

The Role of Polymeric Immunoglobulin Receptor in Breast Cancer

by

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Declaration

The research presented in this thesis is bonafide original work carried out by the author: Wichitra Asanprakit, PhD student, at the University of Nottingham between July 2017 and March 2021. All work was performed by the author, under the supervision of the late Professor Oleg Eremin, Associate Professor Andrew J Bennett, and Professor Dileep N Lobo.

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Abstract

Introduction

Neoadjuvant chemotherapy (NAC) is used with increasing frequency as the primary treatment for breast cancer. A pathological complete response (pCR), which contributes to survival benefit is achieved in only 25-30% of patients with little benefit and significant morbidity in the remainder. Therefore, predicting the patients who are likely to achieve a pCR before commencing NAC is desirable. A pilot study of 10 large and locally advanced breast cancer biopsy specimens, using next generation RNA sequencing and validated by real-time quantitative polymerase chain reaction (real-time qPCR) and Western blotting, demonstrated that the expression of polymeric immunoglobulin receptor (PIGR) was significantly increased in breast cancers achieving a pCR compared with non-pCR. PIGR has a major role in mucosal immunity as a transporter of polymeric immunoglobulin across the epithelial cells. Increased PIGR expression has been reported in many cancers and is related to patient outcome and response to chemotherapy. The role of PIGR in breast cancer was expanded in this thesis.

Methods

This thesis consists of preclinical and clinical studies. The preclinical study was performed using MCF7 and MDA-MB468 breast cancer cell lines. The basal level of PIGR mRNA and protein expression in MCF7 and MDA-MB468 cells was evaluated by real-time qPCR and Western blotting. MCF7/PIGR and MDA-MB468/PIGR stable cell lines, overexpressing the *PIGR* gene, were generated using a lentiviral vector with tetracycline dependent induction of expression. Cell viability, cell proliferation and chemo-sensitivity of PIGR transduced cells were evaluated and compared with un-transduced cells to determine the effect of PIGR overexpression on cell

phenotype. The effect of PIGR expression in breast cancer cells on macrophage polarisation was investigated by treatment of bone marrow derived macrophages (BMDMs) with MDA-MB468/PIGR conditioned media. Real-time qPCR was performed to evaluate the expression of M1 (interleukin [IL]-1 β and inducible nitric oxide synthase [iNOS]) and M2 (IL-10 and arginase [ARG] 1) macrophage markers which demonstrated polarisation of macrophages. On the other hand, M1, M2 macrophage conditioned media and recombinant human cytokines: interferon (IFN)- γ , IL-1 β , tumour necrosis factor (TNF)- α , IL-10 and transforming growth factor (TGF)- β were used to determine the role of macrophages and cytokines on PIGR expression in breast cancer cell lines. For the clinical study, PIGR mRNA expression was determined by the RNA *in situ* hybridization assay using formalin-fixed paraffin-embedded specimens of pre-treatment breast cancer core biopsy from 46 women with breast cancer who received NAC. The expression of PIGR and its associations with pCR and overall survival was examined.

Results

The levels of PIGR mRNA and protein expression were significantly higher in MDA-MB468 cells than in MCF7 cells (380-fold, $p < 0.0001$). However, the differential expression of PIGR in these two cell lines did not lead to significant differences in chemo-sensitivity. Viral overexpression of PIGR was also not found to change any of the parameters measured in either cell line. There was no significant difference in M1 and M2 markers between BMDMs treated with conditioned media of MDA-MB468/PIGR with or without induction of PIGR expression. In contrast, M1 macrophage conditioned media induced a striking dose-dependent increase in PIGR mRNA expression in MDA-MB468 cells, up to 20-fold in 100% conditioned media. IFN- γ and IL-1 β were able to increase PIGR expression in MDA-MB468 cells while TNF- α , IL-10 and TGF- β were not. IL-1 β mRNA expression increased significantly in M1 macrophages, however, IFN- γ mRNA

