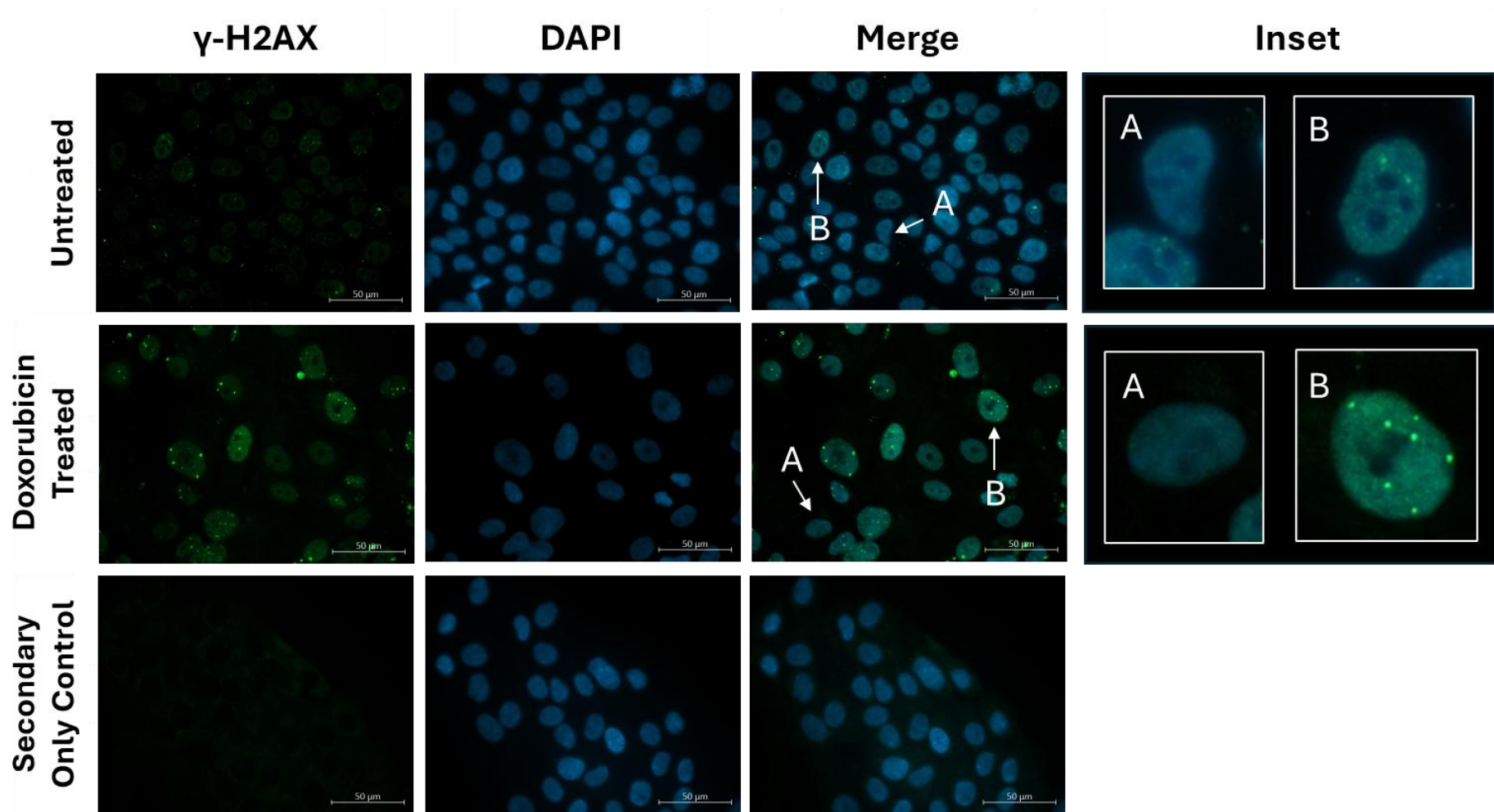


Figure 3.23. Representative fluorescence microscopy images illustrating γ -H2AX staining in MCF10A cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: untreated cells, cells treated with 100nM doxorubicin, and a secondary antibody only control. γ -H2AX fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm). Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell and B) a positive cell.

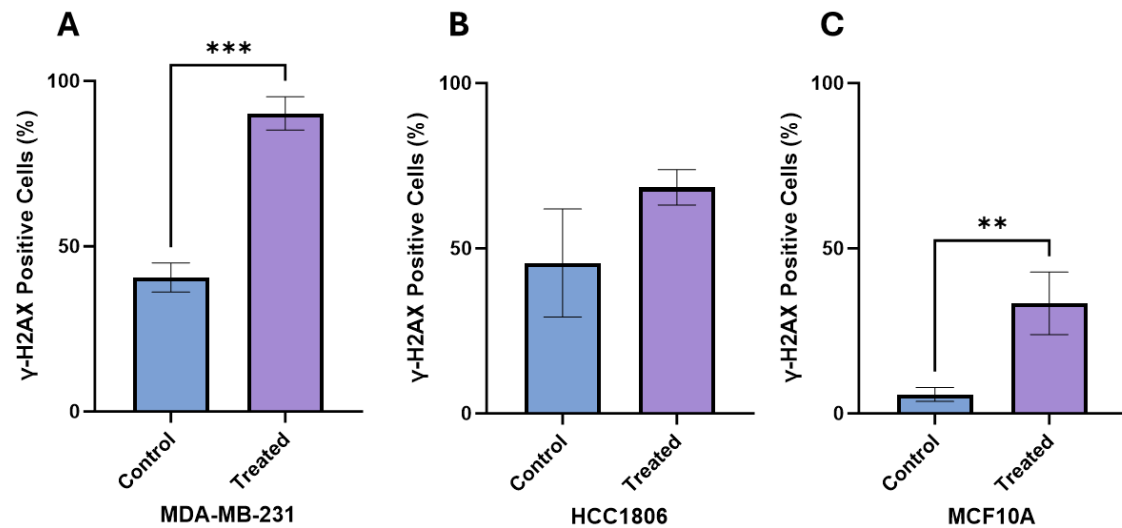


Figure 3.24. γ -H2AX staining following TIS induction. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of positive cells. A statistically significant increase in positive cells was observed in doxorubicin treated MDA-MB-231 and MCF10A cells, compared to their respective untreated controls. No significant difference in γ -H2AX staining was observed in HCC1806 cells. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests for MDA-MB-231 and MCF10A. For HCC1806 the Mann Whitney test was used. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

3.4.2.4 Nuclear area

As a consequence of aberrant signalling from both DDR and SASP, TIS cells can have an abnormal appearance characterised by increased cell size (hypertrophy), a flattened appearance and nuclear distortion, which can be quantified and used as a morphological marker of senescence (Beck, Horikawa and Harris, 2020; Herranz and Gil, 2018). Nuclear area (μm^2) was measured, using ImageJ software and results for each cell line are presented in Figure 3.25. Although all doxorubicin treated samples showed an increase in nuclear area, this was only statistically significant in MDA-MB-231, which had the greatest increase of ~ 3.5 fold. No other statistically significant differences were observed in HCC1806 and MCF10A, which had ~ 1.5 fold increases in nuclear area.

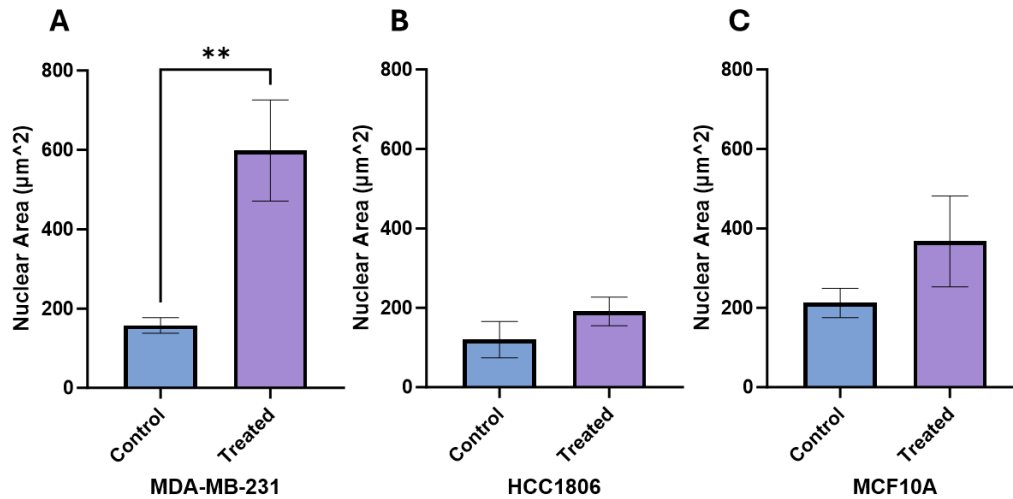


Figure 3.25. Nuclear area analysis following TIS induction. Nuclear area (μm^2) was measured, using ImageJ software, across 150 cells per treatment group, in (A) MDA-MB-231, (B) HCC1806, and (C) MCF10A cells. A statistically significant increase in nuclear area was observed in doxorubicin treated MDA-MB-231 cells, however no significant differences in nuclear area were observed in HCC1806 or MCF10A cells. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using unpaired T-tests for MDA-MB-231 and MCF10A. For HCC1806 the Mann Whitney test was used. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

3.4.2.5 Gene expression analysis of senescence associated genes

Gene expression analysis was performed using qPCR to assess key TIS markers, including components of the SASP (*CXCL1*, *IL1A*, *IL6* and *IL8*), regulators of senescence induction pathways (*CDKN1A* and *TP53*), and nuclear lamina components (*LMNB1*). Results are represented as fold change and were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, normalised to two housekeeping genes, *GAPDH* and *PUM1*, validated in BC cell lines.

Given the heterogeneity of the SASP, the gene expression of several components was assessed. In MDA-MB-231, HCC1806 and MCF10A all SASP components were elevated following doxorubicin treatment, indicating inflammatory pathway activation (Figure 3.26-Figure 3.28). This increase was significant for *CXCL1* and *IL1A* in MDA-MB-231 and additionally for *IL6* in HCC1806. In MCF10A, significant increases were observed for *IL1A* and *IL8*. Of the SASP components analysed *IL1A* exhibited the greatest fold increase in all cell lines. Overall, a more pronounced SASP response was observed in the TNBC cell lines compared to MCF10A, with HCC1806 displaying higher levels of SASP components than MDA-MB-231.

The P53/P21^{WAF/CIP1} pathway induces senescence by inhibiting cyclin activation, functioning alongside the P16^{INK4A}/Rb pathway (Mijit et al., 2020). In all three cell lines, *CDKN1A* expression was significantly increased in doxorubicin treated samples (Figure 3.26-Figure 3.28). In contrast, *TP53* expression was significantly decreased in MCF10A cells, compared to TNBC cell lines which showed a modest, non-significant increase in expression.

LMNB1 encodes one of the two B-type Lamin proteins, forming a key structural component of the nuclear lamina. As an intermediate filament, it provides cellular structural integrity, and its downregulation is associated with structural weakening of the nuclear envelope in TIS (Martins et al., 2020). *LMNB1* expression was significantly reduced in MCF10A and MDA-MB-231 cells but remained unchanged in HCC1806 cells (Figure 3.26-Figure 3.28). *LMNB1* expression decreased to ~10% of control levels in MCF10A and ~50% in MDA-MB-231.

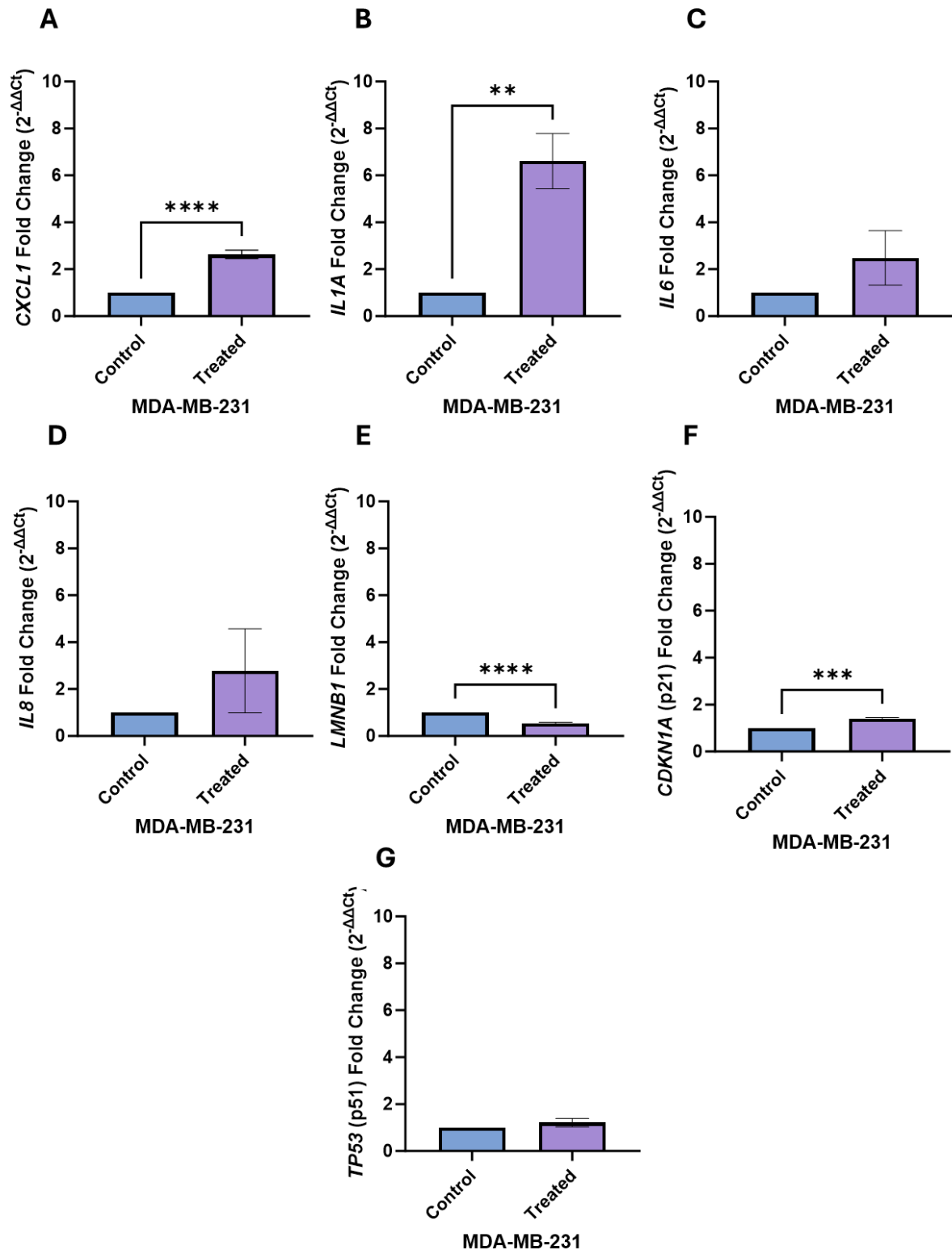


Figure 3.26. qPCR analysis of senescence associated genes in MDA-MB-231 following TIS induction. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6*, (D) *IL8*, (E) *LMNB1*, (F) *CDKN1A* and (G) *TP53*. A statistically significant increase in gene expression was observed in *CXCL1*, *IL1A* and *CDKN1A*. A statistically significant decrease of *LMNB1* expression was also observed. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $N_s = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

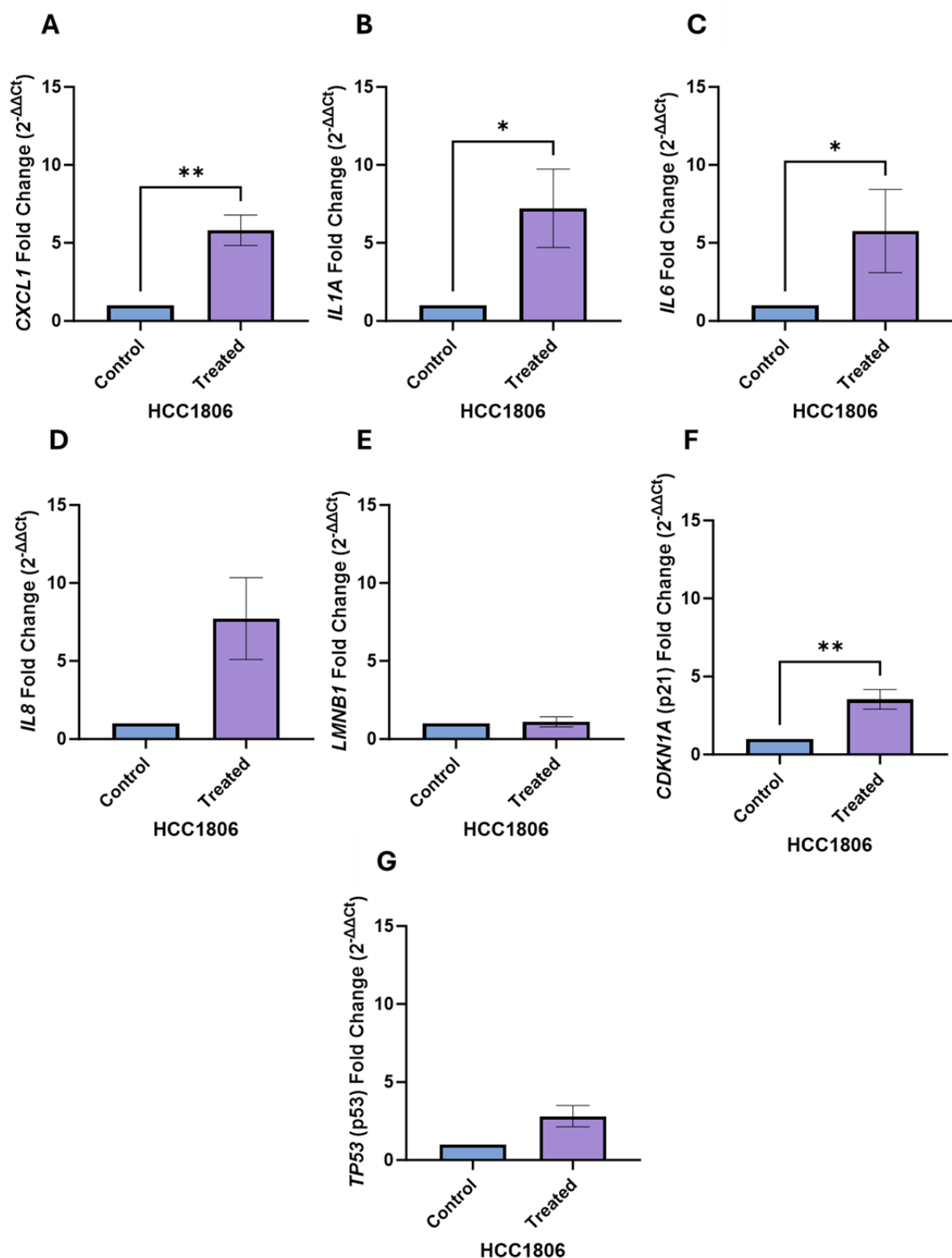


Figure 3.27. qPCR analysis of senescence associated genes in HCC1806 following TIS induction. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6*, (D) *IL8*, (E) *LMNB1*, (F) *CDKN1A* and (G) *TP53*. A statistically significant increase in gene expression was observed in *CXCL1*, *IL1A*, *IL6* and *CDKN1A*. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests for *CXCL1*, *IL1A*, *IL6*, *LMNB1* and *CDKN1A*, and Mann Whitney for *IL8* and *TP53*, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

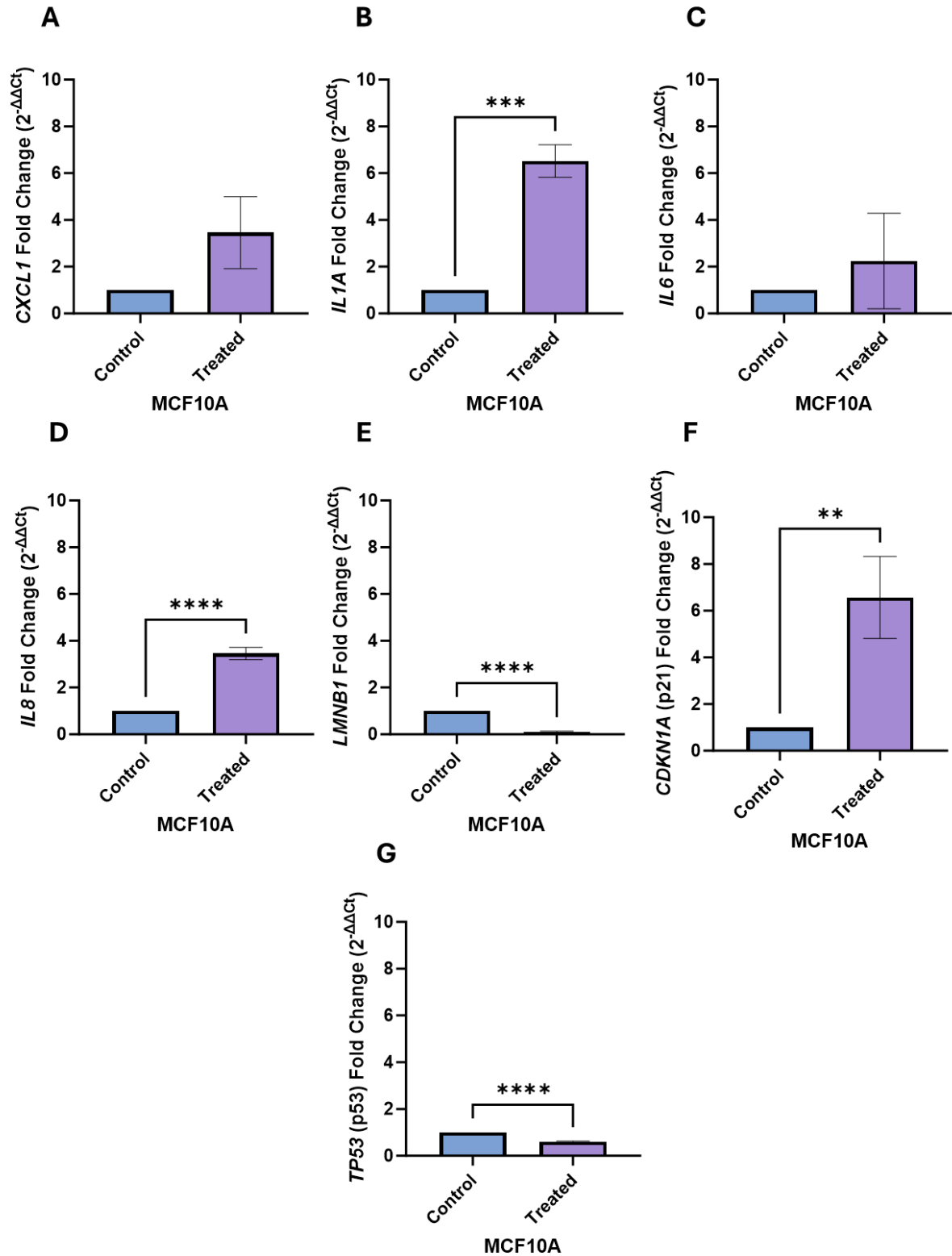


Figure 3.28. qPCR analysis of senescence associated genes in MCF10A following TIS induction. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6*, (D) *IL8*, (E) *LMNB1*, (F) *CDKN1A* and (G) *TP53*. A statistically significant increase in gene expression was observed in *IL1A*, *IL8*, *CDKN1A* and *TP53*. A statistically significant decrease of *LMNB1* expression was also observed. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

3.4.3 Gene expression analysis of candidate genes

qPCR analysis of candidate genes identified in bioinformatic analysis was performed to determine whether differential expression in patient datasets was recapitulated in TIS cell models (Figure 3.29-Figure 3.34). Results are represented as fold change and were calculated using the $2^{-\Delta\Delta Ct}$ method, normalised to two housekeeping genes, *GAPDH* and *PUM1*, previously validated in BC cell lines. For each cell line, TIS cells were normalised to their respective untreated controls. Untreated controls were normalised to a baseline fold change of 1. A statistically significant decrease in expression was observed for *CDCA5*, *KIFC1*, *LMNB1*, *SMC6* and *TUBG1* in MCF10A TIS. In contrast, TNBC TIS cell lines generally had increased expression of candidate genes (*CDCA5*, *KIFC1*, *SMC6* and *STIM1*), with the exception of *LMNB1* which was decreased in MDA-MB-231 and *TUBG1* which was fractionally decreased in HCC1806. These changes reached statistical significance for *STIM1* and *SMC6* in HCC1806 TIS and *LMNB1* in MDA-MB-231 TIS. *STIM1* expression was increased in both TNBC and MCF10A TIS models, although this increase was only statistically significant in HCC1806.

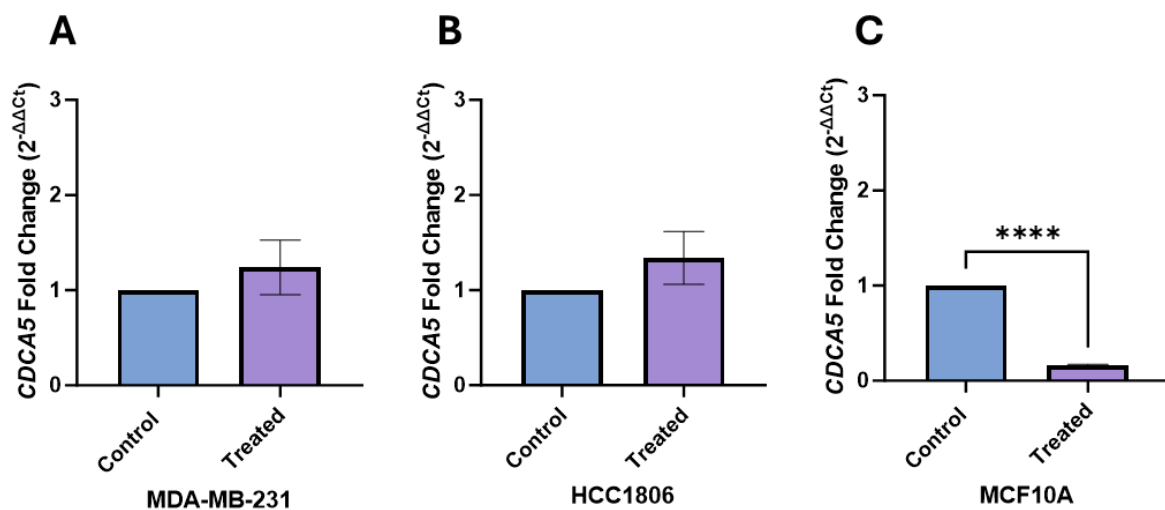


Figure 3.29. qPCR analysis of *CDCA5* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant decrease in gene expression was observed in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

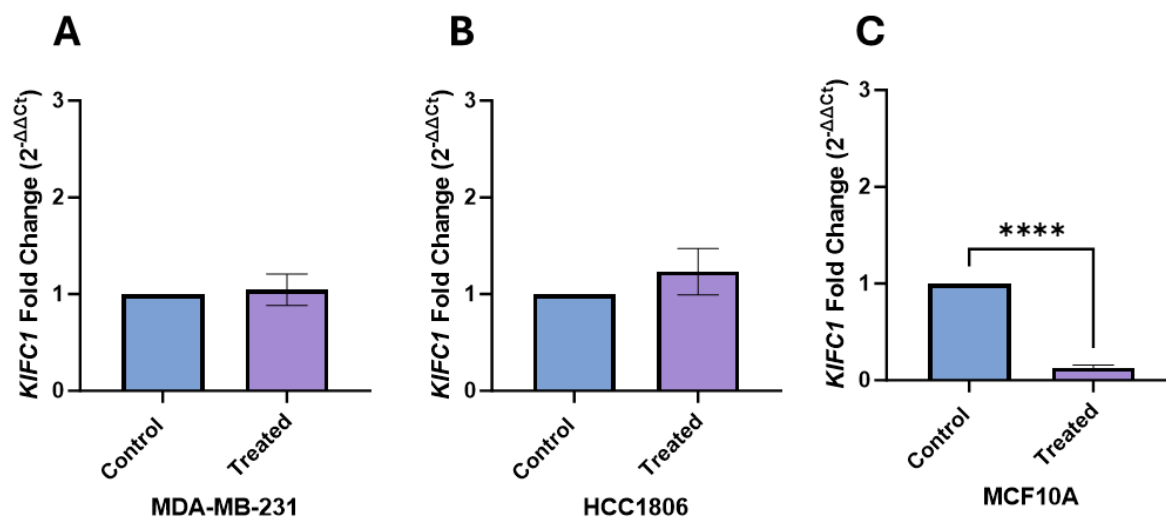


Figure 3.30. qPCR analysis of *KIFC1* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant decrease in gene expression was observed in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

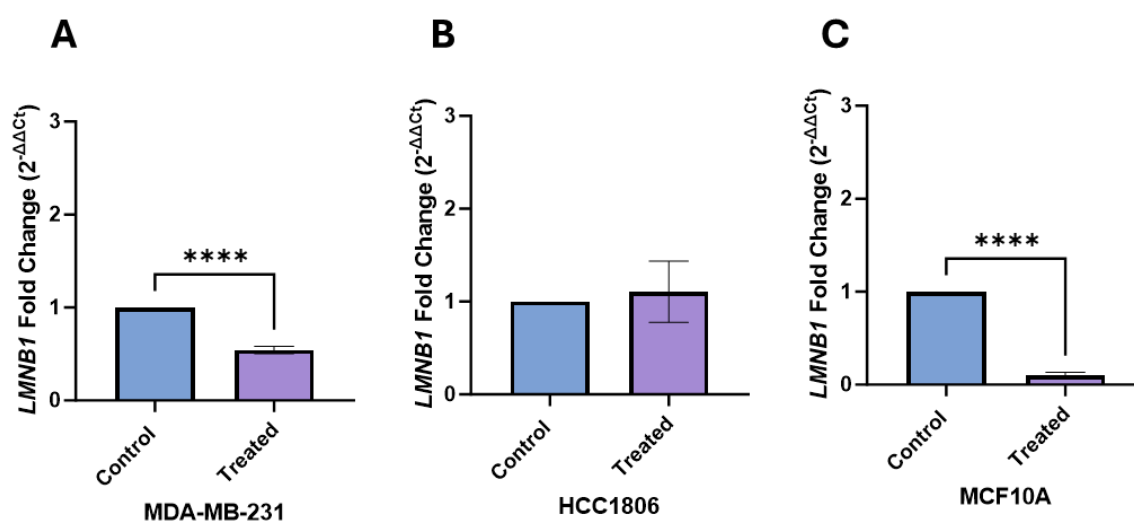


Figure 3.31. qPCR analysis of *LMNB1* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant decrease in gene expression was observed in MDA-MB-231 and MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

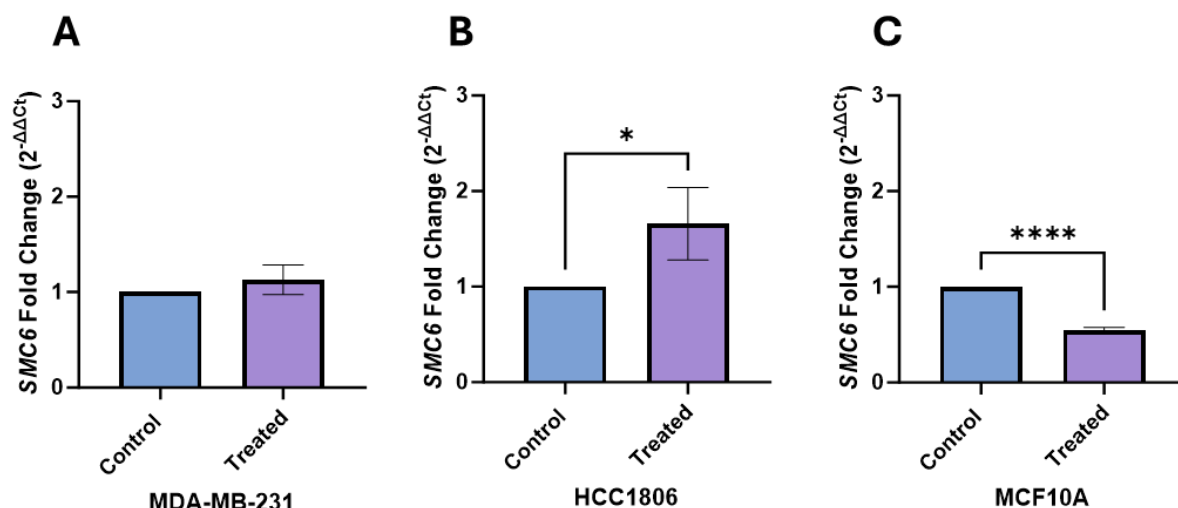


Figure 3.32. qPCR analysis of *SMC6* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant increase in gene expression was observed in HCC1806, and a statistically significant decrease was observed in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $Ns = P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

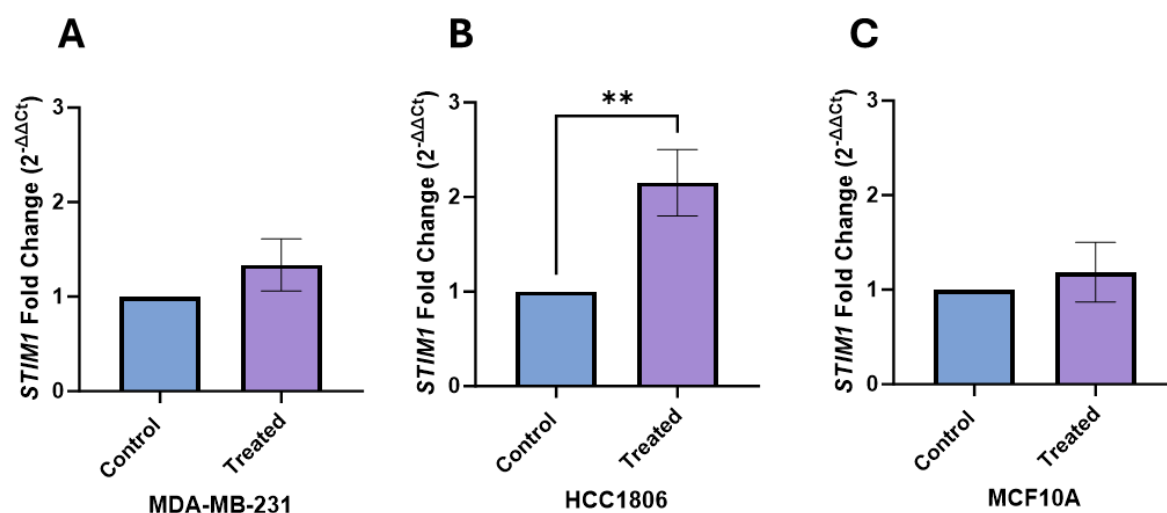


Figure 3.33. qPCR analysis of *STIM1* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant increase in gene expression was observed in HCC1806. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $Ns = P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

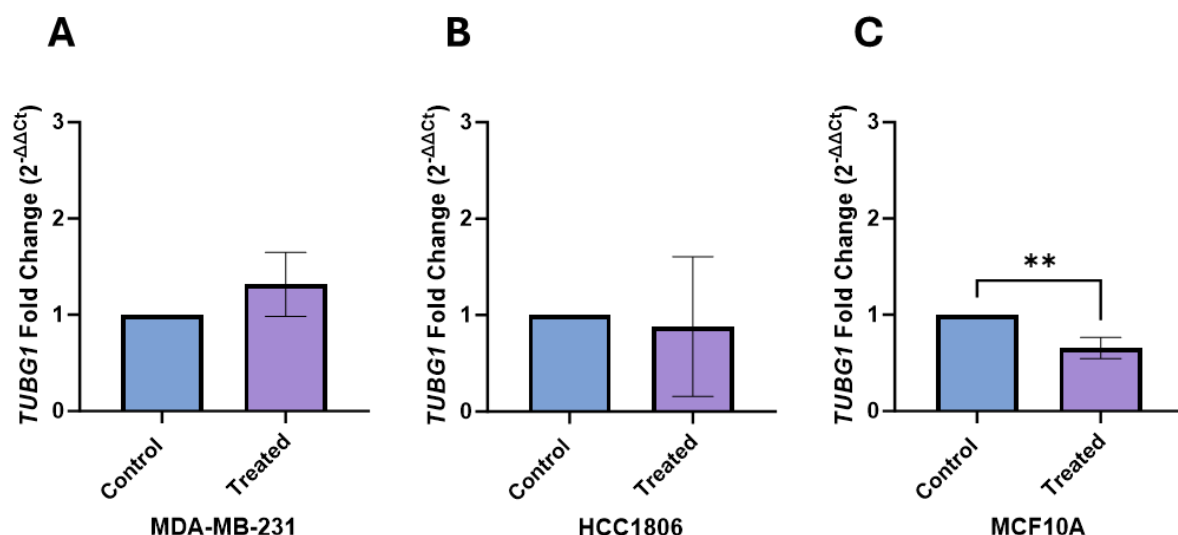


Figure 3.34. qPCR analysis of *TUBG1* in TIS models. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control samples were untreated, normalised to a baseline fold change of 1. Treated samples, were treated with TIS inducing doxorubicin concentrations. Data is shown for (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A. A statistically significant decrease in gene expression was observed in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

3.5 DISCUSSION

The primary aims of this chapter were to identify potential biomarkers of TIS in TNBC, using bioinformatics analysis and test for their presence in TIS TNBC cell models. As part of this, robust TIS models needed to be developed in the cell lines used. The development and validation of this model are discussed initially, followed by the identification and evaluation of candidate biomarkers.

3.5.1 Generating TIS models

There are many hallmarks of senescence, which can vary between cells. Therefore, no single assay is sufficient to define senescence, and a combination of methods is required (Faragher, 2021; Hernandez-Segura, Nehme and Demaria, 2018). It is generally recommended to identify a number of traits including morphological changes and the presence of DNA damage and SASP factors (González-Gualda et al., 2021). In this study, two TNBC cell lines (MDA-MB-231 and HCC1806) were used alongside a non-tumorigenic epithelial cell line (MCF10A) to determine whether observed responses were TNBC specific or a more general feature of TIS. Although previous studies have modelled TIS in MDA-MB-231, few, if any evaluate TIS in HCC1806 and MCF10A. Therefore, TIS was characterised in

each cell line. Results indicated that TIS inducing drug concentrations were appropriate and that TIS was induced in each cell line. Although an increase in lysosomal content, DNA damage, nuclear area, and SASP genes was observed in each cell line, it is important to consider that a marker of cell cycle arrest was not assessed in this chapter. To address this, BrdU staining was incorporated in subsequent experiments, using the same models, and confirmed the absence of proliferation (4.4.2.2).

3.5.1.1 Doxorubicin concentration optimisation

To identify suitable doxorubicin treatments for further study, a range of concentrations were tested in each cell line, to account for their differing drug sensitivities. MDA-MB-231 tolerated higher doxorubicin doses, whereas HCC1806 cells exhibited detachment and cell death at equivalent levels. β -galactosidase staining was used as a preliminary indicator of senescence when optimising treatments. Apoptosis and MTT assays were performed to confirm that senescence inducing doxorubicin concentrations were suitable for subsequent experiments, ensuring that cells remained viable and were entering TIS rather than undergoing cell death. Although not a hallmark of senescence, it was important to assess the fate of TIS induced cells to ensure results were a consequence of TIS rather than cell death. As no significant reduction in viability or increase in apoptosis was observed in any cell line, following treatment, the selected doxorubicin concentrations were deemed suitable (Figure 3.12, Figure 3.16, Figure 3.17 and Figure 3.18). A small proportion of apoptotic and necrotic cells was detected in each line, as expected in healthy cell populations (Abcam, 2025).

3.5.1.2 β -galactosidase staining

β -galactosidase staining is a widely used method for detecting senescence (Hernandez-Segura, Nehme and Demaria, 2018). Senescent cells have increased lysosomal content and upregulate several lysosomal proteins, leading to characteristic blue cytoplasmic staining at pH 5.9-6.1, indicative of TIS (Hernandez-Segura, Nehme and Demaria, 2018; Park et al., 2018). The precise mechanism behind this is not fully understood, although lysosomal accumulation may arise from increased lysosomal biogenesis (synthesis) or lack of lysosomal degradation, and an increase in pH may reflect a decrease in the function of proton pumps responsible for acidifying intracellular compartments (Bagh et al., 2017; Colacurcio and Nixon, 2016; Hernandez-Segura, Nehme and Demaria, 2018; Park et al., 2018). In this study, comparable levels of β -galactosidase staining were observed between cell lines, and a significant increase in staining after doxorubicin treatment was consistent with the lysosomal increases seen in senescent cells (Figure 3.19). However, β -galactosidase is limited as a senescence

detection method as increased levels are also detectable in quiescent cells, driven by high confluency (Cho and Hwang, 2012). It is therefore important to use this stain in conjunction with other senescence detection assays.

3.5.1.3 γ -H2AX staining

Chronic DDR is a characteristic of TIS cells, commonly induced by DNA damaging drugs such as doxorubicin (Hernandez-Segura, Nehme and Demaria, 2018). Although not a direct marker of senescence, DDR is essential for TIS induction, which is induced by chronic DDR pathway activation (Kumari and Jat, 2021). When DSBs occur ATM and ATR phosphorylate H2AX at serine 139, forming γ -H2AX (Tanaka et al., 2009). γ -H2AX (Ser139) forms nuclear foci at sites of DNA damage, facilitating the recruitment of DDR proteins. In cells undergoing TIS, DNA damage is not fully repaired, γ -H2AX foci are persistent, and cells enter stable cell cycle arrest (Sutanto et al., 2025). Sustained DDR signalling further contributes to the maintenance of cell cycle arrest through ATM/ATR activation (Ayrapetov et al., 2014; Lukas et al., 2003).

In this study, an increase in γ -H2AX (Ser139) staining, indicative of increased DDR and TIS, was observed in each cell line, significantly for MDA-MB-231 and MCF10A (Figure 3.24). The largest increase was observed in MDA-MB-231, followed by MCF10A and HCC1806. Basal staining was detected in all samples, likely reflecting endogenous DNA damage. The higher basal staining in TNBC cell lines is likely due to their inherently higher levels of DNA damage and genomic instability. As defects in DDR are a characteristic of TNBC, impaired repair mechanisms may have contributed to this increased DNA damage (Asleh, Riaz and Nielsen, 2022).

As expected for a histone modification, γ -H2AX (Ser139) staining was confined to the nucleus. A combination of punctate and pan γ -H2AX staining was observed, likely reflecting heterogeneity in the DDR among individual cells (Figure 3.21, Figure 3.22 and Figure 3.23). In some cells, large, distinct punctate foci were observed, whereas in others, staining was more diffuse and less defined. Generally, in untreated cells, staining was more diffuse and appeared less bright than in doxorubicin treated cells which appeared brighter. This indicated increased levels of DNA damage were associated with doxorubicin treatment. Punctate γ -H2AX staining is commonly associated with DSBs and therefore indicates the DNA damage associated with TIS (Baure et al., 2009; Marti et al., 2006). In MCF10A, doxorubicin treatment resulted in visibly fewer punctate foci than in MDA-MB-231, which had more

abundant and widespread punctate staining. In HCC1806, foci were more difficult to distinguish, and staining appeared more diffuse. This may to some extent be due to the foci in a 3D cell, being compressed into a 2D image.

Any cell which fluoresced with γ -H2AX (Ser139) staining was interpreted as positively stained. Whilst this approach was beneficial in assessing overall DNA damage, it did not take into consideration more subtle differences in foci number or intensity. Ideally, these nuances would have been quantified using flow cytometry or foci counting; however, equipment limitations and the high density, overlapping nature of γ -H2AX foci prevented this, particularly where staining was diffuse and couldn't be resolved. To address this limitation, subsequent chapters categorised γ -H2AX (Ser139) staining into unstained, weakly positive (diffuse staining or <50% of the nucleus with foci) and strongly positive (numerous discrete foci across >50% of the nucleus), to improve comparative analysis. Regardless, doxorubicin treated cells had consistently higher levels of γ -H2AX (Ser139), indicative of DNA damage and TIS.

3.5.1.4 Nuclear area

An increase in nuclear size (hypertrophy) is a characteristic of senescence and can be quantified as a morphological marker (Beck, Horikawa and Harris, 2020; Herranz and Gil, 2018). Nuclear hypertrophy can arise due to the loss of nuclear integrity caused by the sustained DNA damage from doxorubicin and chronic SASP signalling (Santos et al., 2021). All doxorubicin treated samples showed an increase in nuclear area, indicative of TIS, although this was only statistically significant in MDA-MB-231, which had the greatest increase of ~3.5 fold, compared to HCC1806 and MCF10A, which both had ~1.5 fold increases in nuclear area (Figure 3.25).

Cell type may have influenced the extent of nuclear hypertrophy, in response to doxorubicin treatment, whereby HCC1806 are more basal and MDA-MB-231 are more mesenchymal (Kvokačková et al., 2021). Basal cells have an epithelial origin and are defined by the expression of basal cytokeratins, whereas mesenchymal cells are characterised by a loss of epithelial adhesion and increased motility (Lehmann et al., 2011). MCF10A and HCC1806 typically formed compact colonies which may have physically limited the extent of their nuclear hypertrophy. In contrast, MDA-MB-231 has a more mesenchymal phenotype, characterised by reduced cell adhesion and increased cytoskeletal plasticity. This likely facilitates nuclear remodelling on a larger scale and contributed to the larger nuclear size observed in these cells, compared with MCF10A or HCC1806 (Kvokačková et al.,

2021). Regardless of the extent of nuclear hypertrophy, ultimately an increase in nuclear area was observed in all cell lines, indicating TIS induction.

3.5.1.5 Gene expression analysis of senescence associated genes

The secretory profiles of senescent cells vary dramatically and are often shared with other cell types, such as endothelial or cancer cells, however, some SASP markers, such as IL6 and IL8 are highly conserved (Hernandez-Segura, Nehme and Demaria, 2018). Gene expression analysis was performed using qPCR to assess key TIS markers, including components of the SASP (*CXCL1*, *IL1A*, *IL6* and *IL8*), regulators of senescence induction pathways (*CDKN1A* and *TP53*), and nuclear lamina components (*LMNB1*).

Given the heterogeneity of the SASP, the gene expression of several components was assessed (Figure 3.26-Figure 3.28). In all three cell lines all SASP components were elevated following doxorubicin treatment, indicating activation of inflammatory pathways, consistent with TIS (Hernandez-Segura, Nehme and Demaria, 2018). This increase was significant for *CXCL1* and *IL1A* in MDA-MB-231 and additionally for *IL6* in HCC1806. In MCF10A, significant increases were observed for *IL1A* and *IL8*. The variability observed between cell lines is unsurprising, as SASP composition is known to differ depending on cellular context, stress response, and cell type (Hernandez-Segura, Nehme and Demaria, 2018). Despite these differences, the overall upregulation of multiple SASP components in each cell line, indicates cells are senescent. Of the SASP components analysed *IL1A*, a potent cytokine, had the greatest fold increase in all cell lines. Interestingly, *IL1A* is an upstream SASP regulator which reinforces senescence both autonomously and in neighbouring cells (Chou, Ho and Hung, 2022). It activates NF- κ B, sustaining transcription of downstream SASP components (Chou, Ho and Hung, 2022). As IL6 and IL8 expression is often dependent on IL1A signalling, their upregulation is consistent with its expression (Davalos et al., 2010; Orjalo et al., 2009). Overall, a more pronounced SASP response was observed in the TNBC cell lines compared to MCF10A, with HCC1806 displaying higher levels of SASP components than MDA-MB-231. The comparatively lower levels of DNA damage observed in MCF10A may limit activation of NF- κ B signalling resulting in a weaker SASP response (Chien et al., 2011). Additionally, *TP53* (p53) mutation in TNBC may result in exaggerated NF- κ B activation and an increased SASP response (Cooks et al., 2013; Lee et al., 2025). Furthermore, as SASP contributes to a microenvironment that favours tumour progression, its components may be maintained to support processes such as angiogenesis, invasion, and metastasis in TNBC cells (Luo et al., 2025). Collectively,

these results show doxorubicin treated cells express higher levels of SASP components than their untreated controls, indicating TIS has been successfully induced.

The p53/p21^{WAF/CIP1} pathway is a key mediator of senescence induction, functioning alongside the p16^{INK4A}/Rb pathway to induce cell cycle arrest (Mijit et al., 2020). In all three cell lines, *CDKN1A* expression was significantly increased in doxorubicin treated samples, consistent with TIS pathway activation (Figure 3.26-Figure 3.28) (Chehab et al., 1999). The extent of *CDKN1A* expression was greatest in MCF10A, followed by HCC1806 and MDA-MB-231. This may reflect differences in p53 function, as MCF10A retains wild type p53, which may more efficiently activate p21, than in TNBC cell lines harbouring p53 mutations. *TP53* expression was modestly increased in TIS TNBC cell lines, indicating activation of the p53/p21^{WAF/CIP1} pathway, whilst in MCF10A it was significantly downregulated (Figure 3.26-Figure 3.28). As MCF10A displayed several other hallmarks of senescence, it is possible that *TP53* downregulation was an indicator of late stage senescence (Johmura and Nakanishi, 2016). Differences in senescence activation and maintenance pathways are also likely, given *CDKN2A* (p16) is deleted in both MDA-MB-231 and MCF10A. Although increases in *CDKN2A* and *TP53* expression suggest activation of the p53/p21^{WAF/CIP1} pathway, changes in gene expression do not always correspond to protein abundance or function. Therefore, it may be valuable in future studies to determine whether these transcriptional changes are reflected at a protein level.

LMNB1 is a key structural component of the nuclear lamina, and its downregulation in TIS is associated with weakening and structural deformation of the nuclear envelope (Martins et al., 2020). *LMNB1* expression was significantly reduced in MCF10A and MDA-MB-231 cells, consistent with senescence associated nuclear envelope weakening (Figure 3.26 and Figure 3.28). Given the presence of several TIS markers in HCC1806, the maintenance of *LMNB1* expression likely reflects a differential senescence response rather than an absence of senescence (Figure 3.28).

3.5.2 Analysis of candidate biomarkers

The aim of this research was to identify a biomarker with predictive or prognostic significance, associated with TIS. Ultimately, these biomarkers could be used to identify patients who may benefit from senotherapeutic interventions. A bioinformatics approach was taken, prior to validation in TNBC TIS models, to enable large scale screening of candidates. Similar bioinformatics approaches have proven successful, when analysing genes of interest and their impact on cancer development,

progression, and response to treatment (Houghton-Gisby et al., 2022; Hu et al., 2022c; Phan et al., 2018; Qin et al., 2022). Initially, biomarkers were assessed using bioinformatics tools, to determine whether their expression correlated with treatment response, patient survival and cancer stage, or subtype. To evaluate their association with TIS, the expression of candidates were examined in TIS models. Given that transcriptional changes often precede protein alterations, qPCR was used as a sensitive method to examine the gene expression of candidates. Gene expression profiling quantifies molecular signatures associated with senescence (Hernandez-Segura, Nehme and Demaria, 2018). These changes could potentially be captured using transcriptomic analysis of tumour biopsies or surgical resections, which are routinely used to stage and grade tumours.

The cytoskeleton is an attractive therapeutic target due to its role in maintaining senescence (Ghosh et al., 2020; Molina et al., 2025; Moujaber et al., 2019; Mu et al., 2020). However, whilst drugs targeting the cytoskeleton, such as microtubule inhibitors, are used clinically, the highly conserved nature of cytoskeletal components limits the likelihood of selectively targeting TIS cells, without inducing widespread toxicity (Huang, Mei and Han, 2022; Jordan and Wilson, 2004). It is therefore more probable that TIS specific, cytoskeletal biomarkers would be used to identify patients who could benefit from senotherapies, rather than acting as direct therapeutic targets themselves. Below, potential biomarkers of TIS and their relevance to TIS TNBC models are discussed.

3.5.2.1 CDCA5

CDCA5 is associated with microtubule dynamics, through its predicted roles in chromatid separation and mitotic spindle organisation (Uniprot, 2022). Although not a cytoskeletal component, CDCA5 stabilises chromatin cohesion (joining of sister chromatids by cohesin to ensure organised chromosome segregation), enabling kinetochores to establish and maintain attachment to spindles (formed of microtubules) (Schmitz et al., 2007). Loss of *CDCA5* results in premature cohesin release, defective microtubule kinetochore coupling and aberrant chromatid segregation (Rankin, Ayad and Kirschner, 2005; Schmitz et al., 2007).

Results from bioinformatics analysis showed, *CDCA5* was downregulated in both patients who did not respond to treatment, and those who had poorer survival (Table 3.2, Table 3.3 and Table 3.6). It was decreased significantly in both CPR and RFS cohorts, indicating *CDCA5* expression could serve as a predictive biomarker of therapeutic response and likelihood of relapse (Table 3.2 and Table 3.3).

Although, patients with low expression had a lower OS rate of ~7 months, in comparison to the high expression cohort, this was not statistically significant, therefore *CDCA5* is unlikely to be a reliable predictor of patient survival (Table 3.6 and Figure 3.5). In contrast, literature suggests that increased *CDCA5* expression in breast and colorectal cancer is associated with poor prognosis and survival respectively, where *CDCA5* knockdown suppresses BC progression in cell models (Shen et al., 2019; Wang et al., 2022; Xiong et al., 2024). One potential explanation for this is that *CDCA5* downregulation may promote senescence and suppress proliferation. Consistent with this, previous studies have reported that *CDCA5* is downregulated in senescent cells and promotes inflammatory signalling (Collin et al., 2018; Orfanidis et al., 2017). The downregulation of *CDCA5* during senescence may be a consequence of permanent cell cycle exit which negates the need for chromatid cohesion and spindle organisation.

CDCA5 is overexpressed in several cancers, however expression is particularly high in BC compared to healthy tissue (Hu et al., 2022c; Nguyen et al., 2010; Phan et al., 2018). GEPIA analysis supported these findings, suggesting *CDCA5* may have utility in differentiating between cancerous and healthy samples (Table 3.8). Furthermore, as *CDCA5* expression statistically differed in all BRCA subtype comparisons, it may also be useful for distinguishing between BC subtypes (Figure 3.6). Although, *CDCA5* expression increased proportionately to BC stage, this was not statistically significant, therefore *CDCA5* expression is unlikely useful for staging tumours.

In cell models, *CDCA5* was slightly upregulated in TNBC cells and significantly downregulated in TIS MCF10A, suggesting that *CDCA5* regulation in TIS is context dependent (Figure 3.29). In non-tumorigenic cells, it is possible a decrease in *CDCA5* is indicative of TIS. However, it is unlikely to serve as a reliable biomarker of TIS in TNBC, as a significant change in expression was not observed between untreated and TIS samples (Figure 3.29). In TNBC it is possible that the observed *CDCA5* upregulation may reflect higher basal expression levels, which were unchanged by TIS. Supporting this, elevated *CDCA5* expression has been associated with increased aggression of cancers (Shen et al., 2019; Wang et al., 2022; Xiong et al., 2024). As *CDCA5* is differentially expressed in healthy and cancerous cells, it is unlikely to be a conserved feature of TIS. Instead, it may be that differences in *CDCA5* expression, between healthy and cancerous cells, provide an opportunity to distinguish between TIS in normal and malignant tissue. However, as *CDCA5* expression in TNBC cells was not significantly altered, it is unlikely *CDCA5* is a robust biomarker of TIS in TNBC. Therefore, it was not further pursued as a candidate biomarker in this study.

3.5.2.2 KIFC1

KIFC1 is a C-terminal kinesin motor, which uses ATP to move towards the minus end of microtubules and facilitates protein transport, centrosome clustering and mitosis (Khwaja et al., 2021; Teng et al., 2019). Whilst KIFC1 is not a direct cytoskeletal component, it functions in both healthy and cancerous cells to facilitate the function of spindles, comprised of microtubules, a key cytoskeletal component. KIFC1 seems more essential for proper spindle formation and survival in cancer where it maintains spindle architecture by converting multipolar spindles into pseudo-bipolar spindles, enabling cancer cells to survive mitotic aberrations (Li et al., 2015; Tiwari et al., 2025). Unlike healthy cells, with bipolar spindles, cancer cells can have extra centrosomes and create multipolar spindles. KIFC1 clusters centrosomes together, to form two clustered spindles, which prevents chromosome mis-segregation and apoptosis (Tiwari et al., 2025).

An increase in *KIFC1* has been associated with tumour initiation, progression, metastasis, recurrence, and reduced survival (Fu et al., 2018; Li et al., 2015; Lu et al., 2024; Mittal et al., 2016). This is consistent with GEPIA and UALACAN analysis, showing *KIFC1* transcripts were increased ~6 fold in BRCA, compared to healthy tissue and increased in all BRCA stages compared to normal tissue (Table 3.8 and Figure 3.7). Together these findings suggest *KIFC1* expression may have utility in differentiating between cancerous and healthy samples. Furthermore, as *KIFC1* expression statistically differed in all BRCA subtype comparisons, it may also be useful for distinguishing between BC subtypes (Figure 3.7). However, because its expression remains consistent across BRCA stages, it is unlikely to be useful for predicting cancer stage (Figure 3.7).