expression was not detected. The role of IL-1 β secreted from M1 macrophages in increasing expression of PIGR was confirmed by IL-1 receptor blockade indicating that IL-1 β is the M1 macrophage cytokine that enhances PIGR expression in breast cancer cells. The study of PIGR expression in breast cancer tissues from the patients demonstrated that PIGR mRNA expression is lower in breast cancer compared with normal breast tissue (median H score: 0 vs.120, $p < 0.0001$). There was no statistically significant difference in the proportion achieving a pCR in PIGR positive compared with PIGR negative tumours (22.2% vs. 32.1%, $p = 0.466$). Five-year overall survival of the patients with PIGR positive and negative tumours was 88.9% and 78.3%, respectively. However, overall survival was not significantly different ($p = 0.406$).

Conclusions

PIGR did not affect cellular behaviour and chemo-sensitivity of these breast cancer cell lines and did not polarise macrophages. Nevertheless IL-1 β , which is the M1 macrophage cytokine, enhanced PIGR expression in breast cancer cells. These results imply that increased PIGR expression in breast cancer *in vivo* may reflect the polarisation state of tumour-associated immune cells. However, the expression of PIGR did not appear to be associated with a pCR after NAC and did not correlate with overall survival in this group of breast cancer patients.

Presentations

Oral Presentations

1. Predictive Factors of a Pathological Complete Response in Breast Cancer in Women Undergoing Neoadjuvant Chemotherapy
W. Asanprakit, D. N. Lobo, A. J. Bennett, O. Eremin
The Sue Watson Postgraduate Oral Presentation Prize, University of Nottingham Medical School, Queen's Medical Centre, 24th May 2019
2. Expression of Polymeric Immunoglobulin Receptor (PIGR) and the Effect of PIGR overexpression on Breast Cancer Cells
W. Asanprakit, D. N. Lobo, O. Eremin, A. J. Bennett
Surgical Research Society Virtual Meeting, 24th March 2021
3. Predictive and Prognostic Roles of Polymeric Immunoglobulin Receptor in Breast Cancer
W. Asanprakit, A. M. Zaitoun, D. N. Lobo, O. Eremin, A. J. Bennett
Surgical Research Society Virtual Meeting, 24th March 2021
4. M1 Macrophages Evoke an Increase in PIGR Expression in MDA-MB468 Breast Cancer Cells Through Interleukin-1 β
W. Asanprakit, D. N. Lobo, O. Eremin, A. J. Bennett
Surgical Research Society Virtual Meeting, 25th March 2021

Poster Presentation

1. Predictive Factors of a Pathological Complete Response in Breast Cancer in Women Undergoing Neoadjuvant Chemotherapy
W. Asanprakit, D. N. Lobo, A. J. Bennett, O. Eremin
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List of Abbreviations

5-FU	5-fluorouracil
AEE	apical early endosomes
AJCC	American Joint Committee on Cancer
ALN	axillary lymph node
ALND	axillary lymph node dissection
ANOVA	analysis of variance
AP2	activator protein-2
AR	androgen receptor
ARE	apical recycling endosomes
ARG1	arginase 1
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BCP	1-bromo-3-chloropropane
BCS	breast conserving surgery
BEE	basolateral early endosomes
BMDM	bone marrow derived macrophage
BRCA1	breast cancer susceptibility gene 1
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation
CE	common endosomal compartment

CEP17	centromeric region of chromosome 17
CM	conditioned medium
cm	centimetre
CT	computerized tomography
CTLA-4	cytotoxic T lymphocyte antigen 4
DapB	4-hydroxy-tetrahydrodipicolinate reductase
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DEPC	diethyl pyro-carbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DPX	dibutylphthalate polystyrene xylene
DTT	dithiothreitol
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EC ₅₀	half maximal effective concentration
EDTA	ethylene-diamine-tetraacetic acid
EGFR	epidermal growth factor receptor
EMEM	Eagle's Minimum Essential Medium
ER	oestrogen receptor
FBS	foetal bovine serum
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded

FISH	fluorescence <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
fmol	femtomole
FNAC	fine needle aspiration cytology
FOXP3	forkhead box protein 3
GAPDH	glyceraldehyde-3-Phosphate Dehydrogenase
GDP	guanosine diphosphate
GFP	green fluorescent protein
GGI	genomic grade index
gm	gram
HEK	Human embryonic kidney
HER2	human epidermal growth factor receptor 2
HPLC	high-performance liquid chromatography
hr	hour
HRP	horseradish peroxidase
H score	histo score
H&E	haematoxylin and eosin
IF	immunofluorescence
IFN- γ	interferon gamma
Ig	immunoglobulin
IHC	immunohistochemistry
I κ B	inhibitory kappa B

IKK	inhibitory kappa B kinase
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
iNOS	inducible nitric oxide synthase
IQR	interquartile range
IRF1	interferon regulatory factor 1
ISH	<i>in situ</i> hybridization
ISRE	interferon stimulated response element
JAK	Janus kinase
kb	kilobase
kDa	kilodalton
L	litre
LABC	locally advanced breast cancer
LB	lysogeny broth
LLABC	large and locally advanced breast cancer
LP	lentiviral particle
LPS	lipopolysaccharide
M	molar
mA	milliampere
MDSC	myeloid-derived suppressor cell
mg	milligram
min	minute

miRNAs	microRNAs
ml	millilitre
mM	millimolar
mm	millimetre
MOI	multiplicity of infection
MRI	magnetic resonance imaging
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MWCO	molecular weight cutoff
MyD88	myeloid differentiation primary response 88
μg	microgram
μl	microlitre
μM	micromolar
NAC	neoadjuvant chemotherapy
NCBI	National Center for Biotechnology Information
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NK	natural killer
nm	nanometre
NSABP	National Surgical Adjuvant Breast and Bowel Project
NST	no special type
PAMP	pathogen associated molecular pattern

PAM50	prediction analysis of microarray 50
PB	phosphate buffer
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Triton X-100
PCR	polymerase chain reaction
pCR	pathological complete response
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PEG	polyethylene glycol
PES	polyethersulfone
PET	positron emission tomography
pg	picogram
PIGR	polymeric immunoglobulin receptor
PI3K	phosphoinositide 3-kinase
PPIB	cyclophilin B
PR	progesterone receptor
RCPATH	Royal College of Pathologists
real-time qPCR	real-time quantitative polymerase chain reaction
RECIST	Response Evaluation Criteria in Solid Tumours
RIPA	radioimmunoprecipitation assay
ROR	risk of the relapse
rpm	revolutions per minute

RPMI	Roswell Park Memorial Institute
RS	recurrence score
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SC	secretory component
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of mean
SEPT3	septin 3
SIg	secretory immunoglobulin
SLN	sentinel lymph node
SLNB	sentinel lymph node biopsy
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TAE	Tris-acetate-ethylene-diamine-tetraacetic acid
TAM	tumour-associated macrophage
TBST	Tris-buffered saline in Tween 20
TDLU	terminal duct-lobular unit
TE	Tris-ethylene-diamine-tetraacetic acid
TGF- β	transforming growth factor beta
TGN	trans-Golgi network
TIL	tumour-infiltrating lymphocyte

TLR	toll-like receptors
TNBC	triple negative breast cancer
TNF- α	tumour necrosis factor alpha
Tregs	T regulatory cells
TRITC	tetramethylrhodamine-isothiocyanate
TU	transformation unit
UK	United Kingdom
USF	upstream stimulation factor
V	volt
WHO	World Health Organization

Chapter 1 General Introduction

Chapter 1 General Introduction

1.1 Breast cancer: Background

1.1.1 Incidence

Cancer is a major public health problem and is the second leading cause of death worldwide (1). In 2018, there were 17 million new cases and 9.6 million cancer-related deaths globally (1, 2). Breast cancer is the most common cancer in women and was responsible for 2.09 million new cases and 627,000 deaths in 2018 (1, 3). Although the incidence of breast cancer is higher in developed countries, it is increasing worldwide.