Bioinformatics analysis revealed *KIFC1* expression was decreased in both patients who did not respond to treatment, and those who had poorer survival (Table 3.2, Table 3.3 and Table 3.6). It was more strongly downregulated in the CPR cohort than in the RFS cohort, indicating it may be a better indicator of response to therapy rather than likelihood of relapse (Table 3.2 and Table 3.3). Survival analysis revealed that patients with low *KIFC1* expression had a shorter OS of ~9 months, in comparison to the high expression cohort (Table 3.6). However, as this was not statistically significant *KIFC1* is unlikely to be a reliable predictor of patient survival (Figure 3.5). In contrast, high *KIFC1* expression is associated with poor survival of sarcoma patients (Lu et al., 2024). Its knockdown increased senescence and reduced tumour malignancy (Lu et al., 2024). However, bioinformatics

analysis of BRCA showed that low *KIFC1* expression was associated with poor treatment response and survival (Table 3.2, Table 3.3 and Table 3.6). This discrepancy may reflect differences between cancer types or the context dependent effects of senescence. In sarcomas, a lack of *KIFC1*, may beneficially contribute to patient survival by limiting the survival of cancer cells with multipolar spindles, but not in BRCA. Alternatively, in BRCA, low *KIFC1* could induce senescence and potentially increase SASP secretion, impairing treatment response.

In cell models, *KIFC1* was slightly upregulated in TNBC cells and significantly downregulated in TIS MCF10A, suggesting that its regulation in TIS is context dependent (Figure 3.30). In non-tumorigenic cells, it is possible a decrease in *KIFC1* is indicative of TIS, consistent with literature linking its downregulation to senescence (Kim & Song, 2013; Lu et al., 2024). This downregulation may reflect cell cycle exit, where centrosome clustering is no longer required to facilitate mitosis. However, *KIFC1* is unlikely to serve as a reliable biomarker of TIS in TNBC, as a significant change in expression was not observed between untreated and TIS samples. The modest upregulation of *KIFC1* in TIS TNBC cells may reflect higher basal *KIFC1* levels, which are likely required for pseudo-bipolar spindle formation and survival of cancerous cells (Tiwari et al., 2025) (Table 3.8 and Figure 3.30). Supporting this, increased *KIFC1* expression has been associated with increased tumour progression and metastasis (Fu et al., 2018; Li et al., 2015; Lu et al., 2024; Mittal et al., 2016). Similarly to *CDCA5*, *KIFC1* was differentially expressed in healthy and cancerous cells, and is therefore unlikely to be a conserved feature of TIS. Differences in *KIFC1* expression, may aid in differentiating between TIS in normal and malignant tissue. However, as *KIFC1* expression in TNBC cells was not significantly altered, it is unlikely *KIFC1* is a robust biomarker of TIS in TNBC. Therefore, it was not further pursued as a candidate biomarker in this study.

3.5.2.3 LMNB1

LMNB1 encodes for one of the two B-type Lamin proteins, forming the nuclear lamina. It is an intermediate filament which acts as a scaffold, providing structural integrity to the nucleus (Lv et al., 2024; Meng et al., 2025). Consistent with literature reporting elevated *LMNB1* expression in BC, GEPIA and UALCAN analysis showed that *LMNB1* transcripts were nearly ~7 fold higher in BRCA compared with healthy tissue and increased in all BRCA stages compared to normal tissue (Table 3.8 and Figure 3.8) (Lv et al., 2024; Meng et al., 2025). Together these findings suggest *LMNB1* expression may have utility in differentiating between cancerous and healthy tissue. *LMNB1* expression statistically differed in most BRCA subtype comparisons and therefore may be useful for distinguishing between TNBC and

other BC subtypes (Figure 3.8). However, *LMNB1* expression showed very little association with BC severity, therefore is unlikely to be useful for predicting cancer stage (Figure 3.8).

Bioinformatics analysis revealed *LMNB1* expression was decreased in patients who did not respond to treatment. It was more strongly downregulated in the CPR cohort than in the RFS cohort, indicating it may be a better indicator of response to therapy rather than likelihood of relapse (Table 3.2 and Table 3.3). Interestingly loss of *LMNB1* is considered a hallmark of senescence where it is a key regulator of nuclear size, shape, stability (Freund et al., 2012; Martins et al., 2020; Meng et al., 2025). Therefore, decreased *LMNB1* expression in non-responding patients may reflect increased levels of senescence. Consistent with this, in BC tumours which showed little or no response to chemotherapy 40% showed markers of TIS, including decreased *LMNB1* expression, potentially indicating that TIS contributes to poor therapeutic response (Saleh et al., 2021).

KM Plotter mRNA analysis also indicated that low *LMNB1* expression was associated with a ~2 month shorter OS compared with the high expression cohort, suggesting that reduced *LMNB1* expression correlates with poorer outcomes (Table 3.6). In contrast, KM Plotter protein analysis showed that patients with low *LMNB1* expression had an ~18 months longer OS than those with high expression (Table 3.5). These discrepancies may reflect impaired mRNA translation, increased protein degradation, or, alternatively, differences in sample size, as the KM Plotter protein database was still under development. However, neither database showed a significant association between *LMNB1* mRNA or protein expression and OS, therefore it is unlikely to be a reliable prognostic biomarker.

Cell models showed that TIS significantly reduced *LMNB1* expression in both TNBC and non-tumorigenic breast cell lines, consistent with literature suggesting its loss is a hallmark of senescence (Figure 3.31) (Freund et al., 2012; Martins et al., 2020; Meng et al., 2025). This reduction was more pronounced in MCF10A (~90% reduction in expression compared to untreated control) compared to MDA-MB-231 cells (~50% reduction in expression compared to untreated control) (Figure 3.31). Given bioinformatics data suggested that reduced *LMNB1* expression correlated with worse response to treatment, it is possible that this may be attributed to the accumulation of senescent cells (Table 3.2). Supporting this, one study reported that 40% of BC tumours that showed little or no response to chemotherapy exhibited markers of TIS, including reduced *LMNB1* expression (Saleh et al., 2021). A decrease in *LMNB1* expression, in breast tumours, following chemotherapy treatment, compared with

untreated breast tissue has also been observed, which is consistent with the induction of TIS (Saleh et al., 2022). Given that senescent cells can promote tumour progression and treatment resistance via SASP signalling, identifying them *in vivo*, using LMNB1 could help identify patients likely to benefit from anti-senescence therapies. However, it is essential to first establish whether the gene expression changes observed in this study are functionally translated. *LMNB1* expression was modestly, but not significantly, increased in HCC1806 TIS cells, (~10% increase in expression compared to untreated control) (Figure 3.31). This may reflect cell line specific differences in TIS regulation, which is highly heterogeneous (Hernandez-Segura, Nehme and Demaria, 2018). As *LMNB1* expression was reduced in 2 out of 3 TIS models, including one TNBC and one non-tumorigenic cell line, this suggests that *LMNB1* downregulation may be a conserved feature of TIS. Based on these findings, *LMNB1* was included as a hallmark of TIS in TNBC in subsequent analysis, to clarify its role in TIS and how its effects may be modulated by anti-senescence drugs.

3.5.2.4 SMC6

SMC6 primarily functions as part of the SMC5-SMC6 complex, to maintain genomic stability. The SMC5-SMC6 complex contributes to genome maintenance by facilitating DNA repair and enabling chromosome segregation during mitosis (Diaz and Pecinka, 2018; Gallego-Paez et al., 2014; Mangone et al., 2018). Although SMC6 is not a cytoskeletal protein it is indirectly linked to the cytoskeleton by its role in mitosis, a process dependent on microtubule dynamics. The SMC5-SMC6 complex acts to stabilise microtubules, promote spindle formation and maintain chromosome integrity during mitosis (Bermudez-Lopez et al., 2010; Laflamme et al., 2014; Torres-Rosell et al., 2005). Defects in the SMC5-SMC6 complex have been shown to impair spindle integrity, when microtubules are stressed (Laflamme et al., 2014).

Bioinformatics analysis showed *SMC6* expression was reduced in patients who did not respond to treatment. It was more strongly downregulated in the CPR cohort than in the RFS cohort, indicating it may be a better indicator of response to therapy rather than likelihood of relapse (Table 3.2 and Table 3.3). Survival analysis revealed that patients with low *SMC6* expression had a fractionally shorter OS of ~0.6 months, in comparison to the high expression cohort (Table 3.6). As this change was not statistically significant it is unlikely that *KIFC1* expression is a reliable predictor of prognosis (Figure 3.5). However, this result is consistent with reports indicating that increased *SMC6* expression, in BC, is associated with improved patient survival (Mangone et al., 2018; Mangone et al., 2025). As SMC mutation and dysregulation promotes genomic instability, it is likely that it contributes to a decreased

OS in BRCA patients (Ma et al., 2019; Mangone et al., 2025; Rossi et al., 2020; Roy et al., 2023; Tran et al., 2025; Zhou et al., 2021). Interestingly, the aberrant function of the SMC5-SMC6 complex, leads to the accumulation of unresolved DNA damage, activating p53 and p21 signalling pathways, capable of inducing senescence (Chavez et al., 2010; Noël and Wellinger, 2011; Venegas et al., 2020). It is therefore possible that decreased *SMC6* expression contributes to poor therapeutic response by promoting senescence induction and its associated consequences.

Like other members of the SMC family, GEPIA analysis indicated that *SMC6* expression was elevated in BRCA compared with normal tissue, although this increase was not statistically significant (Table 3.8) (Ma et al., 2019; Rossi et al., 2020; Roy et al., 2023; Tran et al., 2025; Zhou et al., 2021). UALCAN analysis suggested that *SMC6* expression may distinguish normal tissue from early stage BRCA (Figure 3.9). However, when GEPIA and UALCAN results are considered together, *SMC6* is unlikely to reliably differentiate between cancerous and healthy tissue, particularly at later stages. *SMC6* gene expression was consistent between BC subtypes, suggesting it is unlikely a reliable indicator of BC subtype (Figure 3.9).

Similar to other candidate genes assessed in this study, *SMC6* showed inconsistent expression in TNBC and non-tumorigenic TIS models (Figure 3.32). It was slightly upregulated in TNBC cells and significantly downregulated in TIS MCF10A, suggesting that its regulation in TIS is context dependent (Figure 3.32). Given that *SMC6* is essential for proper mitosis its downregulation in non-tumorigenic MCF10A TIS cells may reflect the induction of cell cycle arrest. In contrast *SMC6* expression increased in both TIS TNBC cell lines, significantly for HCC1806 TIS (Figure 3.32). As both MDA-MB-231 and HCC18806 have *TP53* mutations, *SMC6* upregulation may be an adaptive mechanism to tolerate the high levels of basal DNA damage associated with increased genomic instability (Mangone et al., 2018; Mangone et al., 2025). Supporting this, GEPIA analysis showed *SMC6* expression was higher in BRCA than normal tissue (Table 3.8). It is possible that this is a response exacerbated by increased DNA damage in TIS. Furthermore, a reduction of *SMC6* expression may result in an increase in unresolved DNA damage, promoting senescence through p53 and p21 signalling pathways in cells with functional *TP53*, like MCF10A (Venegas et al., 2020). However, this mechanism may be impaired in *TP53* mutant cells like HCC1806 or MDA-MB-231 (Venegas et al., 2020). Although *SMC6* expression was significantly altered in 2 TIS models, the expression pattern was not consistent (Figure 3.32). As the change in expression was not significant in both TIS TNBC cell lines, *SMC6* was not pursued further in this study.

3.5.2.5 STIM1

STIM1 encodes for a transmembrane protein, localised in the endoplasmic reticulum (ER), a protein folding compartment and calcium store. *STIM1* senses calcium levels and mediates store operated calcium entry (calcium influx) to maintain its homeostasis (Sanchez-Collado et al., 2022). Through regulation of calcium signalling, *STIM1* influences several processes including ER stress and inflammation (Gross et al., 2023; Pluquet et al., 2015).

Results from bioinformatics analysis showed, *STIM1* was upregulated in both patients who did not respond to treatment, and those who had poorer survival. It was increased significantly in both CPR and RFS cohorts, indicating *STIM1* expression could serve as a predictive biomarker of both therapeutic response and likelihood of relapse (Table 3.2 and Table 3.3). KM Plotter mRNA analysis indicated that high *STIM1* expression was associated with a marked ~86 month shorter OS compared with the low expression cohort (Table 3.6). The association between increased *STIM1* expression and adverse clinical outcomes aligns with previous reports linking *STIM1* with cancer aggression, tumour development and metastasis (Karacicek, Erac and Tosun, 2019; Kulkarni et al., 2019; Liu et al., 2015; Xu et al., 2015; Xu et al., 2016; Yang et al., 2009). In contrast, KM Plotter protein analysis showed that patients with high *STIM1* expression had a ~22 months longer OS than those with low expression (Table 3.5). These discrepancies may reflect impaired mRNA translation, increased protein degradation, or, alternatively, differences in sample size (mRNA cohort n=7462 > protein cohort n=126), as the KM Plotter protein database was still under development. However, neither database showed a significant association between *STIM1* mRNA or protein expression and OS, therefore it is unlikely to be a reliable prognostic biomarker.

GEPIA analysis showed that *STIM1* expression was lower in BRCA compared to healthy breast tissue by ~13 TPM, however this difference was not statistically significant, suggesting that *STIM1* expression is unlikely to serve as a diagnostic biomarker (Table 3.8). Consistent with this, UALCAN analysis showed that *STIM1* downregulation was similar across both cancer stages and BC subtypes, indicating that its expression is unlikely to serve as a reliable indicator of either parameter (Figure 3.10).

In TIS models, *STIM1* expression was increased in all cell lines, significantly for HCC1806 and to a lesser extent in MDA-MB-231 and MCF10A (Figure 3.33). Given that GEPIA analyses showed reduced *STIM1* expression in BRCA patients, this increase may be a specific response to TIS rather than a general

feature of BC (Table 3.8). Mechanistically, an increase in *STIM1* expression may be a compensatory response to, perturbed calcium homeostasis, ER stress, or genomic instability in TIS cells. It is possible that TIS models upregulate *STIM1* to enhance calcium influx, supporting NF- κ B mediated SASP transcription and pathways that promote proliferation, migration, and metastasis (Gross et al., 2023; Sanchez-Collado et al., 2022). Loss of function *TP53* mutations have been associated with reduced intracellular calcium levels (Rabab et al., 2025). Therefore, upregulation of *STIM1* may be a compensatory mechanism to increase calcium influx in MDA-MB-231 and HCC1806 which harbour *TP53* mutations. Furthermore, as calcium levels are depleted by ER stress, TIS cells may increase *STIM1* expression to restore calcium homeostasis and support the ER dependent production of SASP factors (Pluquet et al., 2015). *STIM1* also functions independently of calcium signalling. In response to DNA damage, it translocates to the nucleus and supports DNA repair (Sanchez-Lopez et al., 2024). Its loss has been shown to increase DNA damage and promote senescence, therefore, increased *STIM1* in TIS models may be a compensatory response to resolve DNA damage (Sanchez-Lopez et al., 2024; Wang et al., 2024b). Literature also reports that *STIM1* is associated with the formation of focal adhesions, which connect the ECM and cytoskeleton (Gammons et al., 2021; Schäfer et al., 2012). Therefore, it is possible increased *STIM1* expression reflects enlargement of focal adhesions, typical of senescent cells (Chantachotikul et al., 2025). Although *STIM1* expression was increased in TIS models, the change in expression was only significant in one cell line therefore it was not pursued further in this study.

3.5.2.6 TUBG1

TUBG1 encodes for γ -tubulin. It is a member of the tubulin superfamily and plays an important role in microtubule nucleation and cell cycle progression by anchoring microtubules to the gamma-tubulin ring complex (γ -TuRC) at microtubule organising centres (Dráber and Dráberová, 2021). However, γ -tubulin and its complexes also have essential functions outside of mitosis. During interphase, γ -Tubulin complexes can localise outside of centrosomes, at other sites including the Golgi apparatus (which also acts as an MTOC) and the cytoplasm (Gavilan et al., 2018). Microtubule polarity establishes cellular polarity as microtubule minus ends typically anchor to the MTOC and plus ends extend towards the cell periphery (Fourriere et al., 2020). This polarity enables directional intracellular transport along microtubules which serve as tracks. For example, the transport of secretory vesicles from the Golgi to the plasma membrane (Fourriere et al., 2020). Microtubules also physically interact with organelles, including mitochondria and the Golgi, where they generate tension and guide their positioning, maintaining spatial organisation of the cell (Goodson and Jonasson, 2018).

Consistent with literature reporting increased *TUBG1* expression in several cancers, GEPIA and UALCAN analysis showed that *TUBG1* transcripts were ~2 fold higher in BRCA compared with healthy tissue and increased in all BRCA stages and subtypes, compared to normal tissue (Table 3.8 and Figure 3.11) (Alvarado-Kristensson, 2018; Blanco et al., 2015; Chng et al., 2008; Dráber and Dráberová, 2021; Nami and Wang, 2018). Together these findings suggest *TUBG1* expression may have utility in differentiating between cancerous and healthy tissue. However, *TUBG1* expression showed little association with BC severity or BC subtype, therefore its expression is unlikely to serve as a reliable indicator of either parameter (Figure 3.11).

Bioinformatics analysis revealed *TUBG1* expression was decreased in both patients who did not respond to treatment, and those who had poorer survival. It was more strongly downregulated in the CPR cohort than in the RFS cohort, indicating it may be a better indicator of response to therapy rather than likelihood of relapse (Table 3.2 and Table 3.3). This corresponds with KM plotter results showing decreased expression *TUBG1* expression resulted in a ~2 month lower survival than the high expression cohort (Table 3.6). However, as this was not statistically significant *TUBG1* is unlikely to be a reliable predictor of patient survival (Figure 3.5). Interestingly, loss of γ -tubulin has been associated with microtubule alterations in senescent cells and itself can induce senescence, therefore a decrease in *TUBG1* expression may reflect an increase in senescence (Manning and Kumar, 2010; Moujaber et al., 2019).

TUBG1 was differentially expressed in TIS cell models, where in TIS MCF10A, *TUBG1* expression was significantly reduced, in TIS HCC1806 it was slightly reduced and in TIS MDA-MB-231 it was slightly increased (Figure 3.34). A reduction in *TUBG1* expression may be consistent with senescence associated microtubule alterations or impaired DNA repair (Corvaisier et al., 2021; Manning and Kumar, 2010; Moujaber et al., 2019). In fibroblasts, a loss of γ -tubulin function at the centrosome has been associated with the induction of premature senescence, whilst in renal cells senescence has been associated with a loss of γ -tubulin expression and distribution (Manning and Kumar, 2010; Moujaber et al., 2019). Reduced γ -tubulin has also been shown to impair nuclear localisation of proliferating cell nuclear antigen (PCNA), a key regulator of DNA replication and repair (Corvaisier et al., 2021; Lesca et al., 2005). It results in the cytoplasmic accumulation of PCNA, which disrupts DNA replication and decreases proliferation. This mechanism may therefore contribute to the stable cell cycle arrest seen in senescent cells (Corvaisier et al., 2021; Lesca et al., 2005). In contrast, *TUBG1* expression was increased in TIS MDA-MB-231 cells (Figure 3.34). This may reflect inherently higher basal expression,

which could contribute to tumorigenesis by supporting proliferation (Alvarado-Kristensson, 2018). Alternatively, overexpression of *TUBG1* may be an adaptive response to senescence, required to enhance cytoskeletal stability in larger cells. It is also important to consider that, γ -tubulin performs essential roles outside of mitosis, like vesicle trafficking and cellular spatial organisation. Therefore, *TUBG1* expression may be increased as these functions are likely retained in TIS cells. As *TUBG1* expression in TNBC cells was not significantly altered, it is unlikely *TUBG1* is a robust biomarker of TIS in TNBC. Therefore, it was not further pursued as a candidate biomarker in this study.

3.5.2.7 Gene Summary

The primary aim of this study was to identify potential biomarkers of TIS in TNBC. Bioinformatic analysis identified several candidate genes associated with senescence, the cytoskeleton, treatment response, and patient survival. Reduced expression of *CDCA5*, *KIFC1*, *LMNB1*, *SMC6* and *TUBG1* was associated with poor therapeutic response and reduced OS in BC patients, whereas increased *STIM1* expression correlated with poor OS and treatment response. These findings suggest that the candidates may serve as useful predictive biomarkers. *CDCA5* and *KIFC1* expression also showed potential for differentiating between BC subtypes. However, gene expression changes in these candidates was not consistent across TIS models.

The MCF10A TIS model appeared to show a more canonical senescence associated suppression of mitotic genes, including *KIFC1*, *TUBG1*, *CDCA5* and *SMC6*, than TNBC TIS models. These genes contribute to centrosome clustering, microtubule nucleation, chromatid separation and chromosome segregation respectively; therefore, their downregulation is consistent with senescence, during which cells undergo cell cycle arrest (Bermudez-Lopez et al., 2010; Dráber and Dráberová, 2021; Hernandez-Segura, Nehme and Demaria, 2018; Khwaja et al., 2021; Laflamme et al., 2014; Teng et al., 2019; Torres-Rosell et al., 2005; Uniprot, 2022). In contrast, TIS TNBC cell lines frequently maintained or upregulated these genes, potentially reflecting their high basal expression in BC. Alternatively, *TP53* alterations in TNBC cell lines may have influenced senescence induction and maintenance, contributing to differences in candidate gene expression. It is also important to consider that genes such as *SMC6* and *TUBG1* encode for proteins which function outside of mitosis, so their differential expression may reflect differences in genomic instability or microtubule organisation (Fourriere et al., 2020; Gavilan et al., 2018; Goodson and Jonasson, 2018; Mangone et al., 2018; Mangone et al., 2025).

More consistent results were observed for *STIM1* and *LMNB1*, where *STIM1* expression increased across all TIS models and *LMNB1* expression decreased in a majority of TIS models. Mechanistically, elevated *STIM1* may reflect compensatory responses to perturbed calcium homeostasis, ER stress, or genomic instability in senescent cells (Pluquet et al., 2015; Sanchez-Collado et al., 2022; Sanchez-Lopez et al., 2024). Among the candidates assessed, *LMNB1* was the only gene significantly altered in both a TIS TNBC and TIS non-tumorigenic line. A decrease in *LMNB1* expression was consistent with TIS induction and its associated structural weakening of the nuclear envelope (Freund et al., 2012; Martins et al., 2020; Meng et al., 2025). Further research is required to establish the clinical utility of *LMNB1* as a biomarker of TIS in TNBC. However, studies have reported decreased *LMNB1* expression following chemotherapy treatment, highlighting its potential relevance as a biomarker of TIS (Saleh et al., 2022). Given *LMNB1* loss is associated with an increase in SASP components, strategies to mitigate this would also be beneficial (Meng et al., 2025). Based on these findings, *LMNB1* was included in subsequent experiments, as a marker of TIS in TNBC, to clarify its role in TIS and how its effects may be modulated by anti-senescence therapy.

Together, these results suggest that gene expression changes in the candidate biomarkers are generally consistent with TIS, but in a cell line specific manner. However, in the models used, transcriptional changes were not always conserved within TIS TNBC cells or between TIS TNBC and TIS non-tumorigenic cells. Among the candidates, *LMNB1* was the most consistently downregulated and was therefore selected for use in subsequent experiments.

3.6 CONCLUSION

TIS models were successfully developed in two TNBC cell lines (MDA-MB-231 and HCC1806) and a non-tumorigenic cell line (MCF10A). The absence of extensive apoptosis, and retention of viability confirmed that doxorubicin concentrations were appropriate, whilst increases in multiple senescence hallmarks such as lysosomal content, DNA damage, nuclear area, and expression of senescence associated genes, indicated that all cell lines had undergone TIS. These results confirmed that the models were suitable for subsequent experiments.

A literature review identified 108 potential genes of interest, which were refined to 6 candidates (*CDCA5*, *KIFC1*, *LMNB1*, *SMC6*, *STIM1* and *TUBG1*) with predictive and prognostic significance, using

bioinformatics tools. These genes were then assessed for their presence in TIS models, however, few showed significant and consistent expression changes either between the TIS TNBC cell lines or when comparing TIS TNBC with TIS non-tumorigenic cell lines. Given its well-established role in senescence and downregulation in a majority of TIS models, *LMNB1* was selected as a TIS biomarker in subsequent studies.

Chapter 4

Metformin as a senotherapy in TNBC

4 METFORMIN AS A SENOTHERAPY IN TNBC

4.1 INTRODUCTION

Doxorubicin, a first line chemotherapy treatment for TNBC, is a well-established inducer of TIS (Kumari and Jat, 2021). The accumulation of senescent cells is associated with numerous pro-tumorigenic consequences, therefore developing strategies to mitigate this is essential (Luo et al., 2025). Metformin was selected as a candidate TIS attenuating drug as it is well-tolerated and has senotherapeutic potential, extending beyond SASP attenuation (Barzilai et al., 2016; Foretz, Guigas and Viollet, 2023). As research evaluating its effectiveness in cancer models, particularly at clinically relevant concentrations is limited, this study investigates the capacity of metformin to reduce TIS in cell models. Initially by establishing optimal metformin concentrations, followed by assessing its impact on TIS in TNBC using multiple senescence detection methods.

4.2 AIMS AND OBJECTIVES

The primary aim of this chapter was to (4.1) Determine whether metformin attenuates TIS formation in TNBC models.

To fulfil aim (4.1) the following objectives were completed:

- Objective 4.1A: Establish effective working concentrations of metformin by performing MTT assays to assess cell viability, and apoptosis assays to determine the proportion of live cells within the population.
- Objective 4.1B: Determine whether metformin attenuates TIS in cell models (MCF10A, HCC1806 and MDA-MB-231), by assessing various markers of senescence such as:
 - Gene expression changes in SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*), markers of senescence pathway activation (*CDKN1A* and *TP53*) and nuclear integrity (*LMNB1*).
 - β -galactosidase staining as a marker of increased lysosomal content.
 - Nuclear area as a marker of morphological changes.
 - BrdU staining as a measure of cell proliferation.
 - γ -H2AX staining as a marker of DNA damage.

4.3 MATERIALS AND METHODS

Materials and methods specific to this chapter are described below. Unless otherwise stated, protocols used were those outlined in Chapter 2 (Universal Materials and Methods). This includes methods of TIS induction and detection, metformin preparations and statistical analysis.

4.3.1 γ -H2AX Staining classification

γ -H2AX staining was performed as described in materials and methods (section 2.10.1). In this chapter, γ -H2AX (Ser139) staining patterns were classified as unstained, weakly positive (less than half the nucleus stained), or strongly positive (more than half the nucleus stained, in an attempt to identify more subtle differences in staining patterns than by using a positive or negative classification system (Figure 4.1). Cells were also classified as weakly stained if their staining was diffuse, and fewer foci were observed. In contrast, cells were classified as strongly positive when many discrete foci were observed across the nucleus.

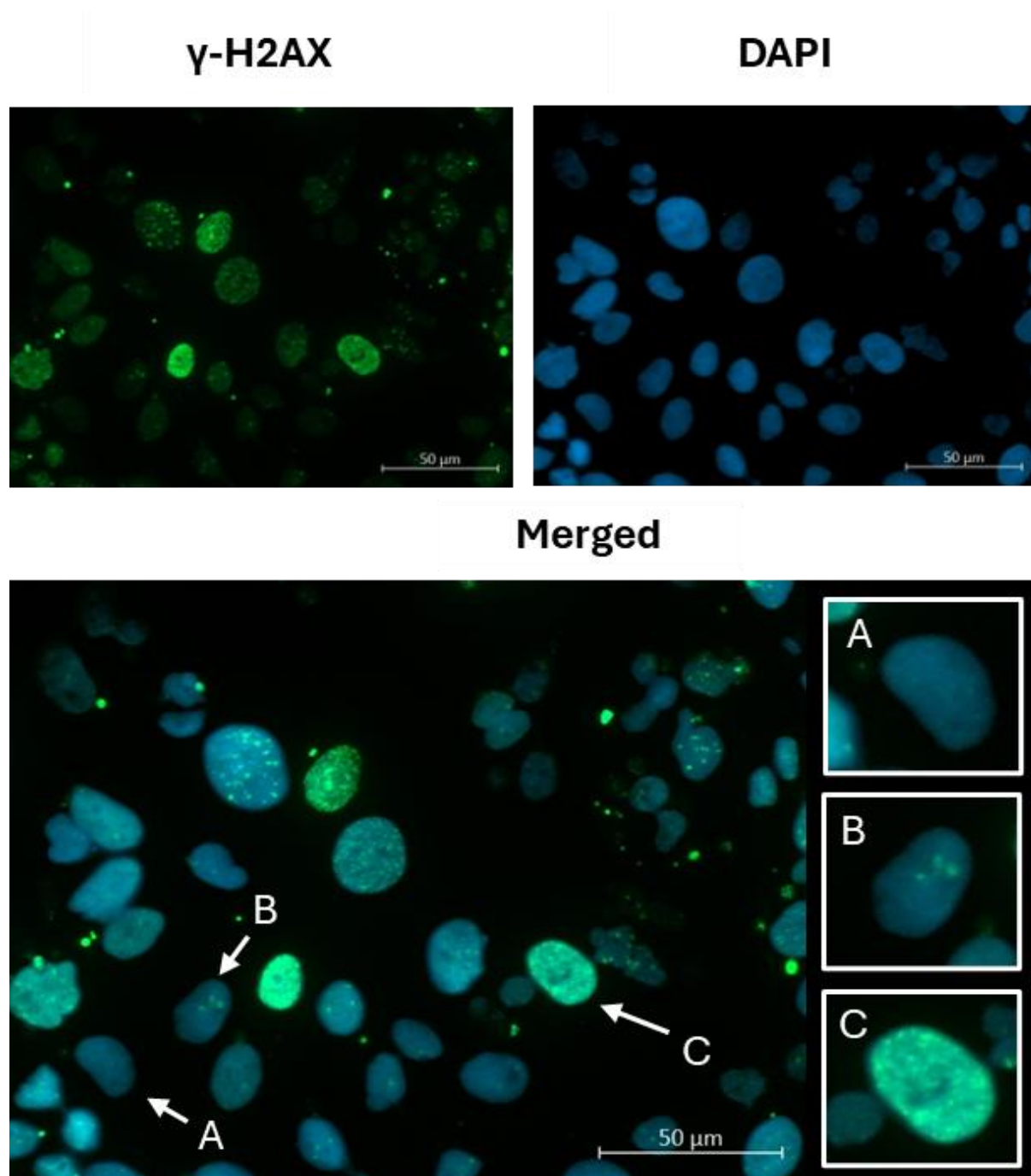


Figure 4.1. Chapter 4 γ -H2AX Staining classification. Representative staining is captured in HCC1806 cells, treated with 25 nM doxorubicin. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. γ -H2AX fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm) for 150 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 380ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell, B) a weakly positive cell and C) a strongly positive cell.

4.4 RESULTS

4.4.1 Establishing metformin dosage

Initially MTT assays were conducted to assess cell viability across a range of metformin concentrations (5nM to 50 mM) and were used to determine appropriate working concentrations for subsequent experiments. Once these concentrations were defined, apoptosis and MTT assays were conducted to confirm that the majority of the cell population remained viable. This ensured that the observed effects in later experiments were due to TIS induction rather than stress induced apoptosis.

4.4.1.1 Establishing a working metformin concentration

MTT assays were performed following 24 and 96 hour drug incubations to assess the impact of treatment duration on metabolic activity, as a measure of cytotoxicity. Metformin induced a stronger cytotoxic response in both TNBC cell lines at 96 hours compared to 24 hours, suggesting that its cytotoxicity was time dependent (Figure 4.2 and Figure 4.3). In contrast, MCF10A cells showed a significant decrease in viability at 5 nM and 50 nM concentrations after 24 hours, which was not observed at 96 hours (Figure 4.4).

As the TIS models used in this study became senescent over 96 hours, it was preferable to maintain metformin exposure throughout this period. Therefore, 96 hours was chosen as the most appropriate time point for determining working concentrations. A statistically significant reduction in viability was observed after 96 hours at concentrations $\geq 500 \mu\text{M}$ for all 3 cell lines. Interestingly, above $500 \mu\text{M}$ metformin treatment, the viability of MCF10A was a lot lower than for the two TNBC cell lines.

There is a gap in the literature regarding the effects of metformin at physiologically relevant levels; therefore, it was important to compare both a clinically relevant concentration and a supra-physiological dose to fully assess metformin's impact on TIS in TNBC. Two concentrations ($50 \mu\text{M}$ and $500 \mu\text{M}$) were selected for subsequent experiments, $50 \mu\text{M}$ represents a clinically relevant dose based on reported plasma levels, and $500 \mu\text{M}$ was identified as the minimum effective concentration (MEC). In MDA-MB-231, treatment with $50 \mu\text{M}$ metformin for 96 hours resulted in a 6% reduction in viability, whereas $500 \mu\text{M}$ metformin reduced viability by 22%. In HCC1806, viability was reduced by 1% at $50 \mu\text{M}$ and by 20% at $500 \mu\text{M}$, after 96 hours treatment. In MCF10A, after 96 hours treatment, $50 \mu\text{M}$ metformin resulted in a 2% reduction in viability, whilst $500 \mu\text{M}$ metformin led to a 39% reduction. As

metformin is generally reported to have plasma concentrations of ~4-70 μM , a concentration of 50 μM was selected as a clinically relevant midpoint (Foretz, Guigas and Viollet, 2023; Graham et al., 2011; He and Wondisford, 2015; LaMoia and Shulman, 2021). The MEC was defined as the lowest metformin concentration which induced a statistically significant reduction in cell viability, after 96 hours of treatment. This concentration was chosen to both induce a substantial metabolic effect in cells and ensure adequate cell viability for subsequent assays.

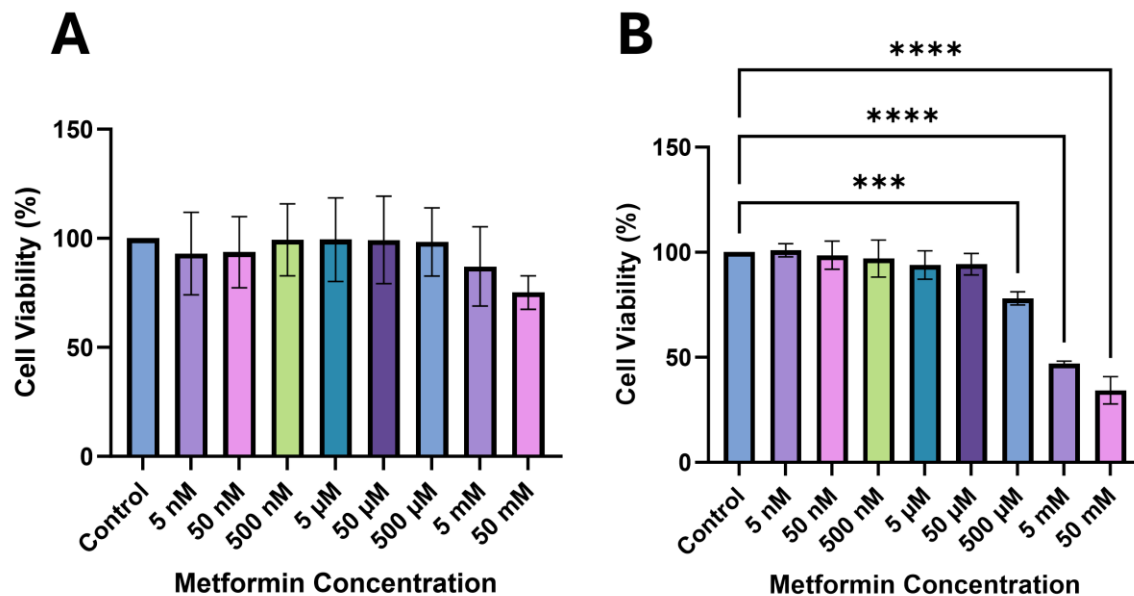


Figure 4.2. Effect of Metformin on MDA-MB-231 cell viability, assessed using MTT assays. Cell viability following A) 24 hour and B) 96 hour metformin treatment. Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed after 96 hours at concentrations $\geq 500 \mu\text{M}$. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$. Comparisons were made between each treatment and the control.

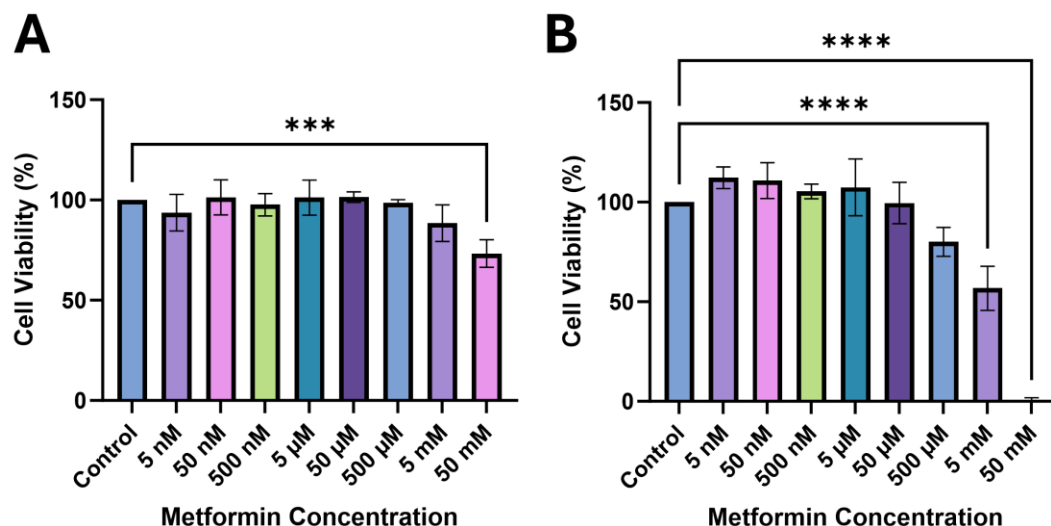


Figure 4.3. Effect of Metformin on HCC1806 cell viability, assessed using MTT assays. Cell viability following A) 24 hour and B) 96 hour metformin treatment. Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed after 24 hours at 50 mM and 96 hours at concentrations $\geq 500 \mu\text{M}$. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$. Comparisons were made between each treatment and the control.

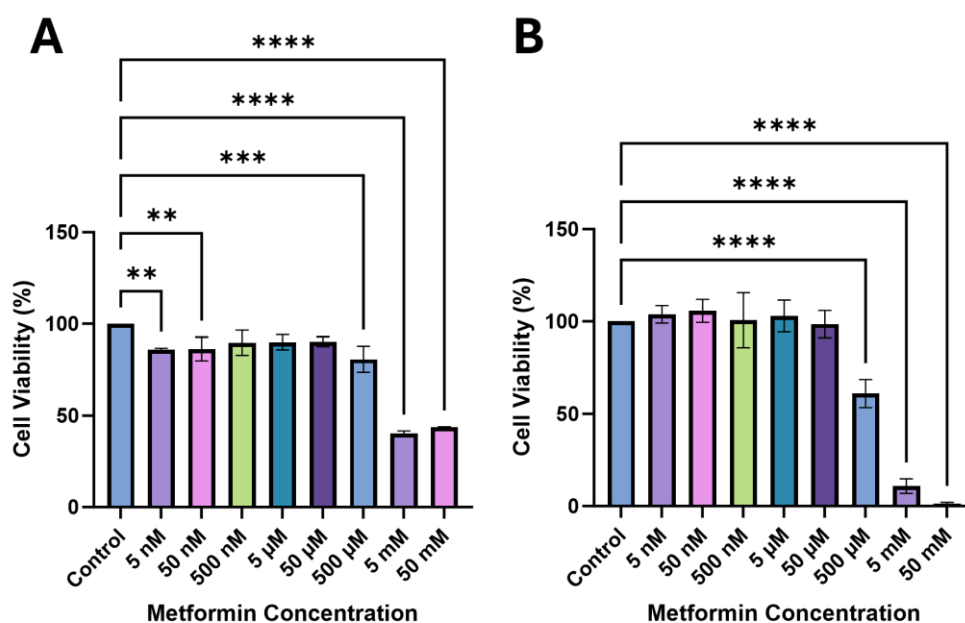


Figure 4.4. Effect of Metformin on MCF10A cell viability, assessed using MTT assays. Cell viability following A) 24 hour and B) 96 hour metformin treatment. Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed after 24 hours at 5 nM, 500 nM and $\geq 500 \mu\text{M}$, and at 96 hours $\geq 500 \mu\text{M}$. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$. Comparisons were made between each treatment and the control.

4.4.1.2 Cell viability confirmation

Apoptosis and MTT assays were performed following TIS induction and metformin treatment to ensure cell populations remained viable. Comparisons were made between untreated and metformin treated cells to ensure that metformin did not induce cell death. Similarly, untreated and doxorubicin treated cells were compared to assess any death as a result of TIS induction. Finally, doxorubicin treated cells were compared with those treated with both doxorubicin and metformin to determine whether metformin induced death in doxorubicin treated cells. Results from the apoptosis assay showed there was no statistical significance between live, apoptotic and necrotic cells across treatment groups in all 3 cell lines (Figure 4.5, Figure 4.6 and Figure 4.7). Slight variations in viability were observed, although not statistically significant. Staining patterns were as expected, where cytochalcein Violet 450 stained the cytoplasm of live cells, apoxin green stained the cytoplasm of apoptotic cells and 7-AAD stained the nucleus of necrotic cells. Representative images of each cell fate are shown in Chapter 3 (Figure 3.13, Figure 3.14 and Figure 3.15). These findings were supported by the MTT assay results, which similarly showed no statistically significant differences in cell viability between treatment groups (Figure 4.8). Consistent with the findings in section 4.4.1.1, treatment with 500 μ M metformin resulted in a greater reduction in cell viability than 50 μ M.

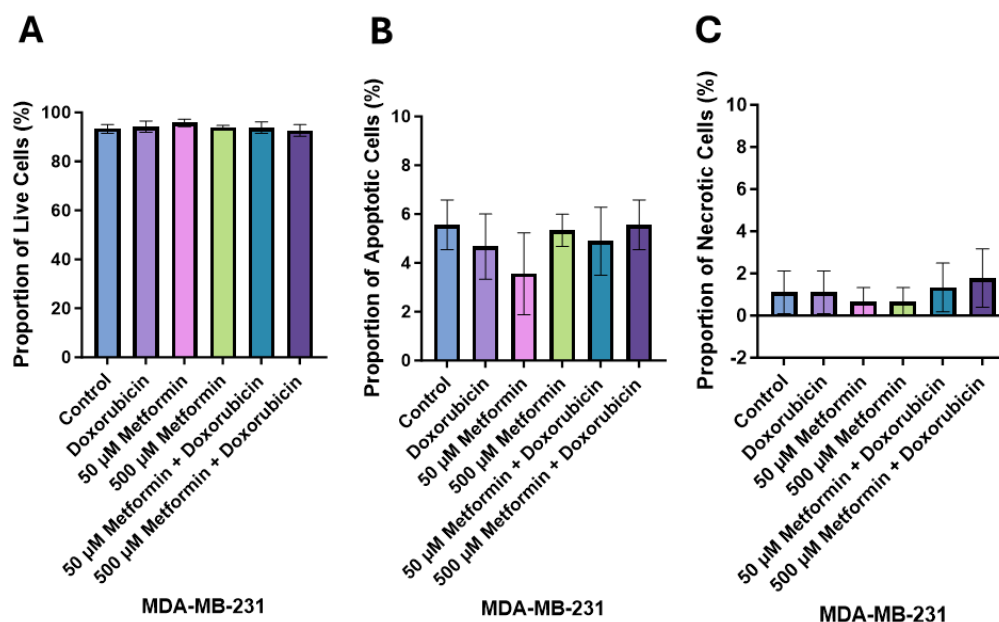


Figure 4.5. Effect of combined doxorubicin and metformin treatment on MDA-MB-231 cell apoptosis. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

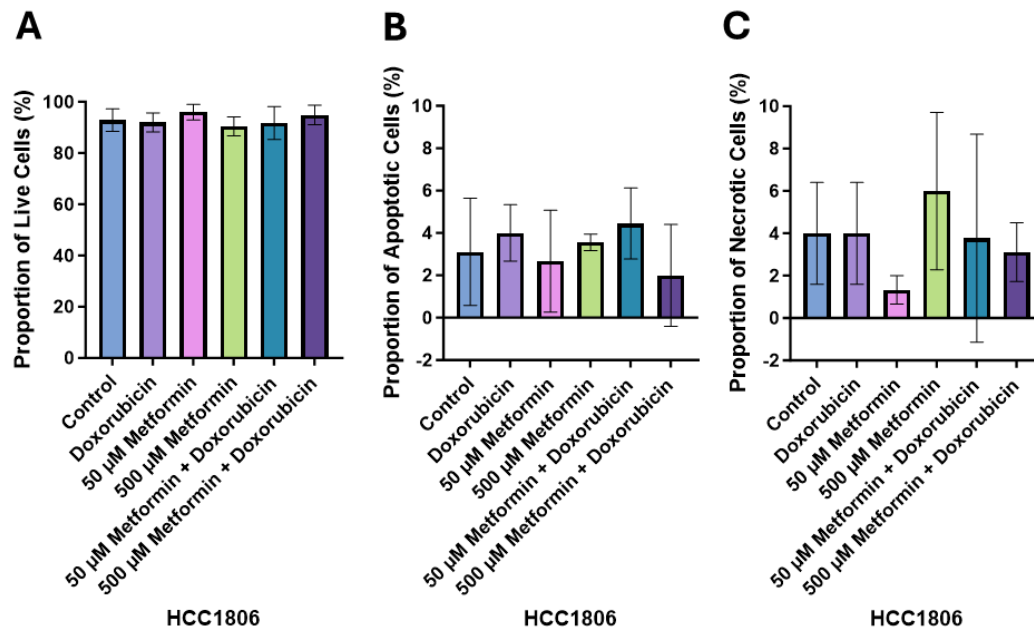


Figure 4.6. Effect of combined doxorubicin and metformin treatment on HCC1806 cell apoptosis. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA. . Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

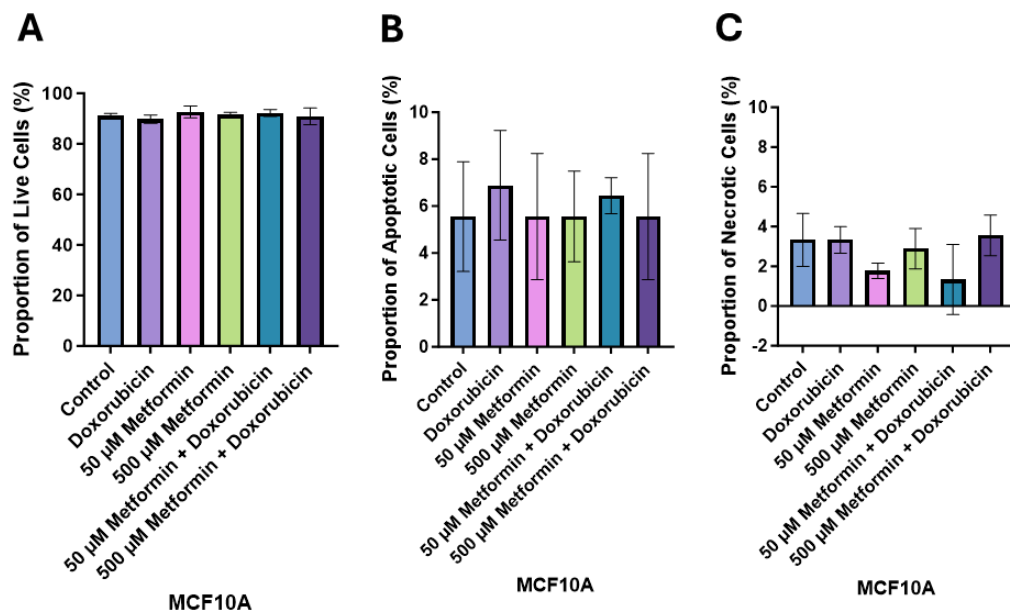


Figure 4.7. Effect of combined doxorubicin and metformin treatment on MCF10A cell apoptosis. Cells were stained using an apoptosis assay kit, and a total of 150 cells were counted to determine the percentage of (A) live, (B) apoptotic, and (C) necrotic cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant differences were observed between treatment groups for any staining category. Apoptosis assays were performed in three independent biological replicates ($n = 3$) and is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA. . Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

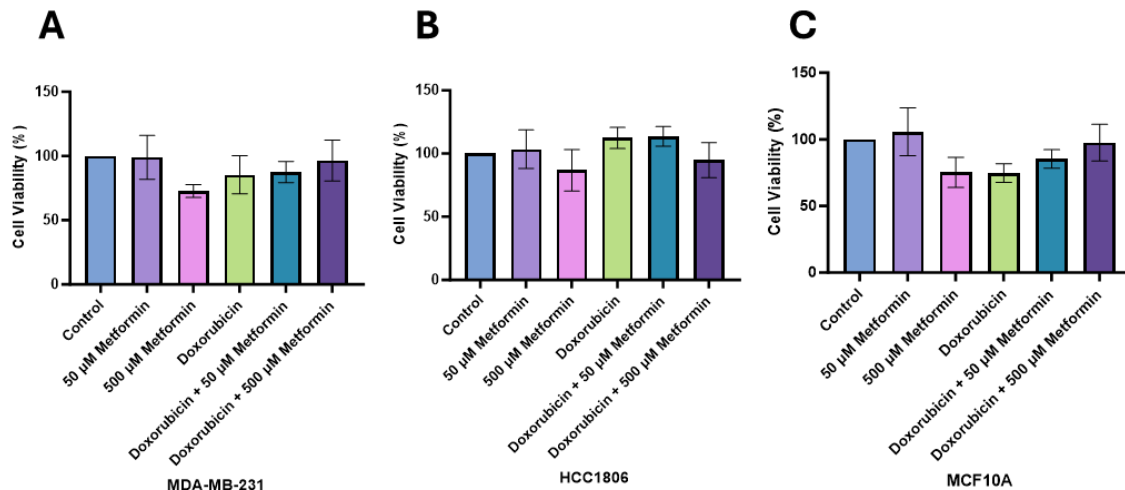


Figure 4.8. Effect of combined doxorubicin and metformin treatment on cell viability of immortalised cells. Cell viability of (A) MDA-MB-231, (B) HCC1806 and (C) MCF10A was assessed using MTT assays. Cells were seeded at densities equivalent to those used in subsequent senescence experiments (MDA-MB-231 untreated: 2083 cells/cm², doxorubicin treated 10417 cells/cm², HCC1806 untreated: 5833 cells/cm², doxorubicin treated 20833 cells/cm² and MCF10A untreated: 1667 cells/cm², doxorubicin treated 20833 cells/cm²). Viability (%) is expressed as a proportion of the untreated control (100%). No statistically significant differences were observed between any comparisons. MTT assays were performed in 3 independent biological replicates (n = 3), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

4.4.2 Evaluating metformin's effect on senescence attenuation

A number of experiments were used to assess the impact of metformin on various markers of senescence. These included β -galactosidase staining, BrdU staining, γ -H2AX staining, nuclear area analysis and qPCR of key senescence hallmarks (*CXCL1*, *IL1A*, *IL6*, *IL8*, *LMNB1*, *CDKN1A1* and *TP53*).

Comparisons were made to confirm the induction of senescence and to evaluate the effects of metformin on TIS. Untreated cells were compared with doxorubicin treated (TIS) cells to confirm TIS was induced. Untreated and metformin treated cells were compared to confirm that metformin did not have any adverse effects on non-senescent cells. This ensured that any observed effects were specific to TIS. Finally, doxorubicin treated cells were compared with those treated with both doxorubicin and metformin to determine whether metformin reversed TIS.

4.4.2.1 β -galactosidase staining indicates no reduction in lysosomal staining following metformin treatment

β -galactosidase staining is a widely used method for detecting senescence. Senescent cells have increased lysosomal content and upregulate several proteins, leading to characteristic blue cytoplasmic staining at pH 5.9-6.1 indicative of TIS (Hernandez-Segura, Nehme and Demaria, 2018; Park et al., 2018).

The % of β -galactosidase positive cells, for each cell type and treatment is presented in Figure 4.9. Representative images of β -galactosidase positive and negative cells are shown in Chapter 3 (Figure 3.20). Lysosomal staining was frequently observed surrounding the nuclei of cells. Doxorubicin treated samples showed a significant increase in β -galactosidase staining compared to untreated controls, confirming the induction of TIS in all cell lines. However, there was no statistically significant reduction in β -galactosidase staining between metformin treated and doxorubicin + metformin treated samples, at either physiological or supraphysiological concentrations, indicating that metformin did not reverse TIS. A low level of staining was observed in untreated controls, which can likely be attributed to basal senescence within the population, this is higher for HCC1806 than for MDA-MB-231 and MCF10A.

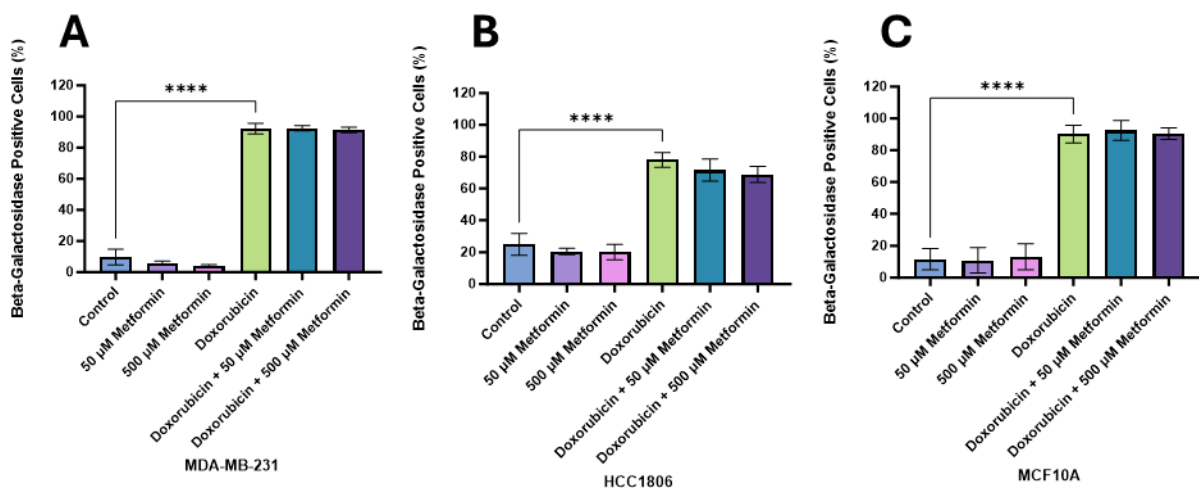


Figure 4.9. Effect of combined doxorubicin and metformin treatment on β -galactosidase staining in TIS cells. Cells were stained using a β -galactosidase staining kit, and a total of 150 cells were counted to determine the percentage of β -galactosidase positive (A) MDA-MB-231, (B) HCC1806, and (C) MCF10A cells. The proportion of positive cells (%) was calculated relative to the total cell population. A statistically significant increase in staining was observed in all doxorubicin treated (TIS) samples compared to untreated controls. However, no significant differences were detected in other comparisons. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test. $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$, and $**** = P \leq 0.0001$.

4.4.2.2 BrdU staining shows no increase in proliferation in metformin treated TIS cells

A lack of proliferation and DNA synthesis is indicative of cell cycle arrest and can be assessed using BrdU staining. BrdU is a thymidine analog, incorporated into newly synthesised DNA during replication, which is detectable via fluorescent labelling (González-Gualda et al., 2021; Hernandez-Segura, Nehme and Demaria, 2018). As TIS cells enter a stable cell cycle arrest, they do not undergo DNA replication and BrdU incorporation was not detected in these cells. However, as BrdU staining can be detected as a result of DNA repair, positive staining was observed in doxorubicin treated samples, in some instances. Staining was primarily punctate, with many foci distributed across the nucleus. Representative images of BrdU positive (A) and negative (B) cells are shown in Figure 4.10, Figure 4.11 and Figure 4.12.

The % of BrdU positive cells, for each cell type and treatment is presented in Figure 4.10. Doxorubicin treated samples showed a significant decrease in BrdU staining compared to untreated controls, confirming a reduction in proliferation, indicative of TIS, in all cell lines. There was no statistically significant increase in BrdU staining between metformin treated and doxorubicin + metformin treated samples, at either physiological or supraphysiological concentrations.

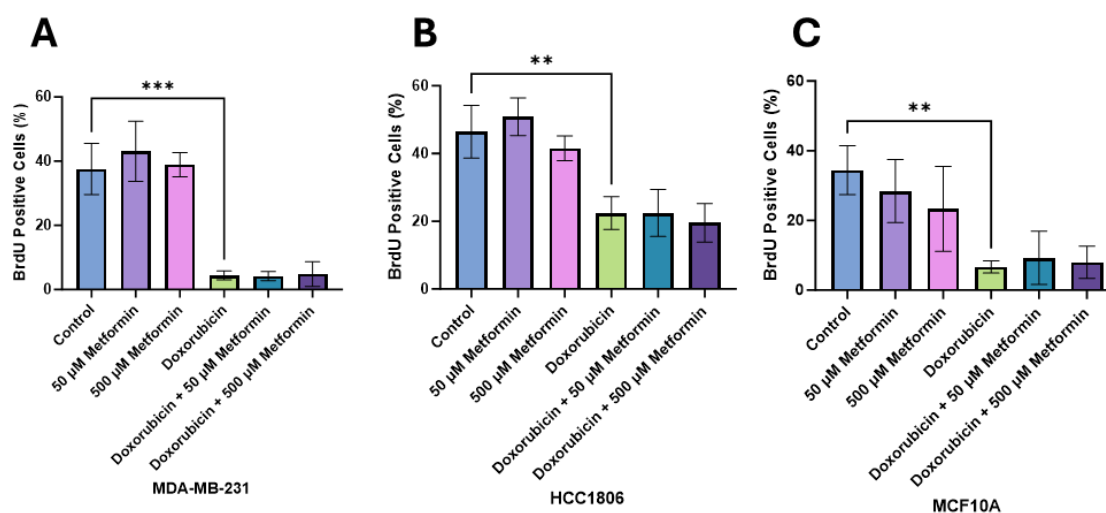


Figure 4.10. Effect of combined doxorubicin and metformin treatment on BrdU staining in TIS cells. Cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of BrdU positive (A) MDA-MB-231, (B) HCC1806, and (C) MCF10A cells. A statistically significant decrease in BrdU staining was observed in doxorubicin treated (TIS) cells compared to untreated controls, across samples. No significant differences were detected in other comparisons. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

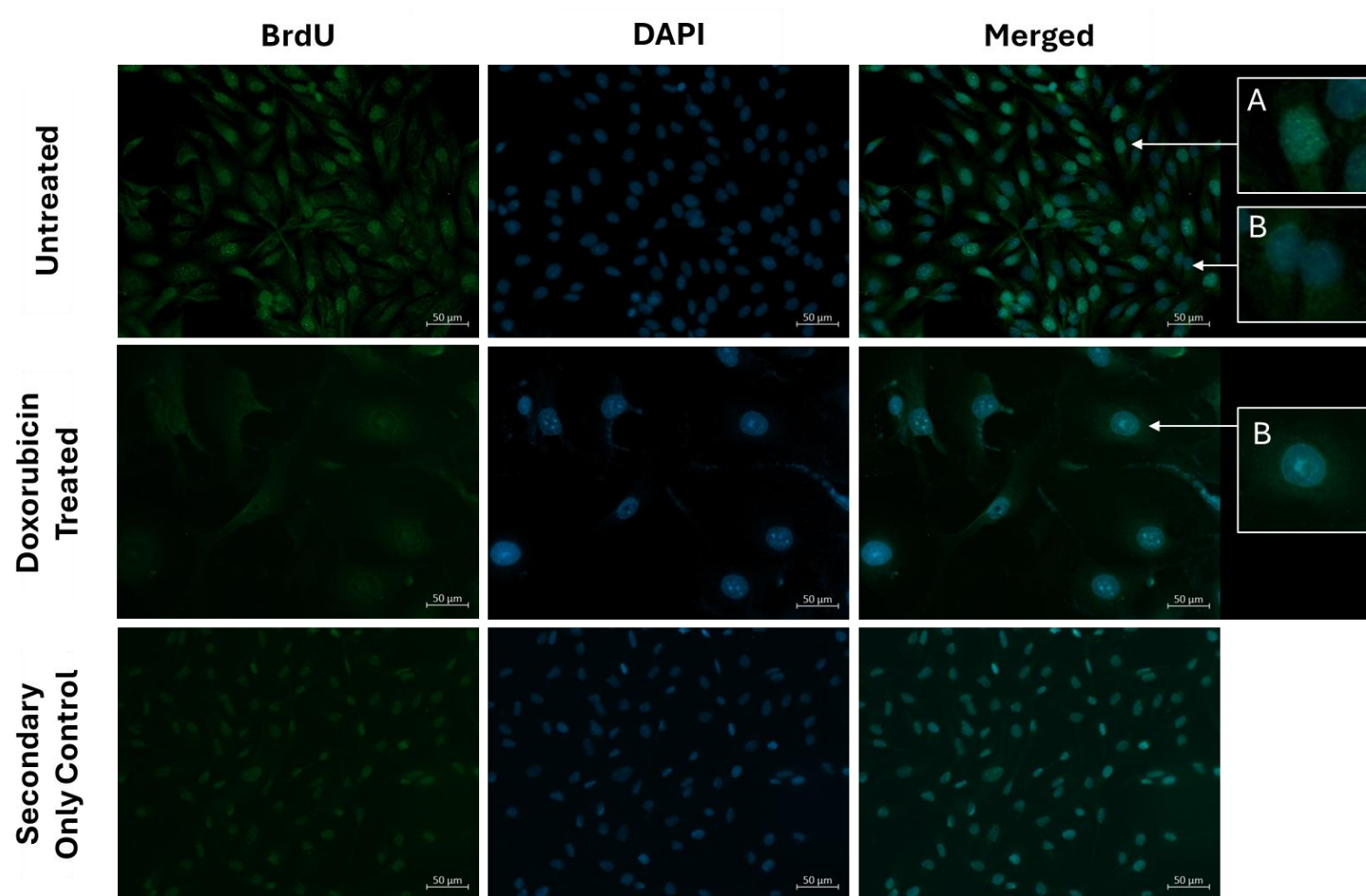


Figure 4.11. Representative fluorescence microscopy images illustrating BrdU staining in MDA-MB-231 cells. Untreated cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 20× magnification and have a scale bar of 50 µm. Representative images show untreated, and doxorubicin treated MDA-MB-231. As well as a secondary antibody only control on an untreated sample. BrdU fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), for 800 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 220 ms. Individual fluorescence channel images are shown alongside the merged images. The merged images combine both fluorescence channels, where A) represents a BrdU positive cell and B) a negative cell.

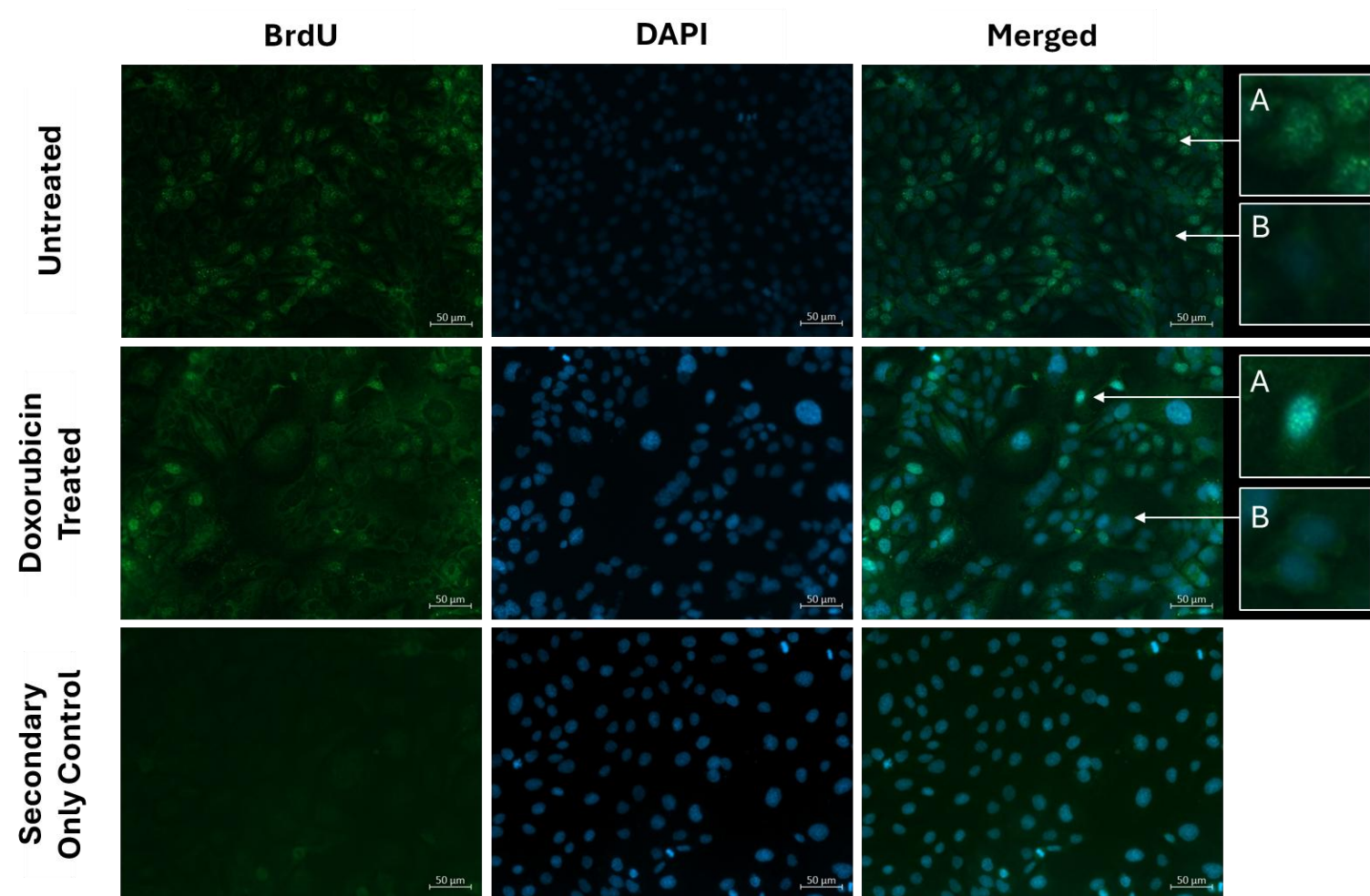


Figure 4.12. Representative fluorescence microscopy images illustrating BrdU staining in HCC1806 cells. Untreated cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 20× magnification and have a scale bar of 50 µm. Representative images show untreated, and doxorubicin treated HCC1806. As well as a secondary antibody only control on an untreated sample. BrdU fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm) for 800 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 220 ms. Individual fluorescence channel images are shown alongside the merged images. The merged images combine both fluorescence channels, where A) represents a BrdU positive cell and B) a negative cell.

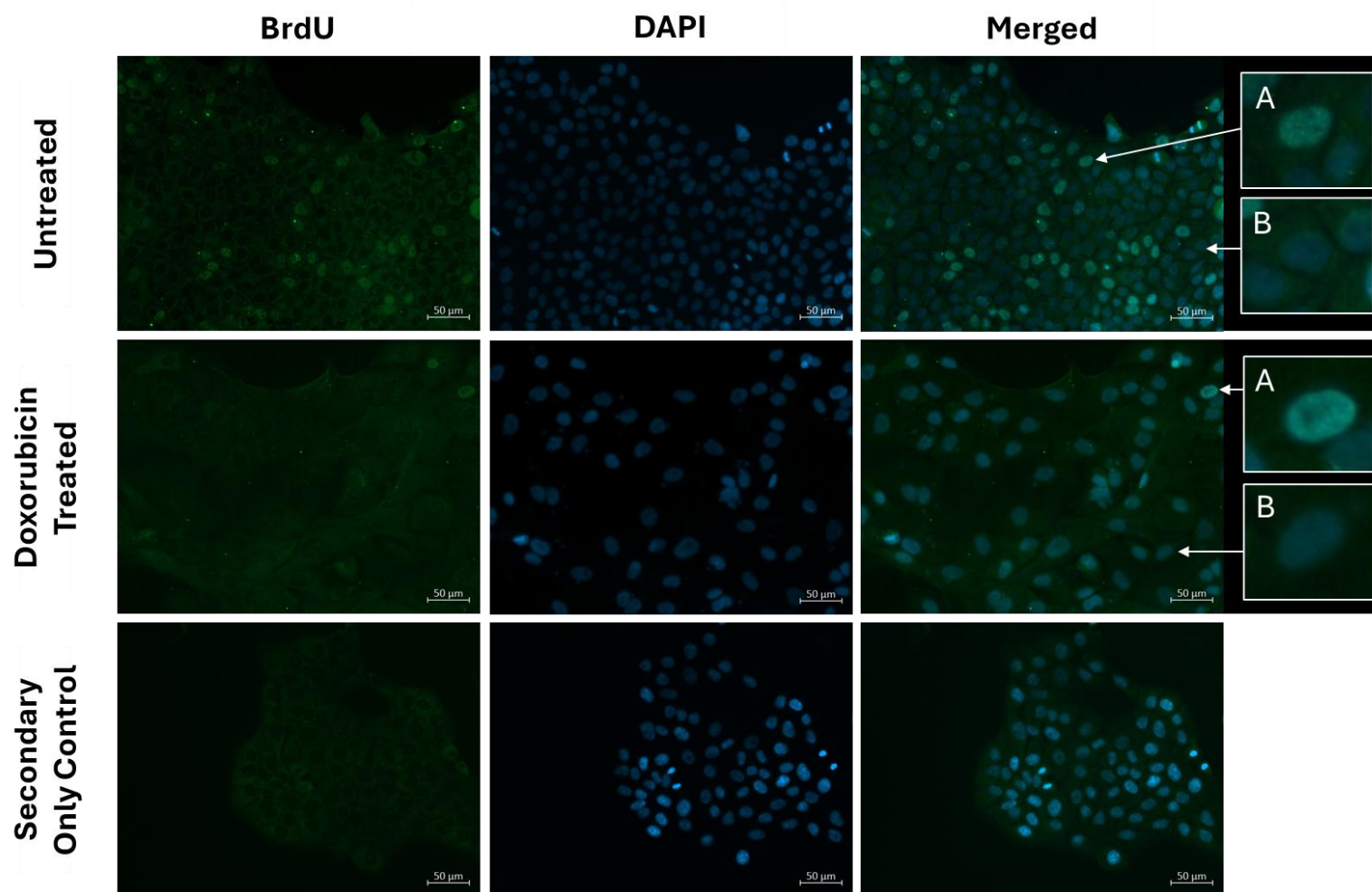


Figure 4.13. Representative fluorescence microscopy images illustrating BrdU staining in MCF10A cells. Untreated cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 20× magnification and have a scale bar of 50 μm . Representative images show untreated, and doxorubicin treated MCF10A. As well as a secondary antibody only control on an untreated sample. BrdU fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm), for 800 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 220 ms. Individual fluorescence channel images are shown alongside the merged images. The merged images combine both fluorescence channels, where A) represents A) a BrdU positive cell and B) a negative cell.

4.4.2.3 γ -H2AX staining reveals persistent DNA damage in metformin treated TIS cells

Chronic DDR is a characteristic of TIS cells, commonly induced by DNA damaging drugs such as doxorubicin (Hernandez-Segura, Nehme and Demaria, 2018). γ -H2AX (Ser 139), the phosphorylated form of the histone variant H2AX, is a well-established marker of DSBs, and when increased indicates DNA damage, as expected in cells undergoing TIS. As expected for a histone modification, γ -H2AX (Ser 139) staining was confined to the nucleus. A combination of punctate and pan γ -H2AX (Ser 139) staining was observed, reflecting heterogeneity in the DDR among individual cells. In some cells, large, distinct punctate nuclear foci were observed, whereas in others, smaller and less defined foci with more diffuse staining were present. In general, stronger staining was observed in cells with a large number of puncta.

Overall, stronger nuclear staining was detected in doxorubicin treated samples compared to controls. To quantify changes in γ -H2AX (Ser 139) expression, cells were categorised based on the extent and pattern of their staining: cells were classified as negative, when no staining was observed, weakly positive when less than half of the nucleus was stained and strongly positive when more than half of the nucleus was stained. Cells were also classified as weakly stained if their staining was diffuse, and fewer foci were observed. In contrast, cells were classified as strongly positive when many discrete foci were observed across the nucleus. In MCF10A and HCC1806 weak positive staining occurred more often than strong positive staining in untreated controls. In contrast MDA-MB-231 had more strong positive staining across treatments. In many instances, weak staining appeared more diffuse, with fewer and less distinct foci compared to strong positive staining. A summary of total staining (the sum of both weak and strong) is also shown. Representative images of γ -H2AX (Ser 139) staining in each cell type is shown in Figure 4.14, Figure 4.15 and Figure 4.16.

The % of γ -H2AX positive cells, for each cell type and treatment are presented in (Figure 4.17, Figure 4.18 and Figure 4.19). In all cell lines, doxorubicin treated (TIS) samples showed an increase in γ -H2AX staining (either weak or strong positive) compared to untreated controls, confirming elevated DNA damage in response to treatment. In MDA-MB-231 and HCC1806 cells, this increase was statistically significant for strong positive staining, whereas in MCF10A, the significant increase was observed in weakly positive cells. For MCF10A total γ -H2AX positive staining was significantly increased in doxorubicin treated samples, compared to untreated samples.

No significant differences were detected in other comparisons, except in MDA-MB-231 where treatment with 50 μ M metformin resulted in a significant decrease in strong positive cells compared to untreated controls. However, this effect was not observed in weak positive or doxorubicin treated samples. A similar decrease in weakly positive γ -H2AX staining was observed in the 50 μ M metformin + doxorubicin treatment group, although this was not statistically significant, likely due to the variation between biological repeats.

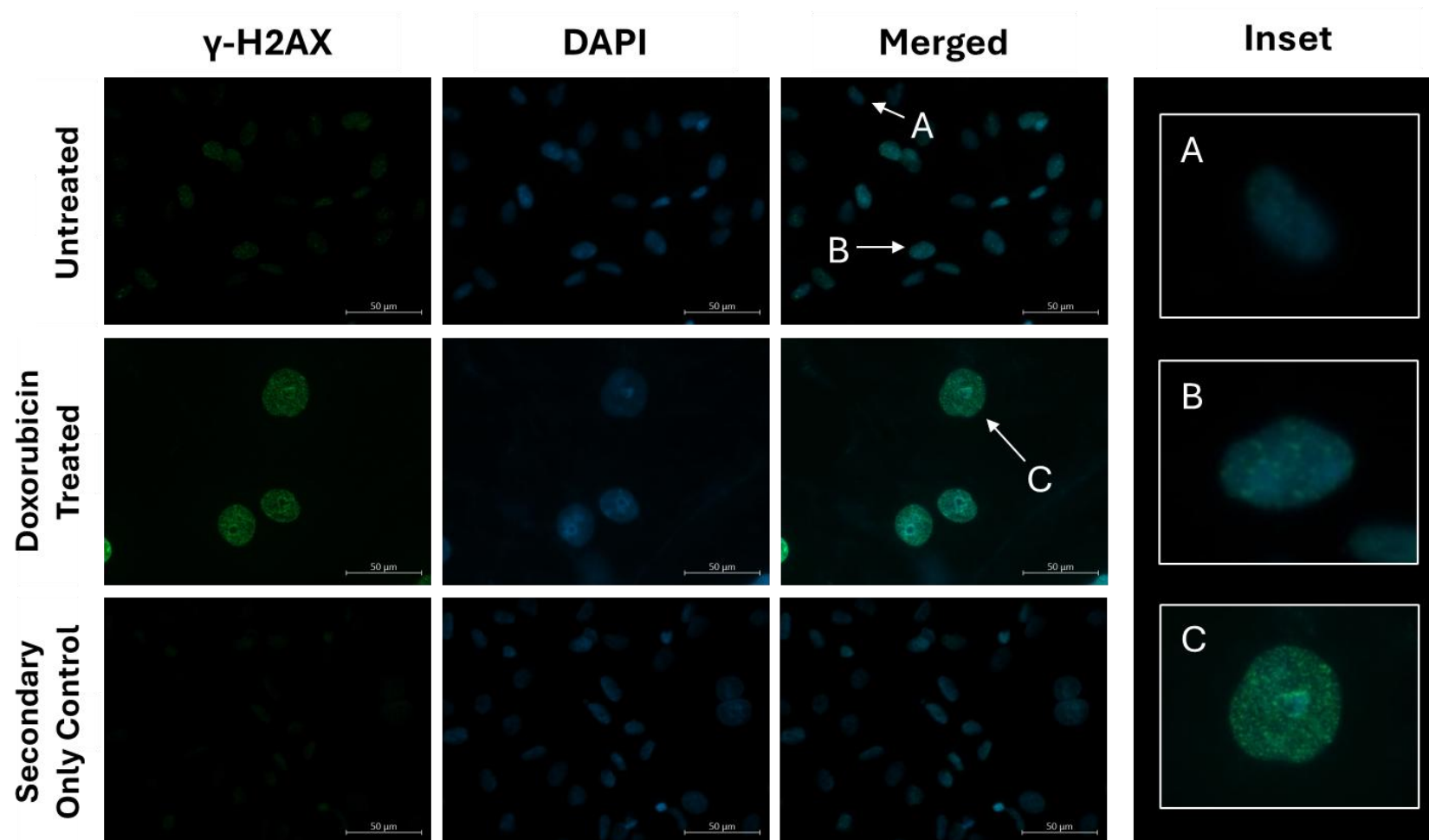


Figure 4.14. Representative fluorescence microscopy images illustrating γ -H2AX staining in MDA-MB-231 cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: (1) untreated cells, (2) cells treated with 175 nM doxorubicin, and (3) a secondary antibody only control. Cells were classified as weakly positive if less than half of the nucleus was stained and strongly positive if more than half of the nucleus was stained. γ -H2AX fluorescence was visualized using the AF488 channel (ex/em: 492/519 nm), for 95 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 140 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell , B) a weakly positive cell and C) a strongly positive cell. The inset shows magnified images of labelled merged channel cells.

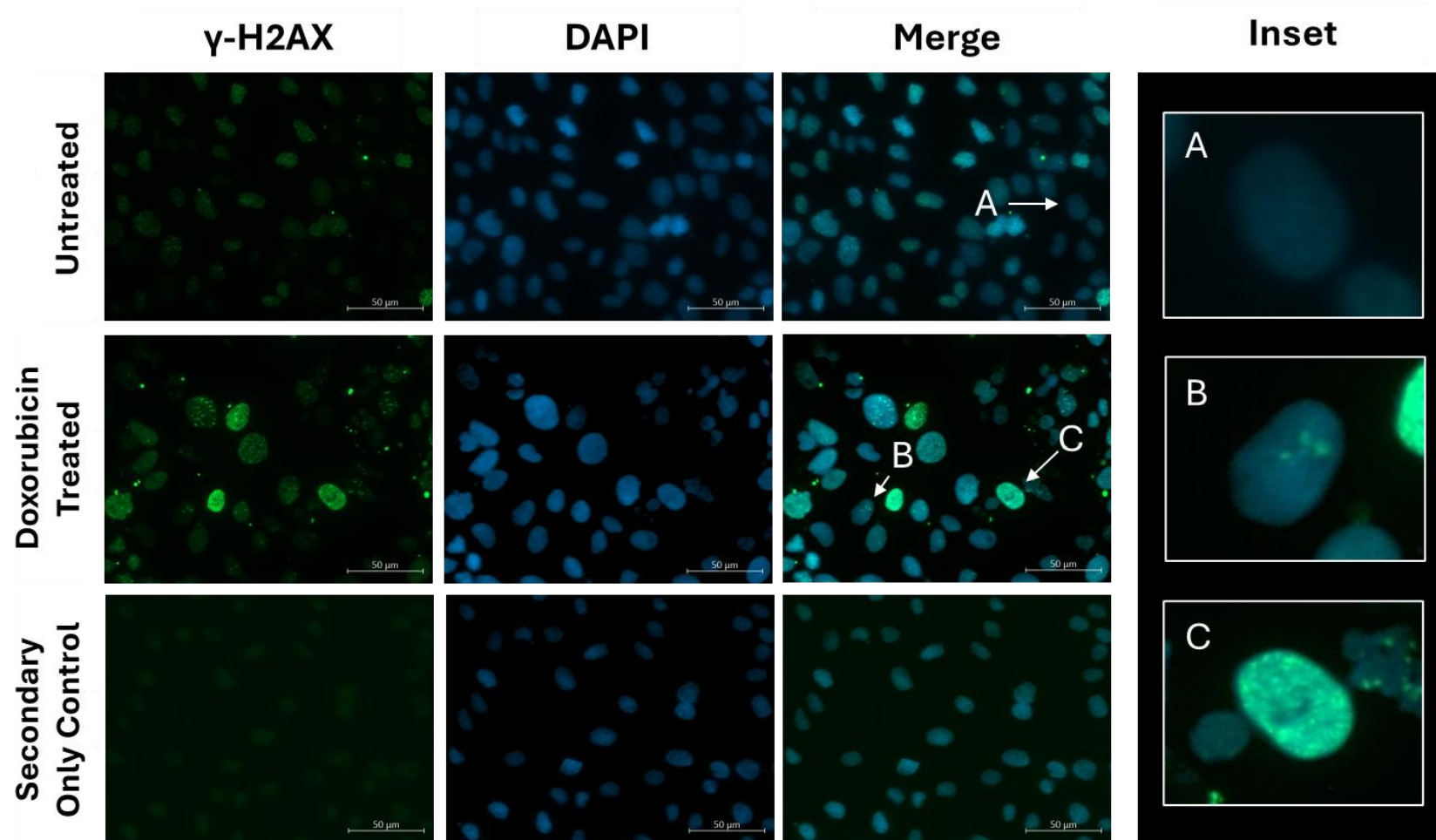


Figure 4.15. Representative fluorescence microscopy images illustrating γ -H2AX staining in HCC1806 cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: (1) untreated cells, (2) cells treated with 25 nM doxorubicin, and (3) a secondary antibody only control. Cells were classified as weakly positive if less than half of the nucleus was stained and strongly positive if more than half of the nucleus was stained. γ -H2AX fluorescence was visualized using the AF488 channel (ex/em: 492/519 nm) for, 150 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 380 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell , B) a weakly positive cell and C) a strongly positive cell. The inset shows magnified images of labelled merged channel cells.

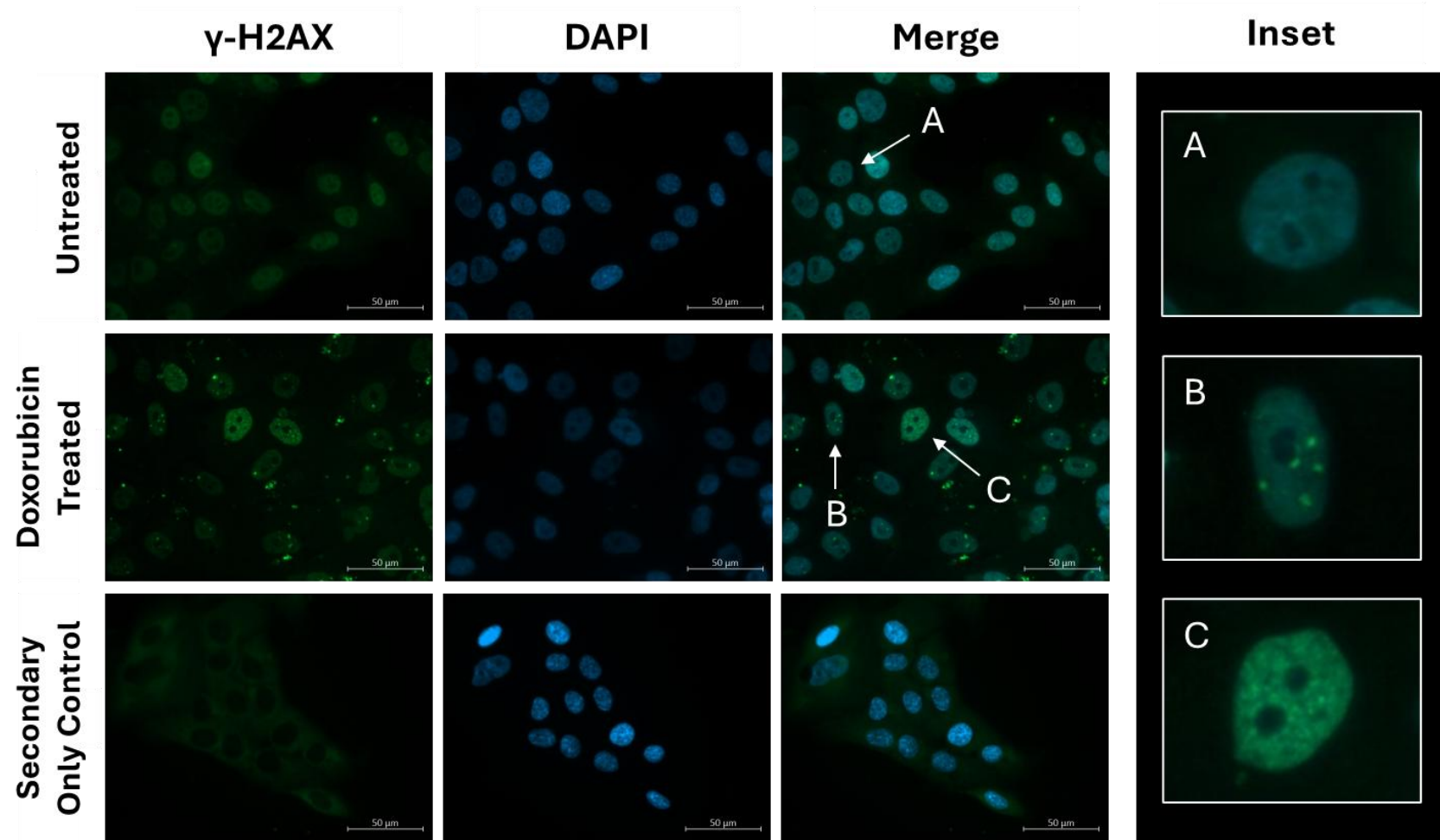


Figure 4.16. Representative fluorescence microscopy images illustrating γ -H2AX staining in MCF10A cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40 $\times$ magnification and have a scale bar of 50 μ m. Samples included: (1) untreated cells, (2) cells treated with 100 nM doxorubicin, and (3) a secondary antibody only control. Cells were classified as weakly positive if less than half of the nucleus was stained and strongly positive if more than half of the nucleus was stained. γ -H2AX fluorescence was visualized using the AF488 channel (ex/em: 492/519 nm) for 360 ms, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 200 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels, where A) represents a γ -H2AX negative cell, B) a weakly positive cell and C) a strongly positive cell. The inset shows magnified images of labelled merged channel cells.

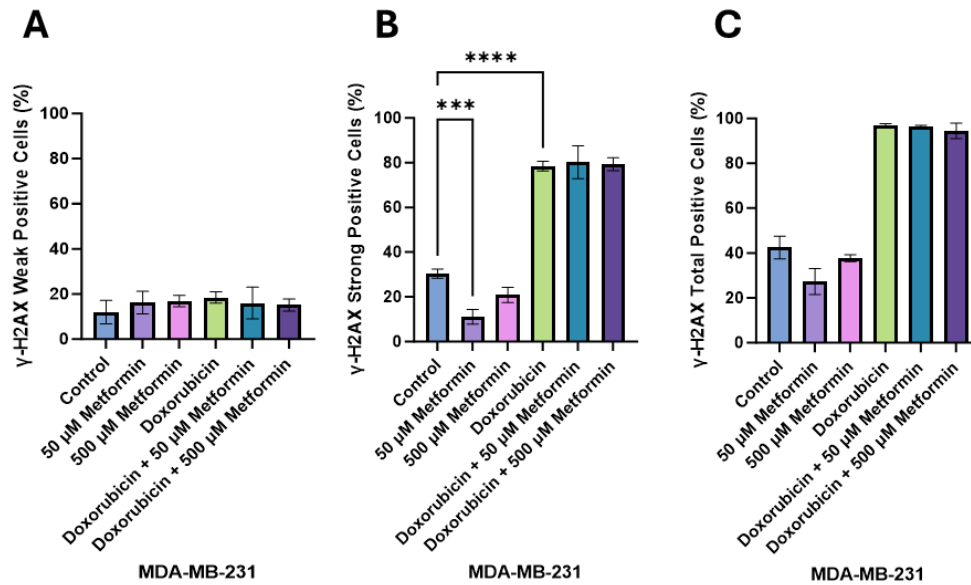


Figure 4.17. Effect of combined doxorubicin and metformin treatment on γ -H2AX staining in MDA-MB-231. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of (A) weakly positive cells, and (B) strongly positive cells. A summary of total stained cells is also shown (C). A statistically significant increase in strongly positive cells was observed in doxorubicin treated (TIS) cells compared to untreated controls. Treatment with 50 μ M metformin resulted in a significant decrease in strongly positive cells compared to untreated controls. A statistically significant increase of staining in doxorubicin treated cells, compared to untreated cells, was observed for strong positive cells. No significant differences were detected in other comparisons. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test for strong staining. For total staining the Kruskal-Wallis test was used. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

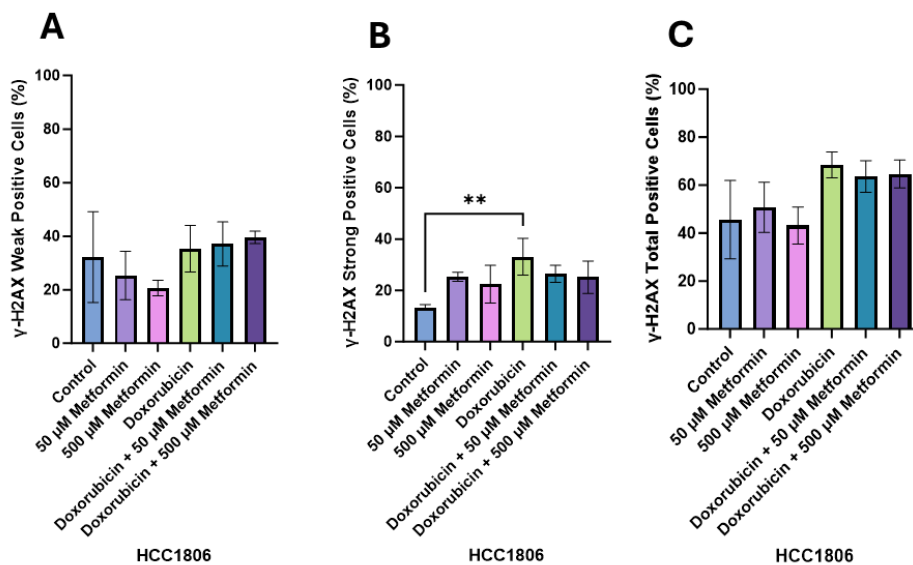


Figure 4.18. Effect of combined doxorubicin and metformin treatment on γ -H2AX staining in HCC1806. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of (A) weakly positive cells, and (B) strongly positive cells. A summary of total stained cells is also shown (C). A statistically significant increase in strongly positive cells was observed in doxorubicin treated (TIS) cells compared to untreated controls but no significant differences were detected between other comparisons. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

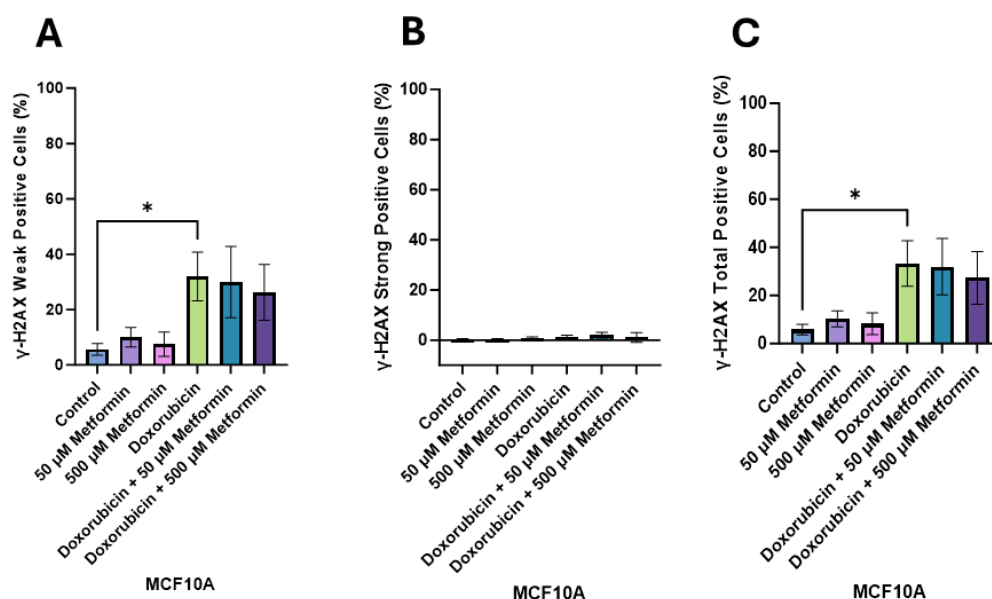


Figure 4.19. Effect of combined doxorubicin and metformin treatment on γ -H2AX staining in MCF10A cells. Cells were stained using γ -H2AX primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of (A) weakly positive cells, and (B) strongly positive cells. A summary of total stained cells is also shown (C). A statistically significant increase in weakly positive cells and total positive cells was observed in doxorubicin treated (TIS) cells compared to untreated controls. No significant differences were detected between other comparisons. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test for weakly positive and positive cells. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

4.4.2.4 Nuclear area analysis shows no attenuation of TIS associated hypertrophy by metformin

As a consequence of aberrant signalling from both DDR and SASP, TIS cells can have an abnormal appearance characterised by increased cell size (hypertrophy), a flattened appearance and nuclear distortion, which can be observed microscopically and qualified as a morphological marker of senescence (Beck, Horikawa and Harris, 2020; Herranz and Gil, 2018).

Nuclear area (μm^2) was measured, using ImageJ software and results for each cell line are presented in Figure 4.20. Although all doxorubicin treated samples showed an increase in nuclear area, this was only statistically significant in MDA-MB-231, where doxorubicin treated cells had a ~ 3 -fold increase in nuclear area relative to the untreated control. In HCC1806 and MCF10A nuclear area increased ~ 1.5 fold in doxorubicin samples relative to the untreated control. No other statistically significant differences were observed between treatment groups. Despite the average nuclear area being reduced in doxorubicin + metformin treated MCF10A samples, in comparison to doxorubicin alone, this difference was not statistically significant.

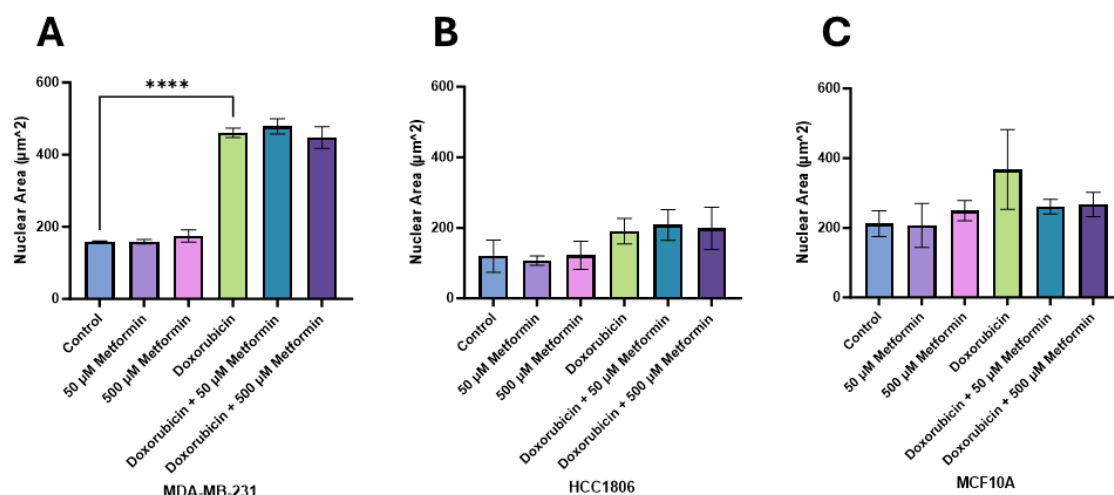


Figure 4.20. Effect of combined doxorubicin and metformin treatment on the nuclear area of TIS cells. Nuclear area (μm^2) was measured, using ImageJ software, across 150 cells per treatment group, in (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in nuclear area was observed in doxorubicin treated (TIS) MDA-MB-231 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one-way ANOVA with Tukey's test. Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

4.4.2.5 Gene expression analysis shows no significant differences between TIS and metformin treated cells

Gene expression analysis was performed using qPCR to assess key TIS markers, including components of the SASP (*CXCL1*, *IL1A*, *IL6*, *IL8*), regulators of senescence induction pathways (*CDKN1A*, *TP53*), and nuclear lamina components (*LMNB1*). Results are represented as fold change and were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, normalised to two housekeeping genes validated as being stable in BC cells, *GAPDH* and *PUM1*. Comparisons were made to confirm the induction of senescence and to evaluate the effects of metformin on TIS. Untreated cells were compared with doxorubicin treated (TIS) cells to confirm TIS was induced. Untreated and metformin treated cells were compared to confirm that metformin did not have any adverse effects on non-senescent cells. Doxorubicin treated cells were compared with those treated with both doxorubicin and metformin to determine whether metformin reversed TIS.

Given the heterogeneity of the SASP, the gene expression of several components was tested (Figure 4.21, Figure 4.22, Figure 4.23 and Figure 4.24). Whilst some factors, such as *IL6* and *IL8*, are widely conserved, SASP expression is dynamic and not uniformly present in all TIS cells (Kumari and Jat, 2021). Out of the 4 SASP components analysed, both TNBC cell lines exhibited a statistically significant

increase in *CXCL1*, *IL1A*, and *IL8*. Only MDA-MB-231 cells showed a statistically significant *IL6* increase in doxorubicin treated samples compared to untreated controls. Although SASP components were elevated in MCF10A cells relative to controls, these changes were not statistically significant. No significant differences were detected in other comparisons.

The p53/p21^{WAF/CIP1} pathway induces senescence by inhibiting cyclin activation, functioning alongside the p16^{INK4A}/Rb pathway (Mijit et al., 2020). In all 3 cell lines, *CDKN1A* (p21) expression was significantly increased in doxorubicin treated samples compared to untreated controls (Figure 4.25). However, only the TNBC cell lines exhibited a significant increase in *TP53* (p53) expression following doxorubicin treatment (Figure 4.26). Unusually, in MCF10A cells, *TP53* expression was reduced in doxorubicin treated samples relative to untreated controls. There was a similar reduction in *TP53* expression in the 50 μ M metformin + doxorubicin treatment group, but this was not statistically significant and was not replicated in the metformin only control. No other significant differences were observed between other comparisons.

LMNB1 encodes one of the two B-type Lamin proteins, forming a key structural component of the nuclear lamina. As an intermediate filament, it provides cellular structural integrity, and its downregulation is associated with nuclear deterioration in TIS (Martins et al., 2020). No significant reduction in *LMNB1* expression was observed in doxorubicin treated TNBC cell lines compared to controls. However, in MCF10A cells, doxorubicin treatment resulted in a statistically significant ~10-fold reduction in *LMNB1* expression compared to untreated controls (Figure 4.27).

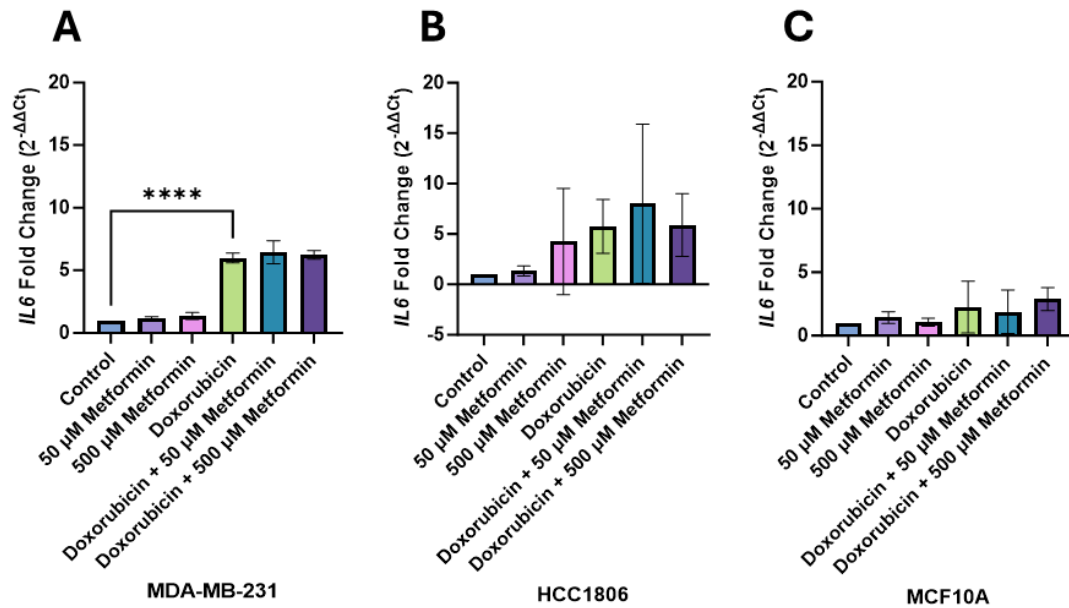


Figure 4.21. qPCR analysis of *IL6* in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

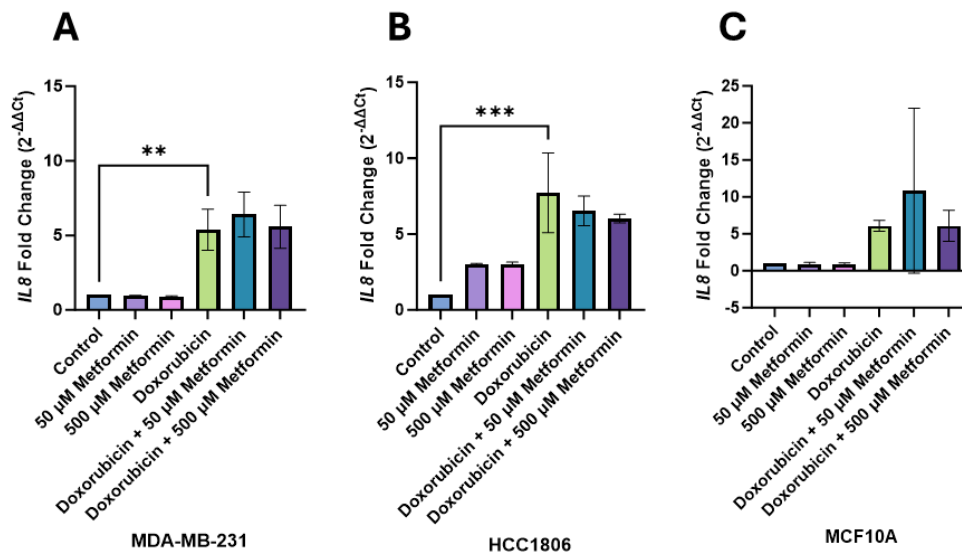


Figure 4.22. qPCR analysis of *IL8* in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231 and HCC1806 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

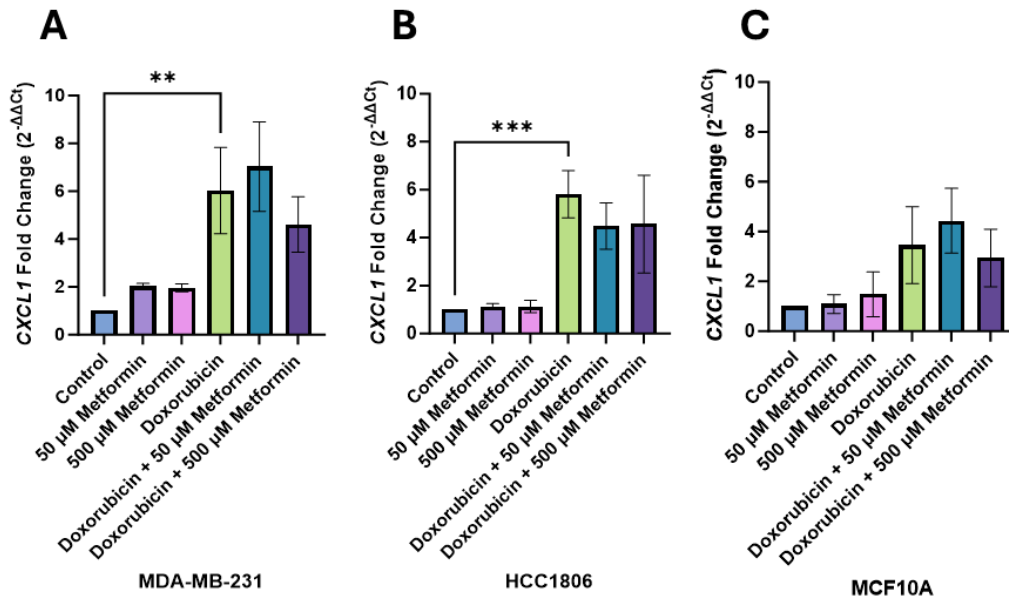


Figure 4.23. qPCR analysis of *CXCL1* in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231 and HCC1806 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.000.1$.

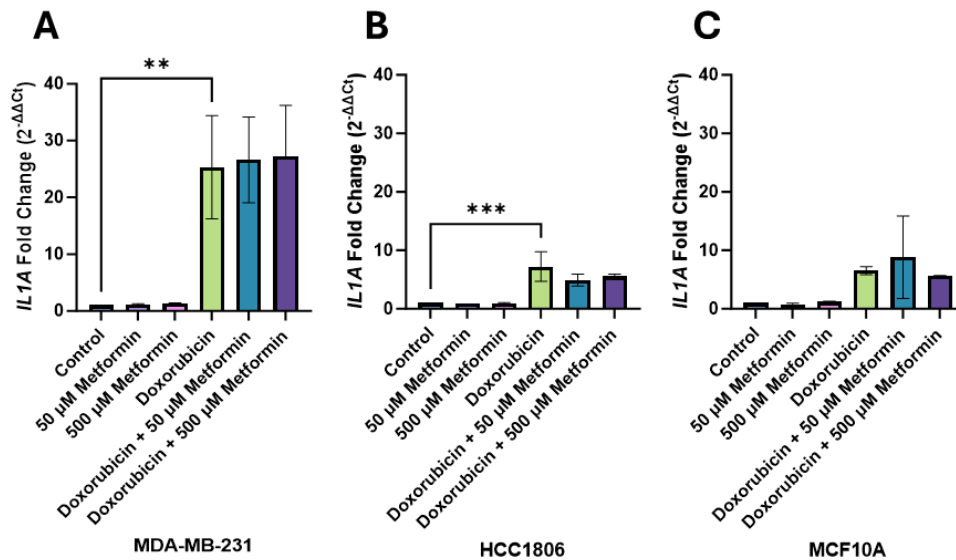


Figure 4.24. qPCR analysis of *IL1A* in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231 and HCC1806 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.000.1$.

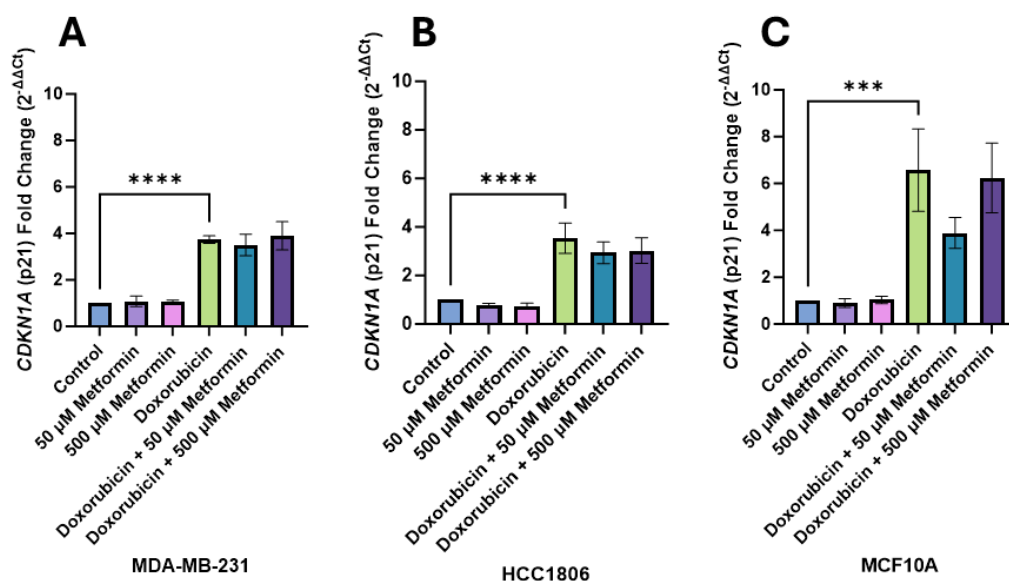


Figure 4.25. qPCR analysis of *CDKN1A* (p21) in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231, HCC1806 and MCF10A cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.000.1$.

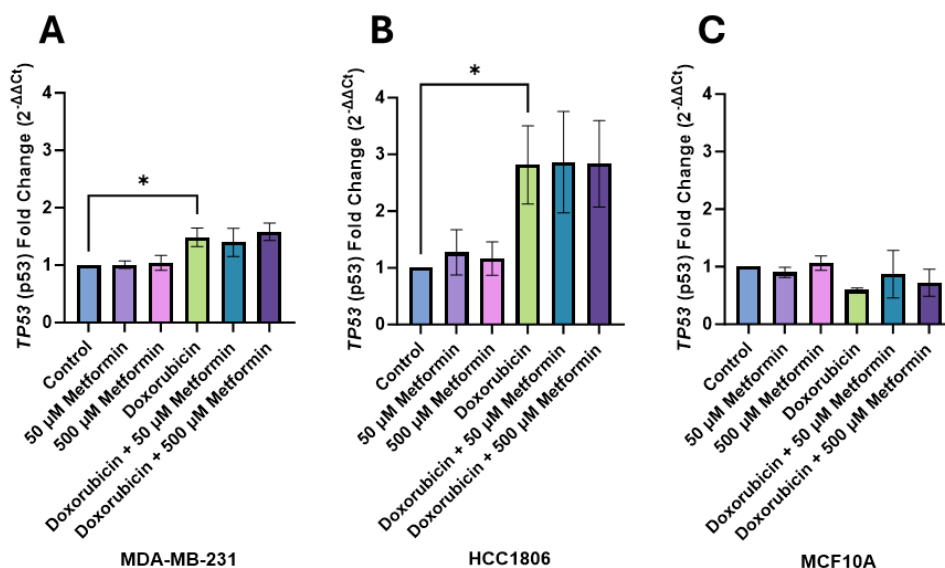


Figure 4.26. qPCR analysis of *TP53* (p53) in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MDA-MB-231 and HCC1806 cells compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.000.1$.

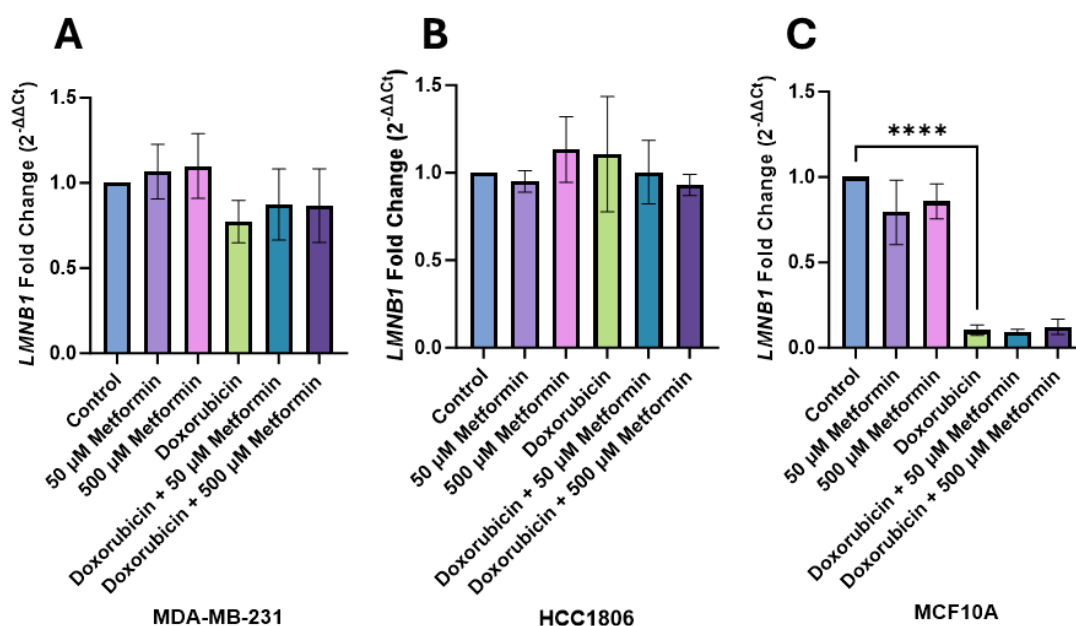


Figure 4.27. qPCR analysis of *LMNB1* in immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is normalised to a baseline fold change of 1. Data is shown for (1) MDA-MB-231, (2) HCC1806, and (3) MCF10A cells. A statistically significant increase in gene expression was observed in doxorubicin treated MCF10A compared to untreated controls. However, no significant differences were detected in other comparisons for MDA-MB-231, HCC1806, or MCF10A cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

4.5 DISCUSSION

The overall aim of this research was to assess the potential of metformin to attenuate TIS in TNBC. As metformin has known senomorphic effects, understanding its role as a senotherapy in TNBC is essential to determine its therapeutic potential. Three immortalised cell lines were induced with TIS, using doxorubicin, and treated with both physiological and supraphysiological metformin concentrations, to determine whether TIS was reversible. Metformin dosage was first optimised, and TIS attenuation was subsequently assessed using several senescence markers. Although, these results indicated no attenuation of senescence, subsequent chapters will investigate why this may have been and use co-culture to provide additional insights into the broader effects of TIS and metformin on the tumour microenvironment.

4.5.1 Dosage optimisation of metformin for investigation of TIS attenuation in TNBC

A key objective of this study was to establish appropriate drug concentrations for assessing the role of metformin in TIS attenuation. To address gaps in the literature, both physiological and supraphysiological concentrations were investigated.