In the United Kingdom (UK), breast cancer is the most common cancer and accounted for 15% of all new cancer cases in 2017 (Figure 1.1) (4). Approximately 55,200 new cases of breast cancer were diagnosed each year (2015-2017) which was around 150 cases per day (5). The incidence of breast cancer in women has increased by 5% over the last decade and reached 167 cases per 100,000 in 2017 (Figure 1.2) (6). As a result of the effective screening program for early detection and the advances in therapeutic modalities, mortality rate has declined by 21% in the last decade (Figure 1.3) (5). Five- and ten-year survival rates for the women diagnosed with breast cancer in England (including Wales) are 87% and 78%, respectively (5).

1.1.2 Classification

Breast cancer is a heterogeneous disease consisting of multiple entities associated with diverse biological features, histopathological profiles, clinical presentations, clinical course, response to treatment, and prognosis (7, 8). Generally, breast cancers can be classified according to histopathological grade, type of tumour, genomic profile and clinical staging. The classification determines the most suitable individual treatment options.

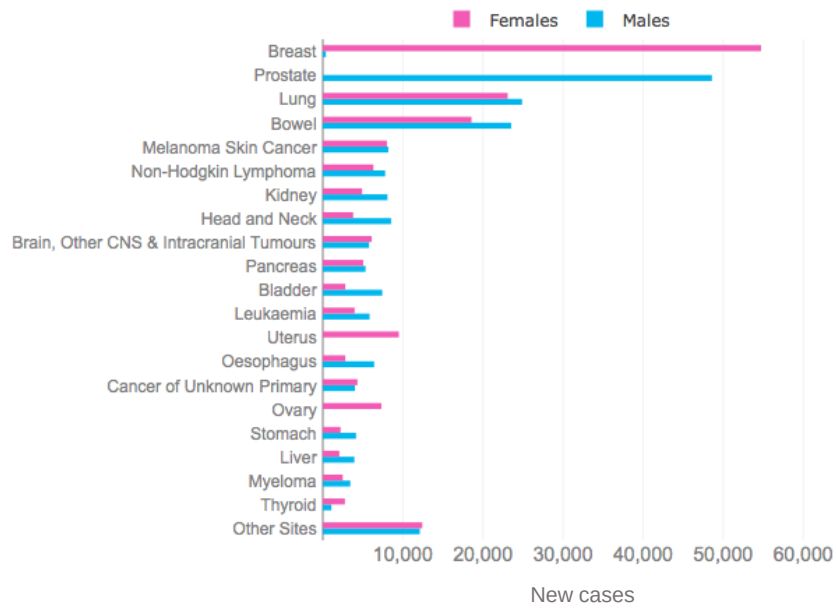


Figure 1.1 The 20 most common cancers in the UK in 2017 (exclude non-melanoma skin cancer)

Credit: Cancer Research UK (4)