MTT assays were used to indirectly assess cell viability, by measuring metabolic activity (Figure 4.2, Figure 4.3 and Figure 4.4). Both TNBC cell lines (MDA-MB-231 and HCC1806) had a time dependent response to metformin treatment, where cells were less metabolically active after 96 hours incubation in comparison to 24 hours. In contrast, MCF10A cells showed a significant decrease in viability at 5 nM and 50 nM metformin after 24 hours, which was not observed at 96 hours. This could indicate an initial response to metformin, followed by a potential metabolic adaptation of the cells to the drug over time (Andrzejewski, Siegel and St-Pierre, 2018). Although, this is more likely to occur at higher metformin concentrations, over a longer time period. Therefore, effects may have been due to biological variability.

Given that the TIS models used in this study became senescent over 96 hours, continuous metformin exposure throughout this period was preferable, which led to the selection of 96 hours incubation time for subsequent experiments. As current literature has investigated the mechanism of metformin in cell models, incubated for 24-48 hours, this suggests that metformin's effects can be observed within shorter time frames and are not necessarily cumulative (Chen et al., 2016; Halicka et al., 2011). Clinically, a steady plasma concentration of metformin is typically reached within 48 hours, therefore using a 96 hour incubation period, allows sufficient time for metformin to exert its effects, in a way that could potentially mimic *in vivo* conditions (Corcoran and Jacobs, 2018). The use of an acute incubation period ensured that any observed effects were due to metformin's impact on TIS cells rather than long term drug adaptations (Andrzejewski, Siegel and St-Pierre, 2018).

When selecting a supra-physiological concentration, it was important to consider the dual role of metformin, which is capable of both inducing and reversing TIS (Williams et al., 2013; Zhang et al., 2023). MTT assays showed a significant reduction in metabolic activity at metformin concentrations $\geq 500 \mu\text{M}$, in all three cell lines, after 96 hours. Therefore, to minimise unintended metformin induced senescence, 500 μM was selected for use in further studies as it was the lowest concentration which induced a statistically significant reduction in cell metabolic activity (Williams et al., 2013). In this

context, a reduction in viability, as a representation of metabolic activity, was desirable, as it indicated that metformin was actively influencing cellular metabolism.

Based on these findings, two metformin concentrations (50 μM and 500 μM) were selected for subsequent experiments. 50 μM represents a clinically relevant dose based on reported levels (He and Wondisford, 2015; LaMoia and Shulman, 2021), whilst 500 μM was a supraphysiological concentration identified as the MEC for inducing a metabolic response. Given metformin primarily inhibits gluconeogenesis, the chosen concentrations were expected to exert a substantial metabolic effect whilst maintaining sufficient cell viability for further assays.

As MTT assays do not distinguish between reduced proliferation and apoptosis, additional viability assessments were needed to assess cell health. Apoptosis assays were used as a direct measurement of cell viability, to confirm that metformin induced changes were due to TIS rather than apoptosis (Figure 4.5, Figure 4.6 and Figure 4.7). In all cell lines, results showed that neither 50 μM nor 500 μM metformin significantly reduced viability in any treatment group, when compared to their respective controls. Although a small percentage of the population was apoptotic, this was to be expected, as up to 6% of cells in culture naturally undergo apoptosis. In addition to this, as a live-cell stain was used, some apoptosis may have resulted from cellular stress.

Interestingly, whilst initial MTT assays indicated reduced viability, apoptosis assays confirmed that the majority of the cell population remained viable. Additionally, no significant changes in BrdU staining between metformin treated and untreated samples also indicated that a majority of the population was proliferating (Figure 4.10). This suggests that metformin was primarily impairing metabolic activity rather than inducing apoptosis or cell cycle arrest. This aligns with existing literature, which demonstrates that metformin reduces metabolic activity. Although its exact mechanism is debated, it is evident that metformin exerts its effects through mechanisms such as mitochondrial complex inhibition or alterations in redox reactions (LaMoia and Shulman, 2021; Rena, Hardie and Pearson, 2017). Given these findings, metformin appears to exert a measurable metabolic effect that may be beneficial for reversing TIS in TNBC.

To further investigate the metabolic effects of metformin in the context of TIS, MTT assays were repeated using untreated, doxorubicin treated (TIS), metformin treated, and doxorubicin + metformin treated samples (Figure 4.8). Unlike initial MTT assays, where 500 μ M metformin alone significantly reduced metabolic activity, the repeated experiments showed no statistically significant reduction in any treatment group, despite an observed decrease in metabolic activity. This could at least, in part, be attributed to biological variability as similar reductions in viability were observed across repeat experiments. In initial MTT assays, treatment with 500 μ M metformin reduced MDA-MB-231 viability by ~22% however, upon repetition, viability was reduced by ~28%. For HCC1806, viability was initially reduced by ~20% and by ~14% in the repeat experiment. Similarly, MCF10A viability was reduced by ~39% initially and ~25% upon repetition. As literature suggests metformin is clinically effective at concentrations up to ~70 μ M and can exert anti-senescence effects <200 μ M, these metformin concentrations were deemed suitable for subsequent experiments (Chen et al., 2016; Fang et al., 2018; Kuang et al., 2020).

The metabolic activity of TIS cells was not significantly reduced compared to controls, which may be due to their characteristically altered metabolic state (Figure 4.8). TIS cells are known to exhibit mitochondrial dysfunction, increased lysosomal activity, and a reliance on alternative energy pathways, meaning they may remain metabolically active despite undergoing senescence (Hernandez-Segura, Nehme and Demaria, 2018). This is expected, as metabolic activity is a key hallmark of TIS cells. Additionally, these metabolic adaptations may have made them less responsive to metformin's inhibitory effects.

Metformin's impact on TIS cells cannot be fully explored through MTT assays, as these are a measure of overall metabolic activity rather than direct TIS reversal. Metformin may influence TIS through mechanisms beyond metabolic reduction, therefore, it is crucial to assess additional senescence markers to fully evaluate metformin's role in TIS attenuation.

4.5.2 Exploring the senotherapeutic potential of metformin in TNBC

Several markers of TIS were assessed to evaluate metformin's role as a senotherapy in TNBC. A panel of markers was chosen to assess multiple markers of TIS, as its dynamic and heterogenous phenotype, makes it difficult to characterise.

4.5.2.1 β -galactosidase staining as a marker of increased lysosomal content

β -galactosidase staining was assessed as it is a widely used method of senescence detection (González-Gualda et al., 2021; Hernandez-Segura, Nehme and Demaria, 2018). All TIS cells, exhibited a significant increase in β -galactosidase staining, indicating successful TIS induction (Figure 4.9). Some basal staining of controls was also observed, which was to be expected as cells can enter senescence, naturally, in response to damage (Hernandez-Segura, Nehme and Demaria, 2018). Metformin treatment did not significantly alter lysosomal content, as shown by the lack of change in the % of β -galactosidase positive cells (Figure 4.9). As expected, lysosomal staining was frequently observed surrounding the nuclei of cells, consistent with their typical localisation which facilitates lysosomal-nuclear signalling (Zhao, Gao and Wang, 2020).

Previous studies have demonstrated that metformin reduces β -galactosidase staining in various cell types (Chen et al., 2016; Fang et al., 2018; Jiang et al., 2020; Kuang et al., 2020; Śmieszek et al., 2017). It is possible that metformin selectively reverses specific forms of senescence, such as OSIS, at lower metformin concentrations in ≤ 48 hours (Chen et al., 2016; Kuang et al., 2020). OSIS primarily relies on ROS production, which metformin can attenuate through an AMPK-independent mechanism involving redox modulation and autophagy upregulation (Figure 1.8). Similarly, another study using replicative senescent fibroblasts demonstrated that low doses of metformin (100-200 μ M over 6 hours) reduced senescence via GPX7 upregulation, an AMPK-independent mechanism (Fang et al., 2018). GPX7 is predicted to counteract the increased ROS levels characteristic of senescent cells, thereby promoting senescence attenuation.

In high glucose induced senescent renal tubular epithelial cells (a model for diabetic nephropathy), 1 mM metformin reduced β -galactosidase staining via STAT3 downregulation, which is thought to reduce cytokine production, potentially mitigating paracrine senescence (Jiang et al., 2020; Mikyskova et al., 2023). As metformin directly acts to reduce gluconeogenesis, it may be more effective at mitigating senescence under high glucose conditions.

These findings suggest that metformin's ability to reverse senescence may depend on the underlying mechanism of senescence induction. Although mitochondrial DNA can be damaged during TIS, increasing ROS, the predominant TIS response may be more closely associated with DSBs. Whilst metformin effectively targets ROS-driven senescence, such as OSIS or metabolic stress-induced

senescence, it may be less effective in reversing DNA damage induced TIS and also in reducing β -galactosidase staining.

4.5.2.2 BrdU staining as a marker of decreased proliferation

Although senescence is generally considered a state of irreversible cell cycle arrest, some studies have demonstrated that cells can re-enter the cell cycle, under certain conditions (Fitsiou, Soto-Gamez and Demaria, 2022). A key feature of TIS is cell cycle arrest, therefore, to assess whether metformin promoted cell cycle re-entry, BrdU staining was used to determine the proportion of actively proliferating cells. Staining was predominantly punctate, with distinct observable foci distributed across the nucleus, as expected for proliferating cells (Figure 4.11, Figure 4.12 and Figure 4.13) (Hernandez-Segura, Nehme, and Demaria, 2018).

Initially, Ki-67 was considered for assessing proliferation; however, due to its instability in TIS cells, BrdU was deemed a more reliable alternative (Bojko et al., 2019; Oijen et al., 1998). It is important to note that BrdU incorporation can also occur as part of DDR. However, since the results indicate that doxorubicin treatment reduced BrdU incorporation and increased γ -H2AX levels, this suggests that the BrdU incorporation observed is primarily associated with cellular proliferation rather than DNA damage. If BrdU incorporation were linked to DNA damage, higher BrdU staining would be expected in the treated samples. A significant reduction in BrdU incorporation, indicative of reduced proliferation, in doxorubicin treated samples compared to controls, indicated successful TIS induction (Figure 4.10). However, metformin treatment did not promote any increase in cell cycle re-entry. Several factors may have contributed to the lack of increased proliferation observed in this study, including post-treatment recovery time, drug concentration, or the ploidy status of the cells.

It is likely both post-treatment recovery time and drug concentration influence cell cycle re-entry. In one study, fibroblasts induced into TIS, using topoisomerase inhibitors ICRF-193 (0-3 μ M) and camptothecin (0-100 nM) over a 10 day period, resumed growth after the drugs were removed, despite exhibiting TIS markers such as increased β -galactosidase staining and p16 expression (Michishita et al., 1998). As doxorubicin is also a topoisomerase inhibitor, it is possible that, given a longer recovery period, beyond the 96 hours used in this study, cells might have re-entered the cell cycle. Another study treating MCF7 (luminal BC) cells with 0.7-1 M doxorubicin for two hours found that subclones, when re-exposed to a TIS inducing concentration over 16 days, were able to evade

senescence (Elmore et al., 2005). This suggests repeated drug exposure may be necessary for senescence evasion; therefore, it is possible a second metformin treatment may have promoted proliferation. However, it is important to consider that cell cycle re-entry over a longer period of time may reflect the proliferation of a subpopulation of cells that were not initially senescent. Therefore, monitoring cell-cycle re-entry in combination with additional markers, such as β -galactosidase staining, is essential to distinguish senescence escape from the expansion of non-senescent cells.

Results from the BrdU assay indicated that cells did not re-enter the cell cycle, after being treated with metformin. Another possible explanation is that cells were stabilised in TIS due to their ploidy status or failed to undergo additional polyploidisation. In some models, additional polyploidisation was required for cell cycle re-entry (Mosieniak et al., 2015; Sikora et al., 2022). However, in the models used cell lines in this study had varying baseline ploidy states (MDA-MB-231 near triploid, HCC1806 aneuploid, MCF10A near diploid), therefore it is possible that the lack of additional polyploidisation prevented cell cycle re-entry in this model. Increased ploidy is also associated with elevated metabolic demand, and as metformin disrupts cellular metabolism, it may reinforce cell-cycle arrest rather than permit re-entry (Coward and Harding, 2014).

4.5.2.3 γ -H2AX Staining as a marker of increased DNA damage

As metformin has been shown to reduce DNA damage, γ -H2AX (Ser 139) staining was used as an indicator of DNA damage and predominantly reflects DSBs (Najafi et al., 2018). Although not a direct marker of senescence, TIS is commonly associated with increased DNA damage; therefore, this assay was used to determine whether metformin reduced DNA damage levels. An increase in γ -H2AX (Ser 139) staining (either weak or strong positive) was observed in all doxorubicin treated samples compared to untreated controls, suggesting activation of DNA damage pathways, indicative of TIS.

In MDA-MB-231 and HCC1806 cells, the increase in strong positive γ -H2AX staining was statistically significant (Figure 4.17 and Figure 4.18). Although the magnitude of this increase, relative to control, was comparable between cell lines (~2.7-fold in MDA-MB-231 and ~2.5-fold in HCC1806), the proportion of strong γ -H2AX positive cells was higher in treated MDA-MB-231 cells (~80%) than HCC1806 cells (~30%). This difference may reflect cell line specific variations in basal DNA damage where, in untreated controls, HCC1806 showed higher levels of weak γ -H2AX staining and MDA-MB-231 cells showed higher levels of strong γ -H2AX staining. The increased level of DNA damage, reflected

by increased strong γ -H2AX staining in MDA-MB-231, may indicate they have a higher level of genomic instability than HCC1806. This may be attributed to the near-triploid chromosome arrangement of MDA-MB-231 cells, which is associated with increased genomic instability and the accumulation of DNA damage (Hayashi et al., 2024). In contrast, in MCF10A cells, a significant increase was observed in weakly positive staining (Figure 4.19). This difference may be attributed to aberrant DNA repair pathways in TNBC cell lines, which enable them to better withstand higher levels of DNA damage. In contrast, healthy cells rely more on DDR to actively resolve damage (Alhmoud et al., 2021; Huang and Zhou, 2021). Basal γ -H2AX staining was detected across all samples, which is expected as cells naturally undergo DNA damage. Higher basal levels in TNBC cell lines are likely due to their inherently higher levels of DNA damage and mutations. Metformin did not attenuate DNA damage in doxorubicin treated samples.

It is important to note the differences in staining patterns between samples, which included both pan-nuclear and punctate staining. In general, higher levels of punctate staining was more frequently observed in the strong staining category, consistent with literature indicating that punctate γ -H2AX foci reflect the presence of DSBs (Baure et al., 2009; Marti et al., 2006). Strong pan-nuclear staining was rarely observed, aligning with previous studies that link this to pre-apoptotic cells (Moeglin et al., 2019). This observation is further supported by the apoptosis data in this study, which indicates a low level of apoptotic cells (Figure 4.8). It is important to note that lower levels of punctate staining were also observed in treated samples, however, weak staining was predominantly diffuse, with fewer and less well-defined foci compared to strongly positive staining. In HCC1806 cells, weak positive γ -H2AX staining was reduced at both 50 μ M and 500 μ M metformin in a dose dependent manner (Figure 4.18). This is consistent with findings in lung cancer cells, where 100 μ M to 20 mM metformin treatments reduced γ -H2AX levels in a dose dependent manner, after 24 hours (Halicka et al., 2011). The same effect was not observed in strong positive staining for HCC1806. In contrast, for MDA-MB-231 cells, treatment with 50 μ M metformin resulted in a significant decrease in strong positive γ -H2AX staining compared to untreated controls (Figure 4.17). However, in MDA-MB-231 cells, this dose dependent effect was not observed at 500 μ M metformin, nor was it evident in weak positive staining. This may reflect cell line specific differences in responses to metformin, where in MDA-MB-231 low doses reduce DNA damage whilst higher doses contribute to increased metabolic stress, sustaining DNA damage responses. However, as the same effect was not observed in weak stained cells, it is unlikely this response is dose dependent.

Interestingly, metformin treatment reduced γ -H2AX staining across all cell lines in doxorubicin treated samples, compared to doxorubicin treatment alone, when measuring the total number of positive cells (Figure 4.17, Figure 4.18 and Figure 4.19). However, this reduction was not statistically significant, likely due to variability between samples and the limited difference between samples. The limited reduction in γ -H2AX by metformin, may be attributable to the senescence induction method. It is possible that metformin is more effective at attenuating the sources of DNA damage in other forms of senescence, such as OSIS, which is primarily driven by oxidative stress (Kuang et al., 2020). In OSIS, metformin may more readily reduce ROS levels, preventing downstream DNA damage, whereas in TIS models doxorubicin directly induces DNA lesions, which may be less susceptible to modulation by metformin. Furthermore, ROS are also elevated in TNBC, at higher levels than in other BC subtypes (Sarmiento-Salinas et al., 2019). Therefore, excessive ROS levels, arising from both dysfunctional mitochondria in TIS and the inherently high ROS levels in TNBC, may exceed the capacity of metformin to reduce oxidative stress induced DNA damage in combination with doxorubicin induced DNA damage.

4.5.2.4 Nuclear Area as a marker of hypertrophy

Alterations in cell morphology, such as nuclear hypertrophy, are a hallmark of senescence (Beck, Horikawa and Harris, 2020; Hernandez-Segura, Nehme, and Demaria, 2018; Herranz and Gil, 2018). Although it remains unclear whether nuclear distortion is a cause or consequence of senescence, it is generally accepted that nuclear hypertrophy arises due to aberrant DDR and sustained SASP signalling (Belhadj et al., 2023; Hernandez-Segura et al., 2018). The accumulation of DNA damage and prolonged activation of stress response pathways, such as p53/p21^{WAF/CIP1}, lead to chromatin reorganisation, loss of nuclear integrity, and increased nuclear area (Santos et al., 2021). *LMNB1* loss and SASP secretion drive nuclear alterations by reducing nuclear integrity and promoting persistent DDR signalling respectively (Hernandez-Segura, Nehme, and Demaria, 2018). Although metformin has not been shown to directly influence nuclear morphology, it is known to modulate the SASP and DDR and was therefore assessed as a general marker of TIS, to determine whether metformin could alter cell morphology indirectly.

Although all doxorubicin treated samples showed an increase in nuclear area, this was only statistically significant in MDA-MB-231 (Figure 4.20). This could be due to differences in cell growth characteristics, where MCF10A and HCC1806 cells tend to form compact colonies with strong cell-cell adhesions, potentially reducing their capacity to increase nuclear area. MDA-MB-231 and MCF10A TIS cells had

larger nuclear area's than HCC1806 (Figure 4.20). This difference may in part, be attributed to changes in *LMNB1* expression, as both cell lines showed reductions in *LMNB1* levels (~30% reduction in MDA-MB-231 and ~90% reduction in MCF10A, Figure 4.27), which may have compromised nuclear integrity and contributed to nuclear hypertrophy.

Metformin treated MCF10A samples showed a slight reduction in nuclear area compared to doxorubicin alone, however this difference was not statistically significant. This may be attributed to the lower levels of DNA damage observed in metformin treated MCF10A TIS cells (Figure 4.20). Despite metformin's senomorphic effects being well documented, results showed that metformin did not reduce TIS associated nuclear hypertrophy. As previously discussed, this could be due to the senescence induction method or metformin dose, whereby metformin may not effectively target the DNA damage induced by doxorubicin, or the concentrations used may have been insufficient to suppress the SASP and its subsequent DDR signalling.

4.5.2.5 Gene expression analysis of key TIS hallmarks

Changes in gene expression are commonly used as indicators of TIS, particularly in pathways associated with senescence induction and the SASP. Key SASP components, including *CXCL1*, *IL1A*, *IL6*, and *IL8*, are proinflammatory cytokines and chemokines involved in immune modulation, inflammation, and angiogenesis. *p21* and *p53* are central to TIS induction and maintenance, whilst *LMNB1* loss is associated with nuclear alterations in senescent cells.

4.5.2.5.1 SASP

The SASP is highly heterogeneous, with variation between different cell lines and individual cell populations. Whilst *IL6* and *IL8* are widely considered conserved SASP components, their expression is not always uniform across models (Hernandez-Segura, Nehme, and Demaria, 2018). To provide a broader assessment, expression of *CXCL1* and *IL1A* was also analysed. *CXCL1* is a potent neutrophil chemoattractant, playing a role in inflammation and immune regulation, while *IL1A*, *IL6*, and *IL8* are key mediators of inflammatory responses.

When analysing SASP components, in general TNBC cell lines (MDA-MB-231 and HCC1806) exhibited a stronger response to doxorubicin treatment than MCF10A (Figure 4.21, Figure 4.22, Figure 4.23, and

Figure 4.24). Although an increase in all SASP markers was observed in MCF10A, these changes were not statistically significant. In contrast, MDA-MB-231 and HCC1806 showed a significant upregulation of *CXCL1*, *IL1A*, and *IL8*, with MDA-MB-231 also exhibiting a significant increase in *IL6*. Despite metformin being a well-documented senotherapy with SASP reducing properties, it did not reverse the SASP components analysed in this study. However, it is important to note that metformin may modulate other SASP components, not assessed in this study.

It is possible that the metformin concentration used in this study was insufficient to inhibit IKK/NF- κ B activation, which is a key driver of SASP expression in senescent cells. Previous studies have reported SASP attenuation, via IKK/NF- κ B inhibition, under different experimental conditions. For instance, oncogene induced senescent fibroblasts, treated with 5 mM metformin, for 7 days, exhibited a reduction in SASP component expression, including *IL6*, *IL8*, and *CXCL1* (Moiseeva et al., 2013), and mouse hepatocytes treated with 5 mM metformin showed decreased expression of *CXCL1* and *IL6* (Cameron et al., 2016). Whilst metformin has been shown to attenuate markers of senescence in cancer cells (Hu et al., 2020) and also modulate their function (Moiseeva et al., 2013; Sabit et al., 2018; Sahra et al., 2011) these effects are observed at millimolar concentrations. This raises the question of whether metformin was acting similarly in the current study, as such concentrations were cytotoxic under the conditions used.

Interestingly, another study demonstrated that fibroblasts exposed to ionising radiation exhibited reduced *IL6*, *IL8*, and *CXCL1* levels 7 days post-induction, following a 1 hour treatment with 500 μ M metformin (Hooten et al., 2016). This effect was linked to an increase in DICER-1, an RNA-processing enzyme that is often downregulated in senescence. Since DICER-1 loss has been associated with persistent SASP activation, its restoration by metformin might contribute to SASP attenuation in cells treated with lower metformin concentrations (Zhao et al., 2015). Given that ionising radiation and doxorubicin both induce DNA damage, a comparable SASP reduction may have been expected in this study (Borrego-Soto, Ortiz-López and Rojas-Martínez, 2015). However, metformin did not significantly suppress SASP components in MDA-MB-231, HCC1806, or MCF10A. This may be due to more DNA damage being induced by doxorubicin, or differences in TIS induction pathways between immortalised cells and fibroblasts.

4.5.2.5.2 Pathways of TIS

DNA damage induced senescence (TIS) is primarily driven by the p53/p21^{WAF/CIP1} pathway (Figure 1.6). DNA damage signals are detected by ATR/ATM and propagated through p53, leading to an upregulation of p21^{WAF/CIP1}, which ultimately inhibits cell cycle progression (Chehab et al., 1999). In cases of chronic DDR increased p16 expression can also indicate TIS. Activation of senescence pathways is context dependent and varies depending on the inducer and stage of senescence (Saul et al., 2025). Although p53/p21^{WAF/CIP1} is reported to be induced early in senescence and p16^{INK4A}/Rb associated with its maintenance, opposing patterns have been reported (Beauséjour et al., 2003; Dolan et al., 2015; Kumari and Jat, 2021; Mijit et al., 2020; Saul et al., 2025).

In this study, *CDKN1A* (p21) expression was significantly elevated in all three doxorubicin treated cell lines, indicating activation of senescence associated pathways (Figure 4.25). Despite, MDA-MB-231 and HCC1806 having mutations in *TP53* (p53), its expression was significantly increased in these cell lines, also suggesting the p53/p21^{WAF/CIP1} pathway was activated (Figure 4.26). However, it is important to consider that the cell lines used in this study had mutations in *TP53* (p53) (MDA-MB-231 and HCC1806) and a *CDKN2A* (p16) deletion (MCF10A and MDA-MB-231), which may have influenced both the induction and maintenance of senescence, as well as the ability of metformin to attenuate the senescent phenotype. Ultimately, although *TP53* and *CDKN1A* expression were increased in TNBC cell lines, this may not have been as a result of p53/p21^{WAF/CIP1} pathway activation. Mutations in *TP53* can lead to aberrant p53 expression, which may have partial or no functionality, depending on whether it has been translated. Therefore, in future it would be beneficial to determine p53 and p21 protein expression, alongside gene expression. If p53 functionality is impaired, it is likely that p21 expression is being driven, or enhanced, by factors independent of p53 such as mothers against decapentaplegic (SMAD). SMAD proteins act as transcription factors, downstream of TGF- β , which when activated, induce p21 expression (Karimian, Ahmadi and Yousefi, 2016). The observed lack of senescence attenuation in response to metformin may therefore reflect variation in pathways driving senescence. Studies demonstrating a reduction in senescence markers, following metformin treatment, have been conducted in fibroblasts, epithelial cells, and ligament cells, relatively healthy, non-immortalised cell types, with functional p53 and p16 (Hooten et al., 2016; Jiang et al., 2020; Kuang et al., 2020). In contrast, the TNBC and immortalised breast cell lines used in this study have mutations, likely altering senescence induction and making metformin less effective at attenuating senescence.

Previous studies have also reported that metformin can reduce p21 expression, however this effect may be dependent on the method of senescence induction (Jiang et al., 2020; Kuang et al., 2020). It is possible that in this study, the DNA damage pathways activated in TIS models (as indicated by increased γ -H2AX staining) are less susceptible to modulation by metformin than the pathways activated in OSIS and high glucose induced senescence, where p21 expression is reduced (Chakrabarty et al., 2021; Jiang et al., 2020; Kuang et al., 2020).

4.5.2.5.3 Nuclear integrity

LMNB1 encodes one of the two B-type Lamin proteins that form the nuclear lamina. As an intermediate filament, it provides structural integrity to the cell and acts as a scaffold supporting the nucleus. Although metformin does not appear to influence *LMNB1* expression, it was measured as an indicator of nuclear integrity, which is commonly reduced in TIS (Martins et al., 2020). *LMNB1* expression was analysed to determine whether metformin selectively targeted specific aspects of senescence, such as the SASP, or if it had a broader impact.

Unlike with the other gene expression markers assessed, MCF10A cells exhibited a strong decrease in *LMNB1* expression (~90% reduction in expression) (Figure 4.27). The TNBC cell lines which showed no significant decrease in *LMNB1* expression, despite MDA-MB-231 having a ~10% reduction in *LMNB1* (Figure 4.27). This difference suggests that *LMNB1* regulation may be cell type dependent. In MCF10A, which have functional p53, TIS may be induced via more canonical p53/p21^{WAF/CIP1} signalling pathways, leading to stronger downregulation of *LMNB1*, than in p53 mutant TNBC. Previous bioinformatics analysis using GEPIA indicated that *LMNB1* expression is elevated in breast invasive carcinoma compared to normal tissue. Therefore, the maintenance of *LMNB1* expression in TNBC cells may be due to its inherently high basal expression. It's role in BC remains controversial, as some studies associate higher *LMNB1* levels with improved patient outcomes, while others suggest the opposite (Lv et al., 2024). As metformin failed to sufficiently influence markers of nuclear integrity, such as *LMNB1* expression and nuclear area, this reinforces the idea that its effects may be restricted to specific types of senescence.

4.6 CONCLUSION

This study aimed to address a gap in the literature by investigating the role of metformin as a modulator of senescence in TNBC, at both physiological and supra-physiological levels. A comprehensive panel of senescence markers were assessed, including β -galactosidase staining, BrdU staining, γ -H2AX staining, nuclear area analysis, and qPCR of key senescence-associated genes (*CXCL1*, *IL1A*, *IL6*, *IL8*, *LMNB1*, *CDKN1A* (p21), and *TP53* (p53)). Metformin did not attenuate any of these markers, suggesting it did not effectively reverse TIS in TNBC, under the conditions of this study. Several factors could account for this, including metformin concentration, duration of recovery time, cell type, ploidy status, or the method of senescence induction. Subsequent chapters will investigate other possible reasons for metformin's limited efficacy, including drug uptake and pathway activation. Despite this, these findings provide insights into TIS in TNBC and contribute to the broader understanding of metformin's role in senescence. Given that only a subset of SASP components were analysed in this study, metformin may have influenced other factors which were not assessed. Therefore, subsequent chapters will explore this through the indirect co-culture of metformin treated TIS TNBC with fibroblasts.

Chapter 5

Mechanisms Limiting Metformin Efficacy in TIS Models

5 MECHANISMS LIMITING METFORMIN EFFICACY IN TIS MODELS

5.1 INTRODUCTION

Metformin is widely recognised as a drug with senomorphic properties; however, its anti-senescence effects extend beyond this, influencing a number of hallmarks associated with senescence (Nehlin, 2023; Zhang et al., 2023). Despite the promising anti-senescence effects of metformin being reported in previous studies (Figure 1.7 and Figure 1.8), comparable results were not observed in the models used in this study. This variability may have been due to a number of reasons, as discussed in chapter 4. However as much of the existing literature uses supra-pharmacological metformin concentrations, to investigate TIS reversal, it was essential to first confirm metformin was capable of entering the cells and inducing downstream effects in the models used, to determine whether this was responsible for the lack of TIS attenuation observed.

5.1.1 Determinants of metformin sensitivity

5.1.1.1 Pathway Activation

A number of metformin's senotherapeutic properties, such as reduced DNA damage and SASP secretions are associated with AMPK activation (Figure 1.7). AMPK is a serine/threonine protein kinase complex comprised of 3 subunits α (catalytic), β (scaffold) and γ (regulatory), responsible for activation, complex structure and energy level sensing respectively (El-Masry, Brown and Dobson, 2012; Kim et al., 2016). High AMP levels, driven by mitochondrial complex I inhibition, activate AMPK by binding allosterically, triggering a conformational change and facilitating phosphorylation of Thr-172 in AMPK α (Hardie, Ross and Hawley, 2012; Kim et al., 2016). Two isoforms of AMPK exist; AMPK α 1 and AMPK α 2, of which AMPK α 1 is detectable in breast tissue and shown to be present in MCF10A, HCC1806, MDA-MB-231 and MCF7, the models used in this study (Yi et al., 2020).

As both a downstream target of AMPK and an independent effector of metformin, mTOR inhibition, quantifiable by a lack of Ser-2448 phosphorylation, can contribute to a reduction of DNA damage, a key feature of TIS (Chiang and Abraham, 2005; Figueiredo, Markworth and Cameron-Smith, 2017). mTOR expression has previously been reported in MDA-MB-231, MCF7, HCC1806 and MCF10A (Alalem, Ray and Ray, 2016; Haga et al., 2020). Previous studies have demonstrated that metformin activates total AMPK α and inhibits mTOR in MCF7 and MDA-MB-231 cells at millimolar concentrations

therefore validating these markers as reliable measures of metformin functionality (Alalem, Ray and Ray, 2016; Liu et al., 2009; Phoenix, Vumbaca and Claffey, 2009; Zhuang and Miskimins, 2008; Zordoky et al., 2014). Given that the functional catalytic subunits of AMPK and mTOR are present in BC models, it raises the question of whether metformin effectively activated these pathways in this study.

5.1.1.2 Drug Transport

Although AMPK activation and mTOR inhibition are key contributors to metformin's senotherapeutic properties, metformin can also act independently of these pathways to elicit its effects. Therefore, it is important to consider whether metformin is entering the cells, in the models used. Metformin is a hydrophilic cation (positively charged) and requires active transport to enter cells (Liang and Giacomini, 2017). The major transporters involved in metformin transport are outlined in Table 5.1. Despite many studies reporting senotherapeutic effects of metformin, most use supra-pharmacological concentrations and provide little information on its cellular transport. Therefore, this study evaluated metformin at both pharmacological and supra-pharmacological concentrations to ensure physiological relevance.

Table 5.1. Summary of key metformin transporters. Table generated using information from Kurlovics et al., 2022; LaMoia and Shulman, 2021; Liang and Giacomini, 2017. Organic cation transporter (OCT), multidrug and toxin extrusion protein (MATE) and plasma membrane monoamine transporter (PMAT).

Transporter	Distribution	Summary
OCT1 (<i>SLC22A1</i>)	Liver, kidney, intestine	Responsible for hepatic absorption and intestinal distribution of metformin. Localised on the basolateral membrane of enterocytes (base of intestinal cells).
OCT2 (<i>SLC22A2</i>)	Kidney	Primarily regulates the secretion of metformin into urine. Localised on the basolateral membrane of renal tubule cells (base of kidney cells).
OCT3 (<i>SLC22A3</i>)	Many tissues including liver, intestinal, skeletal muscle, fat, and brain	Responsible for metformin distribution and absorption in many tissues. Localised on the apical membrane of enterocytes.
MATE1 (<i>SLC47A1</i>)	Kidney and liver	Regulates some uptake and secretion of metformin into urine.
MATE2 (<i>SLC47A2</i>)	Kidney	Regulates secretion of metformin into urine.
PMAT (<i>SLC29A4</i>)	Intestine	Responsible for the intestinal absorption of metformin. Localised on the apical membrane of epithelial cells.

Transporter expression is tissue specific and can influence metformin efficiency. For example, loss of function polymorphisms in OCT transporters have been shown to reduce cellular uptake and therapeutic efficiency of metformin and chemotherapy drugs in patients (Cano et al., 2021; Chowdhury et al., 2016; Naem et al., 2024; Sutherland, Meeson and Lowes, 2020; Wang et al., 2025). Although some findings contradict, previous studies have reported the presence of transporters in MDA-MB-231 and MCF7 (Table 5.2). However, information regarding transporter expression in MCF10A and HCC1806 is lacking. It is therefore important to determine the transporter status of the cell models in this study, to assess whether the absence of an anti-senescence effect was due to limited metformin uptake.

Table 5.2. Summary of transporter expression in MCF7 and MDA-MB-231. Organic cation transporter (OCT), multidrug and toxin extrusion protein (MATE) and plasma membrane monoamine transporter (PMAT).

Transporter	MCF7		MDA-MB-231	
	Expression	Paper	Expression	Paper
OCT1	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020)	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020)
	Yes	(Sutherland, Meeson and Lowes, 2021)	Yes	(Cai et al., 2016; Rajh et al., 2016; Sutherland, Meeson and Lowes, 2021)
OCT2	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020; Sutherland, Meeson and Lowes, 2021)	No	(Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020; Sutherland, Meeson and Lowes, 2021)
			Yes	(Cai et al., 2016)
OCT3	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020; Sutherland, Meeson and Lowes, 2021)	Yes	(Cai et al., 2016; Markowicz-Piasecka et al., 2019; Sutherland, Meeson and Lowes, 2021)
MATE1	Yes	(Cai et al., 2016; Markowicz-Piasecka et al., 2020)	Yes	(Cai et al., 2016; Markowicz-Piasecka et al., 2020)
MATE2	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2020)	No	(Cai et al., 2016; Markowicz-Piasecka et al., 2020)
PMAT	Yes	(Cai et al., 2016; Markowicz-Piasecka et al., 2020)	No	(Cai et al., 2016)
			Yes	(Markowicz-Piasecka et al., 2020)

5.1.2 Cell Models

Alongside the other cell models used in this study, MCF7 cells were included in subsequent experiments as a positive control for metformin function, as they have previously been shown to be metabolically responsive to metformin (Bekezhankyzy et al., 2023; Lopes et al., 2020; Marinello et al., 2016; Queiroz et al., 2014). MCF7 is a luminal A BC, which is oestrogen and progesterone receptor positive. Metformin has been reported to exert antiproliferative effects in MCF7 across a wide range of concentrations (μM -mM) (Bekezhankyzy et al., 2023; Lopes et al., 2020; Marinello et al., 2016; Queiroz et al., 2014). Metformin has also been shown to activate AMPK in MCF7 cells (Kim et al., 2011; Phoenix, Vumbaca and Claffey, 2009; Zhuang and Miskimins, 2008).

5.2 AIMS AND OBJECTIVES

The primary aims of this chapter were to (5.1) investigate the potential reasons for limited metformin efficacy in TIS models and (5.2) assess the relevance of the pre-clinical cell models used in this study to patient gene expression profiles.

To fulfil aim (5.1) the following objectives will be completed:

- Objective 5.1A: Confirm the functional effects of metformin using MTT assays to assess cell viability and BrdU staining to assess replicative alterations in MCF7 cells, as a positive control for metformin function.
- Objective 5.1B: Assess whether key downstream targets of metformin are regulated in accordance with its expected mechanism of action by performing western blots to evaluate activation of key pathway components such as AMPK and mTOR.
- Objective 5.1C: Determine whether cell models express the cation transporters required for metformin uptake using qPCR to assess gene expression and immunofluorescence to examine protein expression. Specifically, this objective aimed to:
 - Assess whether the transporters necessary for metformin uptake are present in cell models.
 - Determine whether transporter expression is altered in TIS samples, potentially affecting metformin uptake.

To fulfil aim (5.2) the following objectives will be completed:

- Objective 5.2A: Perform bioinformatics analysis, using UALCAN and GEPIA to identify relevance of cell models to patient expression profiles.

Two key determinants of metformin sensitivity; pathway activation and cellular uptake will be evaluated to better understand the limited anti-senescence effects observed in the TIS models used in this study. Understanding the limitations of the models used is essential to determine their translational potential, and to ensure they are representative of *in vivo* events.

5.3 MATERIALS AND METHODS

Materials and methods specific to this chapter are described below. Unless otherwise stated, protocols used were those outlined in Chapter 2 (Universal Materials and Methods). This includes TIS induction, MTT assays, BrdU immunofluorescent staining, and statistical analysis. Bioinformatics analysis using GEPIA and UALCAN was performed as described in Chapter 3 (3.3.6 and 3.3.7).

5.3.1 Culture of additional cell lines

In addition to the MDA-MB-231, HCC1806 and MCF10A cell lines previously described, two further immortalised, adherent, cell lines MCF7 and HEPG2 were also cultured (Table 5.3). Both cell lines were kindly donated by staff at the University of Hertfordshire and Anglia Ruskin University. MCF7 and HEPG2 were cultured in High Glucose (GlutaMAX) DMEM (Fisher Scientific, Leicestershire, UK) supplemented with 10% FBS (SLS, Nottinghamshire, UK). Cell lines were maintained as described in Chapter 2.1.

Table 5.3. Summary of additional cell lines used to assess transporter expression.

Cell line	Tissue	Purpose
MCF7	Epithelial cells, isolated from the mammary gland of a female adenocarcinoma patient. Luminal A Breast Cancer.	Positive control for metformin function
HEPG2	Epithelial-like cells, isolated from the liver of a Hepatocellular Carcinoma patient.	Positive control for OCT1 expression

5.3.2 SDS-PAGE and Western Blot

5.3.2.1 Sample preparation

Cells were cultured in 12-well plates as previously described, media was aspirated, and cells were washed once with 1X PBS. Protein lysates were prepared by adding 100 μ L 2x Laemmli buffer (#1610737, Biorad, Hertfordshire, UK) (containing 2-mercaptoethanol, provided by the university, at a 1:20 dilution) to each well. Cells were detached using a cell scraper and lysates were transferred to microcentrifuge tubes where they were denatured, on a heat block, at 100 °C for 5 minutes. Samples were stored at -80 °C until analysis.

5.3.2.2 SDS-PAGE

Protein separation was performed using 4-20% Mini-PROTEAN TGX precast gels (#456-1096, Biorad, Hertfordshire, UK) in a Mini-PROTEAN electrophoresis system (Biorad, Hertfordshire, UK). A total of 15 μ L of each sample and 10 μ L of pre-stained rainbow ladder (Precision Plus Protein Dual Color Standards #1610374, Biorad, Hertfordshire, UK) were loaded per well. AICAR (#28-405-0, Fisher Scientific, Leicestershire, UK) treated cells were used as a positive control as it directly activates AMPK. Gel electrophoresis was run in 1X tank buffer (25 mM Tris, 192 mM Glycine, 0.1% Glycine, supplied by the university) at 60 V for 20 minutes, until samples reached the bottom of the stacking buffer, after which voltage was increased to 100 V for approximately 1.5 hours, until proteins reached the bottom of the resolving gel.

5.3.2.3 Western Blot

Following electrophoresis, the gel, polyvinylidene fluoride (PVDF) membrane, sponges and filter paper were equilibrated in 1X transfer buffer (25 mM Tris base, 192 mM Glycine, 20% methanol, supplied by the university) for 15 minutes. PVDF membranes were pre-activated in methanol for 15 seconds before equilibration. Protein transfer was performed using a Mini-PROTEAN transfer cassette at 100 V for 1 hour. The cassette was assembled from black side to clear with sponge, two sheets of filter paper, PVDF membrane (#88585, Fisher Scientific, Leicestershire, UK), gel, two sheets of filter paper, sponge. Any bubbles were removed using a roller before orientating the black plate of the cassette next to the cathode.

Membranes were cut to isolate regions containing proteins of interest (Table 5.4) and blocked overnight at 4°C in 5% BSA/Tris buffered saline with tween 20 (TTBS) (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, supplied by the university). Membranes were washed three times in TTBS, for 5 minutes each and then incubated with primary antibodies, prepared in 1% BSA/TTBS, for 1 hour at room temperature, on a rocker to ensure complete coverage. Following three 5 minute washes in TTBS, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, prepared in 1% BSA/TTBS, for 1 hour at room temperature, on a rocker. Antibodies used are summarised in Table 5.5. Actin and vinculin were selected as housekeeping proteins as they have been previously validated as loading controls in similar research (Bisso et al., 2013; Kim et al., 2011; Phoenix, Vumbaca and Claffey, 2009).

Table 5.4. Molecular weights of target proteins.

Protein	Molecular Weight (kDa)
Actin	42
AMPK	62
mTOR	289
Vinculin	117

Table 5.5. Antibodies used for western blotting.

Antibody	Type	Dilution	Manufacturer
Actin	HRP-Conjugated Secondary	1:5000	Abcam #ab20272
AMPK	Primary	1:2000	Protein tech #10929-2-ap
Phospho-AMPK alpha-2	Primary	1:500	Invitrogen #MA5-33216
mTOR	Primary	1:5000	Protein tech #66888
Phospho-mTOR (Ser2448)	Primary	1:5000	Protein tech #80596
Vinculin	Primary	1:500	Invitrogen #42H89L44
Goat-anti mouse HRP conjugated	HRP-Conjugated Secondary	1:2000	Protein tech #SA00001-1
Goat-anti rabbit HRP conjugated	HRP-Conjugated Secondary	1:2000	Protein tech #SA00001-2

5.3.2.4 Detection

Proteins were visualised using Novex™ Enhanced Chemiluminescence (ECL) Substrate Reagent Kit (#WP20005, Fisher Scientific, Leicestershire, UK) and imaged using the iBright™ FL1500 Imaging System (Fisher Scientific, Leicestershire, UK). Set exposures of 1 minute were used, to facilitate

comparative band intensity analysis. Band sizes were estimated by overlaying the pre-stained molecular weight marker onto the chemiluminescent image. Images were processed and labelled using Fiji (Image J) (Schindelin et al., 2012) and Microsoft PowerPoint.

5.3.2.5 Band Intensity Analysis

Band intensities were quantified using Fiji (ImageJ). For each blot, individual lanes were selected, and intensity profiles were generated using the Plot Lanes function. The peak area corresponding to each band was measured to determine signal intensity. Band intensities were normalised to the loading control using Equation 7. Phosphorylation change was calculated as the proportion of phosphorylated to total protein using Equation 8. Fold change, relative to the untreated controls was determined using Equation 9. Bar graphs were plotted in GraphPad Prism to visualise the fold change in phosphorylation relative to untreated controls.

Equation 7

$$\text{Normalised Band Intensity} = \frac{\text{Band Intensity}_{\text{Protein of Interest}}}{\text{Band Intensity}_{\text{Loading Control}}}$$

Equation 8

$$\text{Phosphorylation Change} = \frac{\text{Normalised Phosphorylated Protein}}{\text{Normalised Total Protein}}$$

Equation 9

$$\text{Fold Change} = \frac{\text{Phosphorylation Change}_{\text{Sample}}}{\text{Phosphorylation Change}_{\text{Untreated}}}$$

5.3.2.6 Stripping optimisation

Two antibody stripping conditions were evaluated to optimise membrane re-probing. A stringent buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, supplied by the university) was applied for 15 or 30 minutes at 50°C, and a mild buffer (200 mM glycine, 3.5 mM SDS, 1% Tween-20, pH 2.2, supplied by the university) for 10 minutes at room temperature. Membranes were

subsequently washed in TTBS and re-imaged with ECL substrate to confirm complete antibody removal.

5.3.3 qPCR

RNA extraction, cDNA synthesis and qPCR were performed as described in Chapter 2 (Universal Materials and Methods). Gene expression was analysed using the primers shown in Table 5.6, which were designed to detect the transporters present in MCF7, MDA-MB-231, HCC1806 and MCF10A, as well as HEPG2 which was included as a positive control for OCT1 expression. Primers for the housekeeping genes *GAPDH* and *PUM1* are shown in Table 2.8.

Table 5.6. Primers used to assess expression of key transporters.

Transporter	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Product Size (bp)
<i>SLC22A1</i> (OCT1)	TGT CAC CGA AAA GCT GAG CC	TCC GTG AAC CAC AGG TAC ATC	96
<i>SLC22A3</i> (OCT3)	AGG TAT GGC AGG ATC GTC ATT	GCA GGA AGC GGA AGA TCA CA	112
<i>SLC47A1</i> (MATE1)	GCC CTC GCC AAC TAT CTG TT	AGA GGA GTA GAG CCA GGG TG	100
<i>SLC29A4</i> (PMAT)	GAT GAT CTC TCT GAG CCG CAT	CAC CAG GAA GAA GAT GAG CGT	79

5.3.3.1 Normalisation of gene expression in untreated cells

Expression of transporters in untreated cells was normalised to housekeeping gene expression. Relative quantification was performed using the ΔC_t method (Equation 10), with *GAPDH* and *PUM1* serving as housekeeping genes. Fold change in transporter expression between doxorubicin treated and untreated cells was calculated as described in Chapter 2.16.1 using $2^{-\Delta\Delta C_t}$.

Equation 10

$$\text{Fold Change } (\Delta C_t) = 2^{-[C_{tGOI} - C_{tHKG}]}$$

GOI: Gene of interest, HKG: Housekeeping gene

5.3.4 Fluorescent staining: OCT3 and PMAT

PMAT and OCT3 were selected as transporters for investigation of protein expression. Staining was performed as described in Chapter 2.10 with the following amendments. Cells were washed once with PBS and fixed with 4% paraformaldehyde (diluted in PBS) for 15 minutes, followed by three 5 minute PBS washes. For OCT3 staining, cells were permeabilised with 0.1% Triton X-100, provided by the university, for 10 minutes, then washed three times with PBS. For PMAT staining, cells were instead permeabilised throughout the protocol with 0.02% saponin, which was included in the blocking solution and during both primary and secondary antibody incubations.

Cells were blocked in 10% BSA in PBS for 1 hour and subsequently washed three times with PBS. OCT3 primary antibody (#PA5-101735, Fisher Scientific, Leicestershire, UK) was supplied at 1 mg/mL and diluted 1:100 in PBS to a final concentration of 10 µg/mL. PMAT primary antibody (#PA-65742, Fisher Scientific, Leicestershire, UK) was supplied at 0.1 mg/mL and diluted 1:50 in 5% BSA PBS, containing 0.02% saponin, to a final concentration of 2 µg/mL.

Following an hour's incubation with primary antibody, at room temperature, slides were incubated with, Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594 (#SA5-10040, Fisher Scientific, Leicestershire, UK) for 1 hour, in the dark, due to the light sensitivity of the antibody. The secondary antibody was supplied at 0.5 mg/mL and diluted to 5 µg/mL in PBS for OCT3 or in PBS containing 0.02% saponin for PMAT.

After incubation, coverslips were washed nine times with PBS, before being rinsed in deionised water and mounted on microscope slides using DAPI mountant (Fisher Scientific, Leicestershire, UK). Slides were cured overnight, at room temperature, before visualisation. Microscope slides were stored in the dark, due to their light sensitivity.

5.3.4.1 Stain visualisation and interpretation

Slides were imaged using a Zeiss Axioimager M2 microscope (fitted with an Axiocam 503 imaging device), paired with Zen Blue software (Zeiss, Cambridge, UK). The DyLight™ 594 fluorophore appeared red and was detected using the AF594 channel

(Excitation/Emission: 587/614 nm). All samples were imaged at 40X magnification with set exposure times of 2 seconds for AF594 and 70 milliseconds for DAPI, to facilitate comparative intensity analysis.

Quantification of staining intensity was performed using Fiji (Image J) (Schindelin et al., 2012). Single channel images, from at least 3 fields of view, for DAPI and AF594 were exported from Zen Blue software and converted to 16-Bit Greyscale images in Image J. The Mean Grey Intensity (MGI) of the AF594 channel was measured and normalised to the number of cells, determined from DAPI stained nuclei (Equation 11).

Equation 11

$$\text{Mean Grey Intensity (per cell)} = \frac{\text{Mean Grey Transporter Intensity}}{\text{Number of Cells}}$$

In greyscale images, each pixel represents an intensity value, 0 (black) to 255 (white). The MGI corresponds to the average pixel intensity across the image, where higher values indicate greater overall fluorescence intensity. Results are expressed in arbitrary units (AU). Expression of transporters was also analysed in doxorubicin treated cells to establish whether their expression was maintained in TIS cells. Protein expression in doxorubicin treated cells was normalised to untreated controls by dividing the treated MGI per cell by the untreated MGI per cell for each sample. Results are presented as fold change. Normalised MGI per cell and fold change MGI was plotted using GraphPad Prism, SD was displayed to represent sampling variability.

5.4 RESULTS

5.4.1 Confirming functional effects of metformin in MCF7

MTT assays were performed to assess the cytotoxic effects of metformin on MCF7, following a 96 hour exposure, which corresponds to the treatment duration used in TIS models. A statistically significant reduction in viability was observed at metformin concentrations $\geq 500 \mu\text{M}$ (Figure 5.1). Viability is presented as a proportion of the untreated control to illustrate the relative cytotoxicity of metformin treatment.

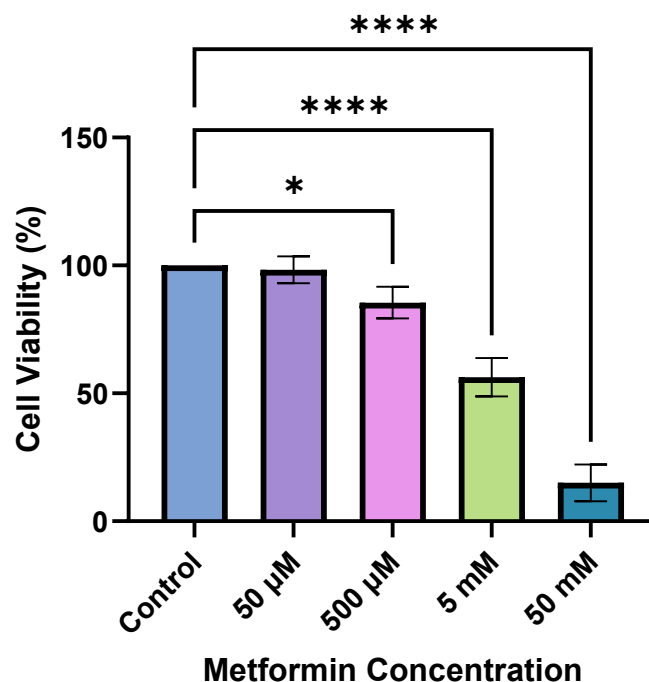


Figure 5.1. Effect of Metformin on MCF7 cell viability, assessed using MTT assays. Cell viability was assessed following a 96 hour metformin treatment. Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed at concentrations $\geq 500 \mu\text{M}$. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

BrdU staining was to assess proliferation changes in response to metformin. As 50 mM metformin resulted in a substantial loss of viability, 10 mM metformin was selected as the upper concentration limit, as this concentration has previously been reported to induce cell cycle arrest in MCF7 (Queiroz et al., 2014). Representative images of BrdU positive (A) and negative (B) cells are shown in Figure 5.2. Although a concentration dependent decrease in proliferation was observed this was not statistically significant (Figure 5.3).

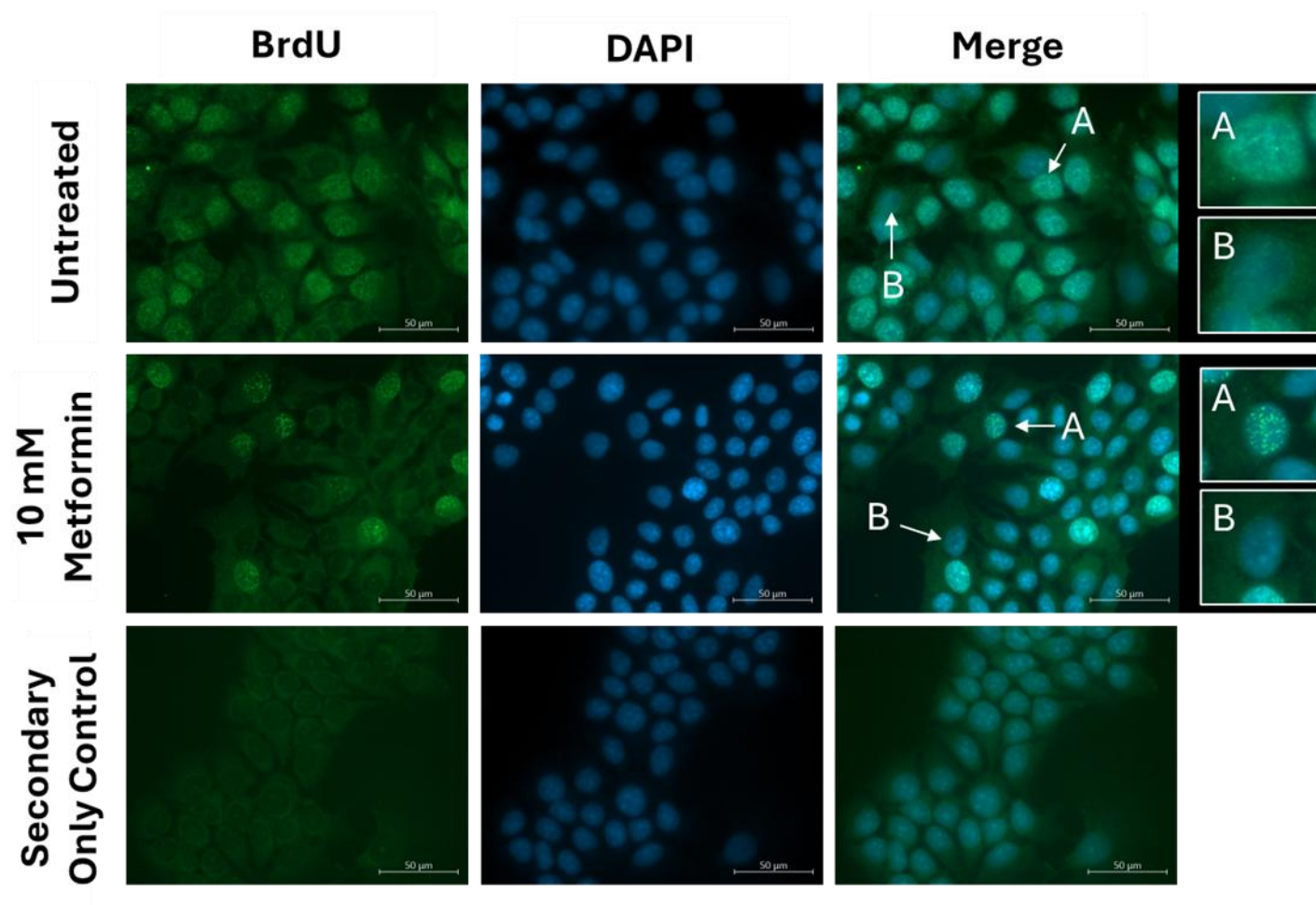


Figure 5.2. Representative fluorescence microscopy images illustrating BrdU staining in MCF7 cells. Untreated cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. Images were captured at 40× magnification and have a scale bar of 50 µm. Representative images show untreated MCF7, 10 mM metformin treated MCF7 and an untreated secondary antibody only control. BrdU fluorescence was visualised using the AF488 channel (ex/em: 492/519 nm) for 1.6 s, while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 420 ms. Individual fluorescence channel images are shown alongside the merged images. The merged images combine both fluorescence channels, where A) represents a BrdU positive cell and B) a negative cell.

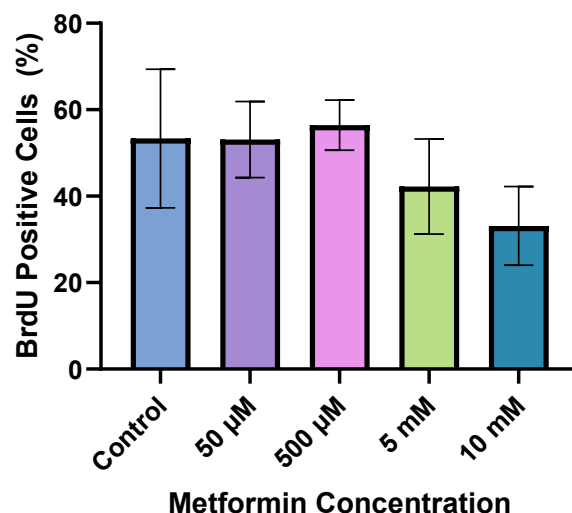


Figure 5.3. Effect of metformin treatment on BrdU staining in MCF7 cells. Cells were stained using BrdU primary antibody and DyLight™ 488 Goat Anti-Mouse IgG secondary antibody. A total of 150 cells were counted to determine the percentage of BrdU positive cells. No statistical differences were observed in any samples compared to the untreated control. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using one way ANOVA.

5.4.2 Regulation of key downstream targets in response to metformin treatment

Phosphorylation of AMPK and dephosphorylation of mTOR are both expected downstream effects of metformin (Alalem, Ray and Ray, 2016; Liu et al., 2009; Phoenix, Vumbaca and Claffey, 2009; Zhuang and Miskimins, 2008; Zordoky et al., 2014). Western blotting was performed to evaluate these effects. AICAR treated cells served as a positive control for AMPK activation, as AICAR has previously been shown to induce a strong phosphorylation response (El-Masry, Brown and Dobson, 2012; Fodor et al., 2016; Kaur et al., 2021; Lützner et al., 2012; Tripathi et al., 2022).

The data shown represents preliminary findings obtained during optimisation of the western blot protocol, as limited reagent availability and time constraints prevented additional replicates. Optimisation of membrane stripping resulted in protein loss, making these membranes unusable. As a result, only the original blots, probed once with either total or phosphorylated antibodies, were analysed. The data presented is derived from a single experiment, therefore statistical analysis could not be applied.

Samples were normalised to the loading controls vinculin and actin, as protein loading was not normalised prior to electrophoresis. Both loading controls showed variable expression between cell

lines, and in some incidences between samples. Although previously validated as loading controls in similar research (Bisso et al., 2013; Kim et al., 2011; Phoenix, Vumbaca and Claffey, 2009), vinculin and actin were not analysed for stability in this study, due to resource limitations. To minimise variability, the average intensity of both loading controls was used for normalisation. GAPDH would have been the preferred loading control, as this would have been consistent with the housekeeping gene used in qPCR analysis, however due to reagent limitations, this was not possible.

For each cell line, the phosphorylation ratio (proportion of phosphorylated total protein) relative to untreated controls is presented in Figure 5.4, Figure 5.5, Figure 5.6 and Figure 5.7 alongside representative blot images. Bands corresponding to total and phosphorylated forms were detected at the expected molecular weights: actin (42 kDa), AMPK (62 kDa), mTOR (289 kDa), and vinculin (117 kDa). Results showed distinct patterns of mTOR and AMPK phosphorylation between TNBC (HCC1806 and MDA-MB-231), luminal A (MCF7) and non-tumorigenic (MCF10A) cell lines.

In HCC1806 cells (Figure 5.4) TIS samples had increased phosphorylation of both mTOR, and AMPK compared to untreated controls. Slight increases in AMPK and mTOR phosphorylation were observed in 50 μ M metformin treated samples; however, this effect was not evident at 500 μ M. AMPK phosphorylation in metformin + TIS samples was slightly lower than in TIS samples, whilst mTOR phosphorylation remained relatively consistent across TIS conditions. AICAR treatment strongly induced AMPK phosphorylation but did not reduce mTOR phosphorylation below basal levels.

In MDA-MB-231 cells (Figure 5.5) TIS samples had increased phosphorylation of both mTOR and AMPK compared to untreated controls. Unlike in HCC1806, mTOR phosphorylation did not decrease below basal levels in any treatment group. Phosphorylation of mTOR was higher in TIS samples and further increased with rising metformin concentrations. Similarly, AMPK phosphorylation was higher in metformin treated samples compared to the untreated control and increased in a dose dependent manner, where a stronger effect was observed at 500 μ M than at 50 μ M metformin. This trend was also observed when comparing metformin treated TIS samples to TIS. As in HCC1806, AICAR treatment strongly induced AMPK phosphorylation but did not reduce mTOR phosphorylation below basal levels.

In MCF10A cells (Figure 5.6) TIS samples had increased AMPK phosphorylation and similar mTOR phosphorylation relative to the untreated control. Treatment with 50 μ M metformin resulted in decreased phosphorylation of both AMPK and mTOR, whilst at 500 μ M, mTOR phosphorylation was slightly reduced and AMPK phosphorylation increased, compared to the untreated control. Although AMPK phosphorylation was elevated in TIS samples, compared to the untreated control, both 50 μ M and 500 μ M metformin treatments reduced AMPK phosphorylation relative to the TIS only sample. AICAR treatment reduced both mTOR and AMPK phosphorylation below basal levels.

In MCF7 cells (Figure 5.7) 50 μ M and 500 μ M metformin treatment increased AMPK phosphorylation, indicative of metformin pathway activation, despite also increasing mTOR phosphorylation. Phosphorylation levels decreased below those of the untreated control at 5 mM and 10 mM metformin treatment, suggesting a loss of pathway activation at cytotoxic doses (Figure 5.1). AICAR failed to induce AMPK phosphorylation.

Overall, TIS, metformin and AICAR treatments elicited cell line specific effects on AMPK and mTOR phosphorylation. TIS consistently increased AMPK phosphorylation in all cell lines. Relative to their respective untreated controls, HCC1806, MDA-MB-231 and MCF7 showed an increase in AMPK phosphorylation at 50 μ M metformin, with MDA-MB-231 and MCF7 showing stronger activation at 500 μ M. Whilst both TNBC cell lines (HCC1806 and MDA-MB-231) either maintained or enhanced mTOR phosphorylation across treatment groups, mTOR phosphorylation was decreased in metformin treated MCF10A and in MCF7 at higher concentrations. AICAR strongly induced AMPK phosphorylation in the TNBC cell lines HCC1806 and MDA-MB-231 but failed to activate AMPK in MCF7 and MCF10A.

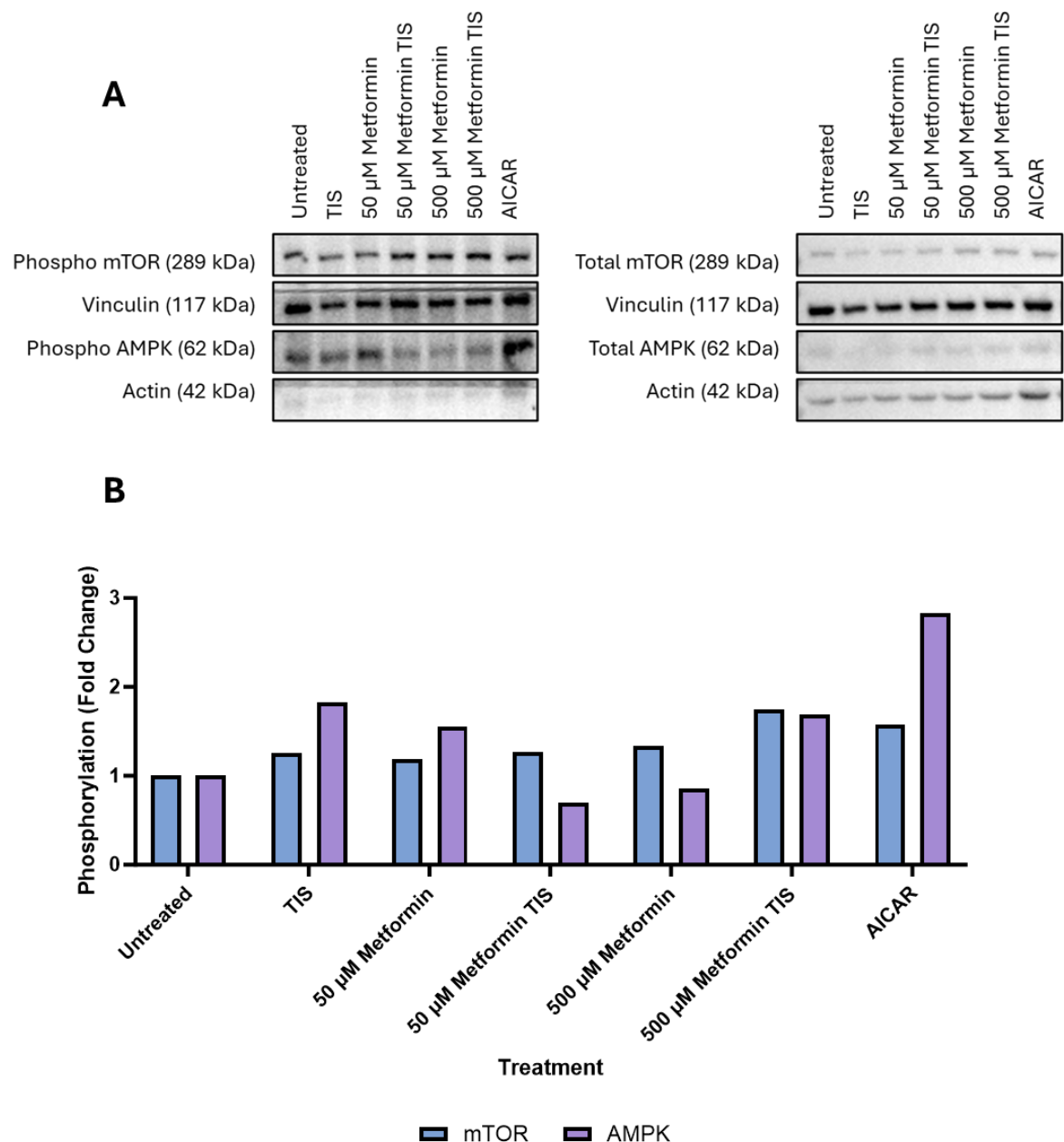
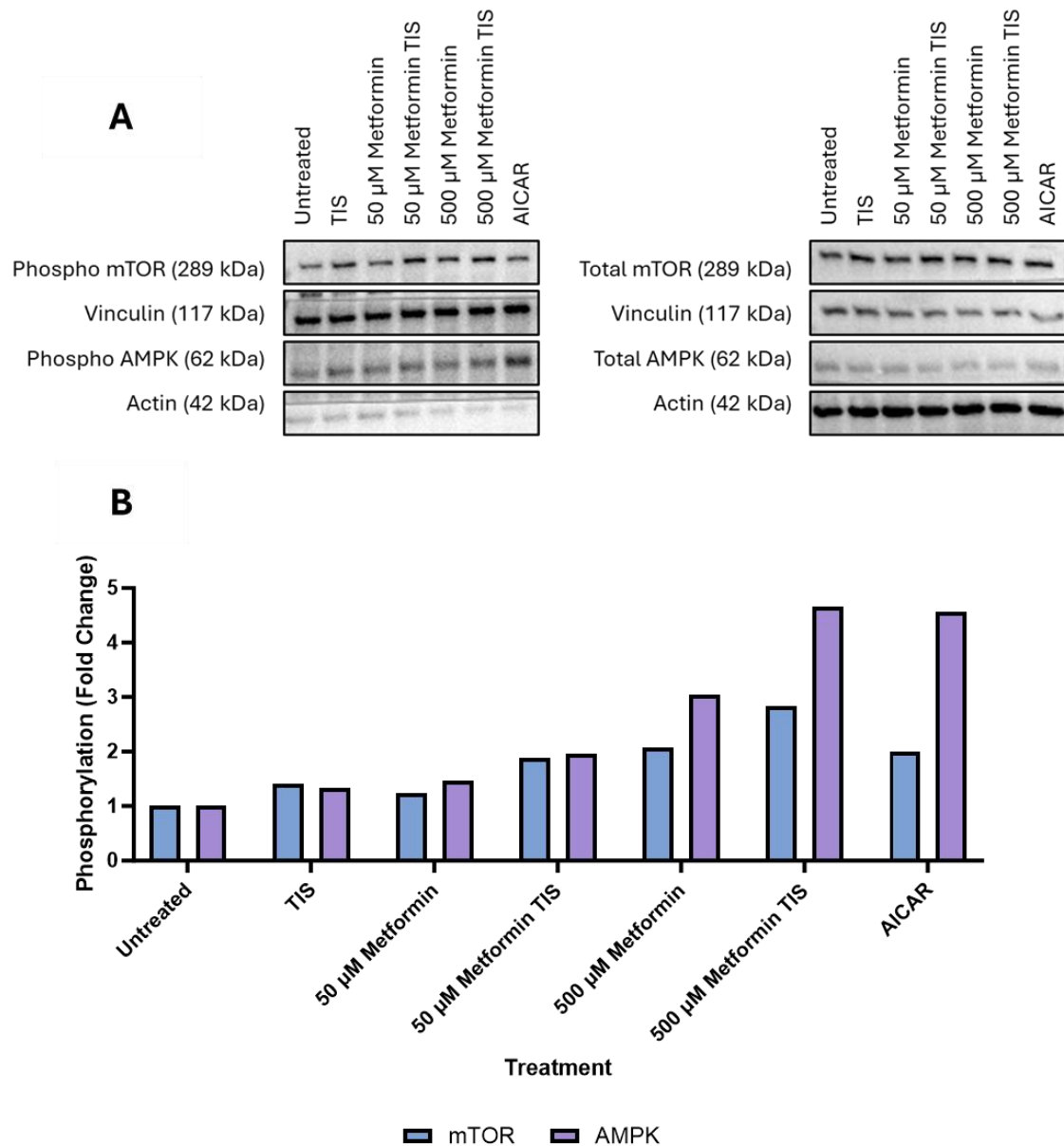
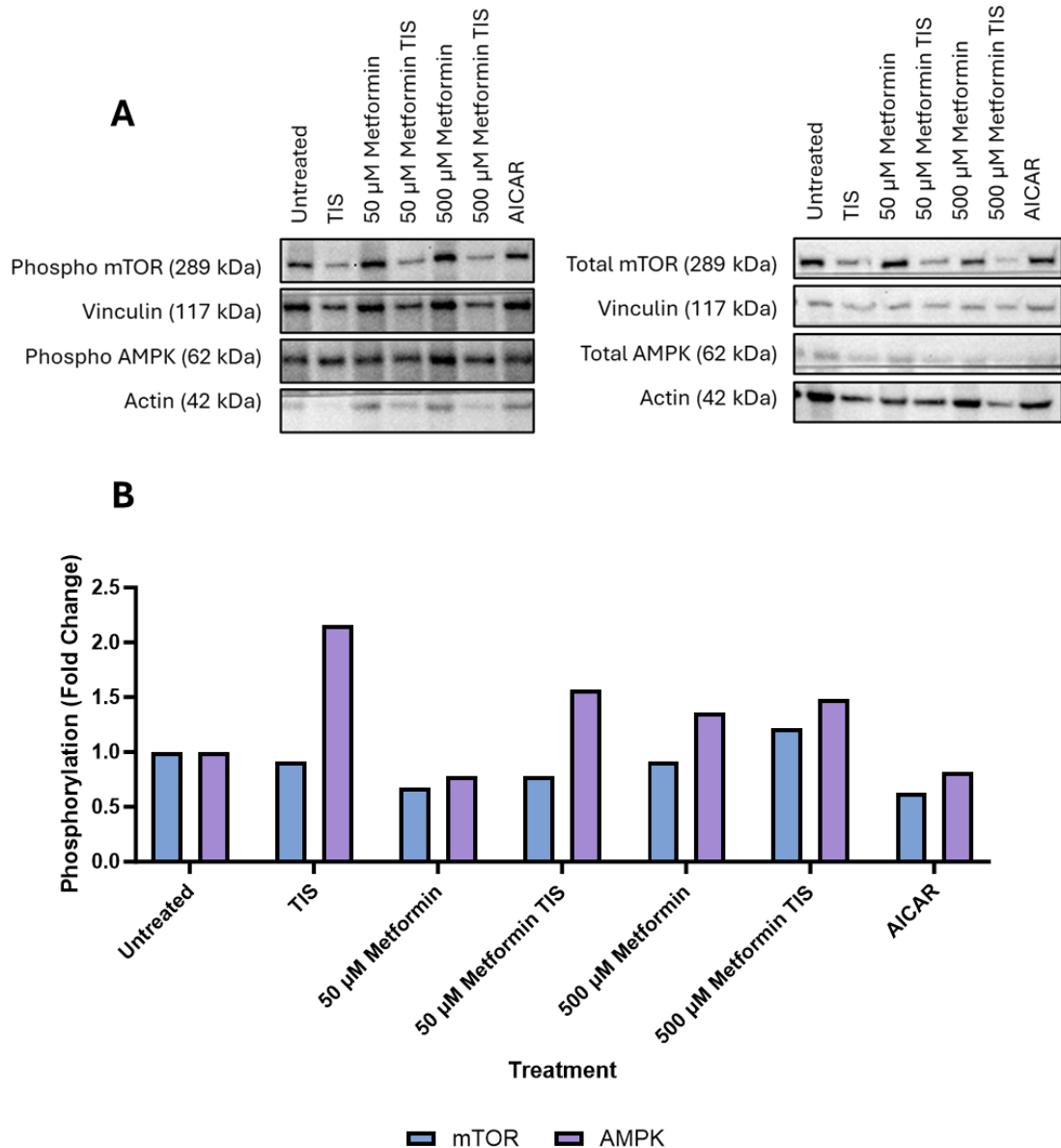


Figure 5.4. Western Blot analysis of AMPK and mTOR phosphorylation in HCC1806 cells. Untreated, therapy-induced senescent (TIS), 50 μ M metformin, 50 μ M metformin + TIS, 500 μ M metformin, 500 μ M metformin + TIS, and AICAR treated samples were analysed. (A) Chemiluminescent images show expression levels of phosphorylated and total mTOR, phosphorylated and total AMPK, vinculin, and actin in HCC1806 cells. Band sizes were estimated by overlaying the pre-stained molecular weight marker onto the chemiluminescent image. Antibodies were probed on separate blots. (B) Graph shows the phosphorylation ratio (phosphorylated protein relative to total protein) relative to the untreated control (fold change). TIS samples had increased phosphorylation of both mTOR and AMPK compared to untreated cells. Slight increases in AMPK and mTOR phosphorylation were observed in 50 μ M metformin treated samples; however, this effect was not evident at 500 μ M. AMPK phosphorylation in metformin + TIS samples was slightly lower than in TIS samples, whilst mTOR phosphorylation remained relatively consistent across TIS conditions. AICAR treatment strongly induced AMPK phosphorylation but did not reduce mTOR phosphorylation below basal levels. Data represent preliminary findings from a single experiment; therefore, statistical analysis could not be applied.



MDA-MB-231

Figure 5.5. Western Blot analysis of AMPK and mTOR phosphorylation in MDA-MB-231 cells. Untreated, therapy-induced senescent (TIS), 50 μ M metformin, 50 μ M metformin + TIS, 500 μ M metformin, 500 μ M metformin + TIS, and AICAR treated samples were analysed. (A) Chemiluminescent images show expression levels of phosphorylated and total mTOR, phosphorylated and total AMPK, vinculin, and actin in MDA-MB-231 cells. Band sizes were estimated by overlaying the pre-stained molecular weight marker onto the chemiluminescent image. Antibodies were probed on separate blots. (B) Graph shows the phosphorylation ratio (phosphorylated protein relative to total protein) relative to the untreated control (fold change). TIS samples had increased phosphorylation of both mTOR and AMPK compared to untreated cells. mTOR phosphorylation did not decrease below basal levels in any treatment group. Phosphorylation of mTOR was higher in TIS samples and further increased with rising metformin concentrations. AMPK phosphorylation was higher in metformin treated samples compared to the untreated control and increased in a dose dependent manner, where a stronger effect was observed at 500 μ M than at 50 μ M metformin. A similar trend was observed when comparing metformin treated TIS samples to TIS. AICAR treatment strongly induced AMPK phosphorylation but did not reduce mTOR phosphorylation below basal levels. Data represent preliminary findings from a single experiment; therefore, statistical analysis could not be applied.



MCF10A

Figure 5.6. Western Blot analysis of AMPK and mTOR phosphorylation in MCF10A cells. Untreated, therapy-induced senescent (TIS), 50 μ M metformin, 50 μ M metformin + TIS, 500 μ M metformin, 500 μ M metformin + TIS, and AICAR treated samples were analysed. (A) Chemiluminescent images show expression levels of phosphorylated and total mTOR, phosphorylated and total AMPK, vinculin, and actin in MCF10A cells. Band sizes were estimated by overlaying the pre-stained molecular weight marker onto the chemiluminescent image. Antibodies were probed on separate blots. (B) Graph shows the phosphorylation ratio (phosphorylated protein relative to total protein) relative to the untreated control (fold change). TIS samples had increased AMPK phosphorylation and similar mTOR phosphorylation relative to the untreated control. Treatment with 50 μ M metformin resulted in decreased phosphorylation of both AMPK and mTOR, whilst at 500 μ M, mTOR phosphorylation was slightly reduced and AMPK phosphorylation increased, compared to the untreated control. Although AMPK phosphorylation was elevated in TIS samples, compared to the untreated control, both 50 μ M and 500 μ M metformin treatments reduced AMPK phosphorylation relative to the TIS only sample. AICAR treatment reduced both mTOR and AMPK phosphorylation below basal levels. Data represent preliminary findings from a single experiment; therefore, statistical analysis could not be applied.

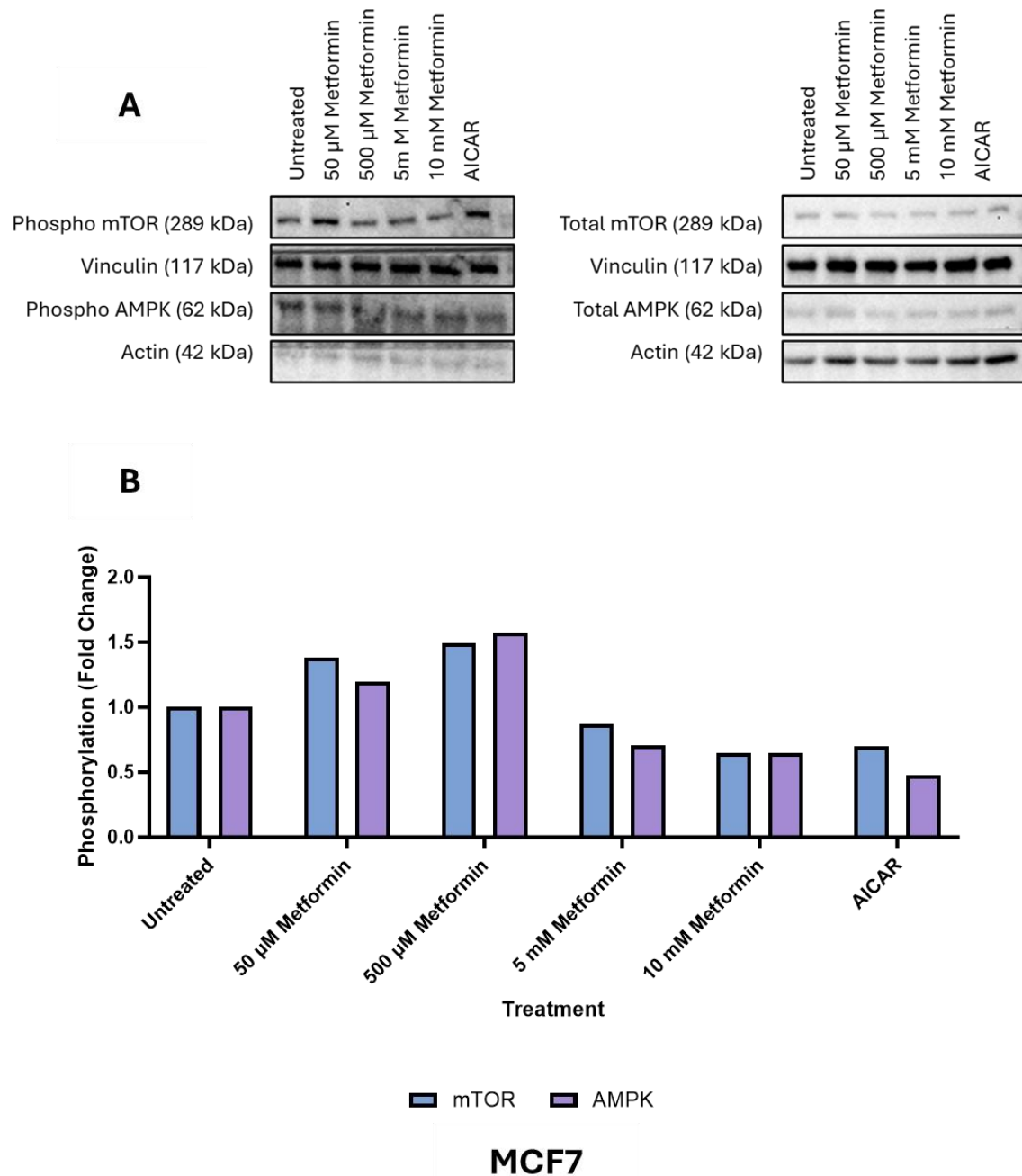


Figure 5.7. Western Blot analysis of AMPK and mTOR phosphorylation in MCF7 cells. Untreated, 50 μ M metformin, 500 μ M metformin, 5 mM metformin, 10 mM metformin, and AICAR treated samples were analysed. (A) Chemiluminescent images show expression levels of phosphorylated and total mTOR, phosphorylated and total AMPK, vinculin, and actin in MCF7 cells. Band sizes were estimated by overlaying the pre-stained molecular weight marker onto the chemiluminescent image. Antibodies were probed on separate blots. (B) Graph shows the phosphorylation ratio (phosphorylated protein relative to total protein) relative to the untreated control (fold change). At 50 μ M and 500 μ M metformin, treatment increased AMPK and mTOR phosphorylation, however phosphorylation levels decreased below those of the untreated control at 5 mM and 10 mM. AICAR did not induce AMPK phosphorylation. Data represent preliminary findings from a single experiment; therefore, statistical analysis could not be applied.

5.4.3 Expression of transporters involved in metformin uptake

Initially gene expression of key metformin transporters *SLC22A1* (OCT1), *SLC22A3* (OCT3), *SLC29A4* (PMAT) and *SLC47A1* (MATE1) were analysed in MDA-MB-231, HCC1806, MCF10A and MCF7, using qPCR. HEPG2 was included as a positive control for *SLC22A1* (OCT1). Based on these results, two transporters, OCT3 and PMAT, were selected for further investigation of protein expression, using immunofluorescence.

5.4.3.1 Gene expression of transporters

Gene expression was first analysed in untreated cells to establish basal expression levels and then in TIS cells to determine whether doxorubicin altered transporter expression. Transporter expression in each cell line is shown in Figure 5.8 (A-E), with panel F comparing expression between cell lines. Consistent with previously published research (Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020), gene expression in untreated cells was normalised to housekeeping genes whilst expression in doxorubicin treated samples was normalised to untreated controls. Expression of transporters therefore appeared low, as their abundance was relatively low compared with the housekeeping genes (transporter Ct ~30 in comparison to the housekeeping gene Ct~20). Melt curve analysis showed a single peak, and no signal was detected in NTCs, indicating that the observed signal was weak, specific expression rather than non-specific amplification.

HEPG2 served as a positive control, confirming that the *SLC22A1* (OCT1) primers were functional (Figure 5.8). In MDA-MB-231, HCC1806, and MCF10A, all transporters were expressed at varying levels: in HCC1806, *SLC47A1* (MATE1) showed the highest expression followed by *SLC29A4* (PMAT); in MDA-MB-231, *SLC22A3* (OCT3) was highest followed by *SLC47A1* (MATE1); and in MCF10A, *SLC29A4* (PMAT) was highest followed by *SLC22A1* (OCT1). MCF7 only expressed PMAT and *SLC47A1* (MATE1). The strongest overall transporter expression was observed in MDA-MB-231, which had the highest *SLC22A3* (OCT3) expression, followed by *SLC29A4* (PMAT) expression in MCF7. Based on these results, PMAT and OCT3 were selected for further investigation. *SLC29A4* (PMAT) was present in MCF7, the positive control for metformin function, and expressed in the other three cell lines, albeit at lower levels. *SLC22A3* (OCT3) was chosen due to its established role in peripheral metformin uptake and its expression in the three breast cell models used in this study (Liang and Giacomini, 2017). In comparison, *SLC22A1* (OCT1) exhibited lower expression and *SLC47A1* (MATE), whilst expressed, was not investigated further due to its primary role in metformin excretion rather than uptake.

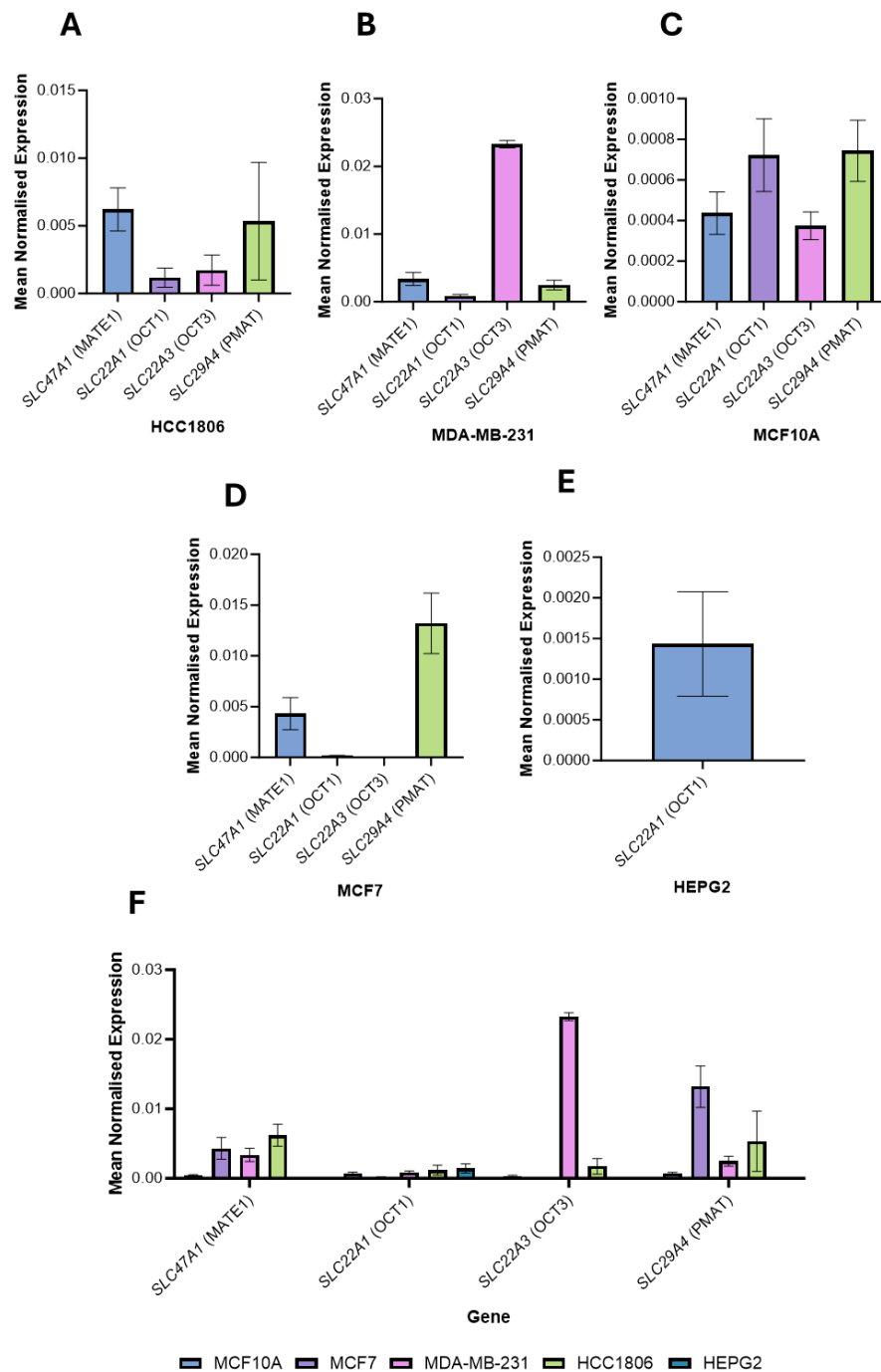


Figure 5.8. qPCR analysis of key metformin transporters in immortalised cell lines. Gene expression is represented as mean normalised expression, quantified using the ΔC_t method and standardised to two housekeeping genes (GAPDH and PUM1). Data is shown for (A) HCC1806, (B) MDA-MB-231, (C) MCF10A, (D) MCF7 and (E) HEPG2 cells. A comparative summary of each cell line and transporter is shown in (F). HEPG2 served as a positive control, confirming that the *SLC22A1* (OCT1) primers were functional. In MDA-MB-231, HCC1806, and MCF10A, all transporters were expressed at varying levels: in HCC1806, *SLC47A1* (MATE1) showed the highest expression followed by *SLC29A4* (PMAT); in MDA-MB-231, *SLC22A3* (OCT3) was highest followed by *SLC47A1* (MATE1); and in MCF10A, *SLC29A4* (PMAT) was highest followed by *SLC22A1* (OCT1). MCF7 only expressed *SLC29A4* (PMAT) and *SLC47A1* (MATE1). The strongest overall transporter expression was observed in MDA-MB-231, which had the highest *SLC22A3* (OCT3) expression, followed by *SLC29A4* (PMAT) expression in MCF7. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates.

Expression of transporters was then analysed in doxorubicin treated cells (Figure 5.9) to establish whether their expression was maintained in TIS cells. As transporter expression was analysed in TIS cells relative to untreated cells, the resulting expression levels differed from those observed when analysing transporter expression in untreated cells relative to housekeeping genes. Overall, expression of all transporters increased in all three BC cell lines, except for *SLC47A1* (MATE1) in MCF10A, which decreased in response to doxorubicin. Interestingly, *SLC29A4* (PMAT), one of the selected candidates, was significantly increased in all three cell lines. *SLC22A3* (OCT3) expression also increased, particularly in MDA-MB-231, whilst *SLC22A1* (OCT1) expression was significantly elevated in MCF10A.

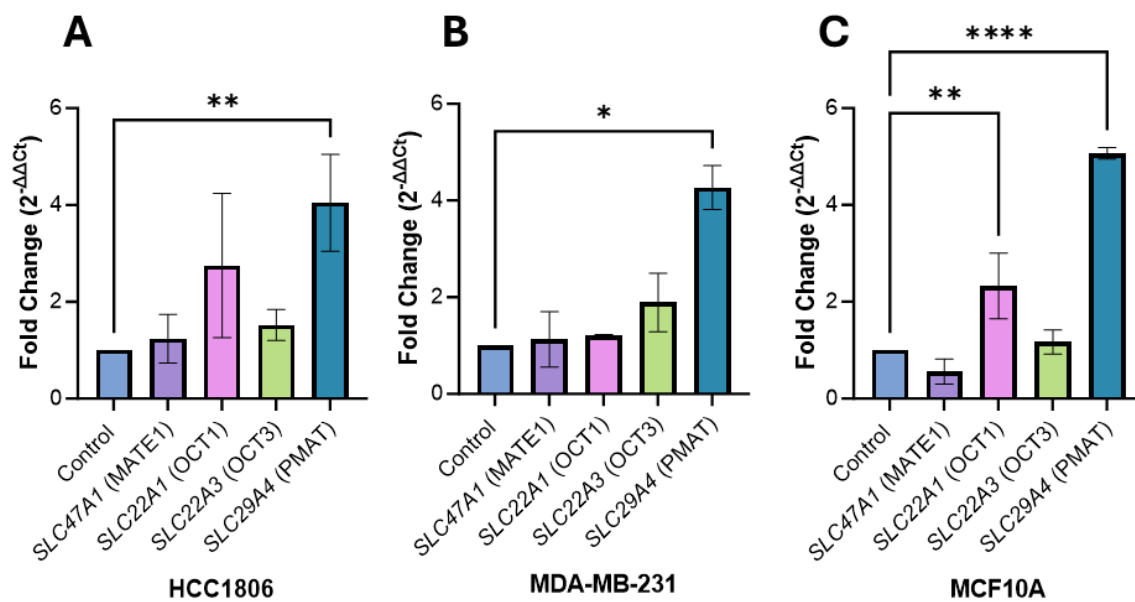


Figure 5.9. qPCR analysis of key metformin transporters in doxorubicin treated immortalised cell lines. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes (GAPDH and PUM1). The control sample is normalised to a baseline fold change of 1. Data is shown for (A) HCC1806, (B) MDA-MB-231, and (C) MCF10A cells. Expression of all transporters increased in all three breast cancer cell lines in response to doxorubicin treatment, except for *SLC47A1* (MATE1) in MCF10A, which decreased. *SLC29A4* (PMAT) was significantly increased in all three cell lines. *SLC22A3* (OCT3) expression also increased, particularly in MDA-MB-231, whilst *SLC22A1* (OCT1) expression was significantly elevated in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test for HCC1806 and MCF10A, and the Kruskal-Wallis test for MDA-MB-231, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

5.4.3.2 Localisation of transporters

Immunofluorescence images were primarily analysed to assess presence of transporters at a protein level. However, transporter localisation was also reviewed to determine whether OCT3 and PMAT were present at the plasma membrane as expected (Liang and Giacomini, 2017). Figure 5.10, Figure 5.11 and Figure 5.12 show representative images of OCT3 expression in MDA-MB-231, HCC1806 and MCF10A cells, whilst Figure 5.13, Figure 5.14, Figure 5.15 and Figure 5.16 show representative images of PMAT expression in MDA-MB-231, HCC1806, MCF10A and MCF7 cells. Secondary only controls showed negligible signal, indicating antibodies were specific to the target proteins. OCT3 staining appeared strongest in doxorubicin treated samples, with the most intense fluorescence observed in MDA-MB-231. OCT3 staining was predominantly cytoplasmic. PMAT fluorescence intensity was visually weaker than OCT3, which aligns with the lower MGI values quantified in Figure 5.17. The strongest PMAT staining was observed in MCF7 cells. PMAT staining appeared more diffuse compared to OCT3, and in MDA-MB-231 was primarily localised to the cytoplasm.

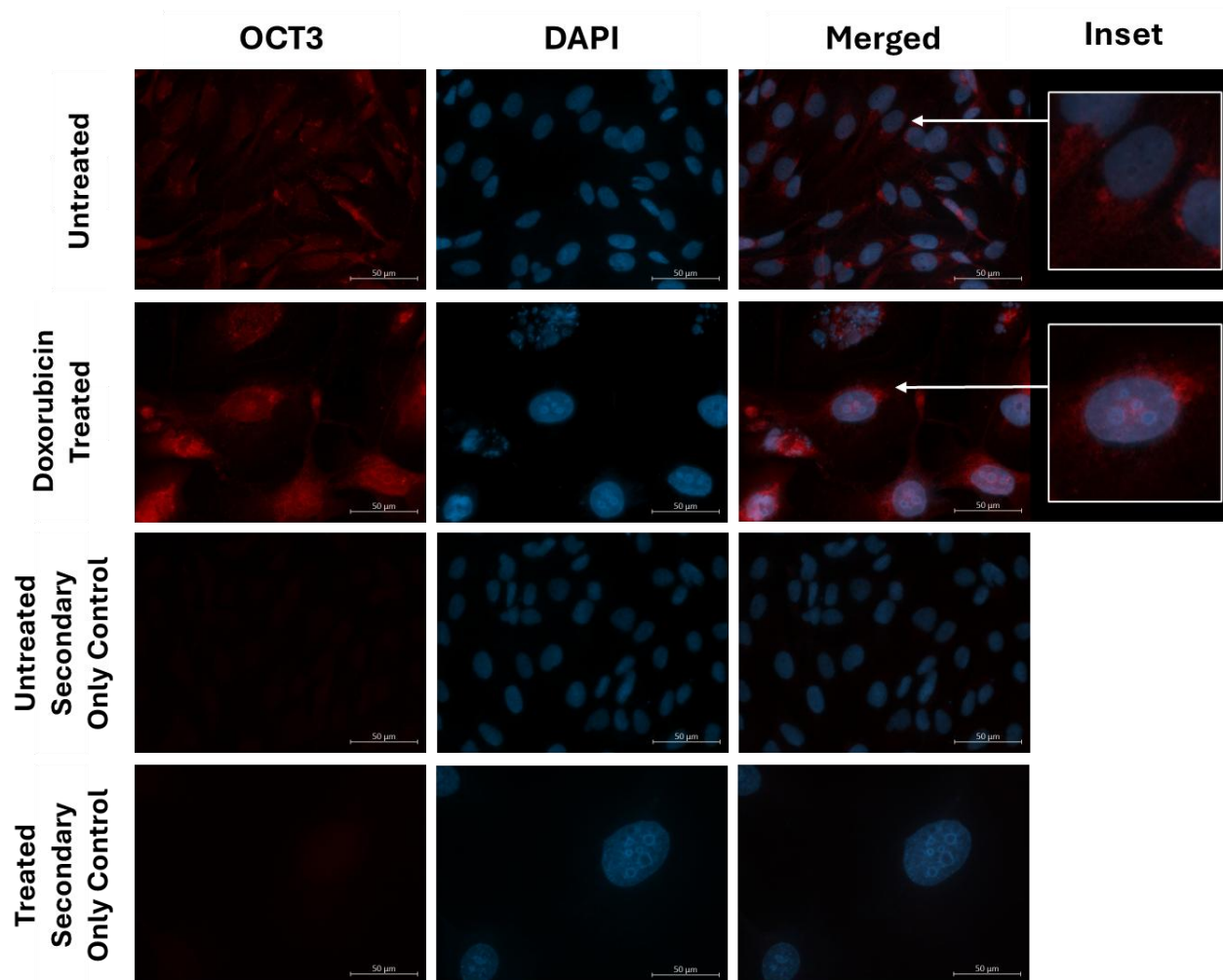


Figure 5.10. Representative fluorescence microscopy images illustrating OCT3 staining in MDA-MB-231 cells. Cells were stained using OCT3 primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 175nM doxorubicin, and corresponding secondary only antibody controls. OCT3 fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm) for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

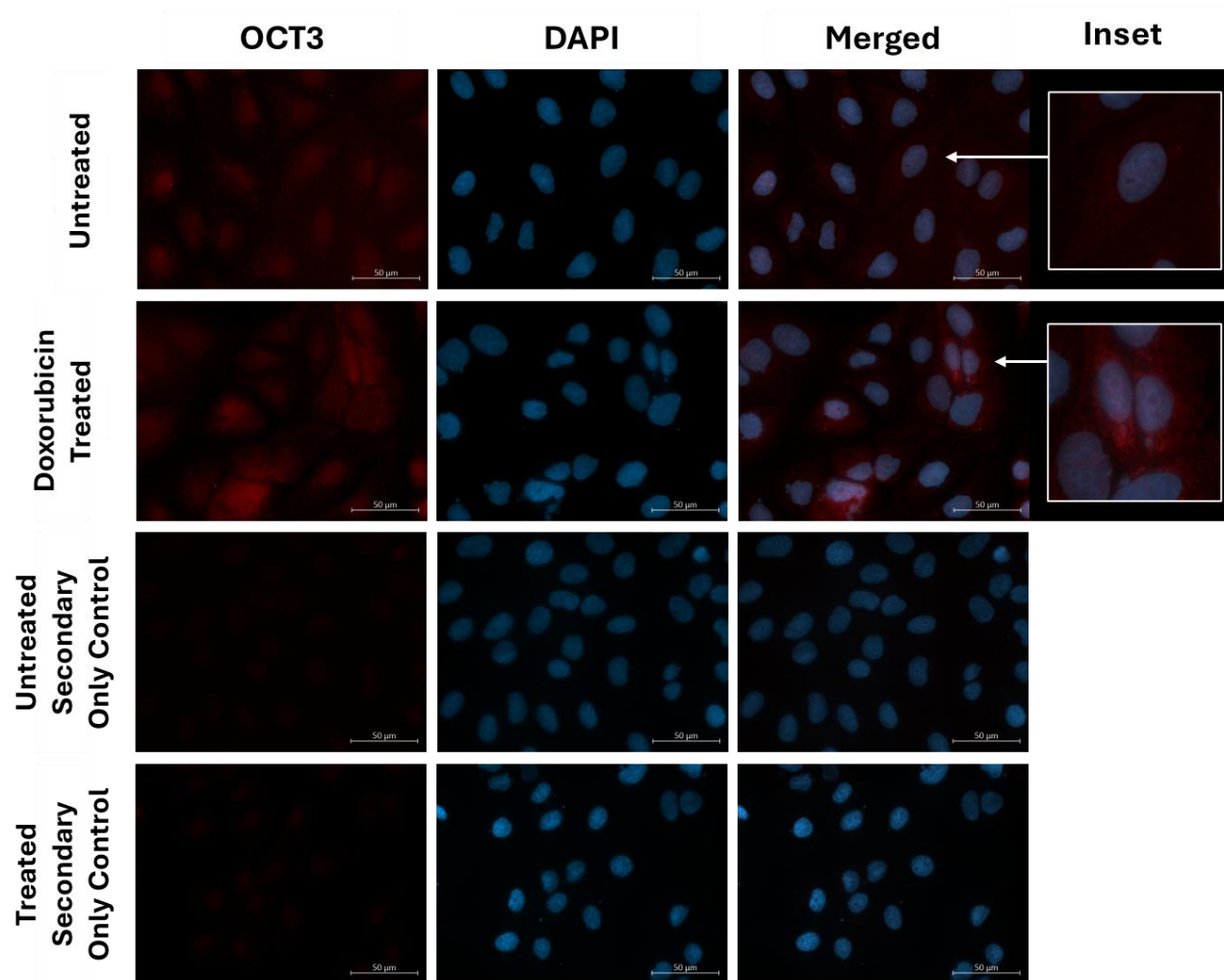


Figure 5.11. Representative fluorescence microscopy images illustrating OCT3 staining in HCC1806 cells. Cells were stained using OCT3 primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 25nM doxorubicin, and corresponding secondary only antibody controls. OCT3 fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm) for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

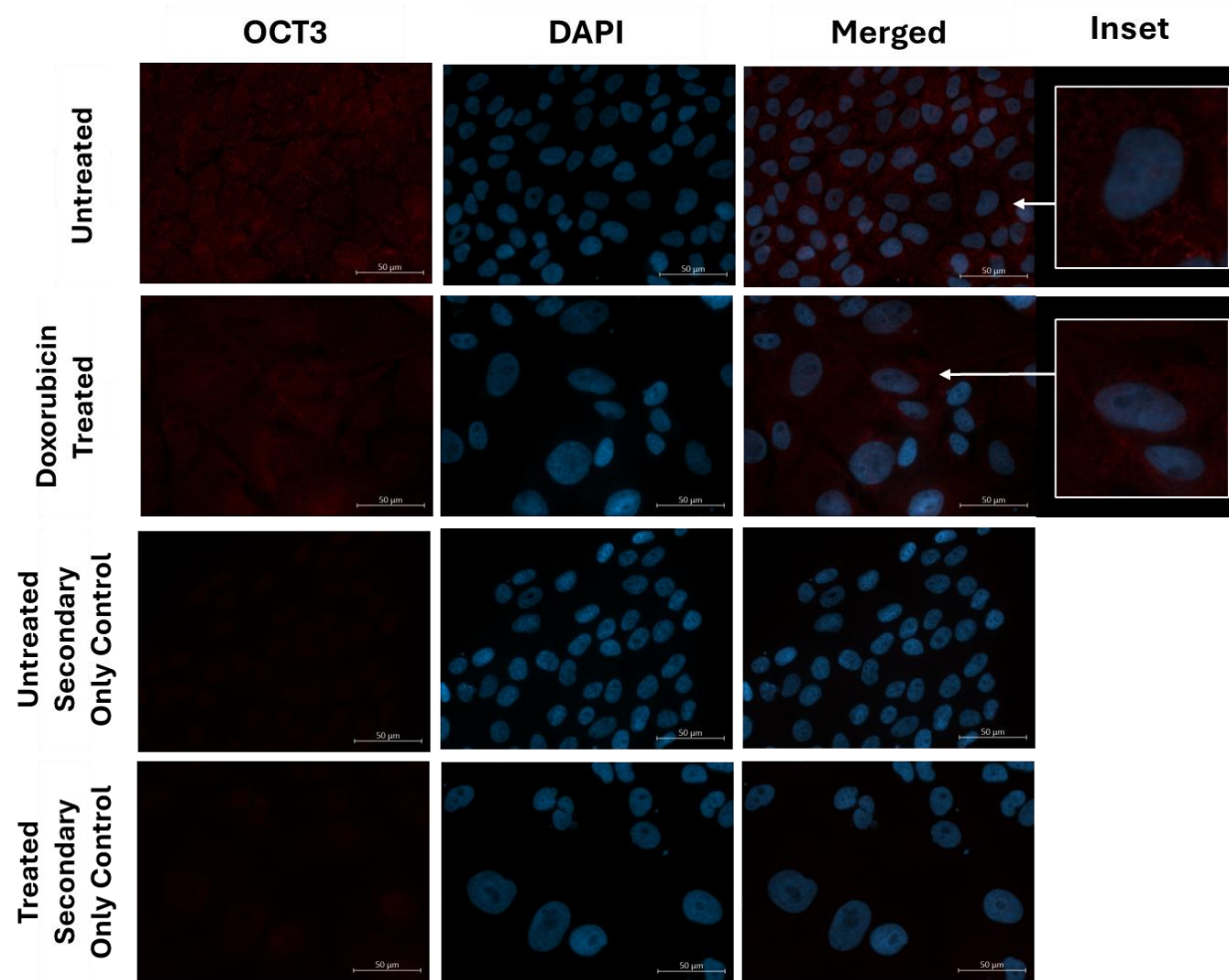


Figure 5.12. Representative fluorescence microscopy images illustrating OCT3 staining in MCF10A cells. Cells were stained using OCT3 primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 100nM doxorubicin, and corresponding secondary only antibody controls. OCT3 fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm) for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

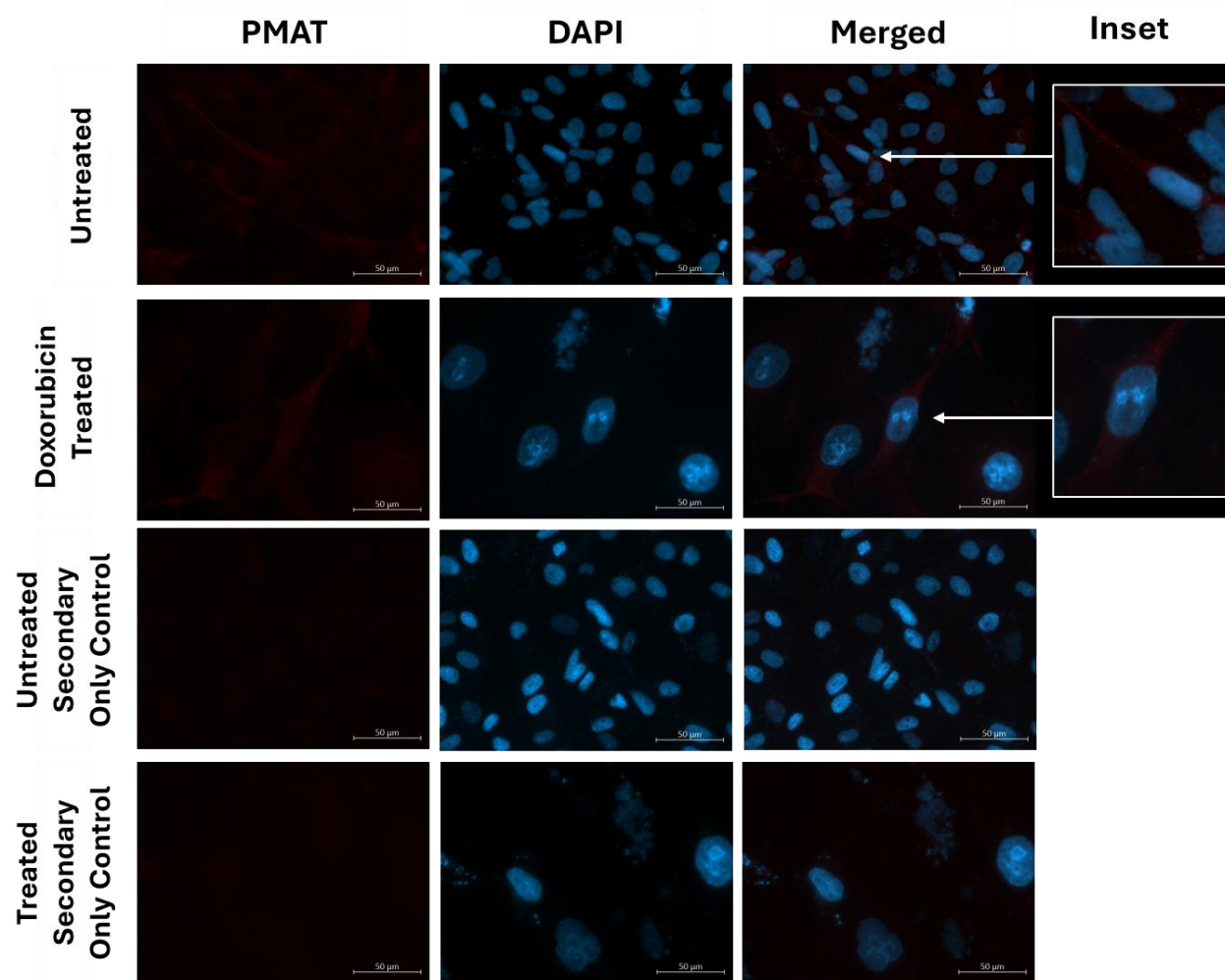


Figure 5.13. Representative fluorescence microscopy images illustrating PMAT staining in MDA-MB-231 cells. Cells were stained using PMAT primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 175nM doxorubicin, and corresponding secondary only antibody controls. PMAT fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm), for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

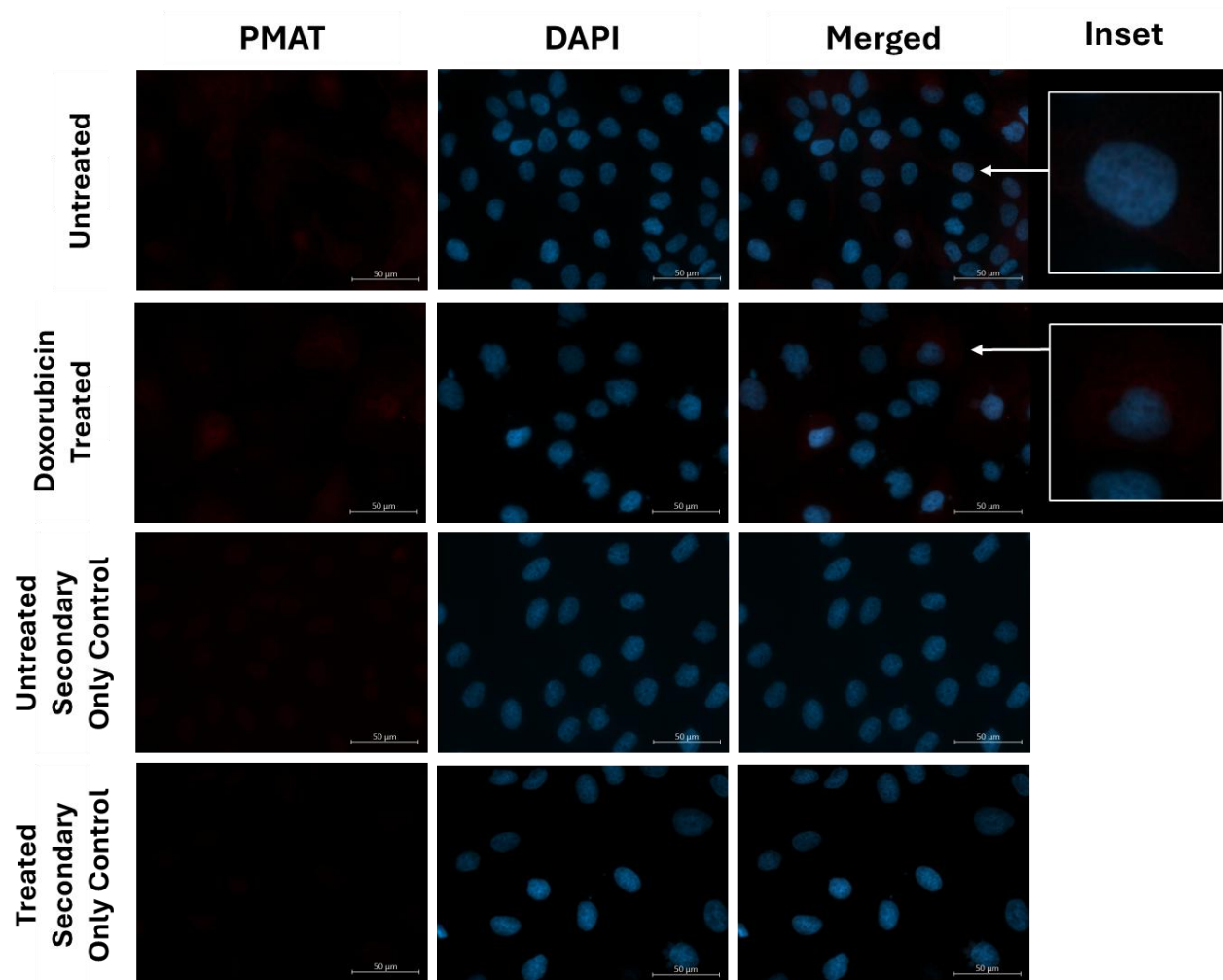


Figure 5.14. Representative fluorescence microscopy images illustrating PMAT staining in HCC1806 cells. Cells were stained using PMAT primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 25nM doxorubicin, and corresponding secondary only antibody controls. PMAT fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm) for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

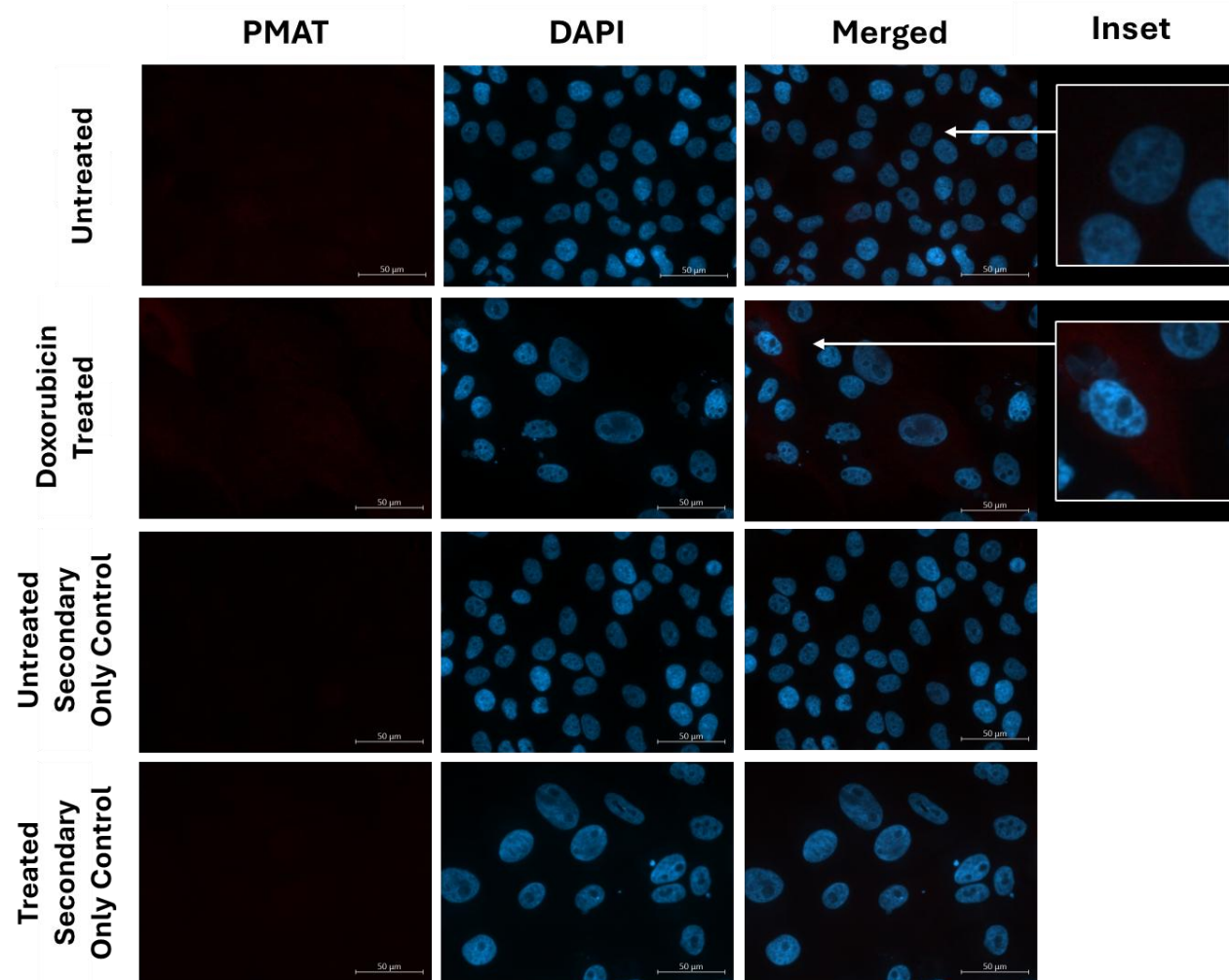


Figure 5.15. Representative fluorescence microscopy images illustrating PMAT staining in MCF10A cells. Cells were stained using PMAT primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50µm. Samples included: untreated cells, cells treated with 25nM doxorubicin, and corresponding secondary only antibody controls. PMAT fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm) for 2 s, whilst nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm) for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is stronger in doxorubicin treated samples and is predominantly cytoplasmic. The inset shows a magnified cell for clarity.