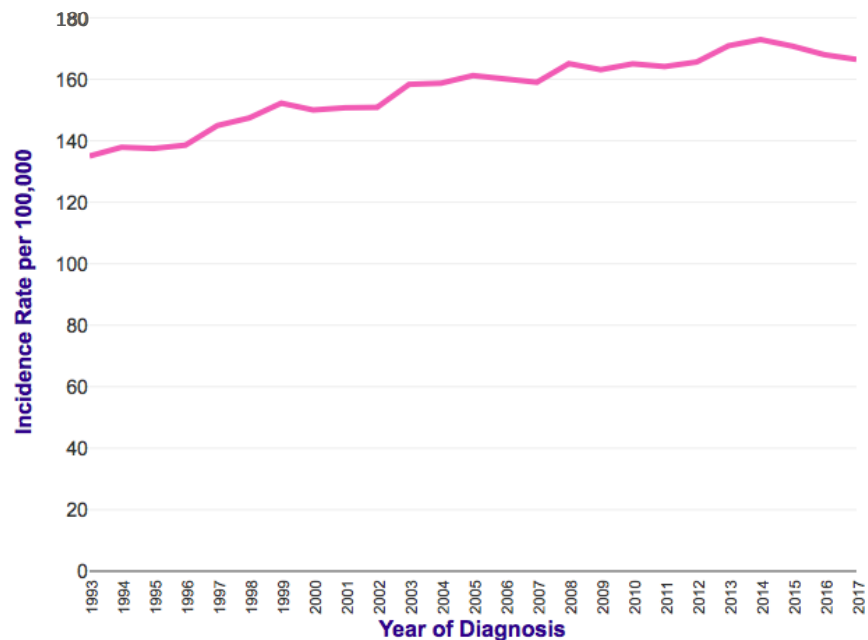


Figure 1.2 Breast cancer incidence in the UK (1993-2017)

Credit: Cancer Research UK (6)

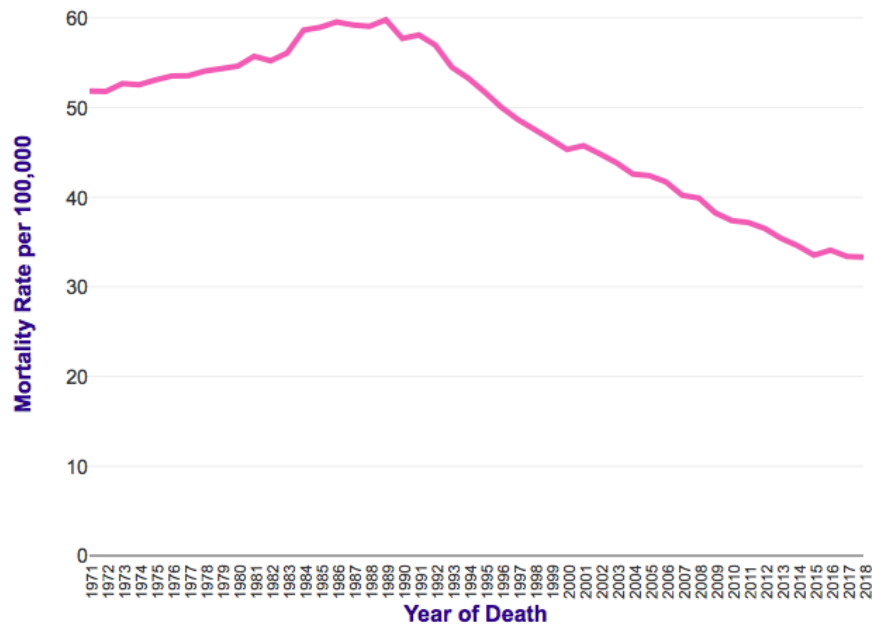


Figure 1.3 Breast cancer mortality rates in the UK (1971-2018)

Credit: Cancer Research UK (9)

1.1.2.1 Histological grade

Histological tumour grade is defined by the morphological characteristics of cancer cells compared with normal cells on the basis of the degree of differentiation (10). It represents the aggressive potentials of the tumour. Low grade cancers tend to be less invasiveness and less likely to metastasize than high grade cancers. The Nottingham grading system (the Elston-Ellis modification of Scarff-Bloom-Richardson grading system) is the most commonly used and is recommended by many professional organisations such as World Health Organization (WHO), the College of American Pathologists, American Joint Committee on Cancer (AJCC) and the UK Royal College of Pathologists (RCPATH) (10-12). By combining the assessment of tumour morphology (tubular/acinar/glandular formation), nuclear atypia/pleomorphism and frequency of mitoses (each scored from 1-3), a total score (ranging from 3-9) determines the histological grade (13). Tumours are classified as well differentiated (grade 1, score 3-5), moderately differentiated (grade 2, score 6-7) and poorly

differentiated (grade 3, score 8-9). The Nottingham grading system was documented to correlate with long-term prognosis and was incorporated in prognostic algorithms and guidelines for breast cancer management (14, 15). The estimated ten-year survival rate for patients with grade 1 tumours is 85% while reduced to 45% for those with grade 3 tumours (13).