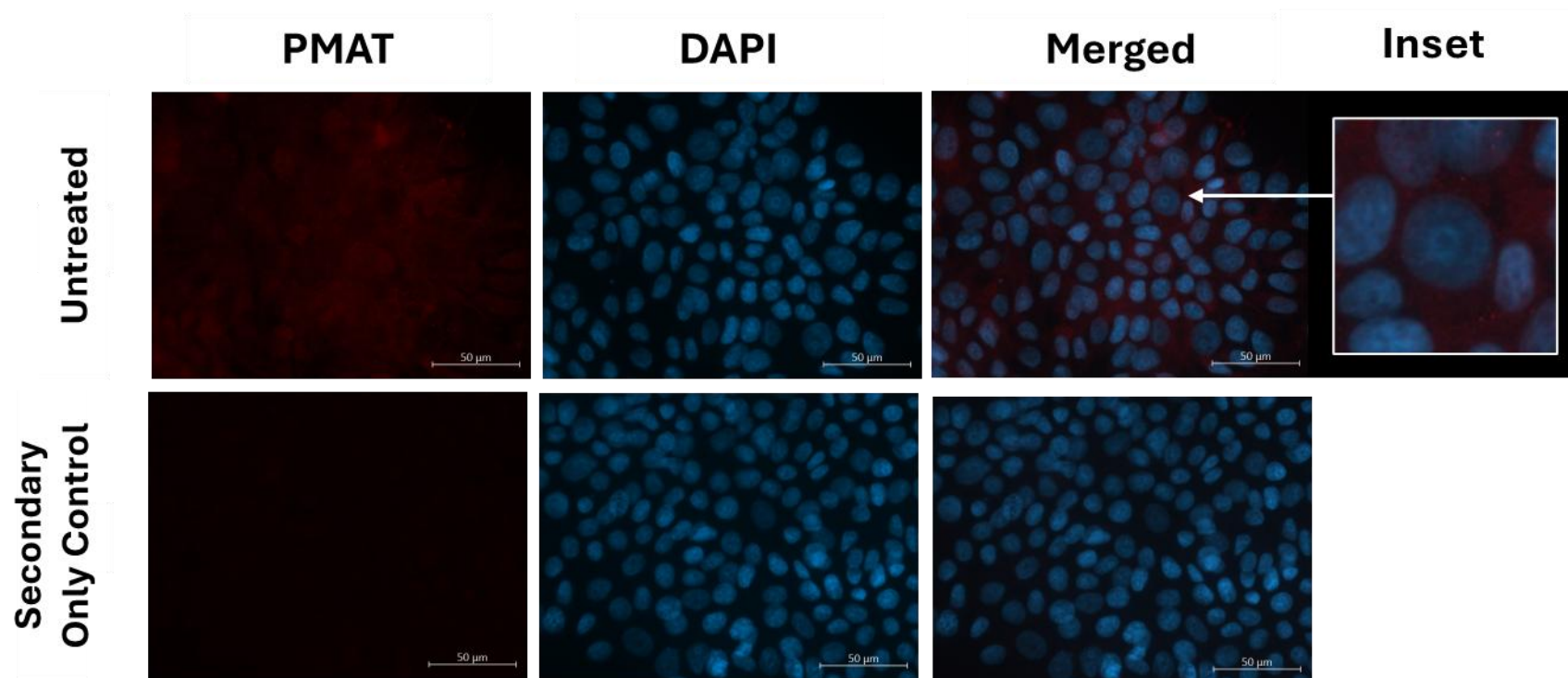


Figure 5.16. Representative fluorescence microscopy images illustrating PMAT staining in MCF7 cells. Cells were stained using PMAT primary antibody and Donkey anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, DyLight™ 594. Images were captured at 40× magnification and have a scale bar of 50μm. Samples included: untreated cells and a secondary antibody only control. PMAT fluorescence was visualised using the AF594 channel (Excitation/Emission: 587/614 nm), for 2 s while nuclei were counterstained with DAPI and visualised using the DAPI channel (ex/em: 360/460 nm), for 70 ms. Individual fluorescence channel images are shown alongside the merged image. The merged image combines both fluorescence channels. Staining is diffuse. The inset shows a magnified cell for clarity.

5.4.3.3 Protein expression of transporters

As with gene expression, protein expression was analysed in untreated cells to establish basal expression levels and then in TIS cells to determine whether doxorubicin altered transporter expression. Immunofluorescence analysis was carried out to confirm that transporters were translated into protein and localised to regions consistent with their role in metformin transport. Transporter mean grey intensity (MGI), representing fluorescence intensity as a measure of protein expression, is shown in Figure 5.17 (A-D), with panel E providing a comparative summary across all cell lines.

Across all cell lines, PMAT exhibited lower MGI values than OCT3 (Figure 5.17). This contrasts with the gene expression data, where *SLC29A4* (PMAT) expression in HCC1806 and MCF10A was higher than *SLC22A3* (OCT3) (Figure 5.8). OCT3 protein expression was highest in HCC1806, followed by MCF10A and MDA-MB-231, whereas *SLC22A3* (OCT3) gene expression was highest in MDA-MB-231, followed by HCC1806 and MCF10A (Figure 5.17 and Figure 5.8). PMAT protein expression was highest in MCF7, followed by HCC1806, MCF10A, and MDA-MB-231 (Figure 5.17). Similarly, *SLC29A4* (PMAT) gene expression was highest in MCF7 and HCC1806 but differed in MDA-MB-231 and MCF10A (Figure 5.8). Overall, MGI values for both OCT3 and PMAT were similar across cell lines.

To assess whether transporter expression was maintained in TIS cells, MGI fold change, relative to untreated cells, was calculated following doxorubicin treatment (Figure 5.18). In HCC1806 and MDA-MB-231, OCT3 MGI increased while PMAT decreased; however, gene expression data showed increases in both. In MCF10A, both OCT3 and PMAT MGI were increased, contrasting with gene expression data where *SLC22A3* (OCT3) decreased and *SLC29A4* (PMAT) increased.

Despite differences between gene and protein expression, immunofluorescence staining confirms that both OCT3 and PMAT are expressed at the protein level and should therefore be capable of contributing to metformin uptake in these models.

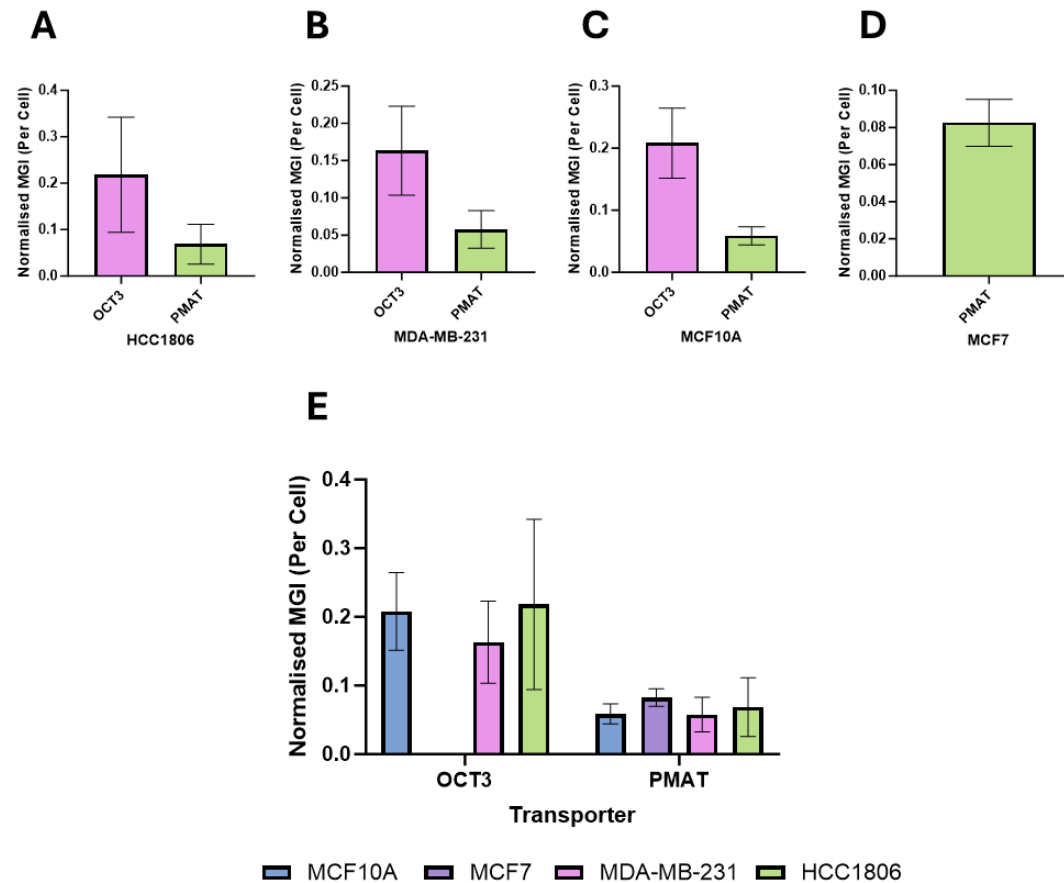


Figure 5.17. Immunofluorescence analysis of key metformin transporters in immortalised cell lines. Normalised mean grey intensity (MGI) was used as a measure of fluorescence intensity. The MGI of the transporter channel was quantified and normalised to the number of cells, determined from DAPI stained nuclei. In greyscale images, each pixel represents an intensity value ranging from 0 (black) to 255 (white), and the MGI reflects the average pixel intensity across the image, with higher values indicating greater fluorescence intensity. Results are expressed in arbitrary units (AU). Data is shown for (A) HCC1806, (B) MDA-MB-231, (C) MCF10A and (D) MCF7 cells. A comparative summary of each cell line and transporter is shown in (E). For all cell lines PMAT had a lower MGI intensity than OCT3. Overall, MGI values for both OCT3 and PMAT were comparable across cell lines. Experiments were performed in 3 independent biological replicates (n = 3), MGI values were calculated from at least 3 independent fields of view. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates.

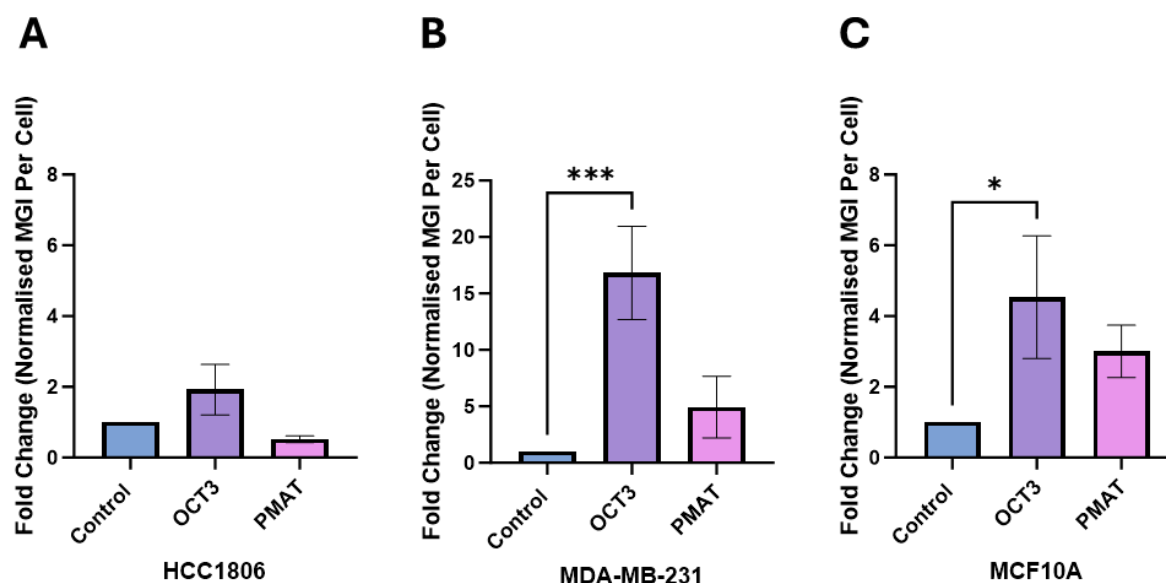


Figure 5.18. Immunofluorescence analysis of key metformin transporters in doxorubicin treated immortalised cell lines. Normalised mean grey intensity (MGI) was used as a measure of fluorescence intensity. The MGI of the transporter channel was quantified and normalised to the number of cells, determined from DAPI stained nuclei. In greyscale images, each pixel represents an intensity value ranging from 0 (black) to 255 (white), and the MGI reflects the average pixel intensity across the image, with higher values indicating greater fluorescence intensity. Gene expression in doxorubicin treated cells was normalised to untreated controls by dividing the treated MGI per cell by the untreated MGI per cell for each sample. Results are presented as fold change MGI per cell. The control sample is normalised to a baseline fold change of 1. Data is shown for (A) HCC1806, (B) MDA-MB-231 and (C) MCF10A cells. For all cell lines doxorubicin treatment increased the MGI of OCT3, which was statistically significant in MDA-MB-231 and MCF10A. The MGI of PMAT was decreased in MDA-MB-231 and HCC1806 but increased in MCF10A. Experiments were performed in 3 independent biological replicates ($n = 3$), MGI values were calculated from at least 3 independent fields of view. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one-way ANOVA with Dunnett's post hoc test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

5.4.3.4 Expression of transporters in patient datasets

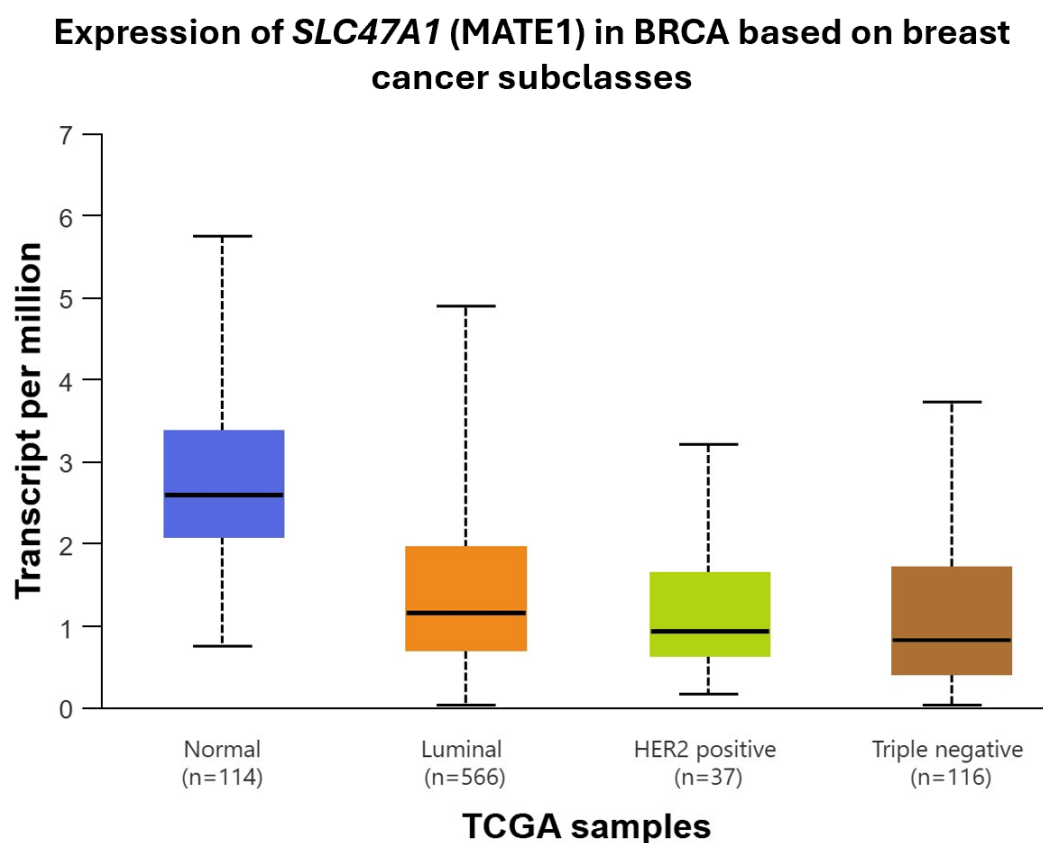
To assess the clinical relevance of the *in vitro* models used in this study, transporter expression was analysed using publicly available patient datasets. GEPIA was used to compare gene expression between healthy breast tissue and breast invasive carcinoma samples (Table 5.7). Transporter expression was higher in normal tissue than in BC. Interestingly, this contrasts with the gene expression analysis in this study, where the non-malignant MCF10A cell line had substantially lower transporter expression than BC cell lines.

Table 5.7. GEPIA expression analysis of transporters across healthy tissue and breast invasive carcinoma. The analysis was restricted to breast invasive carcinoma (BRCA) patients. GEPIA integrates RNA-seq expression data from tumour samples (n = 9,736) and normal tissue samples (n = 8,587). Gene expression was normalised by GEPIA and reported as transcripts per million (TPM). Statistical differences in gene expression were assessed using GEPIA's internal one-way ANOVA. A default q-value threshold of < 0.01 was applied to define statistical significance and minimise the likelihood of false positives. Fold change (FC) in expression was calculated using GEPIA's internal pipeline, which quantified differences in gene expression between tumour and normal tissue. A $|\text{Log}_2\text{FC}| \geq 1$ was applied as a default threshold to filter out genes with expression differences unlikely to be biologically meaningful. GEPIA calculates Log_2FC based on normalised expression data across all samples, not directly from TPM averages. GEPIA reported Log_2FC , which was converted to fold change ($\text{FC} = 2^{\text{Log}_2\text{FC}}$) for ease of interpretation. Differential expression statistics were not available for all genes, where data was unavailable, a hyphen (-) is shown.

Gene Symbol	TPM in BRCA	TPM in normal	$\text{LOG}_2 \text{FC}$	FC	q-value
<i>SLC47A1</i> (MATE1)	0.93	1.73	-	-	-
<i>SLC22A1</i> (OCT1)	0.23	0.52	-	-	-
<i>SLC22A3</i> (OCT3)	0.36	4.37	-1.98	0.25	2.17e-174
<i>SLC29A4</i> (PMAT)	2.34	9.93	-1.71	0.31	1.39e-60

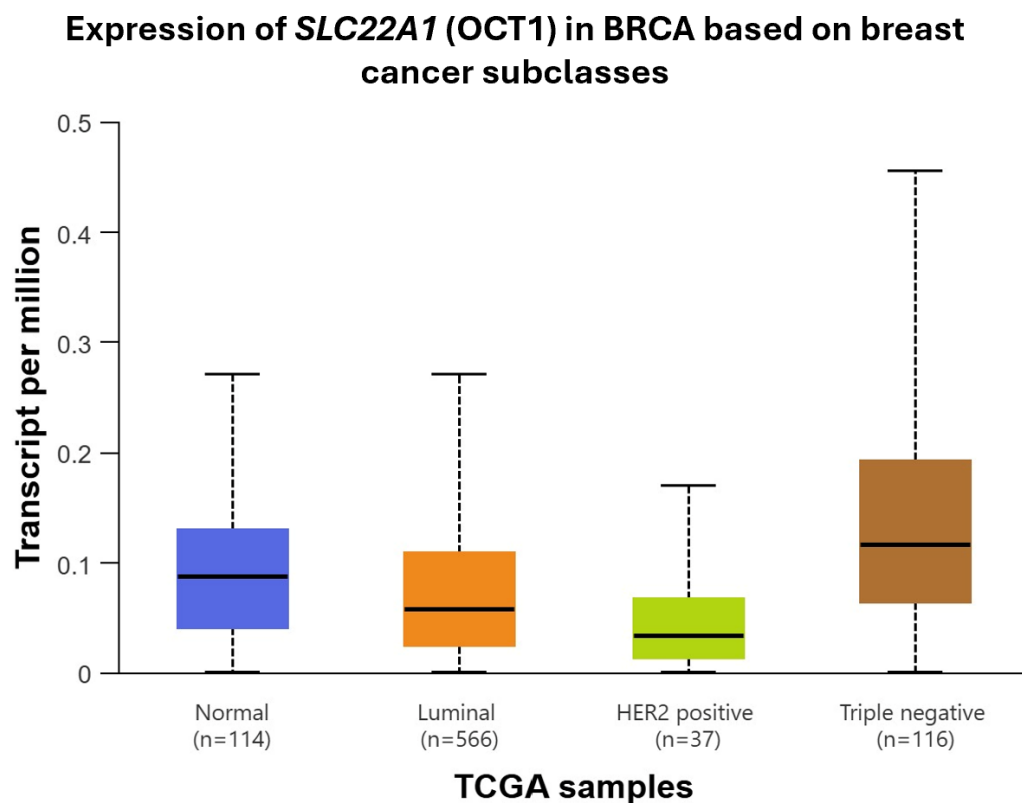
UALCAN was used to compare transporter gene expression across molecular BC subtypes (Figure 5.19- Figure 5.22), TNBC (represented in this study by MDA-MB-231 and HCC1806), luminal A (represented by MCF7), and normal breast tissue (represented by MCF10A). For *SLC47A1* (MATE1), UALCAN analysis indicated highest expression in normal tissue, with similar expression levels between TNBC and luminal A BC (Figure 5.19). In contrast, the *in vitro* data showed lowest *SLC47A1* (MATE1) expression in MCF10A, while the cancer cell lines displayed comparable levels (Figure 5.8). *SLC22A1* (OCT1) expression was highest in TNBC according to UALCAN, followed by normal and then luminal tissue (Figure 5.20). This trend was consistent with the experimental data, where *SLC22A1* (OCT1) expression was elevated in TNBC cell lines but minimal in MCF10A (Figure 5.8). *SLC22A3* (OCT3) was most strongly expressed in normal tissue, with markedly lower expression in both TNBC and luminal BC (Figure 5.21). This pattern only partially aligned with *in vitro* findings, as *SLC22A3* (OCT3) expression was high in MDA-MB-231 and present in HCC1806, but very low in MCF10A and undetectable in MCF7 (Figure 5.8). *SLC29A4* (PMAT) showed highest expression in normal tissue, followed by TNBC and then luminal cancers (Figure 5.22). In contrast, the *in vitro* models showed very low *SLC29A4* (PMAT) expression in MCF10A, whilst MCF7 displayed higher expression than the other TNBC cell lines (Figure 5.8).

Interestingly, UALCAN analysis showed that *SLC29A4* (PMAT) and *SLC22A3* (OCT3) had the highest levels of gene expression, consistent with qPCR findings where these genes were also the most highly expressed. The transporter with lowest expression for both experimental and bioinformatic datasets was *SLC22A1* (OCT1). Whilst *in vitro* models only partially reflected the patterns seen in bioinformatic data, this similarity between experimental and patient data supports the relevance of the models used in this study.



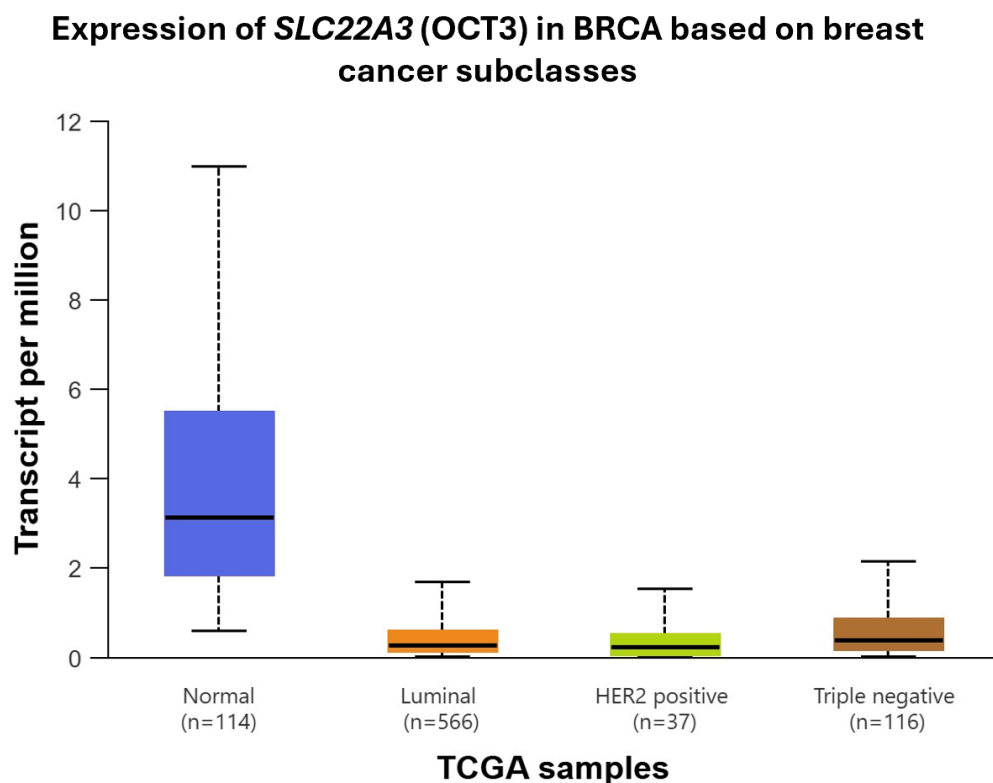
Comparison	Statistical significance	Symbol
Normal-vs-Luminal	1.349610E-02	*
Normal-vs-HER2 Positive	3.2236435743016E-12	****
Normal-vs-TNBC	1.388830E-01	
Luminal-vs-HER2 Positive	9.10829999978269E-07	****
Luminal-vs-TNBC	5.669400E-01	
HER2 Positive-vs-TNBC	2.044600E-01	

Figure 5.19. UALCAN Analysis of *SLC47A1* (MATE1) gene expression in breast invasive carcinoma subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to molecular subtype, as defined in the TCGA clinical metadata. For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was similar in BRCA subtypes, but lower than normal tissue.



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	8.419800E-01	
Normal-vs-HER2 Positive	8.836200E-01	
Normal-vs-TNBC	1.894310E-02	*
Luminal-vs-HER2 Positive	8.016400E-01	
Luminal-vs-TNBC	2.297700E-02	*
HER2 Positive-vs-TNBC	2.798100E-02	*

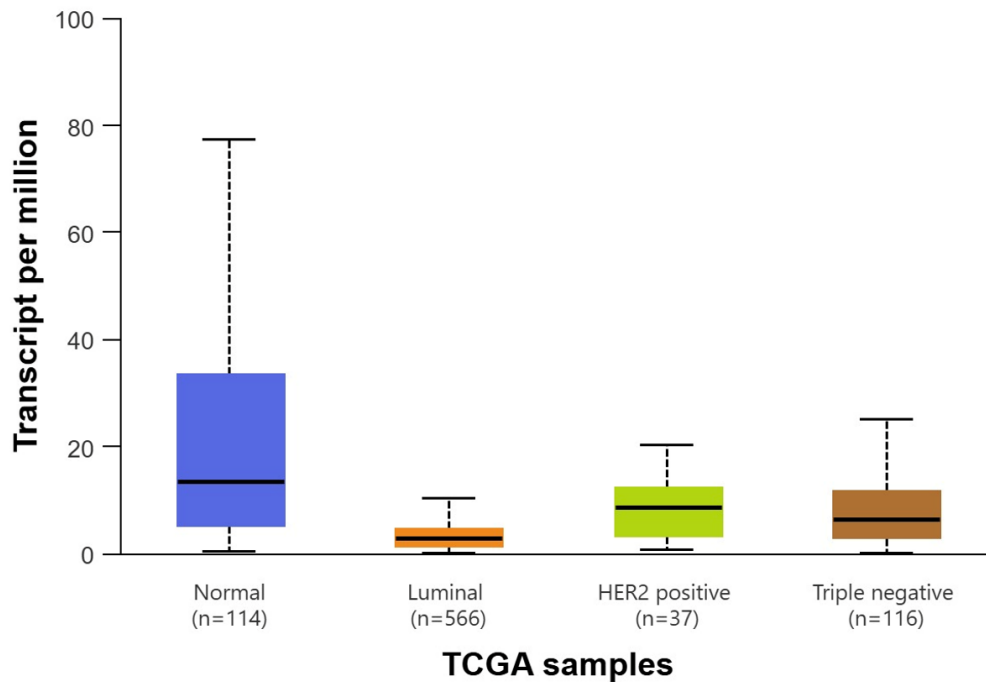
Figure 5.20. UALCAN Analysis of *SLC22A1* (OCT1) gene expression in breast invasive carcinoma subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to molecular subtype, as defined in the TCGA clinical metadata. For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was higher in TNBC than normal tissue similar but lower in other BRCA subtypes.



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	<1E-12	****
Normal-vs-HER2 Positive	<1E-12	****
Normal-vs-TNBC	1.62447832963153E-12	****
Luminal-vs-HER2 Positive	2.319000E-01	
Luminal-vs-TNBC	4.766300E-02	*
HER2 Positive-vs-TNBC	1.285850E-02	*

Figure 5.21. UALCAN Analysis of *SLC22A3* (OCT3) gene expression in breast invasive carcinoma subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to molecular subtype, as defined in the TCGA clinical metadata. For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression was similar in BRCA subtypes and lower than normal tissue.

Expression of *SLC29A4* (PMAT) in BRCA based on breast cancer subclasses



Comparison	Statistical significance	Symbol
Normal-vs-Luminal	5.85719994727896E-09	****
Normal-vs-HER2 Positive	7.759600E-01	
Normal-vs-TNBC	1.00650043854955E-10	****
Luminal-vs-HER2 Positive	2.732300E-02	*
Luminal-vs-TNBC	<1E-12	****
HER2 Positive-vs-TNBC	1.218600E-01	

Figure 5.22. UALCAN Analysis of *SLC29A4* (PMAT) gene expression in breast invasive carcinoma subtypes. Data was derived from the TCGA, PanCancer Atlas dataset and refined to breast invasive carcinoma (BRCA). Gene expression was categorised according to molecular subtype, as defined in the TCGA clinical metadata. For subtype comparisons, the cohort included n = 833 samples: normal (n = 114), luminal (n = 566), HER2-positive (n = 37), and TNBC (n = 116). Expression is reported as transcripts per million (TPM). Box plots display the interquartile range (Q1-Q3) as the coloured box, the median (line inside the box), and the minimum and maximum values (error bars). Statistical significance between subgroups was calculated by UALCAN using Welch's t-test and is shown below the graphs: *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Gene expression in BRCA subtypes was lower than normal tissue, and lowest in Luminal BRCA.

5.5 DISCUSSION

The primary aims of this chapter were to investigate potential reasons for the limited efficacy of metformin in TIS models and to assess the translational relevance of these pre-clinical models to patient gene expression profiles. Metformin function was first validated in MCF7 cells, a commonly used positive control for metformin studies, before investigating two key determinants of metformin sensitivity: pathway activation and cellular uptake. Metformin transporter expression was also reviewed in patient datasets to assess the clinical relevance of the models used in this study.

5.5.1 Confirming functional effects of metformin in MCF7

Two known functional effects of metformin were assessed; reduction in cell viability (assessed using MTT assays) and reduction in proliferation (assessed using BrdU immunofluorescence).

MTT analysis showed that after 96 hours of treatment, metformin concentrations $\geq 500 \mu\text{M}$ induced a significant reduction in cell viability, decreasing to $\sim 85\%$ at $500 \mu\text{M}$, 56% at 5 mM , and 15% at 50 mM (Figure 5.1). Although few studies have assessed metformin's cytotoxicity over longer durations, the reduction observed here is consistent with published literature showing that prolonged exposure enhances the anti-proliferative effects of metformin in MCF7 (Bekezhankyzy et al., 2023; Lopes et al., 2020; Marinello et al., 2016; Queiroz et al., 2014). Although reduction of MCF7 viability was not as potent as expected, $500 \mu\text{M}$ metformin, the upper concentration used in TIS models, induced a significant reduction in viability, confirming that metformin was biologically active under the conditions used in this study.

Previous studies, using MTT assays, have reported variable sensitivity of MCF7 cells to metformin, suggesting its effects are highly context dependent. For example, Lopes et al., (2020) reported that after 24 hours, 1 mM metformin decreased MCF7 viability to $\sim 95\%$ and 5 mM to $\sim 80\%$, whereas Queiroz et al., (2014) showed that 5 mM metformin had a minimal effect after 24 hours but reduced viability by $\sim 50\%$ after 72 hours, suggesting that exposure duration is a key determinant of metformin response. It is evident MCF7 viability can vary in response to metformin, with some studies suggesting $150 \mu\text{M}$ reduces proliferation to $\sim 60\%$ at 24 hours whilst others found that MCF7 maintained $\sim 94\%$ viability at 5 mM (Bekezhankyzy et al., 2023; Phoenix, Vumbaca and Claffey, 2009). There are many factors which may have contributed to the difference in these outcomes, including media composition, glucose concentration, cell density, and metformin batch.

When compared to other cell lines used in this study, treated for 96 hours with metformin, MCF7 appeared unexpectedly less sensitive. At 500 μ M, MCF7 viability was ~85%, compared with 78% in MDA-MB-231, 80% in HCC1806, and 61% in MCF10A. This was surprising given that the reduction of MTT reflects NAD(P)H-dependent oxidoreductase activity in viable, metabolically active cells (Lopes et al., 2020). One explanation may involve differences in cellular NAD⁺ metabolism, which plays a crucial role in metformin sensitivity. Metformin inhibits mitochondrial complex I, suppressing oxidative phosphorylation and leading to ATP depletion (Foretz et al., 2014). This subsequently increases the NADH: NAD⁺ ratio, reducing NAD⁺ availability and cellular energy levels. Metformin induced cytotoxicity has been associated with NAD⁺ depletion (Zhuang et al., 2023). MCF7 are reported to express lower levels of NAMPT (the key enzyme in NAD⁺ biosynthesis), in comparison to MDA-MB-231 (Zhuang et al., 2023).

Assessing NAMPT expression in the cell lines used in this study may help clarify whether differences in NAD⁺ metabolism contributed to the varied metformin responses observed. It is also important to consider that MTT primarily reflects metabolic activity rather than direct cell death, therefore future studies could incorporate apoptosis assays to distinguish between proliferation inhibition and cytotoxicity.

BrdU was used to assess proliferation as a measure of metformin function, following treatment at concentrations published in literature (Figure 5.3). A previous study showed that 10mM metformin for 48 hours reduced BrdU incorporation in MCF7 cells from ~50% to ~30% (Queiroz et al., 2014). Similarly, in this study, 10 mM metformin for 96 hours reduced BrdU positive cells to 33%, from 53%. Although this reduction was over a longer time frame and not statistically significant, the trend supports a reasonable anti proliferative effect of metformin in MCF7.

An additional factor that may have influenced the metformin response is culture medium composition. Previous studies have reported that renewal of culture medium can reduce metformin cytotoxicity by replenishing nutrients and preventing the energy stress which enhances its action (Rajh et al., 2016). In the present study, media was not replaced during treatment, ruling this out as a cause of reduced sensitivity. The same high-glucose DMEM (25 mM glucose) was used for MCF7, MDA-MB-231, and HCC1806 cells, consistent with Queiroz et al., (2014). In contrast, MCF10A cells were

maintained in a lower glucose medium (17mM glucose). Notably, at 5mM metformin, all cancer cell lines displayed comparable viability (~50%), whereas MCF10A showed a stronger response (~10% viability). This finding suggests that metformin efficacy may be glucose dependent.

Ultimately, both MTT and BrdU assays confirmed that metformin exerted biological activity in MCF7 cells, although the response was less pronounced than anticipated. Differences in metabolic state, transporter expression, and culture conditions may have contributed to this variability.

5.5.2 Regulation of key downstream targets in response to metformin treatment

Western blots were performed to assess whether key downstream targets of metformin were regulated in accordance with its expected mechanism, using AMPK and mTOR phosphorylation as indicators of activation (Figure 5.4, Figure 5.5, Figure 5.6 and Figure 5.7). AICAR was included as a positive control for AMPK phosphorylation. It was expected that metformin would activate AMPK via phosphorylation, leading to inhibition of mTOR phosphorylation (Alalem, Ray and Ray, 2016; Liu et al., 2009; Phoenix, Vumbaca and Claffey, 2009; Zhuang and Miskimins, 2008; Zordoky et al., 2014).

5.5.2.1 Technical limitations

It is important to note that, due to time constraints and reagent limitations, western blot results were obtained from a single repeat during method optimisation. Results were therefore interpreted with caution. Initially, to maintain consistency in sample loading, it was intended that membranes would be probed for phosphorylated proteins, stripped and then re-probed for total proteins, which are generally more abundant. However, membrane stripping using both stringent and milder methods resulted in substantial protein loss, rendering membranes unusable. Despite using un-stripped membranes, variability in the intensity of loading controls was also observed, indicating that protein binding was inconsistent. These technical issues limited the interpretation of phosphorylation changes among samples. Given TIS cells undergo extensive cytoskeletal remodelling, and vinculin has been shown to be differentially expressed in senescent stromal cells, it is also important to consider that the housekeeping genes may not have been stable in the TIS models used (Ghosh et al., 2020; Gresham et al., 2024; Molina et al., 2025; Moujaber et al., 2019). Although actin and vinculin have been used as loading controls in literature, GAPDH or PUM1 would have been preferred, as these were stable, housekeeping genes used in qPCR analysis, however due to resource limitations, this was not possible (Bisso et al., 2013; Kim et al., 2011; Phoenix, Vumbaca and Claffey, 2009).

5.5.2.2 AICAR as a positive control for AMPK phosphorylation

AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) was used as a positive control for AMPK phosphorylation. AICAR is a cell permeable nucleotide which enters cells through adenosine transporters (independent to those that transport metformin) (Višnjić et al., 2021). It is converted intracellularly into ZMP, an AMP analogue that directly activates AMPK and leads to downstream mTOR inhibition (Višnjić et al., 2021). Previous studies have shown variable concentrations and durations of AICAR treatment are sufficient to induce AMPK phosphorylation in MDA-MB-231, MCF10A and MCF7, including 0.83 mM for 1 hour, 1 mM for 18 hours and 300 μ M to 3 mM for 24 hours (El-Masry, Brown and Dobson, 2012; Kaur et al., 2021; Lützner et al., 2012).

An initial test blot, using 1 mM AICAR, confirmed the functionality of the phosphorylated AMPK antibody. Although comparisons with total AMPK were not possible, phosphorylation of AMPK was detectable in all cell lines (MCF7, MDA-MB-231, HCC1806, and MCF10A) after 1, 3, and 24 hours of treatment. The strongest band intensity was at 24 hours (data not shown), which was the concentration used in subsequent experiments.

For the TNBC cell lines, HCC1806 and MDA-MB-231, AICAR treatment strongly induced AMPK phosphorylation but did not reduce mTOR phosphorylation below basal levels, indicating partial pathway activation. In contrast, MCF10A and MCF7 cells failed to increase AMPK phosphorylation compared to untreated controls, which was unexpected given the consistent activation reported in previous studies.

These differences could be attributed to differential expression of adenosine transporters, responsible for AICAR uptake (Dolinar et al., 2018; Višnjić et al., 2021). Equilibrative nucleoside transporters ENT1 and ENT2 are the primary transporters mediating AICAR uptake and when blocked prevent AICAR transport (Dolinar et al., 2018). Although ENT1 and ENT2 have previously been reported in MDA-MB-231, MCF10A and MCF7, ENT1 levels are significantly lower in MCF10A and MCF7 compared to MDA-MB-231 (Krys et al., 2019). This may explain the reduced AMPK phosphorylation, by AICAR, in these cell lines. This could be investigated in future work by examining ENT expression and function, using gene expression and protein analysis.

Another potential factor limiting efficacy of AICAR is culture media composition. Nucleosides present in culture media can compete with AICAR for transporter uptake and inhibit its activity (Dolinar et al., 2018). However, the DMEM GlutaMAX media used to culture cancer cell lines does not contain nucleosides, and the DMEM/F12 used for MCF10A contains only trace levels (1.5 μ M thymidine), well below concentrations reported to interfere with AICAR function.

5.5.2.3 mTOR and AMPK signalling in response to metformin

Although in some circumstances AMPK phosphorylation was consistent with expected metformin activity, simultaneous phosphorylation of mTOR suggested incomplete pathway activation. In preliminary data, mTOR phosphorylation was not consistently inhibited relative to untreated controls, despite an increase in AMPK phosphorylation. This suggests that metformin is not eliciting its effects through mTOR inhibition and may be exerting its effects via alternate pathways.

Whilst both TNBC cell lines (HCC1806 and MDA-MB-231) either maintained or increased mTOR phosphorylation across treatment groups, mTOR phosphorylation was decreased in metformin treated MCF10A and in MCF7 at higher concentrations, suggesting cell-type-specific responses (Figure 5.4, Figure 5.5, Figure 5.6 and Figure 5.7). Inhibition of proliferation by metformin in MCF7 has been linked to reduced cyclin D1 and release of p27/p21 (Zhao et al., 2017; Zhuang and Miskimins, 2008). MDA-MB-231 cells, which express low levels of cyclin D1, p27, and p21, did not exhibit the same response, perhaps explaining the differences in mTOR phosphorylation inhibition.

The duration and concentration of metformin may have also influenced AMPK and mTOR activation. Previous studies have reported AMPK phosphorylation and mTOR inhibition, in MCF7, following varied metformin treatments, ranging from 3 mM for 30 minutes to 20 mM metformin for 24 hours (Alalem, Ray and Ray, 2016; Kim et al., 2011; Palma et al., 2021; Phoenix, Vumbaca and Claffey, 2009). However, the same effect was not observed in this study. Lower metformin treatments (50 μ M and 500 μ M) increased AMPK phosphorylation slightly, indicating pathway activation. However, phosphorylation levels decreased below untreated controls at 5 mM and 10 mM metformin, potentially suggesting a loss of pathway activation at cytotoxic doses (Figure 5.7). This is consistent with MTT data showing a significant reduction in viability at $\geq$ 5 mM (Figure 5.1). Exposure time may have played a role in the

response of MCF7 to metformin, where over a longer treatment period, cells were unable to survive these high metformin doses.

In this study, MDA-MB-231 cells appeared to exhibit a dose dependent increase in AMPK phosphorylation in response to metformin (Figure 5.5). This observation is consistent with previous reports suggesting that metformin can activate key signalling pathways across a broad range of concentrations and exposure times (10-40 mM for 8-24 hours) (Ariaans et al., 2017; Liu et al., 2009; Palma et al., 2021). Interestingly, in MDA-MB-231, mTOR phosphorylation also increased with metformin dose. A similar trend was observed for some HCC1806 samples (TIS and 500 μ M metformin TIS) where AMPK and mTOR were simultaneously phosphorylated. This may be a consequence of incomplete pathway activation, where AMPK is partially activated but not to a level which suppresses mTOR. Alternatively, there may be other pathways overriding AMPK, and contributing to an increase in mTOR phosphorylation. For example, Rag-GTPases potentially activate mTORC1, and maybe dysfunctional in TNBC cell lines, preventing the inhibition of signalling by metformin (Lama-Sherpa et al., 2023). Additionally, TSC2 is a negative regulator of mTORC1 and inactivates Rheb, a GTPase that promotes mTORC1 activation. Despite metformin promoting TSC2 activity and inhibiting Rheb, through REDD1 activation, it is possible, dysfunctional or absent TSC2 leads to increased Rheb activity and enhanced mTORC1 signalling. Given *TSC2* is frequently mutated in BC's it is not unreasonable to assume that this may have resulted in the increased mTOR phosphorylation observed alongside AMPK activation, in the western blot analysis (Jiang et al., 2021; Zhu et al., 2023).

Differences in pathway activation between tumorigenic and non-tumorigenic cell lines, may have been due to differences in media composition. Zordoky et al., (2014) showed glucose concentration can modulate metformin activity, where in high glucose (25 mM) conditions, metformin (5 mM for 24 hours) failed to induce mTOR inhibition and AMPK activation, whereas in low glucose (5mM) conditions, AMPK was phosphorylated and mTOR inhibited. All cell lines in this study were maintained in relatively high glucose conditions (25 mM for MCF7, MDA-MB-231, HCC1806 and 17 mM for MCF10A), which may have contributed to the muted AMPK mTOR response.

Variations in AMPK phosphorylation could also be attributed to differences in Liver kinase B1 (LKB1) expression. LKB1 is a kinase responsible for AMPK phosphorylation at Thr172 (Zhao et al., 2017). It is essential for metformin action, and cells lacking it fail to respond to treatment (Shaw et al., 2005;

Zordoky et al., 2014). LKB1 expression data in BC is inconsistent, some indicate that both MDA-MB-231 and MCF7 express LKB1 (Phoenix, Vumbaca and Claffey, 2009), whilst others suggest MDA-MB-231 lacks expression (Shen et al., 2002) or that MCF7 is LKB1 deficient (Linher-Melville, Zantinge and Singh, 2012). To confirm this, future work could examine the LKB1 status of the cell models used in this study. Differences in AMPK phosphorylation may also reflect antibody specificity. Therefore, in future studies it may be beneficial to use an antibody that detects both AMPK α (AMPK α 1 and AMPK α 2) isoforms, to ensure that any isoform-specific changes are being detected.

Ultimately, inconsistencies were observed in the data. In MCF10A, treatment with 50 μ M metformin resulted in decreased AMPK phosphorylation, whereas at 500 μ M, AMPK phosphorylation increased relative to the untreated control (Figure 5.6). In contrast, in HCC1806 cells, AMPK phosphorylation was lower at 500 μ M compared to 50 μ M (Figure 5.4). These discrepancies may also partly reflect technical limitations in the experiments.

5.5.2.4 TIS Response

All TIS cells had higher levels of AMPK phosphorylation compared to their respective untreated controls. Although metformin altered AMPK activity across the cell lines, the patterns were cell type specific. For instance, in HCC1806, 500 μ M metformin increased AMPK phosphorylation above TIS only levels, whereas at 50 μ M phosphorylation remained below basal levels (Figure 5.4). In MDA-MB-231, both 50 μ M and 500 μ M metformin resulted in AMPK phosphorylation above untreated levels, with the higher dose producing a stronger response (Figure 5.5). In contrast, in MCF10A, metformin at both concentrations reduced AMPK phosphorylation relative to the TIS only sample (Figure 5.6). Although MDA-MB-231 and HCC1806 TIS samples, demonstrated some degree of expected AMPK activation, inconsistencies in metformin only treatments, make it difficult to draw firm conclusions. As previously discussed, these variations may reflect variable pathway activation, influenced by treatment duration, media composition or differential expression of LKB1.

AMPK signalling in senescence, seems to be context dependent, with some studies reporting reduced AMPK activity whilst others report an increase in activity (Chembukavu and Lindsay, 2024; Han et al., 2016; Morsceck, Reck and Reichert, 2023). A key result is the elevated AMPK phosphorylation in TIS cells. Given the consistency between cell lines, it is reasonable to assume this may be a genuine outcome. This result aligns with the idea that DNA damage, mitochondrial dysfunction, and increased

metabolic demand from SASP production, all activate AMPK (Han et al., 2023; Herranz and Gil, 2018; Miwa et al., 2022; Zhang et al., 2025).

This baseline elevation could be important in determining the efficacy of metformin in TIS cells. Metformin predominantly acts by inhibiting mitochondrial complex I to stimulate AMPK; however, if AMPK is already highly activated in TIS cells, the pathway may be close to its activation limit. This may explain the limited efficacy of metformin in TIS cells.

5.5.3 Expression of key metformin transporters

Expression of four key metformin transporters (*SLC22A1* (OCT1), *SLC22A3* (OCT3), *SLC29A4* (PMAT), and *SLC47A1* (MATE1)) was examined by qPCR. Based on these results, OCT3 and PMAT were selected for protein level investigation using immunofluorescence.

5.5.3.1 Gene expression

For MCF7 and MDA-MB-231, the existing literature is contradictory; however, the data generated in this study aligned with the most consistently reported findings (Figure 5.8). MCF7 did not express *SLC22A1* (OCT1), whereas MDA-MB-231 expressed *SLC22A1* (OCT1) at low levels (Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020; Rajh et al., 2016; Sutherland, Meeson and Lowes, 2020). Both cell lines expressed *SLC47A1* (MATE1), and *SLC29A4* (PMAT) (Cai et al., 2016; Markowicz-Piasecka et al., 2020). Whilst MDA-MB-231 expressed *SLC22A3* (OCT3) and MCF7 did not (Cai et al., 2016; Markowicz-Piasecka et al., 2019; Markowicz-Piasecka et al., 2020; Sutherland, Meeson and Lowes, 2020). Overall, this supports the validity of the qPCR results in this study.

Transporter expression varied across the cell models; however, all expressed at least one transporter capable of mediating metformin uptake. MCF10A showed the lowest expression of all transporters, consistent with its relatively weak AMPK activation in western blot analyses, whereas MDA-MB-231 displayed the highest expression profile, correlating with stronger AMPK phosphorylation.

The highest transporter expression was *SLC22A3* (OCT3) in MDA-MB-231, followed by *SLC29A4* (PMAT) in MCF7. In contrast, the lowest expression was of *SLC22A1* (OCT1) across cell lines, suggesting

it plays little role in uptake within breast cells, despite being the predominant hepatic transporter for metformin (Figure 5.8). OCT3 is responsible for metformin distribution and absorption in many tissues (Nies et al., 2010). Therefore, the response to metformin observed in western blot analysis of MDA-MB-231 may reflect its high *SLC22A3* (OCT3) expression, in comparison to the other cell models. Supporting this idea, OCT3 overexpression induced in MCF7 has been previously shown to enhance AMPK activation by metformin (Cai et al., 2019a). *SLC22A3* (OCT3) polymorphisms can also influence metformin efficiency, with most variants reducing uptake, some increasing it or having no functional effect (Naem et al., 2024; Wang et al., 2025). It is possible that *SLC22A3* (OCT3) polymorphisms in HCC1806, MDA-MB-231, or MCF10A may reduce transporter function which could contribute to differences in response to metformin. *SLC22A3* (OCT3) expression increased slightly in TIS conditions across all lines, indicating that doxorubicin treatment did not impair metformin uptake (Figure 5.9).

Given that, in this study, MCF7 served as a positive control for metformin function and expressed *SLC29A4* (PMAT), not *SLC22A1* (OCT1) or *SLC22A3* (OCT3), it appears to play an important role in metformin uptake in the breast (Figure 5.8). PMAT is similarly important to intestinal metformin uptake, contributing to approximately 20% of intestinal metformin absorption (Cheng et al., 2024). Interestingly, *SLC29A4* (PMAT) was the only transporter significantly increased in all TIS samples, perhaps suggesting metformin uptake should be increased in senescent cells. However, current evidence does not clearly link differential PMAT expression to altered metformin sensitivity (Beaver et al., 2022).

SLC47A1 (MATE1) expression may also influence intracellular metformin concentration, as it mediates drug efflux. HCC1806 expressed *SLC47A1* (MATE1) at levels comparable to *SLC29A4* (PMAT), raising the question of whether increased metformin export, reduced intracellular drug accumulation (Figure 5.8). Interestingly, inhibition of MATE1 has been shown to increase metformin levels supporting this idea (Toyama et al., 2012; Tsuda et al., 2009). In this study, *SLC47A1* (MATE1) expression decreased slightly in MCF10A TIS samples but remained unchanged in both TNBC lines (Figure 5.8). Although not statistically significant, reduced *SLC47A1* (MATE1) expression could promote metformin retention in TIS cells, which somewhat aligns with the increased AMPK activation observed in 50 μ M and 500 μ M metformin treated MCF10A (Figure 5.6). Although *SLC47A1* (MATE1) was expressed in MCF7 it was not selected for immunofluorescence due to its primary role in metformin excretion rather than uptake.

Whilst differences in transporter expression may influence metformin uptake *in vivo*, patient data shows no clear correlation between *SLC22A1* (OCT1), *SLC22A3* (OCT3), *SLC29A4* (PMAT), and *SLC47A1* (MATE1) expression and metformin tumour concentration (Sundelin et al., 2020). Highest expression was of *SLC47A1* (MATE1) and *SLC29A4* (PMAT), which were both expressed in the models used. In BC patients, with ductal carcinoma, receiving intravenous metformin, metformin was detectable in tumour tissue, though cleared more rapidly than in the liver.

Overall, transporter expression in the cell lines used should not have prevented metformin uptake. Therefore, the limited metformin response observed in some assays is unlikely to be fully explained by transporter expression. Most transporters were either maintained or increased in TIS conditions, suggesting that senescence did not impair metformin import.