1.1.2.2 Histological types

Breast cancer can be divided into two main types: carcinoma and sarcoma. The majority of breast cancers are carcinomas. Breast carcinomas arise from the epithelial lining of the terminal duct-lobular unit (TDLU) of the breast, whilst sarcomas arise from the mesenchymal/stromal tissue of the breast. Breast sarcomas are very rare, accounting for less than 1% of breast cancers (16, 17).

Breast carcinomas are divided, regarding the invasion of the cancer cells, into two groups: *in situ* and invasive carcinoma. Carcinoma *in situ* is pre-invasive carcinoma in which cancer cells are contained within the lining of the basement membrane in the TDLU. In comparison, invasive carcinoma occurs when cancer cells invade beyond the basement membrane into connective stromal tissue. The latest publication of the WHO Classification of Tumours of the Breast in 2019 classified invasive breast carcinomas by their morphologic subtypes which relate to clinical relevance (Table 1.1) (18). Up to 75% of invasive breast carcinomas are classified as invasive ductal carcinoma of no special type (NST) followed by invasive lobular carcinoma (5-15%) (19). The remaining types of invasive carcinomas are less common variants. These different histologic types are characterised by distinct pathological features which relate to tumour biology and prognosis (Table 1.2).

Table 1.1 Histological classification of invasive breast carcinoma (WHO 2019)

Invasive breast carcinoma	
Invasive breast carcinoma of no special type	
Microinvasive carcinoma	
Invasive lobular carcinoma	
Tubular carcinoma	
Cribriform carcinoma	
Mucinous carcinoma	
• Mucinous cystadenocarcinoma	
Invasive micropapillary carcinoma	
Carcinoma with apocrine differentiation	
Metaplastic carcinoma	
Rare and salivary gland-type tumours	
• Acinic cell carcinoma • Adenoid cystic carcinoma • Secretory carcinoma • Mucoepidermoid carcinoma • Polymorphous adenocarcinoma • Tall cell carcinoma with reversed polarity	

Table 1.2 Prognosis of different histologic types of invasive breast carcinoma

Prognosis	Histological type
Excellent prognosis (>80% 10-year survival)	Tubular carcinoma Cribriform carcinoma Mucinous carcinoma Tubulo-lobular variant of invasive lobular carcinoma
Good prognosis (60-80% 10-year survival)	Mixed invasive carcinoma with tubular and other special types Mixed carcinoma with ductal and other special types (invasive carcinoma of NST pattern constitutes 10% to 49% of tumour) Alveolar variant of invasive lobular carcinoma
Moderate prognosis (50-60% 10-year survival)	Invasive papillary carcinoma Invasive lobular carcinoma, classic type Carcinoma with medullary features, including medullary and atypical medullary carcinomas*
Poor prognosis (<50% 10-year survival)	Invasive carcinoma NST Solid variant of invasive lobular carcinoma

NST: no special type

*Currently regarded as part of the spectrum of invasive carcinomas NST with prominent tumour-infiltrating lymphocytes (TILs)

Modified with permission from "Invasive breast cancer: Current perspectives and emerging views" by E. Shea and V. Koh, 2020, Pathology International, 70(5), 242-252. Copyright 2020 by John Wiley and Sons (20).

1.1.2.3 Molecular (intrinsic) subtypes

The advancement of various molecular techniques such as microarray techniques and gene expression profiling has brought additional insights into the complex molecular structure, underlying genetic alteration and biological diversity in breast cancer. This information has led to new strategies in patient stratification, diagnosis and treatment in clinical practice. Gene expression array analysis has resulted in the classification of breast cancer into subtypes according to pervasive differences in their gene expression patterns (21). Four distinct molecular subtypes of breast cancer are widely accepted: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched (HER2 +ve) and basal-like subtype (Table 1.3) (22, 23). These subtypes have been associated with differences in clinical features, incidence, response to treatment and survival (24). The diversity of gene expression profile is associated with phenotypic expression of each subtype. Oestrogen receptor (ER), progesterone receptor (PR), HER2 and Ki-67 are the critical biomarkers used as surrogates for gene expression profiling-based intrinsic subtypes (23, 25, 26).