5.5.3.2 Protein expression

Immunofluorescence was used to assess the protein expression and localisation of OCT3 and PMAT (Figure 5.17). Overall immunofluorescence did not reflect all the changes seen in qPCR data, at a protein level. MGI for OCT3 was higher than PMAT across all cell lines, consistent with qPCR data for MDA-MB-231 but not for MCF10A or HCC1806. In doxorubicin treated samples, OCT3 staining intensity was higher than PMAT despite qPCR showing the opposite pattern (Figure 5.18 and Figure 5.8). This discrepancy may reflect differences in antibody affinity or staining efficiency rather than true differences in protein expression. Regardless, immunofluorescence analysis confirmed that both transporters are expressed at the protein level and therefore could contribute to metformin uptake in these models. In future, it may also be interesting to examine transporter gene and protein expression in metformin treated samples, to determine whether this influenced their expression or localisation.

Localisation was also assessed to determine whether PMAT and OCT3 were present at the plasma membrane, as expected for cells able to uptake metformin (Kurlovics et al., 2022; LaMoia and Shulman, 2021; Liang and Giacomini, 2017)(Figure 5.10, Figure 5.11, Figure 5.12, Figure 5.13, Figure 5.14, Figure 5.15 and Figure 5.16). Although these transporters are typically expressed on the apical membrane of epithelial cells, the breast cell lines used in this study lack clear apical and basal polarity, therefore it was anticipated some transporters would be present at the plasma membrane, if cells

were able to uptake metformin. Despite detectable expression, neither transporter exhibited clear localisation at the plasma membrane. Instead, OCT3 and PMAT predominantly showed cytoplasmic staining in both untreated and TIS cells. This may have contributed to the limited efficacy of metformin observed in TIS models, as transporter internalisation could prevent metformin uptake. It is also important to consider that as cells were fixed with 4% paraformaldehyde, it is likely 3D cell architecture was preserved, making identifying transporter expression at the plasma membrane difficult when viewing on a single focal plane.

Cytoplasmic localisation of OCT3 is not uncommon; images provided by the antibody manufacturer also show cytoplasmic distribution (ThermoFisher, 2025a). However, membrane localisation has been reported in OCT3 transfected KYSE30 cells and in fibroblasts, suggesting that localisation may vary depending on cell type or experimental conditions (Amani et al., 2023; Fu et al., 2017). One explanation for the cytoplasmic localisation of OCT3 in this study, is that cells were permeabilised with 0.1% Triton X-100, which disrupts the plasma membrane and facilitates the intracellular access of antibodies. This may have obscured any membrane specific signal. Staining without permeabilisation may have detected transporters exposed at the membrane, however, attempts to use saponin, a milder permeabilising agent, did not generate a detectable signal.

PMAT staining was considerably weaker than OCT3, potentially due to lower antibody affinity or genuinely lower protein expression (Figure 5.17). As with OCT3, PMAT also showed mainly cytoplasmic staining, consistent with manufacturer images and published research (Gludovacz et al., 2025; ThermoFisher, 2025b). Again, this may reflect true intracellular localisation or may result from membrane disruption during permeabilisation. Although saponin permeabilisation, facilitated some signal from PMAT, it may still have compromised membrane integrity preventing the reliable analysis of transporters at the plasma membrane.

Ultimately, the choice of permeabilisation method likely limited the ability to accurately determine transporter localisation. However, the lack of signal in secondary only controls indicates antibody specificity and confirms that both OCT3 and PMAT are present at the protein level in the cell lines that showed gene expression.

5.5.4 Clinical relevance of cell models

Bioinformatic analysis of transporter expression was performed to assess the clinical relevance of the cell models used in this study. Understanding differences between patient derived datasets and *in vitro* models is essential for determining the translational potential of findings.

Bioinformatics data revealed that transporter expression was generally higher in normal tissue than in BC (Table 5.7). Interestingly, this contrasts with the gene expression analysis in this study, where the non-malignant MCF10A cell line had substantially lower transporter expression than BC cell lines (Figure 5.8). This may have been due to differences between primary tissues and immortalised cell lines. Primary breast tissue is composed of many different cell types, including epithelial, myoepithelial and stromal cells, some of which may express transporters at higher levels than others. In contrast, MCF10A is derived from only immortalised mammary epithelial cells (Jones et al., 2025). Although routinely used as a healthy control, MCF10A has been immortalised, introducing genomic alterations, which change its molecular phenotype and may suppress transporter expression. From a translational perspective, lower transporter expression in healthy tissue may be beneficial, as it could limit metformin uptake and accumulation in normal cells. This would make metformin activity more selective for cancer cells, potentially reducing off target effects in non-cancerous tissue.

Some observations were consistent across bioinformatic and experimental datasets. For example, *SLC29A4* (PMAT) showed the highest overall expression whereas *SLC22A1* (OCT1) had the lowest expression (Table 5.7, Figure 5.20, Figure 5.22 and Figure 5.8). This suggests that the cell models are representative, at least to some degree, of *in vivo* conditions. Some *in vitro* findings, did not correlate with bioinformatic data. However, inconsistencies were expected, as cell lines only represent a small proportion of the tumour microenvironment.

UALCAN analysis indicated that *SLC22A1* (OCT1) expression was highest in TNBC tissue, followed by normal and then luminal tissue (Figure 5.20). This trend was consistent with the experimental data, where *SLC22A1* (OCT1) was elevated in TNBC cell lines but minimal in MCF10A (Figure 5.8). Increased OCT1 expression has been associated with poor prognosis in BC, supporting the plausibility of these findings (Ogura et al., 2021). In contrast, GEPIA analysis suggested higher *SLC22A1* (OCT1) expression in normal tissue (Table 5.7). This discrepancy likely arises from differences in the datasets used by the different software's. UALCAN relies exclusively on TCGA tumour (9736 samples) and tumour adjacent

(726 samples) tissues, whereas GEPIA integrates TCGA and GTEx datasets, which contains a far greater number of healthy, non-adjacent normal tissues (>8000 samples). These differences in sample number and tissue origin may underpin the variations in this bioinformatic study (Chandrashekar et al., 2017; Tang et al., 2017).

5.6 CONCLUSION

MTT assays confirmed that metformin exerted functional effects on MCF7, where reductions in cell viability were observed, consistent with previously reported effects. qPCR and immunofluorescence data indicated that the transporters required for metformin uptake were expressed to varying degrees in the cell models used. Although expression patterns did not fully align with the bioinformatic datasets, it was encouraging that *SLC29A4* (PMAT) showed the highest expression in both experimental and bioinformatic analysis, whilst *SLC22A1* (OCT1) consistently displayed the lowest. qPCR confirmed the presence of transporter gene expression, and immunofluorescence confirmed the presence of PMAT and OCT3 protein expression.

Preliminary western blot data suggested that key downstream targets of metformin were regulated, to some extent, in accordance with its expected mechanism, in TNBC cell lines. However, these findings require validation with repeat experiments and the optimisation of AICAR treatment as a positive control for MCF7 and MCF10A. Initial data suggested MDA-MB-231 had the highest levels of *SLC22A3* (OCT3) expression, which is consistent with its response to metformin, indicated by increased AMPK phosphorylation. *SLC29A4* (PMAT) expression was highest in MCF7 and increased following doxorubicin treatment, although this did not translate into a functional effect on downstream pathway activation, detectable through western blot. Nevertheless, the expression of these transporters at both gene and protein levels confirms that the transporters required for metformin uptake are present in these models.

Chapter 6

Paracrine effects of TIS TNBC and metformin modulation

6 PARACRINE EFFECTS OF TIS TNBC AND METFORMIN MODULATION

6.1 INTRODUCTION

6.1.1 Paracrine effects of TIS

TIS induced cancer cells can have many paracrine effects, through the secretion of their pro-inflammatory SASP, which can modulate the behaviour of cells within the TME (Lopes-Paciencia et al., 2019; Luo et al., 2025). The TME is comprised of many cell types including immune and stromal cells (fibroblasts, mesenchymal stem cells and adipose stem cells) which interact to influence cancer progression (Çakal, Urgancı and Şimşek, 2025). As key mediators within the TME, fibroblasts are particularly important, under healthy conditions they are an essential connective tissue secreting ECM components to facilitate tissue development and repair, but under cancerous conditions can become activated becoming cancer-associated fibroblasts (CAFs) that support tumour progression, metastasis and chemotherapy resistance (Çakal, Urgancı and Şimşek, 2025; Wright et al., 2023). As 90-95% of all BC's are considered invasive, spreading into the surrounding stroma, it is important to understand how fibroblasts, a key stromal component, are influenced by the proinflammatory secretions of SASP from TIS TNBC (Çakal, Urgancı and Şimşek, 2025). The SASP from TIS TNBC cells can influence fibroblasts in many ways including by promoting CAF transformation and inducing paracrine senescence.

6.1.2 Cancer associated fibroblasts

The origin of CAFs is not fully understood, although it is widely accepted that they arise in response to tumour secretions, such as TGF- β , IL1A and platelet-derived Growth Factor (PDGF), which reprogram fibroblasts to be pro-tumorigenic (Caligiuri and Tuveson, 2023). This activation is particularly relevant in the context of TIS, where senescent cells release a SASP enriched with CAF inducing factors (Hernandez-Segura, Nehme and Demaria, 2018). CAFs are tissue specific and highly heterogenous but can be characterised by an altered morphology (increased aspect ratio, the cell is longer than it is wide) and upregulation of markers such as vimentin, alpha-smooth muscle actin (α -SMA) and fibroblast activation protein (FAP), which function in cell contraction, movement and ECM modelling (Chen, McAndrews and Kalluri, 2021; Hu et al., 2022a; Hu et al., 2023; Wright et al., 2023). Although, these markers vary between BC subtypes (Park, Kim and Koo, 2015; Park, Jung and Koo, 2016; Tchou et al., 2012).

CAFs are the primary cell type within the TME of many cancers, including BC, which promote cancer proliferation, epithelial mesenchymal transition (EMT), immune suppression and chemotherapy resistance, all features that negatively impact clinical prognosis (Friedman et al., 2020; Hu et al., 2022a; Yang et al., 2025; Ye et al., 2024). CAFs remodel the TME to facilitate tumour growth, invasion, and metastasis. They secrete ECM proteins such as collagen and fibronectin which synthesise and stiffen the ECM (Hu et al., 2022a). This restricts immune cell infiltration and drug entry whilst simultaneously enhancing pro-tumorigenic signalling, promoting EMT (Çakal, Urgancı and Şimşek, 2025; Hu et al., 2022a; Yang et al., 2025). At the same time, fibroblasts and cancer cells secrete matrix metalloproteinases that degrade the ECM, creating migratory pathways facilitating cancer migration (Erdogan and Webb, 2017; Hu et al., 2022a). CAFs also secrete proinflammatory factors which promote immune suppression, tumour vascularisation and metastasis by sustaining chronic inflammation, recruiting immune cells and inducing CAF transformation in neighbouring fibroblasts, as well as generating a nutrient rich TME beneficial for tumour growth (Çakal, Urgancı and Şimşek, 2025; Hu et al., 2022a; Luo et al., 2025).

Investigating how SASP, from TIS TNBC, influences fibroblasts is important for understanding its contribution to CAF formation, and whether metformin can mitigate this. This may improve patient outcomes by preventing SASP induced TME changes, which promote tumour progression, metastasis, and relapse.

6.1.3 Senescence

Paracrine senescence refers to the process by which senescent cells induce senescence in neighbouring cells, via SASP secretions. As previously described, senescence can have many detrimental impacts on cancer, including promoting relapse, tumour invasion, chemotherapy resistance and metastasis (Luo et al., 2025). It is therefore important to understand the paracrine effects of TIS TNBC and how these can be prevented.

TIS can be induced in many cell types, including fibroblasts which are a primary constituent of the TME, and have been identified in patient tissue (Sirinian et al., 2022; Ye et al., 2024). Senescent fibroblasts have been shown to promote both cancer and untransformed cell proliferation in many models, including of mammary epithelial cells and transplanted BC cells (Gonzalez-Meljem et al., 2018; Liu and Hornsby, 2007; Tsai et al., 2005). Reprogramming of senescent fibroblasts also benefits tumour

growth by increasing nutrient production and improving BC cell viability (Balliet et al., 2011; Fourie et al., 2019). Furthermore, when cells enter senescence, they become more resistant to apoptosis making chemotherapy drugs less efficient (Hu et al., 2022b). Senescence and CAFs are also closely interconnected, influencing both disease reoccurrence and progression. Some CAF populations are senescent and limit immune cell cytotoxicity to enhance tumour growth (Ye et al., 2024). In patients with recurring early-stage BC, senescent CAFs were more abundant than in patients with no recurrence, indicating senescence plays a pivotal role in disease progression (Ye et al., 2024).

It is important to determine if paracrine secretions induce senescence in fibroblasts, as this may result in a pro-inflammatory microenvironment which contributes to cancer aggression. In cases where paracrine TIS is induced, metformin may attenuate SASP induced inflammation, and its associated detrimental consequences.

6.1.4 Co-culture cell models

Few studies have investigated the paracrine effects of senescent BC, of those that do, the majority use conditioned media or isolated SASP components, which fail to recapitulate the complex interactions of cells within the TME (Galindo-Hernandez et al., 2014; Guo et al., 2017; Leal-Orta et al., 2022). Co-culture models are a physiologically relevant system for studying complex intercellular signalling. In direct co-culture models, multiple cell types are grown in contact with one another, whereas in indirect model's cell types are separated by a semi-permeable membrane (Mason and Öhlund, 2023). Indirect co-culture models are beneficial for investigating all components of paracrine signalling, in multiple cell types, within the same environment, by closely mimicking the complexity of *in vivo* interactions, without cells being in direct contact with one another (Mason and Öhlund, 2023).

Indirect co-culture was used to study the effects of TIS TNBC secretions on fibroblasts, as this model allowed the impact of SASP factors to be examined in isolation, by preventing direct cell contact and competition. This approach also allows metformin to be more fully evaluated as a senomorphic, as its effects on the SASP can be investigated as a whole rather than on isolated components. This system also allows the effects of metformin to be assessed across multiple cell types simultaneously, more accurately reflecting its *in vivo* mechanism. In this study patient derived fibroblasts were selected as they are the most abundant TME component, which play key roles in tumour progression and recurrence. As these cells are primary and patient derived, they accurately reflect *in vivo* gene

expression patterns and signalling. Ultimately, co-culture experiments provide insight into how TIS in TNBC shapes the TME and enables the potential benefits of senotherapeutic drugs, like metformin to be studied.

6.2 AIMS AND OBJECTIVES

The purpose of this chapter was to investigate the paracrine effects of TIS and how this may be modulated by metformin. The primary aims of this chapter were to (6.1) determine whether secretions from TIS TNBC cells induce senescence or functional changes in healthy fibroblasts and (6.2) assess whether metformin can mitigate TIS induced inflammation in a co-culture system.

To fulfil aim (6.1) the following objectives will be completed:

- 6.1A: Establish a co-culture system using TIS TNBC cells and patient derived fibroblasts by:
 - Assessing changes in fibroblast and TNBC viability, using MTT assays.
 - Confirming senescence in HCC1806 maintained in fibroblast media, using β -galactosidase staining and gene expression analysis of SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).
- 6.1B: Determine whether TIS secretions induce cancer associated fibroblast formation in fibroblasts by using:
 - Scratch assays to assess migratory changes in fibroblasts co-cultured with TNBC and TIS TNBC.
 - Aspect ratio measurements to assess morphological changes in fibroblasts co-cultured with TNBC and TIS TNBC.
- 6.1C: Determine whether TIS secretions induce senescence in fibroblasts by assessing:
 - β -galactosidase staining as a marker of increased lysosomal content.
 - Gene expression changes in SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).

To fulfil aim (6.2) the following objectives will be completed:

- 6.2A: Determine working metformin concentrations in fibroblasts, using MTT assays to assess cell viability.

- 6.2B: Determine whether metformin reverses the elevated pro-inflammatory cytokine expression in fibroblasts co-cultured with TIS TNBC. by analysing changes in gene expression of SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*).

6.3 MATERIALS AND METHODS

Materials and methods specific to this chapter are described below. Unless otherwise stated, protocols used were those outlined in Chapter 2 (Universal Materials and Methods).

6.3.1 Cell culture of human fibroblasts

Co-culture experiments were performed using fibroblasts obtained from the Breast Cancer Now Biobank, (London, UK). Fibroblasts were derived from normal breast reduction mammoplasty patients and supplied at passage 3. Fibroblasts from the same patient, and passage (5) were used for each biological repeat. Fibroblasts were cultured under conditions recommended by Breast Cancer Now Biobank, in T75 flasks.

Cells were cultured in Gibco™ DMEM/F-12, HEPES (Fisher Scientific, Leicestershire, UK) supplemented with 10% heat inactivated FBS (Fisher Scientific, Leicestershire, UK), 5% penicillin/streptomycin (Fisher Scientific, Leicestershire, UK) and 5% Amphotericin B (Fisher Scientific, Leicestershire, UK). Media was changed twice weekly, leaving a small volume (~1 mL) of conditioned medium in the flask, and replenishing with ~19 mL fresh pre-warmed media. Cells were grown to near confluence before passaging. To subculture, medium was removed, cells were washed once with 10 mL PBS (without calcium and magnesium), supplied by the university and incubated with 3 mL Gibco™ TrypLE™ Express Enzyme, for ~7 minutes, until detachment was observed by microscopy (Fisher Scientific, Leicestershire, UK). Once detached, cells were resuspended in 10 mL fresh medium and centrifuged at $380 \times g$ for 3 minutes at room temperature. The resulting pellet was resuspended in culture medium and seeded as required (5×10^5 - 1×10^6 cells per T75 flask). Cells were maintained at 37 °C with 5% CO₂. As fibroblasts tended to aggregate, a 200 µL pipette was used to triturate the cell suspension before counting and dilution.

6.3.2 Ethical approval for cell studies

The NHS Research Ethics Committee (REC) review concluded that an NHS REC review was not required. Internal ethics approval, at the University of Hertfordshire, was obtained for the use of patient-derived fibroblasts.

6.3.3 Co-culture

6.3.3.1 System optimisation

Indirect co-culture experiments were performed to investigate the paracrine effects of the SASP, from TIS TNBC cells on healthy cells. To enable co-culture, TNBC cell lines (MDA-MB-231 and HCC1806) were grown in fibroblast media (Gibco™ DMEM/F-12, HEPES supplemented with 10% heat-inactivated FBS, 5% penicillin/streptomycin, and 5% Amphotericin B. Cells were gradually acclimatised to the new media by sub-culturing cells weekly in increasing proportions of fibroblast media, starting at a 10:90 ratio of fibroblast media to standard growth media, and increasing in stepwise increments (20:80, 30:70, etc.) until cells were maintained in 100% fibroblast media. MDA-MB-231 did not tolerate media containing >60% fibroblast media and therefore could not be used in subsequent experiments. As a result, only HCC1806 cells were used in co-culture studies.

To confirm HCC1806 was surviving in fibroblast media cell viability was assessed using MTT assays, as described in Chapter 2.6. To ensure the change in media didn't affect TIS induction, TIS was confirmed using β -galactosidase staining and qPCR of SASP components (*CXCL1*, *IL6*, *IL8* and *IL1A*), as described in Chapter 2.9.

6.3.3.2 Indirect co-culture

HCC1806 were cultured in 4 μ m trans-well inserts and placed into 12 well plates containing fibroblasts. A 4 μ m pore size was selected to allow the transfer of SASP components whilst preventing cell contact. On day 1, HCC1806 were seeded in 500 μ L media, onto trans-well inserts, separately to fibroblasts, which were plated in 1 mL media in 12 well plates (10,000 cells/well). As in previous senescence experiments, untreated HCC1806 were seeded at 5833 cells/cm² (5250 cells/insert) whilst cells to be treated with doxorubicin were seeded at 20833 cells/cm² (18750 cells/insert). On day 2, HCC1806 cells were treated with 25 nM doxorubicin, either with or without metformin (50 μ M and 500 μ M). On day 3, doxorubicin containing media was removed, and trans-well inserts were placed into wells

containing fibroblasts. All media was replaced, and fibroblasts were co-cultured with TNBC samples for 96 hours whilst TIS was induced in HCC1806.

On day 7, Fibroblasts were assessed for their response to TIS by comparing the changes in senescence, and migration between HCC1806 and HCC1806 TIS cells. Untreated fibroblasts served as a control for experiments. Where differences were observed, metformin (50 μ M and 500 μ M) was added in subsequent experiments to evaluate whether these effects could be attenuated. Since metformin can pass through the trans-well insert, additional controls were included where fibroblasts were either exposed to metformin or left untreated. A summary of indirect co-culture is shown in Figure 6.1.

6.3.4 Fibroblast MTT assays

Fibroblasts were assessed for changes in viability in response to co-culture with TIS TNBC. After 96-hours of co-culture, trans-wells were removed and 250 μ L of MTT (5 mg/mL MTT in PBS) was added to each well containing fibroblasts, then incubated for 4 hours at 37°C. Plates were kept covered due to the light sensitivity of MTT. Following incubation, media was removed, and 1 mL DMSO was added per well, to dissolve the formazan crystals. Media only wells were included on each plate to account for background absorbance during data analysis. Subsequent steps were performed following the same protocol as for immortalised cell lines (Chapter 2.6).

Fibroblast viability, following metformin treatment was assessed to determine whether metformin cytotoxicity contributed to any changes observed during experiments. To replicate the conditions of the indirect co-culture experiments on day 1, fibroblasts were seeded in 12 well plates (10,000 cells/well). Metformin was added on day 3, and cell viability was measured on day 7. Fibroblasts were either untreated, or incubated with 50 μ M, or 500 μ M metformin. MTT addition, incubation, and absorbance measurements were performed as described above.

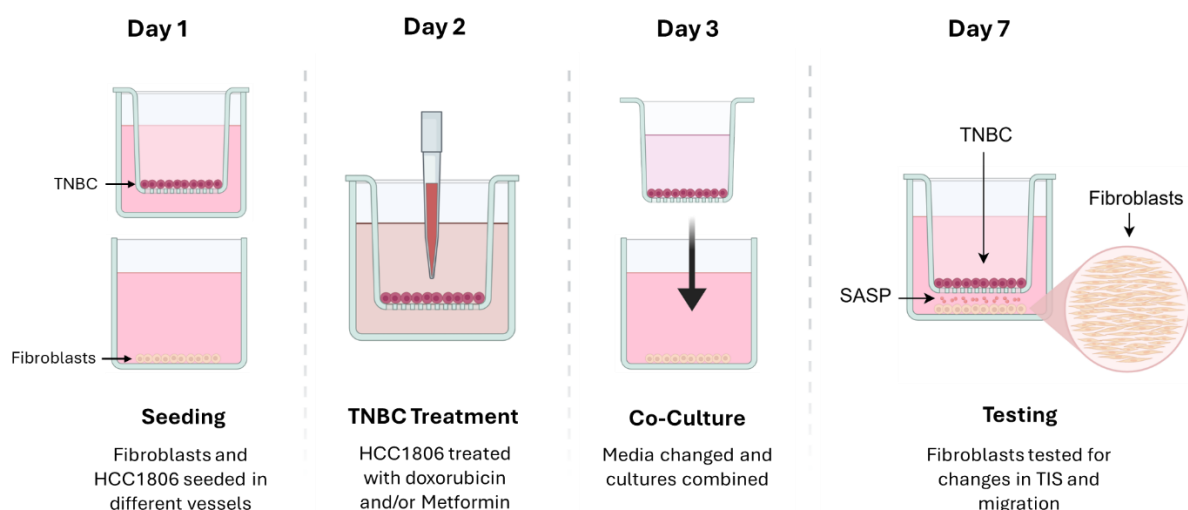


Figure 6.1. Overview of indirect co-culture protocol.

Two approaches were used to investigate the paracrine effects of TIS TNBC on healthy fibroblasts. These included evaluating senescence by β -galactosidase staining and analysing TIS marker expression by qPCR and assessing CAF formation by examining migration and morphological changes (Figure 6.2). Experiments were repeated at least 3 times, independently (biological replicates $n=3$).

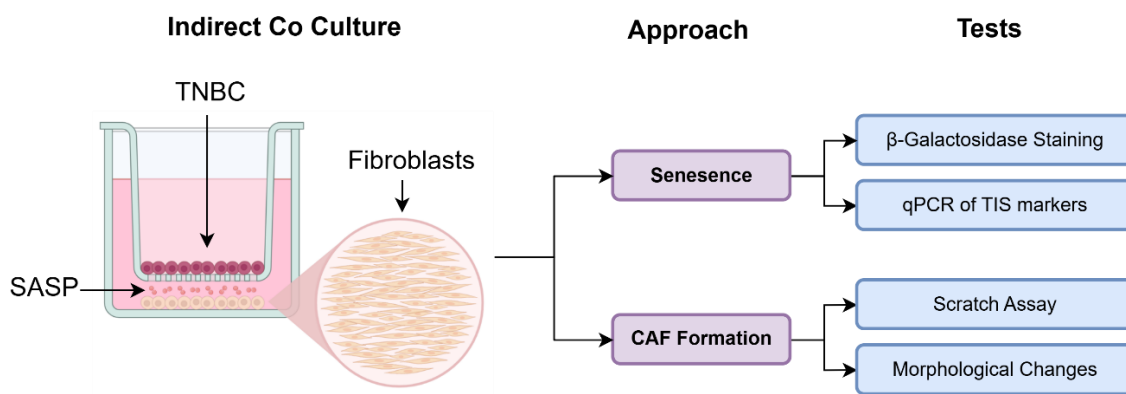


Figure 6.2. Summary of co-culture experiments. Cancer associated fibroblast (CAF).

6.3.5 Fibroblast senescence assays

Fibroblasts were assessed for paracrine senescence using β -galactosidase staining and qPCR analysis of SASP markers. β -galactosidase staining was performed as described in Chapter 2.9. qPCR's of SASP

markers (*CXCL1*, *IL6*, *IL8* and *IL1A*) were performed as outlined in Chapter 2.16, with the amendment that fibroblasts were eluted in 20 µL of RNA elution solution.

6.3.6 Scratch assays

Changes in migration, in response to TNBC TIS co-culture, were assessed using scratch assays. On day 7, trans-well inserts were temporarily removed, and media was replaced. Both horizontal and vertical scratches were created across the fibroblast monolayer, using a p200 pipette tip, to minimise uneven scratch areas. Wells were washed with PBS to remove detached cells, and fresh media was added. Four regions of the scratch per well (horizontal left, horizontal right, vertical up and vertical down) were imaged at 0, 24 and 48 hours, until the wound had closed. Between imaging timepoints, trans-well inserts were re-added to plates and cells were maintained in an incubator set to 37°C with 5% CO₂. The cell-free area at each timepoint was quantified in ImageJ using a wound healing plugin (Schindelin et al., 2012; Suarez-Arnedo et al., 2020). The cell-free area was selected manually to account for the non-uniform morphology of fibroblasts migrating across the scratch, which the automated system could not detect. The plugin then quantified the proportion of the image lacking cells and reported this as the cell-free area (%). Measurements from the four scratch locations, at each time point, were averaged and wound closure (%) at 24 and 48 hours was calculated using Equation 12.

Equation 12

$$Wound\ Closure\ (\%) = (\% Cell\ Free\ Area^{0\ Hours} - \% Cell\ Free\ Area^{Time\ Point}) * 100$$

6.3.7 Morphological changes

Morphological changes were quantified using aspect ratio measurements. In ImageJ, the height and width of 100 fibroblasts was measured manually (µm) and recorded (Schindelin et al., 2012). Fibroblasts were randomly selected for measurement to avoid bias and ensure representative sampling of the population. Aspect ratio was calculated as the height/width for each fibroblast.

6.4 RESULTS

6.4.1 Optimisation of co-culture

After being acclimatised and continuously cultured in fibroblast media, HCC1806 were assessed for viability changes, in the presence and absence of metformin, using MTT assays (Figure 6.3). When normalised to HCC1806 maintained in standard growth media, cells cultured in fibroblast media showed no significant decrease in viability, suggesting cells were suitable for use in subsequent experiments. For 96 hours, in fibroblast media 50 μ M metformin reduced viability to 72%, slightly lower than in standard growth media (99%) however at 500 μ M viability remained at ~80%, consistent with results in standard growth media (Figure 4.3), suggesting these metformin concentrations were appropriate for use in senescence reversal experiments (Figure 6.3).

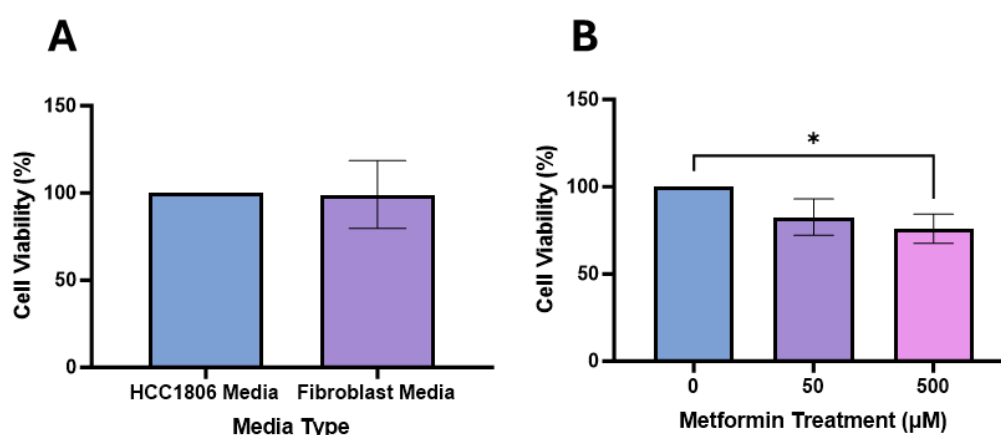


Figure 6.3. Viability of HCC1806 cultured in fibroblast media with and without metformin treatment. Cell viability was assessed following (A) continuous culture in fibroblast media or (B) a 96 hour metformin treatment. Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed at 500 μ M metformin. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using an (A) unpaired t-test or (B) one-way ANOVA with Tukey's post hoc test, where $N_s = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

MTT assays were also performed on co-cultured (TNBC and TIS TNBC) and non-co-cultured fibroblasts, to ensure any observed results were not due to unfavourable culture conditions. No statistically significant reduction in viability was observed between non-co-cultured and co-cultured fibroblasts (Figure 6.4). Further MTT assays were performed to assess the effect of metformin on fibroblast viability (Figure 6.5), as it was important to confirm that any changes observed in subsequent experiments, were due to senescence reversal rather than substantial loss of viability. Similarly to HCC1806, fibroblasts showed a statistically significant reduction in viability, after 96 hours, at 500 μ M

metformin, but not at 50 μM . This is beneficial as 50 μM metformin represents a non-cytotoxic, clinical concentration, whilst 500 μM is a supra-pharmacological concentration. Loss of viability at the higher concentration indicates that metformin is functionally active.

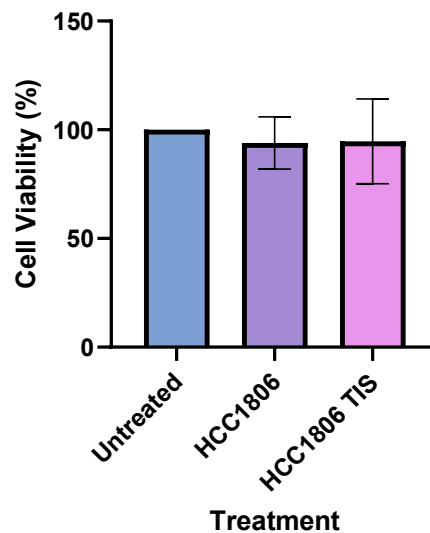


Figure 6.4. Viability changes in fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. Cell viability was assessed following 96 hours of co-culture. Viability (%) is expressed as a proportion of the untreated control (100%). No statistically significant differences were observed. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using a one-way ANOVA with Tukey's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

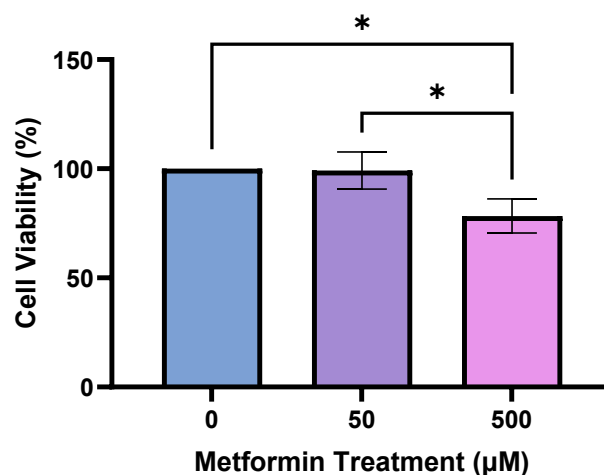


Figure 6.5. Viability of fibroblasts in response to metformin treatment. Cell viability was assessed following a 96 hour treatment with or without metformin (50 μM and 500 μM). Viability (%) is expressed as a proportion of the untreated control (100%). A statistically significant reduction in viability was observed at 500 μM metformin. MTT assays were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using a one-way ANOVA with Tukey's post hoc test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

HCC1806 were also induced with TIS, using the same conditions as described in Chapter 2 (Universal Materials and Methods), to confirm they were senescent, when maintained in fibroblast media. Senescence was assessed using β -galactosidase staining, a widely used hallmark of senescence, and qPCR analysis of key SASP components (*CXCL1*, *IL1A*, *IL6* and *IL8*), as these factors are expected to influence fibroblasts via paracrine signalling. Approximately 80% of cells were β -galactosidase positive (Figure 6.6), indicating senescence, consistent with previous findings in standard growth media (Figure 4.9).

A statistically significant increase in *IL1A*, *IL6* and *IL8* gene expression (fold change in comparison to untreated control) was observed in doxorubicin treated samples, when analysed using qPCR (Figure 6.7). *IL8* expression increased ~6 fold, similar to the response observed in standard media (Figure 4.22 and Figure 6.7). *CXCL1* and *IL1A* expression increased ~2 fold, which was lower than the ~6 fold increase previously observed in standard media (Figure 4.23, Figure 4.24 and Figure 6.7). In contrast, *IL6* expression increased ~25 fold, more than in standard growth media which previously induced a ~5 fold increase in expression (Figure 4.21 and Figure 6.7). These differences highlight the variability of the SASP across conditions; however, as SASP components remained elevated relative to controls, and β -galactosidase was elevated, these cells were considered senescent and suitable for subsequent co-culture experiments.

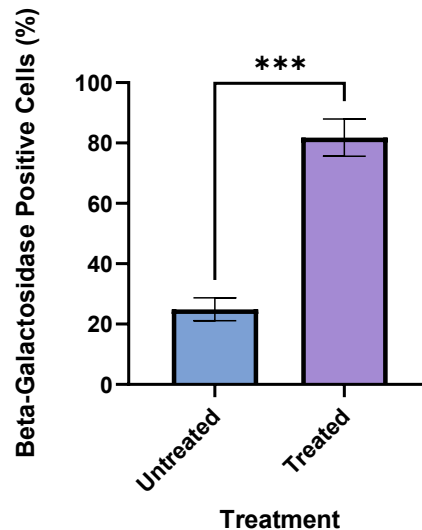


Figure 6.6. Effect of doxorubicin treatment on β -galactosidase staining in HCC1806 cells cultured in fibroblast media. Cells were stained using a β -galactosidase staining kit, and a total of 150 cells were counted to determine the percentage of β -galactosidase positive HCC1806 cells. The proportion of positive cells (%) was calculated relative to the total cell population. A statistically significant increase in staining was observed. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using an unpaired t-test where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

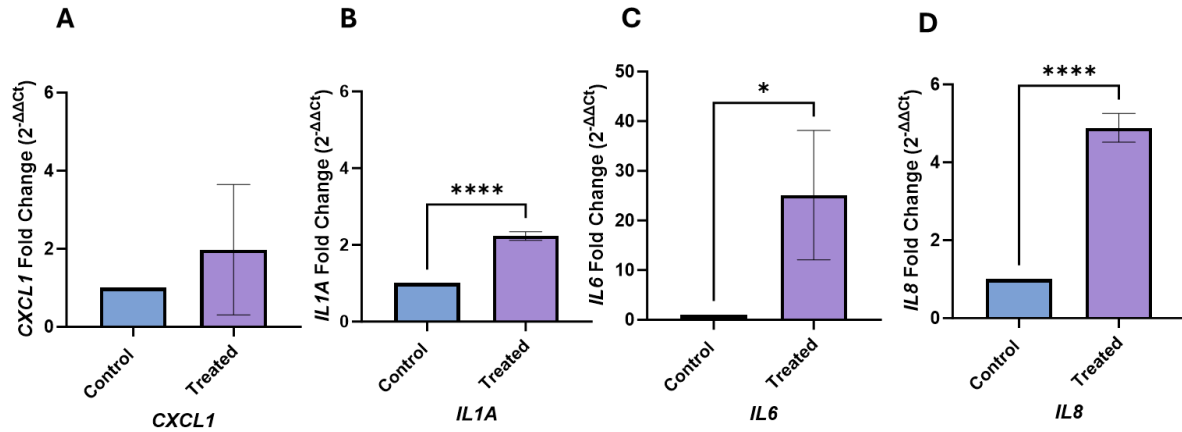


Figure 6.7. qPCR analysis of *CXCL1*, *IL1A*, *IL6* and *IL8* in HCC1806 cultured in fibroblast media. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample is set as a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6* and (D) *IL8*. A statistically significant increase in *IL1A*, *IL6* and *IL8* gene expression was observed in doxorubicin treated samples. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using unpaired t-tests, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

6.4.2 Assessing cancer-associated fibroblast formation in fibroblasts

6.4.2.1 Migratory changes

Scratch assays were performed to determine whether paracrine SASP signalling promoted a more migratory or invasive phenotype in fibroblasts, indicative of CAFs. Fibroblast migration was monitored until the wound closed at 48 hours (Figure 6.8) and the percentage wound closure was quantified (Figure 6.9). No statistically significant differences in migration were observed between treatment groups, indicating TIS TNBC had little paracrine influence on the migratory capacity of fibroblasts.

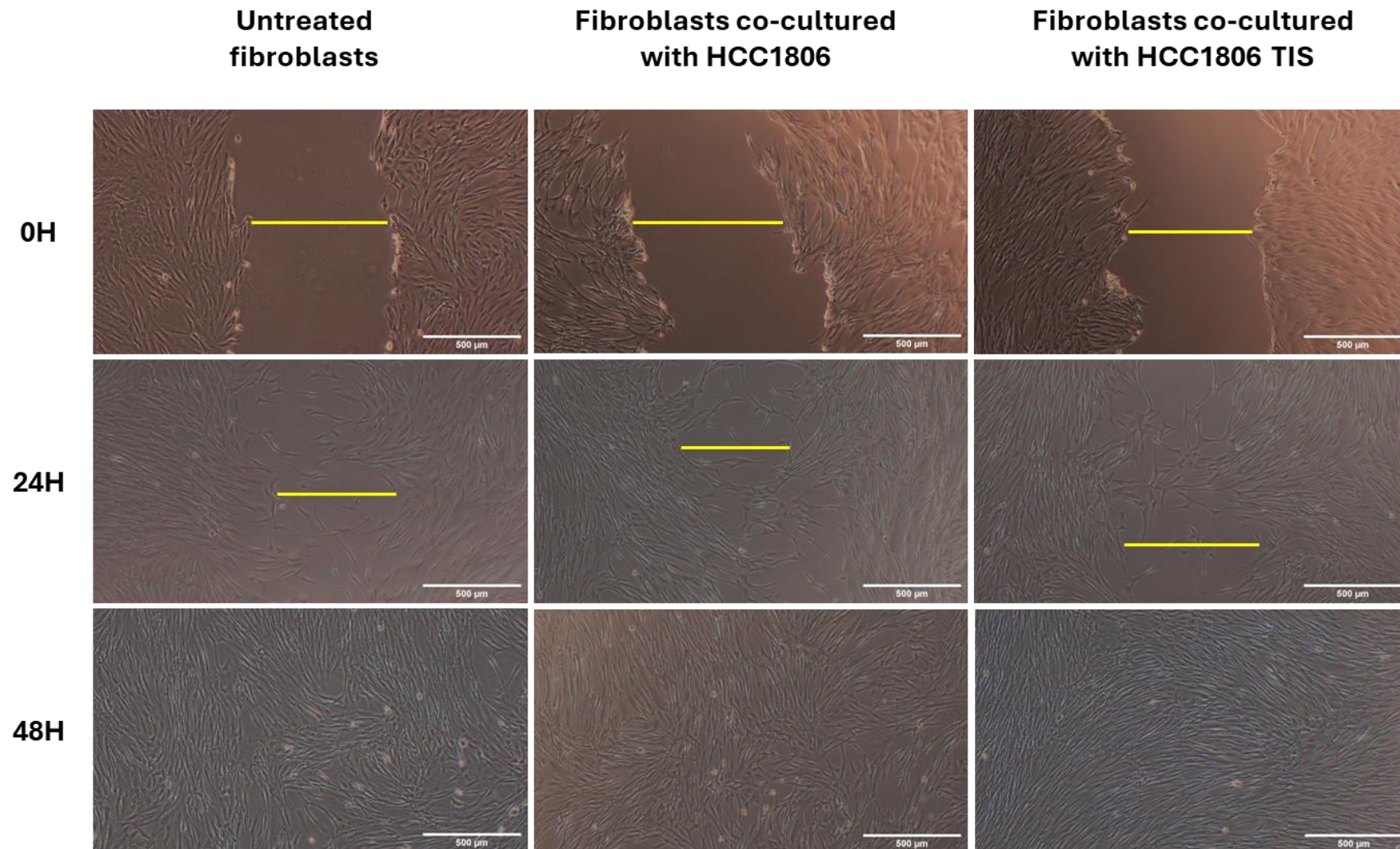


Figure 6.8. Representative scratch assay images of fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. Fibroblasts were either untreated or co-cultured with HCC1806 and HCC1806 TIS, scratched and imaged at 0, 24 and 48 hours (H), in four places per well. Scratch area was measured using a specialised wound healing plugin in ImageJ. The yellow line indicates a section of the cell-free area for reference; however, the software quantified the full cell-free region, not just the line shown. Images were captured at 4× magnification, using a phase contrast microscope and have a scale bar of 500 μm .

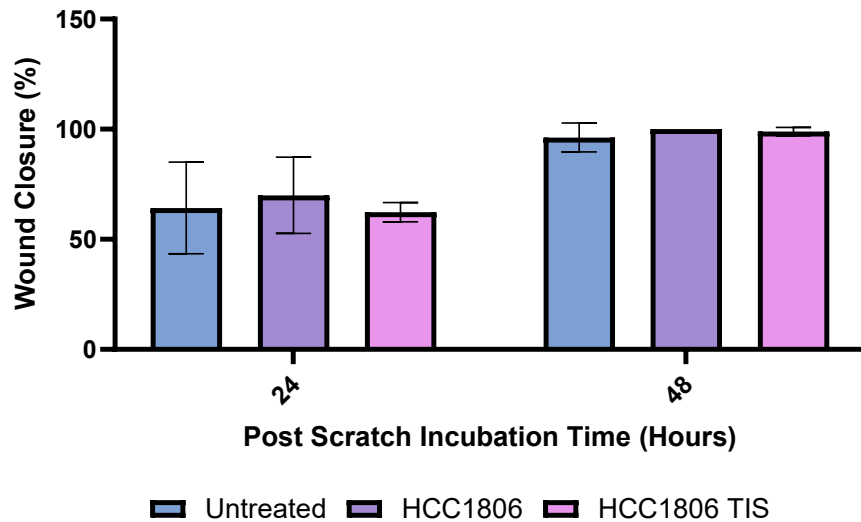
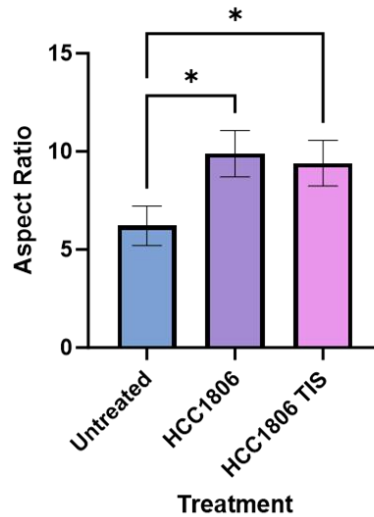


Figure 6.9. Migratory changes in fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. Fibroblasts were either untreated or co-cultured with HCC1806 and HCC1806 TIS, scratched and imaged at 0, 24 and 48 hours, in four places per well. These measurements were quantified using a specialised wound healing plugin in ImageJ. Wound closure was expressed as the percentage cell-free area reduction between the initial scratch at 0 hours and each subsequent time point. Scratch assays were performed in 3 independent biological replicates ($n = 3$). Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using a one-way ANOVA with Tukey's post hoc test for 24 hours and Kruskal-Wallis test with Dunn's post hoc test for 48 hours, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

6.4.2.2 Morphological alterations

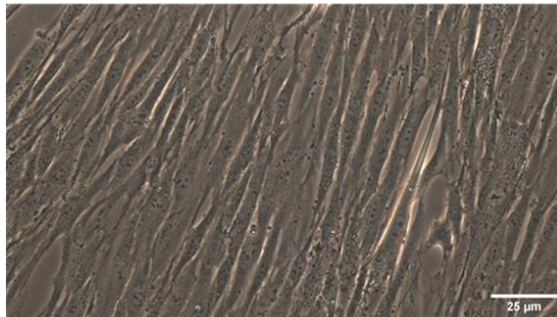
Morphological changes were quantified using aspect ratio measurements, calculated as height/width (AU) for each fibroblast (Figure 6.10). A statistically significant increase in aspect ratio was observed between untreated fibroblasts and those co-cultured with HCC1806 or HCC1806 TIS cells. However, as no significant differences were observed between untreated and TIS HCC1806 samples, these changes were not considered TIS specific. Microscopically, co-cultured fibroblasts appeared more elongated and spindle-like, consistent with aspect ratio measurements. Fibroblasts cultured alone grew in close proximity to one another, whereas co-cultured fibroblasts appeared to have fewer cell-cell interactions, and more gapping between cells.

A

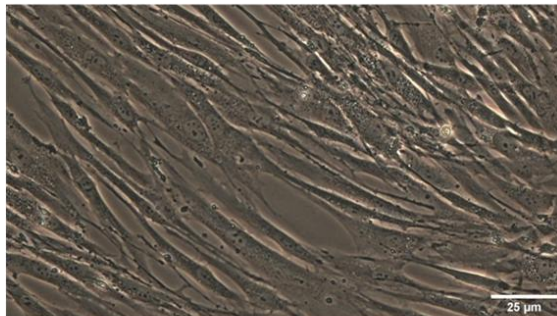


B

Untreated fibroblasts



Fibroblasts co-cultured with HCC1806



Fibroblasts co-cultured with HCC1806 TIS

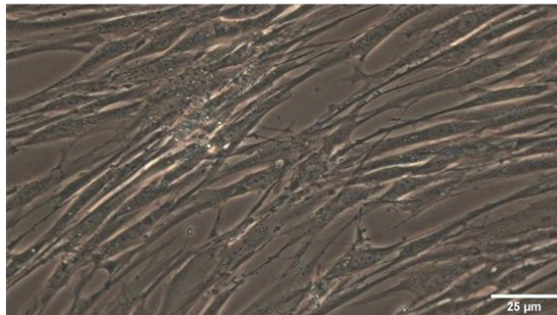


Figure 6.10. Morphological changes in fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. (A) Morphological changes were quantified using aspect ratio measurements. In ImageJ, the height and width of 100 fibroblasts was measured (μm) and recorded. Aspect ratio was calculated as height/width for each fibroblast. A statistically significant increase in aspect ratio was observed between untreated and HCC1806 untreated/TIS samples. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using a one Way ANOVA with Tukey's test where $N_s = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$, and $**** = P \leq 0.0001$. (B) Representative images of untreated and co-cultured fibroblasts. Images were captured at 40 $\times$ magnification, using a phase contrast microscope and have a scale bar of 25 μm . Treated cells appear more elongated than those that are untreated.

6.4.3 Assessing paracrine senescence in fibroblasts

Fibroblasts were assessed for senescence, as a consequence of co-culture treatment, using β -galactosidase staining and gene expression analysis. Comparisons were made between untreated and co-cultured (HCC1806 and HCC1806 TIS) fibroblasts to determine whether SASP secretions from TIS samples influenced fibroblasts in a paracrine manner.

6.4.3.1 β -galactosidase staining

No significant changes in β -galactosidase staining were observed across any treatment group, indicating paracrine senescence was not induced (Figure 6.11). Fibroblasts cultured to passage 14 served as a positive control for staining. Although additional senescence markers were not assessed in passage 14 fibroblasts, their extended time in culture, enlarged morphology, and positive β -galactosidase staining indicated that these cells had likely undergone replicative senescence.

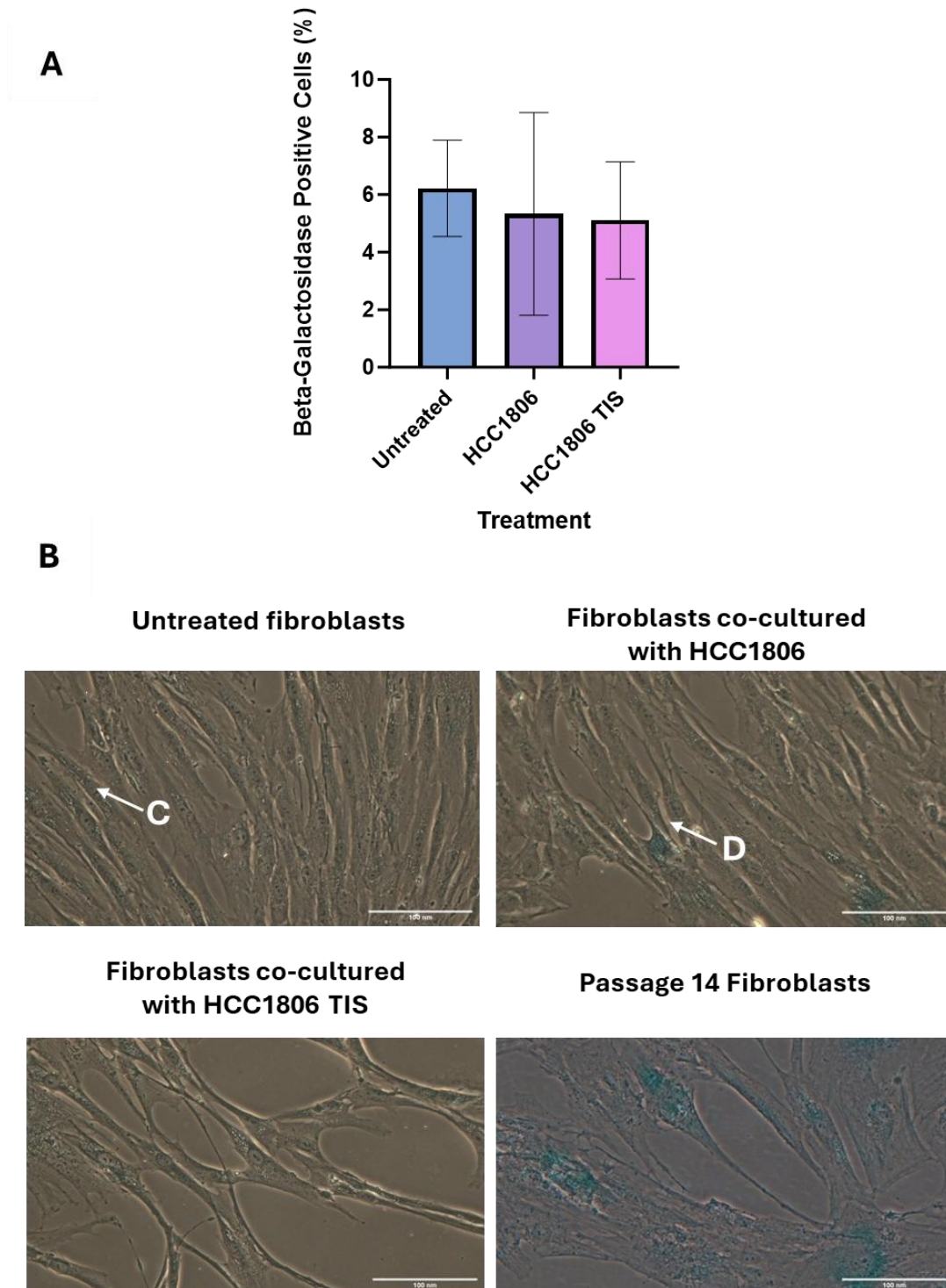


Figure 6.11. β -galactosidase staining in fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. (A) Effect of co-culture treatments on β -galactosidase staining in fibroblasts. Cells were stained using a β -galactosidase staining kit, and a total of 150 cells were counted to determine the percentage of β -galactosidase positive cells. The proportion of positive cells (%) was calculated relative to the total cell population. No statistically significant changes in staining were observed. Assays were performed in three independent biological replicates ($n = 3$) and data is presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using a one Way ANOVA with Tukey's test where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$, and $**** = P \leq 0.0001$. (B) Representative β -galactosidase staining images. Images were captured at 20 $\times$ magnification, using a phase contrast microscope and have a scale bar of 100nm. C) Example of a β -galactosidase negative cell and D) Example of a β -galactosidase positive cell, indicative of senescence. Fibroblasts cultured to passage 14 served as a positive control for β -galactosidase staining.

6.4.3.2 Gene expression changes

Gene expression of key SASP components was assessed using qPCR. Comparisons between untreated and co-cultured fibroblasts showed a statistically significant increase in all SASP markers in fibroblasts treated with HCC1806 TIS compared with the untreated control (Figure 6.12). In contrast, fibroblasts co-cultured with untreated HCC1806 cells showed a significant increase only in *IL8*. Overall, expression of SASP components were elevated in all fibroblasts exposed to HCC1806 cells, which further increased those treated with HCC1806 TIS. Interestingly, expression of *CXCL1*, *IL1A*, and *IL8* was significantly upregulated in fibroblasts co-cultured with HCC1806 TIS compared to those cultured with untreated HCC1806 cells, indicating a TIS specific enhancement of SASP gene expression.

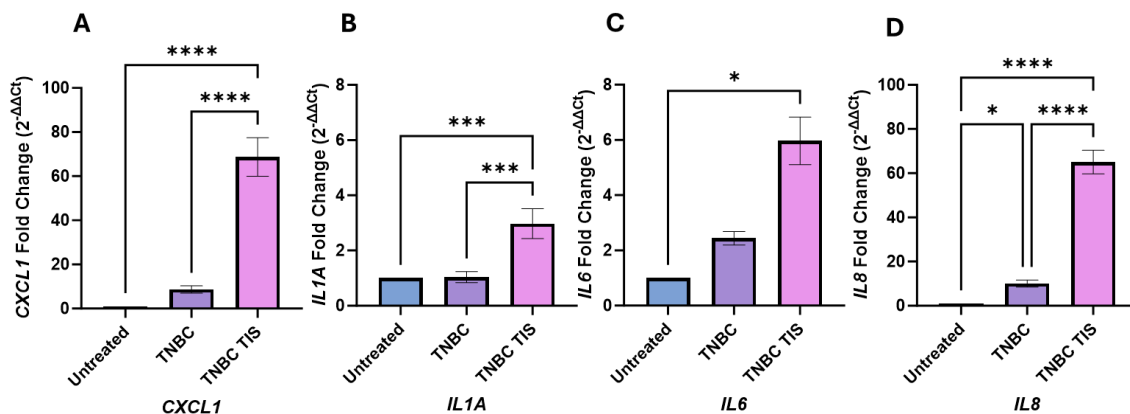


Figure 6.12. Gene expression changes in fibroblasts co-cultured with and without HCC1806 and HCC1806 TIS. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample is set as a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6* and (D) *IL8*. A statistically significant increase in all SASP components was observed in fibroblasts treated with TNBC (HCC1806) TIS compared to the untreated control. When comparing untreated fibroblasts to those co-cultured with untreated TNBC, this trend was only observed for *IL8*. A significant increase in *CXCL1*, *IL1A*, and *IL8* expression was detected in fibroblasts co-cultured with HCC1806 TIS compared with those cultured with untreated HCC1806 cells. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one way ANOVA with Tukey's test for *CXCL1*, *IL1A* and *IL8*, and the Kruskal-Wallis test with Dunn's post hoc test for *IL6*, where $N_s = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

To determine whether a senomorphic drug could attenuate the expression of SASP components in fibroblasts exposed to TIS, fibroblasts were co-cultured with HCC1806 TIS, treated with metformin, and re-evaluated for changes in SASP gene expression (Figure 6.13). The control sample in this incidence was HCC1806 TIS treated fibroblasts. Relative to this control, an increase in all SASP components was observed in 50 μ M metformin treated samples, in contrast to 500 μ M treated samples where SASP component expression decreased in all, but *IL8*. A statistically significant increase in *IL8* expression was observed in metformin treated samples compared to the control.

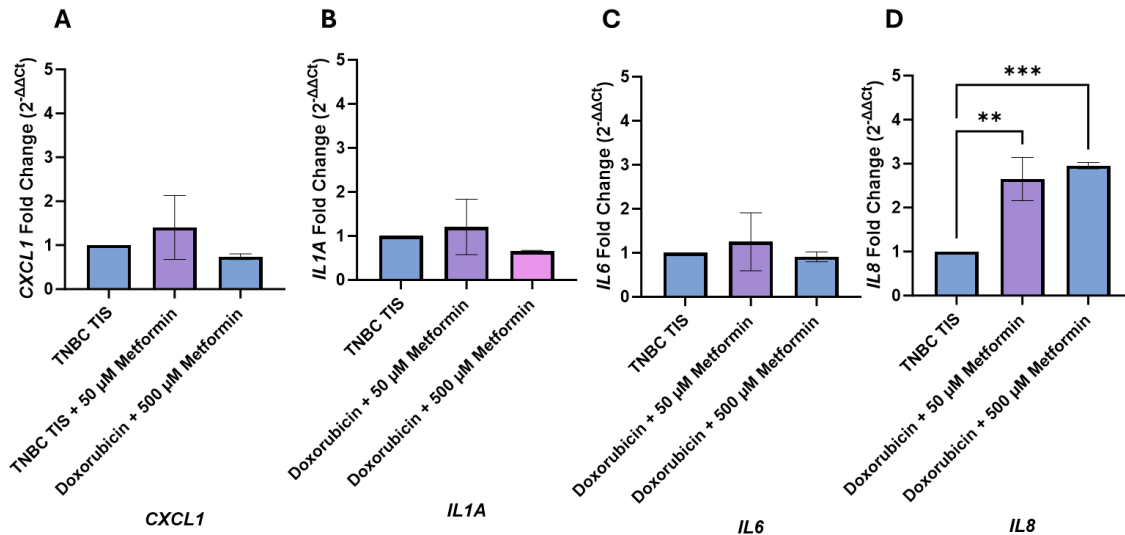


Figure 6.13. Gene expression changes in fibroblasts co-cultured with HCC1806 TIS, treated with metformin. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta Ct}$ method and normalised to two housekeeping genes. The control sample (HCC1806 TIS treated fibroblasts) was set as a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6* and (D) *IL8*. When compared to TNBC TIS fibroblasts, an increase in all SASP components was observed in 50 μ M metformin treated samples, in contrast to 500 μ M treated samples where SASP component expression decreased in all, but *IL8*. A statistically significant increase in *IL8* expression was observed in metformin treated samples compared to the control. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one way ANOVA with Tukey's test, where $Ns = P > 0.05$, $* = P \leq 0.05$, $** = P \leq 0.01$, $*** = P \leq 0.001$ and $**** = P \leq 0.0001$.

To determine whether these changes were specific to metformin treated TIS samples, or were a general response to metformin, fibroblasts that had not been co-cultured, were treated with metformin and assessed for changes in SASP gene expression (Figure 6.14). When compared to untreated fibroblasts, metformin treatments reduced the expression of several SASP components (*CXCL1*, *IL1A*, and *IL6*), however no statistically significant differences were observed between the upper and lower metformin concentrations. *CXCL1* and *IL6* showed the largest reductions, both of which were statistically significant, whilst the modest decrease in *IL1A* expression was not. In contrast, *IL8* expression was significantly increased at both metformin concentrations.

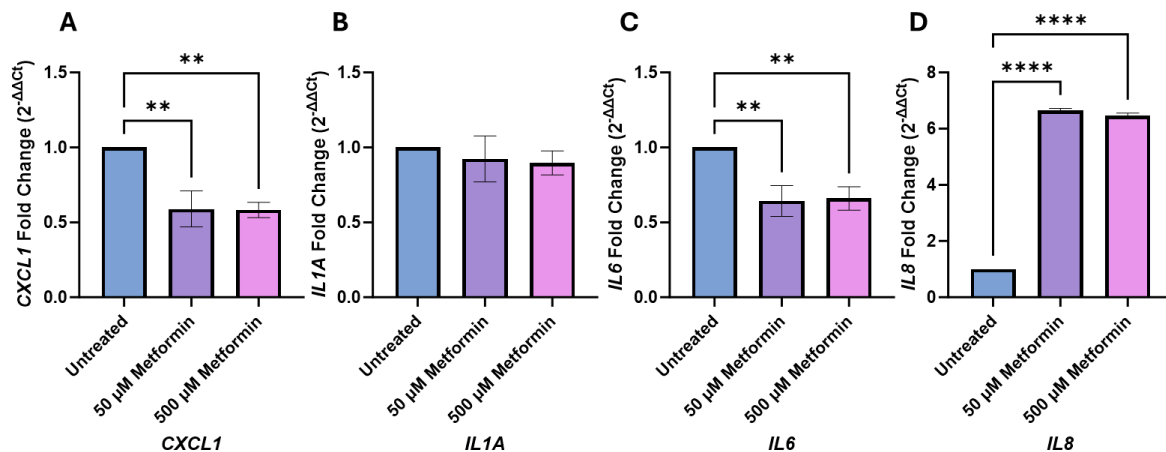


Figure 6.14. Gene expression changes in fibroblasts treated with metformin. Gene expression is represented as fold change, quantified using the $2^{-\Delta\Delta C_t}$ method and normalised to two housekeeping genes. The control sample (untreated fibroblasts) was set as a baseline and has a fold change of 1. Data is shown for (A) *CXCL1*, (B) *IL1A*, (C) *IL6* and (D) *IL8*. When compared to untreated fibroblasts *CXCL1*, *IL1A*, and *IL6* were decreased following both 50 μ M and 500 μ M metformin treatments, *CXCL1* and *IL6* showed the greatest reduction in expression, which was statistically significant. The decrease in *IL1A* expression was non-significant. In contrast, *IL8* expression was significantly increased at both metformin concentrations. No statistically significant differences were observed between metformin concentrations. Experiments were performed in 3 independent biological replicates ($n = 3$), each with technical triplicates per condition. Data is presented as mean $\pm$ standard deviation (SD) of biological replicates. Statistical significance was determined using one way ANOVA with Tukey's test, where Ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

Metformin induced distinct SASP gene expression profiles in co-cultured and non-co-cultured fibroblasts. Expression of *CXCL1*, *IL1A*, and *IL6* was slightly increased in 50 μ M metformin treated, TIS TNBC co-cultured fibroblasts (Figure 6.13). However, the same metformin concentration reduced SASP gene expression in non-co-cultured fibroblasts, significantly for *CXCL1* and *IL6* (Figure 6.14). Furthermore, in non-co-cultured fibroblasts, SASP expression levels were comparable between the 50 μ M and 500 μ M metformin treatments (Figure 6.14), whilst TIS TNBC co-cultured fibroblasts, showed a more pronounced SASP reduction at 500 μ M than at 50 μ M (Figure 6.13). *IL8* expression was significantly increased in both non-co-cultured and co-cultured fibroblasts, treated with metformin (compared with their respective controls). However, the magnitude of this increase differed between conditions where *IL8* expression increased by ~ 2.5 fold in co-cultured fibroblasts (Figure 6.13), compared with ~ 6 fold in non-co-cultured fibroblasts (Figure 6.13).

6.5 DISCUSSION

The primary aim of this chapter was to investigate the paracrine effects of TIS TNBC on fibroblasts and determine how these effects were modulated by metformin. Two ways of which SASP secretions can modulate neighbouring healthy fibroblasts are by activating them, forming CAFs or inducing paracrine

senescence. Where differences were identified between TNBC and TNBC TIS co-cultures, the same assays were repeated with metformin treatment to evaluate whether metformin could attenuate these paracrine effects.

6.5.1 Co-culture system optimisation

To evaluate the paracrine effects of TIS TNBC it was first necessary to establish a suitable co-culture model. Fibroblasts were selected based on their abundance within the TME, and ability to promote tumour progression, metastasis and chemotherapy resistance via CAF differentiation or senescence (Çakal, Urgancı and Şimşek, 2025; Luo et al., 2025; Ye et al., 2024). Patient derived breast fibroblasts were selected as they can recapitulate *in vivo* gene expression patterns and signalling within the breast. The co-culture model was used to assess the indirect functional consequences of paracrine TIS TNBC signalling and metformin treatment in healthy cells. Although metformin did not attenuate senescence in TIS TNBC cells (Chapter 4), co-culture models were used to investigate its potentially protective effects within the TME.

Initially, it was intended that two TNBC cell lines HCC1806 and MDA-MB-231 would be used to model TIS TNBC signalling. However, despite being reported to survive in alternate media formulations, MDA-MB-231 failed to adapt to the fibroblast culture media (Biswenger et al., 2018; Canonici et al., 2020; Gao et al., 2021; Ozawa et al., 2018; Sflomos et al., 2016). Prolonged culture resulted in cell death, cell detachment and altered adherence properties, making them unsuitable for co-culture experiments. When compared composition of standard and fibroblast growth media was similar, although calcium and magnesium content was lower in fibroblast media by 148 mg/L and 152 mg/L respectively (Thermofisher, 2025c; Thermofisher, 2025d). As MDA-MB-231 adhesion is reported to be impaired with decreased calcium and magnesium content, this may explain why they were unable to survive in the fibroblast growth media (Fuhrmann et al., 2017). To confirm this, it would have been necessary to supplement fibroblast media and re-acclimatise MDA-MB-231. However, due to time constraints this was not possible. It is also important to consider, this may have influenced the behaviour of fibroblasts, which grew under very specific conditions. For this reason, preliminary data investigating the paracrine effects of TIS TNBC was modelled in HCC1806 cells, which survived equally as well in both media types. Future studies would benefit from the inclusion of additional TNBC cell lines, such as MDA-MB-468 or BT-549 to ensure results are representative of TNBC as a whole, rather than one specific cell line.

Given the change in culture conditions, senescence was reevaluated in HCC1806 acclimatised to fibroblast media. As the SASP is highly dynamic, variation in the expression of individual SASP components was expected between media compositions (Hernandez-Segura, Nehme and Demaria, 2018). However, HCC1806 cultured in fibroblast medium retained a TIS phenotype, as evidenced by increased β -galactosidase staining (Figure 6.6) and elevated expression of key SASP factors *CXCL1*, *IL1A*, *IL6*, and *IL8* (Figure 6.7).

MTT assays confirmed that alterations in media composition didn't alter HCC1806 viability or metformin sensitivity (Figure 6.3). A comparable decrease in viability, in response to metformin, was observed in both fibroblast media and standard media (Figure 6.3 and Figure 4.3). As the co-culture system allowed media exchange between fibroblasts and HCC1806 it was necessary to examine the response of fibroblasts to metformin, as they too would be exposed (Figure 6.5). This is beneficial as metformin's effects are not cell specific and would also affect healthy cells *in vivo*. At clinically relevant concentrations (50 μ M), metformin didn't adversely affect fibroblast viability, whereas at higher concentrations (500 μ M) resulted in a reduction in viability. However, as the majority of fibroblasts remained viable, both doses were used in subsequent experiments to determine whether any responses to metformin observed, were dose dependent.

MTT assays were also used to assess changes in fibroblast viability to ensure any observed results were not due to unfavourable culture conditions (Figure 6.4). There were no significant differences between the viability of untreated and co-cultured cells suggesting culture conditions were suitable. In future, co-cultures treated with metformin could also be assessed for changes in viability, using MTT assays, to ensure the dual combination of metformin and co-culture does not detrimentally impact fibroblast viability.

6.5.2 SASP induced CAF formation

Changes in fibroblast motility and morphology can be used to indicate fibroblast activation, however, alone are not sufficient to confirm CAF formation. To do this, further tests, would be needed to validate findings. One common marker used to differentiate between naive and activated fibroblasts, is α SMA, a protein associated with cell contraction (Hou et al., 2025). However, as no clear functional

or morphological differences were observed between initial TNBC and TNBC TIS co-cultures, further CAF specific markers were not assessed.

6.5.2.1 Migratory changes

Fibroblast migration was assessed using scratch assays to determine whether co-culture with TNBC or TIS TNBC differentially enhanced fibroblast motility (Figure 6.9). As CAFs are inherently more invasive than fibroblasts it was hypothesised that transformed fibroblasts would be more migratory than naïve fibroblasts (Hu et al., 2023; Peng et al., 2013). However, all scratches had closed by 48 hours, and no significant differences were observed between untreated fibroblasts, TNBC co-cultures, or TIS TNBC co-cultures, indicating fibroblasts were not activated into aggressive CAFs. In future, scratch assays could be performed using shorter time points such as 6 or 12 hours, as the wound closed relatively quickly and earlier measurements may have shown differences between samples. It is also possible that functional changes between samples were more nuanced, and induced changes in contractile force or cytoskeletal arrangement, rather than wound closure. These changes may have been more effectively studied using live cell imaging (Hou et al., 2025). Although alterations in viability are typically not a defining feature of CAFs, they often proliferate more quickly than naïve fibroblasts, therefore, if transformed an increase in metabolic activity may have been expected (Hu et al., 2023; Peng et al., 2013). However, as MTT assays showed no statistically significant changes in viability, it supports the idea that fibroblasts were not transformed into aggressive CAFs.

It would have been beneficial to include a positive control for the scratch assay to validate results. This would have shown whether the similar wound healing times were a genuine response or a limitation of the assay's sensitivity. In future, TGF- β could be used as a positive control to promote fibroblast activation and wound closure (Guo et al., 2017). Future assessment of additional motility markers, including vimentin and FAP, may also be beneficial for understanding the responses of fibroblasts to paracrine TIS signalling (Hu et al., 2022a; Richardson et al., 2018). Both markers are associated with activated CAFs therefore their differential expression may influence the invasion potential of fibroblasts, without directly impacting their migration. In future experiments, trans-well invasion assays could be used to assess invasion, across a porous membrane, as this approach has previously been successful in demonstrating enhanced invasion by CAFs (Hu et al., 2023; Peng et al., 2013).

6.5.2.2 Morphological changes

Co-cultured samples were visibly elongated, and more spindle-like than untreated fibroblasts, this was reflected by a statistically significant increase in cell aspect ratios (Figure 6.10). An elongated morphology has frequently been described as a characteristic feature of CAFs (Devarasou, Kang and Shin, 2024; Hou et al., 2025; Hu et al., 2023; Kang et al., 2024; Stylianou, Gkretsi and Stylianopoulos, 2018). CAFs are a heterogeneous population comprised of multiple subtypes, including inflammatory CAFs (iCAFs), myofibroblastic CAFs (myCAFs), and antigen-presenting CAFs (apCAFs), each of which contributes differently to tumour progression (Yang et al., 2023). iCAFs are primarily responsible for the tumour promoting properties described in literature (increased proliferation, invasion and metastasis), whilst myCAFs behave more similarly to myofibroblasts, and function in ECM remodelling (Yang et al., 2023). Therefore, it is possible the increased aspect ratio of co-cultured samples, without migratory or metabolic changes, may indicate myCAF differentiation rather than an iCAF (Figure 6.10) (Cords, Souza and Bodenmiller, 2024). However, myCAFs typically arise through direct tumour contact, which was not possible in this co-culture model (Sahai et al., 2020; Yang et al., 2023). It is also important to consider that co-cultured fibroblasts developed a pro-inflammatory gene profile, in response to inflammatory signalling, which is more consistent with iCAF differentiation (Figure 6.12) (Cords, Souza and Bodenmiller, 2024; Sahai et al., 2020; Yang et al., 2023). As the co-cultured fibroblasts share features of both iCAF and myCAFs, future studies could assess expression of α SMA, FAP and IL's to differentiate between myCAFs (α SMA+, FAP+, IL-) and iCAFs (α SMA-, FAP+/-, IL+) (Cords, Souza and Bodenmiller, 2024).

Although the aspect ratio of both co-cultured samples was elevated in comparison to the untreated control, and was indicative of CAF activation, no significant differences were observed between HCC1806 untreated and TIS samples. This suggests that morphological changes were likely driven by HCC1806 secretions, rather than those specific to TIS. Given that the objective of this work was to assess paracrine effects driven specifically by TIS, subsequent experiments investigating the role of metformin were not performed. TNBC are inherently pro-inflammatory and secrete many factors which overlap with those secreted within the SASP. This may have been sufficient to maximally alter fibroblast morphology. Therefore, it is important to consider aspect ratio measurements are only one measure of fibroblast activation and may lack the sensitivity required to distinguish between TIS and TIS TNBC co-cultures. More nuanced changes in ECM remodelling or fibroblast secretory profiles could be assessed by staining for collagen and fibronectin or profiling inflammatory gene expression (Erdogan and Webb, 2017).

Differences in cell organisation were observed between non-co-cultured and co-cultured fibroblasts (Figure 6.10). Fibroblasts cultured alone typically grew in close proximity to one another, whereas co-cultured fibroblasts had more gapping between cells. This may indicate a reduction in fibroblast-fibroblast adhesion. Given that disruption of N-cadherin has been shown to diminish fibroblast-fibroblast adhesion, it is possible that co-cultured fibroblasts reduce N-cadherin expression, to enhance motility and invasion (Blaschuk, 2022). To investigate this further, future studies could quantify intercellular spacing or cell density per field of view as indirect measures of adhesion. Assessing adhesion markers, such as N-cadherin, would help clarify the underlying mechanisms driving these changes.

Although the data presented is not sufficient to determine CAFs were induced, it provides evidence for the role of paracrine BC signalling in fibroblast activation. Future work could therefore determine whether the observed morphological changes reflect CAF activation, by assessing markers such as vimentin, α -SMA and FAP (Chen, McAndrews and Kalluri, 2021; Hu et al., 2022a; Hu et al., 2023; Wright et al., 2023). This is particularly important given that CAFs can promote tumour progression through paracrine signalling and ECM remodelling (Cords, Souza and Bodenmiller, 2024; Hu et al., 2022a).

6.5.3 Paracrine senescence

To assess whether paracrine senescence was induced in fibroblasts, β -galactosidase staining and gene expression analysis of key SASP components were assessed. β -galactosidase staining is a widely accepted hallmark of cellular senescence, whilst SASP factors are directly responsible for eliciting paracrine effects.

6.5.3.1 β -galactosidase staining

There were no significant differences between the staining of untreated and co-cultured cells, indicating that SASP exposure did not induce the lysosomal increase typically associated with senescence (Figure 6.11). This is consistent with functional and phenotypic data (from scratch assays and morphological analysis), which demonstrated that fibroblasts were motile and elongated, rather than stationary, enlarged and flattened, features typically associated with senescent cells (Figure 6.9 and Figure 6.10) (Hernandez-Segura, Nehme and Demaria, 2018). Previous studies have used β -

galactosidase staining to identify cells induced with paracrine senescence, proving it to be a useful indicator, in combination with other markers (Acosta et al., 2013; Alessio et al., 2024; Chou, Ho and Hung, 2022). These studies also highlight that the induction of paracrine senescence is context dependent and influenced by SASP composition.

Paracrine senescence induction is likely dependent on SASP composition, as components elicit distinct effects. For example, pro-inflammatory CXCR-2 binding cytokines and IL6 primarily amplify existing senescence, whereas TGF- β has been more directly involved in inducing paracrine senescence through CDK inhibitor regulation (Acosta et al., 2008; Acosta et al., 2013; Herstein et al., 2024). Other components such as insulin-like growth factor-binding protein 7 (IGFBP7) have similarly been shown to induce paracrine senescence and their removal has prevented this (Siraj et al., 2024; Severino et al., 2013). Given the heterogeneity of the SASP, it is possible that secretions from TIS TNBC did not contain the specific combination of factors required to induce paracrine senescence. As a limited number of factors were assessed in this study, in future it would be beneficial to profile the SASP produced by TIS TNBC cells, to determine whether components known to induce paracrine senescence are present. Although multiple SASP components can induce paracrine senescence, these effects often require NF- κ B activation (Acosta et al., 2013; Chou, Ho and Hung, 2022; Saleh et al., 2018). It is therefore possible that insufficient activation of NF- κ B prevented senescence induction. Therefore, future studies assessing NF- κ B activation could also help clarify whether downstream SASP signalling was induced.

An alternative explanation for the lack of β -galactosidase staining, is that fibroblasts did not undergo sufficient DNA damage to activate the DDR pathways driving mitochondrial dysfunction, ROS accumulation, and the subsequent increase in lysosomal content (Faragher, 2021). Instead, more direct cell contact may be required to sustain DDR signalling pathways, as previously demonstrated in co-culture fibroblast models (Nelson et al., 2012; Saleh et al., 2018). It is also important to consider, that *in vivo*, fibroblasts would also be exposed to chemotherapy drugs, like doxorubicin, which elicit DNA damage. Therefore, future studies could treat co-culture models with doxorubicin to establish its effect on senescence.

β -galactosidase staining is considered a marker of stable senescence, as it reflects the increased lysosomal biogenesis required to degrade the dysfunctional organelles which accumulate during

senescence (González-Gualda et al., 2021; Hernandez-Segura, Nehme and Demaria, 2018). As cells may enter senescence without undergoing significant lysosomal expansion, a lack of β -galactosidase staining is not definitive evidence that senescence was not induced. However, when this result is interpreted alongside the observed migratory capacity and elongated morphology, the absence of staining strongly suggests that the fibroblasts in this study did not undergo paracrine senescence (Faragher, 2021; González-Gualda et al., 2021).

6.5.3.2 Senescence associated gene expression changes

Fibroblasts were analysed for their expression of several pro-inflammatory SASP markers, known to be elevated following TIS induction. Co-culture increased inflammatory gene expression in fibroblasts, which was further exaggerated by co-culture with TIS TNBC co-cultures, significantly for *CXCL1*, *IL1A* and *IL8* (Figure 6.12). This suggests that factors released by TIS TNBC cells activate specific paracrine responses in fibroblasts.

Interestingly, when comparing gene expression profiles between HCC1806 TIS and fibroblasts exposed to its paracrine signalling, expression patterns were not mirrored between cell types. TIS TNBC co-cultured fibroblasts expressed the highest levels of *CXCL1* (~60 fold increase compared to TNBC co-culture), despite only being modestly increased (~2 fold) in non-co-cultured TIS TNBC (Figure 6.7 and Figure 6.12). A similar pattern was observed for *IL8*, where co-cultured fibroblasts had a 55 fold increase in expression compared to TIS TNBC cells which showed a more moderate 5 fold change (Figure 6.7 and Figure 6.12). The opposite was true for *IL6* where expression in fibroblasts was lower than in TIS TNBC (4 fold and 55 fold increase respectively), whereas *IL1A* expression was consistent between both cell types (~2 fold increase) (Figure 6.7 and Figure 6.12). This suggests that paracrine signalling does not induce a like-for-like transcriptional programme but instead activates cell specific inflammatory pathways in fibroblasts. Fibroblasts had a more pronounced response to paracrine signalling than TNBC cells undergoing direct senescence induction, which may reflect the higher basal expression of inflammatory components in TNBC cells (Rosado-Sanz et al., 2025). This result is also consistent with iCAF activation, as these cells amplify inflammatory signalling, likely generating a disproportionately large inflammatory response relative to the original stimulus. This inflammation contributes to increased tumour proliferation, invasion and metastasis (Yang et al., 2023).

Expression of key SASP genes was increased in fibroblasts co-cultured with TNBC cells compared with untreated fibroblasts, which likely reflects the pro-inflammatory signalling from TNBC (Figure 6.12). TNBC cells are known to actively remodel the TME through cytokine and chemokine secretion, promoting fibroblast activation and generating conditions favourable for tumour growth (Allavena et al., 2008; Fitsiou, Soto-Gamez and Demaria, 2022; Luo et al., 2025). Interestingly, the expression of SASP components was further increased in TIS TNBC co-cultures when compared to co-cultures of TNBC alone (Figure 6.12). This suggests that TIS TNBC cells are eliciting a senescence specific, paracrine response and are reprogramming neighbouring fibroblasts toward a more pro-inflammatory phenotype. This is consistent with literature demonstrating senescent cells secrete a complex inflammatory SASP, which is sustained to induce chronic inflammation and fibroblast activation (Caligiuri and Tuveson, 2023; Luo et al., 2025). These findings support the idea that TIS secretions contribute to increased inflammation, and likely aggression, in cancer (Luo et al., 2025).

Although elevated SASP gene expression can indicate paracrine TIS, the absence of β -galactosidase staining, together with the spindle like morphology and retained migratory capacity of fibroblasts, indicates that senescence was unlikely to have been induced under the conditions of this study (Figure 6.12, Figure 6.11, Figure 6.10 and Figure 6.9). Instead, cells may have been transitioning into an inflammatory, CAF like state characterised by a spindle like morphology and a pro-inflammatory transcriptional profile (Figure 6.10 and Figure 6.12).

Expression of *IL6*, *IL8*, *IL1A* and *CXCL1*, increased in fibroblasts co-cultured with TIS TNBC, consistent with iCAF activation and inflammatory reprogramming (Figure 6.12) (Bae et al., 2014; Jenkins et al., 2025; Li et al., 2022; Naito, Yoshioka and Ochiya, 2022; Tjomsland et al., 2011). *IL6*, *IL8* and *CXCL1* are responsible for promoting cell migration a characteristic associated with CAFs (Coppé et al., 2008; Davalos et al., 2010; Kim et al., 2018; Ortiz-Montero, Londoño-Vallejo and Vernot, 2017; Vernot, 2020). *IL8*, also drives fibroblast activation, matrix remodelling and tumour invasion (Chen et al., 2024; Jundi and Greene, 2015). *CXCL1* additionally modulates immune signalling (Davalos et al., 2010; Kim et al., 2018). It also reinforces CAF activation and has been shown to convert naïve fibroblasts into CAF like cells (Kim et al., 2018; Yang et al., 2023; Yu et al., 2024). Similarly, *IL1A* has been shown to modulate inflammatory signalling within CAF populations, and acts as a key upstream regulator of cytokine expression and stromal activation (Bae et al., 2014; Tjomsland et al., 2011). It is therefore possible that the increased expression of these components promoted CAF like activation in co-

cultured fibroblasts (Figure 6.12). However, to confirm this, markers of CAF induction must be assessed.

IL6, *IL8*, *CXCL1* and *IL1A* are well-established SASP components, closely linked to NF- κ B activation. As previous studies have shown inhibition of NF- κ B results in a 75% reduction in SASP components, the increased expression of *IL6*, *IL8*, *CXCL1* and *IL1A* suggests NF- κ B signalling may be activated in response to co-culture and contributed to the observed pro-inflammatory phenotype (Davalos et al., 2010). Consistent with this, is the moderate increase in *IL1A* expression compared to *IL8*, observed in this study (Figure 6.12). *IL1A* is a potent cytokine, considered a primary upstream regulator of the SASP, functioning in both an autocrine manner to reinforce senescence within cells and a paracrine manner to induce inflammatory responses in neighbouring cells (Chou, Ho and Hung, 2022). It potentially activates NF- κ B, sustaining transcription of downstream SASP components such as *IL6* and *IL8* (Chou, Ho and Hung, 2022). As *IL8* is highly responsive to NF- κ B its expression can increase rapidly (10-100 fold) in response to cellular stress or inflammation (Davalos et al., 2010; Hoffmann et al., 2002; Orjalo et al., 2009). The significant increase in *IL8* expression may also indicate activation of C/EBP β , a key transcription factor involved in SASP gene regulation. Knockdown of C/EBP β has been shown to markedly reduce *IL6* and *IL8* expression, supporting its role alongside NF- κ B in coordinating inflammatory SASP responses (Gonzalez-Meljem et al., 2018; Kuilman et al., 2008). As gene expression was measured using qPCR, definitive conclusions regarding corresponding changes in protein abundance cannot be drawn. However, the physical separation of cells indicates that soluble factors were responsible for driving the transcriptional changes in fibroblasts.

Given the significant upregulation of inflammatory genes, implicated in both CAF activation and senescence, in TIS TNBC co-cultured fibroblasts, it is unusual that no significant functional differences were observed between TNBC and TNBC TIS co-cultured fibroblasts. However, it is important to consider that cancer and TIS cells share pro-inflammatory secretion profiles, so their respective inflammatory signals may overlap and reinforce one another, potentially driving fibroblast activation (Danforth, 2021; Wang et al, 2024a). Future studies assessing specific CAF markers, ECM remodelling, invasion, a broader range of inflammatory genes, or contractility would be beneficial to potentially differentiate between co-cultures.

Regardless of whether or not the observed phenotype reflects partial CAF activation, fibroblasts exposed to TIS TNBC signalling clearly adopt a more pro-inflammatory state than TNBC cells alone. Given the role of inflammation in promoting tumour progression, it is important to investigate whether this can be mitigated, using metformin.

6.5.3.3 The impact of metformin on gene expression changes

Despite in this study not reversing TIS in TNBC cells (Chapter 4), metformin is classified as a senomorphic and has known senescence attenuating properties. Therefore, it is possible that metformin may limit the paracrine inflammation induced by TIS, in fibroblasts.

In untreated fibroblasts, 50 μ M metformin reduced *CXCL1*, *IL6*, and *IL1A*, however, this effect was not observed in 50 μ M metformin treated cancer cells (Figure 6.13 and Figure 6.14). It is possible that at lower concentrations, metformin is unable to suppress persistent SASP driven inflammation but is capable of reducing inflammatory gene expression in fibroblasts, where basal inflammation is low. Supporting this, many studies report the anti-inflammatory effects of metformin in primary, non-cancerous cell types, similar to the untreated fibroblasts in this study (Hooten et al., 2016; Jiang et al., 2020; Kuang et al., 2020).

Data suggests that response to metformin was not dose dependent in non-co-cultured fibroblasts, as a similar anti-inflammatory response was observed at both 50 μ M and 500 μ M (Figure 6.14). However, 500 μ M metformin promoted an anti-inflammatory response in TIS TNBC co-cultures, which was not observed at 50 μ M (Figure 6.13). In TIS TNBC, metformin reduced expression of *CXCL1* by ~30%, *IL1A* by ~35%, and *IL6* by ~10%. (Figure 6.13). Although these changes were not statistically significant, they suggest that supra-pharmacological concentrations of metformin may be required to produce a senomorphic effect, capable of suppressing persistent SASP driven inflammation in fibroblasts. This corroborates with previously published literature showing SASP components can be attenuated at ≥ 500 μ M metformin (Chung et al., 2017; Hooten et al., 2016; Moiseeva et al., 2013).

IL8 consistently behaved as an outlier, with increased expression observed across treatment conditions, in fibroblasts cultured with and without co-culture (Figure 6.13 and Figure 6.14). Previous studies have shown that metformin can reduce *IL8* expression in senescent fibroblasts at

concentrations $\geq 500 \mu\text{M}$, suggesting that perhaps higher metformin doses are needed to suppress *IL8* expression (Hooten et al., 2016; Moiseeva et al., 2013). It is also important to consider that *IL8* can increase rapidly in response to cell stress, therefore it is possible metformin induced cellular stress in fibroblasts (Davalos et al., 2010; Hoffmann et al., 2002; Orjalo et al., 2009). Given that *IL8* is a potent chemokine associated with tumour progression, poor prognosis, and neutrophil recruitment, its increased expression in response to metformin raises questions regarding its senotherapeutic benefits (Matsushima, Yang and Oppenheim, 2022; Meier and Brieger, 2025; Rosado-Sanz et al., 2025). Whilst higher concentrations of metformin reduce *CXCL1*, *IL1A* and *IL6* expression in both non-co-cultured and TIS TNBC co-cultured fibroblasts, it would be beneficial to establish the effects of metformin on a broader range of SASP components, to determine whether reductions in their expression outweigh the potential pro-tumorigenic effects of increased *IL8* expression (Figure 6.13 and Figure 6.14).

Future studies could include additional TIS TNBC cell lines in this co-culture model, to determine whether the observed effects are specific to HCC1806 or are representative of TIS TNBC as a whole. It would also be interesting to determine metformin's ability to attenuate TIS driven inflammation in these models. Additional assessment of AMPK phosphorylation in these cells, would clarify whether metformin's effects are AMPK dependent or mediated through alternative mechanisms. Further analysis of SASP components, at the protein level, would also be beneficial to determine whether the transcriptional changes observed are functionally translated. Future research could also explore whether the pro-inflammatory phenotype of co-cultured fibroblasts adversely affects TIS TNBC cells.

Persistent SASP secretion creates a pro-inflammatory and pro-tumorigenic microenvironment where SASP components drive angiogenesis, microenvironment remodelling, tumour invasion and metastasis (Luo et al., 2025). Given the clinical relevance of TIS in BC, mitigating the harmful inflammatory effects of the SASP remains an important therapeutic goal (Fleury et al., 2019; Luo et al., 2025). The presented data suggests that targeting the SASP with metformin could be beneficial in preventing the propagation of inflammation through healthy cells, at least to some degree. Although further investigation is required to fully understand metformin's role in TNBC, evidence from large-scale studies such as TAME support its potential clinical benefit (Barzilai et al., 2016; Hua et al., 2023; Tang et al., 2018).

6.6 CONCLUSION

The primary aims of this chapter were to determine whether secretions from TIS TNBC cells induce senescence or functional changes in healthy fibroblasts and assess whether metformin can mitigate TIS induced inflammatory cytokine expression in a co-culture system.

To address these aims, a co-culture model was developed using HCC1806 TNBC cells and patient derived fibroblasts. Fibroblasts were assessed for features associated with CAF activation and paracrine senescence. Whilst co-cultured fibroblasts did not fully meet the criteria required to confirm either phenotype, they displayed a distinct TIS specific gene expression profile, which when considered alongside the observed morphological alterations, suggests that fibroblasts are likely activated to some extent.

Metformin was evaluated as a potential strategy to counteract the paracrine inflammatory effects of TIS TNBC. Treatment of TIS TNBC co-cultures, with a supra-pharmacological concentration of metformin (500 μ M) reduced the expression of several inflammatory cytokines, including *CXCL1*, *IL6* and *IL1A*, although increased expression of *IL8*. The contrasting effects observed between low and high metformin concentrations highlights the complexity of metformin's mechanism and indicates that its effects are both dose and context dependent. Further investigation across a broader panel of SASP components and cell lines is required to fully characterise these responses.

Collectively, this data provides preliminary evidence that TIS TNBC elicits distinct inflammatory changes in neighbouring fibroblasts and suggests that metformin may partially attenuate this. Whilst not definitive, these findings suggest metformin may be beneficial in targeting the SASP to reduce inflammation within the TME.

Chapter 7

General Discussion and Conclusion

7 GENERAL DISCUSSION AND CONCLUSION

7.1 GENERAL DISCUSSION

TIS is a state of cell cycle arrest, commonly induced by chemotherapy drugs which remains challenging to characterise due to its heterogenous phenotype and lack of universal markers (Hernandez-Segura, Nehme and Demaria, 2018). TIS cells secrete a SASP, composed of many pro-inflammatory components, which although initially are beneficial to promoting immune clearance and preventing malignant transformation, become damaging when persistent (Fitsiou, Soto-Gamez and Demaria, 2022; Lannello et al., 2013; Paula et al., 2023). Chronic SASP contributes to a pro-inflammatory tumour microenvironment which can promote cancer progression (Luo et al., 2025). TIS cancer cells can also escape growth arrest and re-enter the cell cycle, which contributes to cancer recurrence (Saleh, 2024).

TNBC was the focus of this study as it is a clinically challenging BC subtype. Although BC is the most common cancer in the UK, TNBC only accounts for 15% of all cases, yet is responsible for 40% of all BC deaths (Cancer Research UK, 2022). As TIS has been detected in BC tumours clinically, understanding its role, in aggressive subtypes like TNBC, with high mortality and relapse rates, is essential. In TNBC models, TIS was induced using doxorubicin, a primary BC treatment. Doxorubicin elicits a potent TIS response and is clinically relevant given 81% of BC patients are reported to be treated with anthracycline based chemotherapy (Asselain et al., 2018).

This thesis specifically aimed to better understand the nuances of TIS in TNBC, by investigating potential biomarkers and reversal strategies. This was achieved by, first, identifying biomarkers associated with TIS in TNBC; second, assessing whether metformin could attenuate TIS in TNBC models; third, investigating why metformin didn't attenuate TIS in TNBC and fourth, identifying the paracrine effects of TIS TNBC on healthy cells and investigating their modulation by metformin.

Initially, TIS models were developed in two TNBC cell lines (MDA-MB-231 and HCC1806) and a non-tumorigenic cell line (MCF10A), to facilitate subsequent senescence studies. Including both malignant and non-malignant cell lines in this research was beneficial to establish whether responses were TNBC specific or applied to TIS more generally. To confirm that the proposed doxorubicin concentrations were suitable for inducing TIS, MTT assays and apoptosis assays were performed. Retention of cell

viability and the lack of extensive apoptosis, following doxorubicin treatment, indicate that cells were not irreversibly damaged or undergoing cell death, supporting the suitability of these models. Assessment of multiple TIS hallmarks, including lysosomal content, DNA damage, nuclear area, and expression of senescence associated genes, indicated that all cell lines had undergone TIS. BrdU staining was also incorporated in subsequent chapters and verified the absence of proliferation. Initially, Ki-67 was considered for assessing proliferation; however, due to its instability in TIS cells, BrdU was deemed a more reliable alternative (Bojko et al., 2019; Oijen et al., 1998). Prior to each experiment, TIS was assessed to ensure that senescence was induced. The optimisation of robust and reproducible TIS systems was essential for this research which aims to study TIS biomarkers and reversal strategies in TNBC.

TIS has been increasingly associated with treatment resistance and tumour progression in TNBC (Fitsiou, Soto-Gamez and Demaria, 2022; Lannello et al., 2013; Luo et al., 2025; Paula et al., 2023). Therefore, the identification of reliable biomarkers, capable of detecting TIS *in vivo*, is essential for improving patient stratification and informing treatment strategies. Improved detection of TIS in tumours may justify integrating senotherapeutic drugs into TNBC therapies to limit the detrimental impacts of TIS. The objectives of this study were to identify candidate biomarkers associated with senescence and the cytoskeleton that have prognostic or predictive relevance in patients, and to assess their expression in TIS models. Given that TIS is characterised by extensive transcriptional reprogramming, this study focused on gene expression changes as potential biomarkers of TIS in TNBC. Cytoskeletal components were of particular interest, given their established roles in TME remodelling, SASP maintenance, and changes in migratory behaviour, processes commonly associated with aggression and therapeutic resistance in TNBC (Ghosh et al., 2020; Malla et al., 2025; Molina et al., 2025).

A literature review identified 108 genes related to senescence, the cytoskeleton and cancer. These candidates were first evaluated for their association with therapeutic response using patient data from ROC plotter. Candidate genes with potential clinical utility in PCR cohorts ($AUC \geq 0.58$) and consistent expression trends in RFS cohorts, were assessed for their impact on OS, using KM plotter. Candidates for further investigation were selected based on whether their gene expression patterns were consistent in ROC plotter and KM plotter datasets. Several candidate genes with potential predictive value were identified using this approach. Reduced expression of *CDCA5*, *KIFC1*, *LMNB1*,

SMC6 and *TUBG1* was associated with poor therapeutic response and reduced OS in BC patients, whereas higher *STIM1* expression correlated with poorer OS and treatment response.

Across the models studied, non-tumorigenic MCF10A TIS cells demonstrated a more classical senescence associated suppression of mitotic genes than TNBC TIS cell lines. Genes involved in centrosome clustering, microtubule nucleation, and chromosome dynamics (including *KIFC1*, *TUBG1*, *CDCA5* and *SMC6*) were consistently downregulated in MCF10A, aligning with the cell cycle arrest expected during senescence (Hernandez-Segura, Nehme and Demaria, 2018). In contrast, TNBC TIS models frequently maintained or upregulated these genes. This may reflect the higher basal expression of these genes in BC, or differences in senescence regulation linked to *TP53* alterations. Furthermore, as genes such as *SMC6* and *TUBG1* encode for proteins which function outside of mitosis, their differential expression may also reflect broader differences in genomic instability or microtubule dynamics (Fourriere et al., 2020; Mangone et al., 2025). More consistent expression patterns were observed for *STIM1* and *LMNB1*. *STIM1* expression increased in all TIS models, potentially reflecting compensatory responses to TIS induced disrupted calcium homeostasis or cellular stress (Pluquet et al., 2015; Sanchez-Collado et al., 2022; Sanchez-Lopez et al., 2024). In contrast, *LMNB1* expression was reduced in both TNBC and non-tumorigenic TIS models. *LMNB1* loss is a well-established feature of senescence and is associated with structural weakening of the nuclear envelope, therefore it was incorporated into subsequent experiments as a marker of TIS in TNBC (Freund et al., 2012; Martins et al., 2020; Meng et al., 2025).

Together, these results indicate that although candidate biomarker expression changes are broadly consistent with TIS in non-tumorigenic models, most are not consistently conserved in TNBC TIS models. As a result, *LMNB1* was the only candidate selected as a biomarker of TIS in subsequent experiments. It is important to consider that whilst senescence is associated with widespread transcriptional alterations, it is also regulated by post-transcriptional and post-translational modifications. Therefore, gene expression analysis may not be indicative of functional protein levels. Future studies could therefore analyse protein expression levels of these candidates (Hernandez-Segura, Nehme and Demaria, 2018).

Another aim of this study was to determine whether the detrimental effects of TIS in TNBC could be reversed. Metformin was selected as a candidate senotherapeutic drug, due to its tolerability,

repurposing potential and broad range of anti-senescence effects. Although a majority of metformin's benefits can be attributed to SASP suppression, it has also been shown to attenuate several hallmarks of senescence (Figure 1.7 and Figure 1.8). These characteristics of metformin support its potential use in a “one-two punch” strategy for TNBC whereby chemotherapy is administered alongside another drug to enhance its benefit (Chembukavu and Lindsay, 2024; Paula et al., 2023). By attenuating SASP driven microenvironment changes, and potentially reversing TIS, metformin may prevent tumour progression whilst simultaneously making TIS cancer cells more susceptible to cell cycle dependent chemotherapies, like doxorubicin. This approach could therefore theoretically improve treatment efficacy of chemotherapy in TNBC.

Much of the existing literature uses supra-pharmacological metformin concentrations, well above systemic levels, to investigate TIS reversal, which limits its translational relevance. Therefore, this study assessed both clinically relevant (50 μ M) and supra-pharmacological (500 μ M) concentrations. MTT assays were used to define suitable metformin concentrations, where the upper supra-pharmacological limit was the MEC for inducing a significant reduction in viability. After TIS induction and metformin treatment, the extent of senescence attenuation was quantified using several markers. These included β -galactosidase, BrdU incorporation, γ -H2AX staining, nuclear hypertrophy and qPCR analysis of SASP and senescence associated genes. At both concentrations metformin did not significantly alter any of these markers, indicating metformin did not effectively attenuate TIS in TNBC.

Several factors may have contributed to the differences between findings in this study and previously published research, including variations in metformin concentration, duration of recovery time, cell model and method of senescence induction. However, before modifying any of these variables, it was essential to confirm that metformin was capable of entering the cells and inducing downstream effects in the models used, to determine whether this was responsible for the lack of TIS attenuation.

MCF7 cells were included as a positive control for metformin function, as they have previously been shown to respond to metformin treatment (Bekezhankyzy et al., 2023; Kim et al., 2011; Lopes et al., 2020; Phoenix, Vumbaca and Claffey, 2009). Preliminary data showed some AMPK activation in TNBC cells exposed to metformin, although the positive control (MCF7) did not display the expected response, limiting the conclusions that can be drawn from this study. Although AMPK phosphorylation in TNBC cells was consistent with metformin activity, simultaneous mTOR phosphorylation suggested

incomplete pathway activation, where AMPK may be partially activated or overridden by other pathways contributing to increased mTOR signalling. Even so, the phosphorylation patterns observed in TNBC cells suggests that metformin activates some canonical pathways, which underpin several of metformin's anti-senescence mechanisms.

To determine whether the lack of a metformin response was caused by insufficient pathway activation, western blot analysis needs further optimisation and repeating. Additional pathways could also be included to investigate metformin's proposed AMPK independent effects. For example, assessing NF- κ B activity would help determine whether metformin reduces pathway activation by suppressing cytokine production. It is important to consider that although SASP was not suppressed by metformin in this study, it may be driven by pathways that are not directly targeted by the drug. Whilst metformin may effectively modulate ROS driven or metabolic stress induced senescence, it may be less effective against DNA damage induced TIS (Chen et al., 2016; Fang et al., 2018; Jiang et al., 2020; Kuang et al., 2020; Mikyskova et al., 2023). Therefore, future studies could aim to determine the mechanisms driving SASP induction in these TIS models, to understand why metformin fails to attenuate this response. To investigate this, inhibition of DDR, p38 MAPK, or cGAS-STING pathways could be combined with secretome profiling to identify the pathways required for SASP induction.

Transporters necessary for metformin uptake were detected to some extent in all cell lines, at gene and protein levels. MDA-MB-231 expressed high levels of *SLC22A3* (OCT3), which is responsible for peripheral metformin uptake, perhaps explaining the stronger AMPK phosphorylation observed in the preliminary western blot. The presence of transporters indicates that metformin is likely able to enter the cells, supporting the idea that the lack of senotherapeutic effects in TIS TNBC models is due to cellular unresponsiveness rather than insufficient drug uptake. However, to confirm this, it would be beneficial to establish whether these transporters were functionally active, and able to facilitate metformin transport. This could be addressed in future studies by inhibiting transporters, treating cells with metformin and reassessing their viability. If cell viability was unaffected by ≥ 500 μ M metformin, it suggests transporters are required for the loss of viability observed in this study.

Some transporter findings were broadly consistent with the bioinformatics analysis of clinical datasets and showed that *SLC29A4* (PMAT) and *SLC22A3* (OCT3) had the highest overall expression, whilst *SLC22A1* (OCT1) exhibited the lowest. Although more nuanced differences were undetectable

between cancer subtypes or between healthy and cancerous samples, these patterns suggest that the models reflected clinical transporter data to some degree.

It is also important to consider the behaviour of MCF10A, the non-tumorigenic control. MCF10A was included to determine whether healthy breast epithelial cells responded differently to metformin or TIS compared to TNBC. However, no major differences were observed. Interestingly, MCF10A expressed comparably low levels of metformin transporters and was not responsive to metformin or AICAR in pathway activation studies. Although MCF10A is commonly used in BC studies, its transporter expression did not align with clinical data, indicating that potentially MCF10A may not be representative of *in vivo* events, limiting its relevance in understanding TIS attenuation by metformin. In future, it may be beneficial to compare transporter expression in MCF10A to those in primary, non-transformed breast epithelial cells to assess how closely MCF10A resembles normal tissue.

To fully evaluate the role of metformin as a senotherapeutic in TIS TNBC, further testing is required. TIS is highly variable, therefore using a broader range of TNBC cell lines would be beneficial to determine whether lack of TIS reversal, by metformin, was consistent across cancers with different molecular backgrounds. Metformin's senomorphic effects were assessed after 96 hours, which may have been insufficient time to induce measurable changes in TIS cells. Therefore, extending the duration of metformin treatment would clarify whether metformin required additional time to elicit its effects. In future studies it may be beneficial to include higher metformin concentrations (>500 μ M) to determine whether the lack of senescence attenuation was due to insufficient dosing. Although not physiologically relevant, using a higher metformin concentration would establish whether TNBC models are inherently unresponsive to metformin's senotherapeutic effects. Furthermore, as SASP attenuation is one of the primary anti-senescence mechanisms through which metformin prevents tumour progression, assessing changes in a broader panel of SASP components would be of use. It is also important to consider that although changes in gene expression can indicate alterations at the transcriptional level, protein expression is also regulated through post-transcriptional and post-translational mechanisms. Given that alterations in gene expression do not necessarily correspond to protein abundance, future studies could evaluate SASP protein levels to validate the findings in this research (Hernandez-Segura, Nehme and Demaria, 2018). In future, the study could be expanded by including alternative senotherapeutic agents. Comparing senotherapy's would be beneficial to determine whether the lack of TIS attenuation in TNBC was specific to metformin.

Despite demonstrating some capacity to reduce inflammatory signalling within the TME at supra-pharmacological concentrations, metformin's senotherapeutic potential in TIS models, at a clinical concentration, was minimal. This suggests that, within the broader context of senotherapy, senomorphic drugs like metformin may be inherently less effective at attenuating TIS in comparison to senolytic drugs like Dasatinib and Quercetin.

Senotherapies are broadly categorised into senomorphics and senolytics, which are distinct in their senescence attenuation mechanisms. Senomorphic drugs aim to modulate senescence whilst senolytic drugs actively induce apoptosis in senescent cells (Zhang et al., 2023). Metformin is a senomorphic drug, which primarily suppresses the SASP and modulates the metabolism of senescent cells. Mechanistically, this is largely induced via AMPK activation, which subsequently dampens mTOR and NF- κ B signalling, reducing senescence associated inflammation (Zhang et al., 2023). In contrast, senolytic drugs like Dasatinib and Quercetin are designed to induce apoptosis in senescent cells by targeting senescent cell anti-apoptotic pathways (SCAPs) (Kirkland and Tchkonja, 2017; Zhang et al., 2023). Senescent cells typically upregulate SCAPs such as BCL-2 and BCL-xL to survive stress and promote survival (Kirkland and Tchkonja, 2017). Dasatinib is a tyrosine kinase inhibitor which primarily disrupts pro-survival signalling through Src family inhibition whilst Quercetin, a flavonoid, inhibits the PI3K/AKT pathway and anti-apoptotic proteins like BCL-2 (Kirkland and Tchkonja, 2017). By simultaneously targeting these SCAPs, Dasatinib and Quercetin increase the likelihood of triggering apoptosis, clearing senescent cells in both pre-clinical and clinical settings (Chaib, Tchkonja and Kirkland, 2022).

The results observed in this study indicate TIS TNBC cells were resistant to the senomorphic effects of metformin at clinical concentrations (50 μ M). Although metformin induced changes indicative of metabolic reprogramming, such as a reduction in metabolic activity and partially activated AMPK signalling, this did not correspond with altered functional outcomes, like SASP attenuation, reduced β -galactosidase staining, or decreased nuclear area. This suggests that the metabolic alterations induced by metformin were insufficient to modulate the senescence phenotype at a clinical concentration. One possible explanation for this is the metabolic plasticity of TNBC cells, which are known to adapt to energetic stress by utilising alternative fuel sources such as fatty acid oxidation and glutaminolysis (Wang, Jiang and Dong, 2020). The reliance of TNBC on anaerobic glycolysis may reduce the effects of metformin in the mitochondria by negating the need for high glucose levels (Wang, Jiang and Dong, 2020). The persistence of viable senescent cells, despite metabolic perturbation by

metformin, indicates the presence of apoptotic resistance, possibly through SCAP networks. As metformin lacks the mechanistic capacity to target these anti-apoptotic defences, senolytics like Dasatinib and Quercetin may be a more effective therapeutic alternative. Furthermore, current evidence suggests that metformin sufficiently alters the metabolism of tumorigenic senescence models at supra-pharmacological concentrations (mM), which far exceed those that are physiologically obtainable (Halicka et al., 2011; LaMoia and Shulman, 2021; Sabit et al., 2018; Sahra et al., 2011; Vazquez-Martin et al., 2011). Given that a clinical metformin dose had limited efficacy in this study, it may be more clinically viable to explore the use of senolytic drugs, which maintain their potency at lower concentrations (Chaib, Tchkonja and Kirkland, 2022; Kirkland and Tchkonja, 2017).

Ultimately, the findings of this study demonstrate that metabolic modulation by metformin is insufficient to directly attenuate TIS in TNBC. The persistence of metformin treated TIS cells, despite metabolic alteration suggests that effective TIS treatment requires an alternative approach, like senolytic therapy. Future research could use the TIS TNBC models established in this study to test for the efficacy of Dasatinib and Quercetin. This would provide insight into whether drugs targeting survival pathways can overcome the persistence of senescent cells more effectively than metabolic interventions like metformin. Future expression analysis of SCAP components like BCL-2 and BCL-xL could clarify why TIS cells were resistant to metformin induced metabolic stress. It is important to consider that although clinical concentrations of metformin failed to modulate TIS cells, it may be beneficial in limiting the spread of paracrine senescence and reduce the inflammatory burden within the TME.

To assess the paracrine effects of TIS in TNBC and metformin's potential as a senotherapeutic, a co-culture system was developed. It was essential to develop a robust model of paracrine TIS, to study interactions with TNBC and the TME, as current research predominantly relies on immortalised cell lines and conditioned media, which are limited by their translational potential (Galindo-Hernandez et al., 2014; Guo et al., 2017; Leal-Orta et al., 2022). Fibroblasts were used to model the effects of TIS TNBC, as they are a primary TME component which can undergo senescence or become activated into CAFs, in response to SASP signalling, both of which can adversely impact patient outcome (Luo et al., 2025; Yang et al., 2025).

To ensure only paracrine signalling was assessed, the co-culture model was designed to allow the transfer of soluble SASP components whilst preventing direct cell contact between cancer cells and fibroblasts. The co-culture model offered a way to study the functional consequences of the SASP and its modulation, in its entirety, which provided a broader overview of the paracrine influences of TIS TNBC on the TME. Although a successful model was optimised, the data collected is preliminary as only one TNBC cell line survived under co-culture conditions and could be included in the study. Future studies would therefore benefit from the inclusion of additional TNBC cell lines to assess the influence of TNBC heterogeneity on results and to ensure the observed data is representative of TNBC.

Fibroblasts co-cultured with TNBC and TIS TNBC cells were assessed for morphological and migratory changes as markers of CAF activation. No significant changes in migration were detected and although fibroblast elongation was observed in both co-culture conditions, this change was not specific to TIS. As direct CAF markers were not measured, these observations alone are insufficient to conclude that fibroblasts underwent CAF activation. Paracrine senescence was also assessed in fibroblasts using β -galactosidase staining and SASP gene expression analysis. Whilst elevated SASP expression was indicative of paracrine TIS, the absence of β -galactosidase staining, retention of migratory capacity and spindle like morphology of fibroblasts, indicated that senescence was unlikely induced under the conditions of this study.

Gene expression analysis showed TIS TNBC co-cultures induced a more pronounced inflammatory response in fibroblasts, compared to TNBC alone. This suggests that TIS may promote paracrine inflammatory signalling within the TME. Whilst co-cultured fibroblasts did not fully meet the criteria for either senescence or CAF activation, their TIS specific inflammatory gene expression profiles and observed morphological changes suggest they underwent partial activation in response to paracrine signalling. Persistent SASP secretion creates a pro-inflammatory and pro-tumorigenic microenvironment where SASP components drive angiogenesis, microenvironment remodelling, tumour invasion and metastasis, which ultimately contributes to poor prognosis and therapeutic resistance (Luo et al., 2025). Given the clinical relevance of TIS in BC, mitigating the harmful inflammatory effects of the SASP remains an important therapeutic goal (Fleury et al., 2019; Luo et al., 2025). Metformin was therefore investigated for its senomorphic properties.

In fibroblasts co-cultured with TIS TNBC, the effects of metformin were concentration dependent. Lower metformin concentrations (50 μ M) induced a pro-inflammatory response, and higher concentrations (500 μ M) induced a partial anti-inflammatory response. At higher doses, reduced expression of *CXCL1*, *IL1A*, and *IL6* was observed, however, the simultaneous increase in *IL8* demonstrates the complexity of SASP modulation. Further investigation is required to determine the overall benefits of metformin. Expanding the gene expression analysis to include a broader panel of SASP components would provide a better overview of the inflammatory components modulated by metformin. Future studies could also explore whether metformin mediated SASP attenuation in fibroblasts occurs through AMPK dependent or independent mechanisms. Assessing SASP components at the protein level would also be beneficial to determine whether the observed transcriptional changes are functionally translated.

Although, metformin did not reverse the expression of the four SASP components in monocultured TIS TNBC cells, it attenuated paracrine inflammatory signalling in fibroblasts, to some extent. This perhaps suggests that metformin's therapeutic potential may lie in limiting the propagation of inflammation, rather than directly reversing TIS within TNBC. Collectively, these findings indicate that targeting the SASP with metformin may reduce inflammation within the TME and mitigate its harmful effects in neighbouring fibroblasts. Given the repurposing potential of metformin, these are encouraging results which may support its use clinically to prevent TIS driven inflammation.

7.2 GENERAL CONCLUSION

This thesis provides insight into the complexity of TIS in TNBC by identifying its potential biomarkers, investigating strategies to mitigate its effects, and exploring its impact on healthy cells. To facilitate senescence studies, robust TIS models were successfully established using doxorubicin, and were validated in both TNBC and non-tumorigenic cell lines.

Several candidate biomarkers, associated with senescence, the cytoskeleton and cancer were identified to have predictive significance, using bioinformatics analysis. Whilst some genes showed similar expression patterns in TIS induced non-malignant cells, these trends were not consistently conserved in TNBC TIS cell lines, highlighting the complexity of identifying a universal TIS biomarker.

Of the candidates assessed, *LMNB1* was identified as a biomarker of TIS and used in subsequent experiments.

Metformin was evaluated for its senotherapeutic effects in TIS models but failed to attenuate several features of senescence. However, cells possessed the transporters required for metformin uptake and preliminary data indicated partial activation of its canonical pathways, consistent with its expected mechanism of action. These findings suggest that metformin was likely functional in TIS models, although further investigation is required to confirm this.

Results from co-culture studies, showed fibroblasts developed a pro-inflammatory gene expression profile when co-cultured with TNBC cells, which was further enhanced by TIS. These findings indicate that SASP signalling may drive pro-tumorigenic TME changes. Supra-physiological concentrations of metformin suppressed several SASP associated genes, in fibroblasts co-cultured with TIS TNBC, highlighting metformin's potential to limit the propagation of inflammation through the TME. However, this effect was not observed at lower concentrations, suggesting that clinically relevant metformin doses may be insufficient to effectively suppress SASP signalling.

Collectively, these findings emphasise that TIS in TNBC is highly complex and challenging to modulate. They underline the need for further research into TIS and the role of senotherapeutics to mitigate its detrimental effects in TNBC.

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