Luminal A and B subtypes

Luminal tumours are the most common subtype of breast cancer and account for 70-83% of invasive cancers (27-29). In general, this group of patients has a good prognosis, good response to hormonal therapy but poor response to chemotherapy. Luminal subtypes express a genetic profile comparable with the luminal epithelial cells of mammary ducts (21). These patterns include the expression of hormone receptors and associated genes. Luminal subtypes are further classified to luminal A and luminal B subtypes.

Table 1.3 Clinical and pathological characteristics of breast cancer categorised by molecular subtype

	Luminal A	Luminal B	HER2 +ve	Basal-like
Frequency	60-73% of all invasive breast cancer	5.5-11% of all invasive breast cancer	12-20% of all invasive breast cancer	15-20% of all invasive breast cancer
Morphology	Grade 1 or 2, most are well differentiated carcinomas	Grade 2 or 3, less well differentiated cancers than Luminal A	Grade 2 or 3	Most are grade 3
Immunohistochemical profile	ER +ve and/or PR +ve HER2 -ve Low Ki-67	ER +ve and/or PR +ve HER2 -ve or +ve Hi Ki-67	ER -ve, PR -ve HER2 +ve	ER -ve, PR -ve HER2 -ve
Locoregional recurrence rate	0.8-8%	1.5-8.7%	1.7-9.4%	3-17%

Luminal A subtype includes 60-73% of invasive breast cancers (27-29). This group is characterised by high expression of oestrogen receptor 1 (*ER1*), oestrogen regulated gene (*LIV-1*), hepatocyte nuclear factor 3-Alpha (*HNF3A*), X-box binding protein 1 (*XPB1*) and GATA-binding protein 3 (*GATA3*) (30). Immunophenotyping of luminal A shows the expression of ER and/or PR (ER +ve and/or PR +ve), absence of HER2 (HER2 -ve) and low Ki-67 (31, 32). Luminal A breast cancer tends to be of low histological

grade (33). Patients with luminal A breast cancer have a low recurrence rate, good prognosis and the best survival (28, 33).

Luminal B subtype, which includes 5.5-11% of invasive cancers, expresses a low to moderate level of ER cluster genes (as mentioned for luminal A) but a high level of *GGH*, *LAPTMB4*, *NSEP1* and *CCNE1* genes (27-30). There are two phenotypic characteristics of luminal B tumours, luminal B/HER2 -ve (ER +ve and/or PR +ve, HER2 -ve and high Ki-67) and luminal B/HER2 +ve (ER +ve and/or PR +ve and HER2 +ve) (31, 32). Luminal B breast cancers are associated with a higher histological grade than the luminal A subtype. Patients with luminal B/HER2 -ve breast cancer have a worse prognosis than those with luminal A but better than luminal B/HER2 +ve (33).

Ki-67 represents proliferation of breast cancer cells and has been used to categorise luminal A and luminal B subtype. High expression of Ki-67 is associated with high tumour grade and adverse prognostic features but indicates a good response to neoadjuvant chemotherapy (NAC) (32, 34). However, there is no analytical validity of Ki-67 immunohistochemical staining. The cutoff point to distinguish between high and low levels of expression varies amongst laboratories (35). The Saint Gallen International Expert Consensus suggested that Ki-67 <14% is the best correlation with the gene-expression definition of luminal A subtype (35, 36).

HER2 enriched (HER2 +ve) subtype

The HER2 enriched (HER2 +ve) subtype includes 4-10% of invasive breast cancers (27-29). This subtype is characterised by high expression of *ERBB2*, *GRB7* and *TRAP100* but low expression of basal and luminal related genes (30, 37). *TP53* and *PIK3CA* mutations are frequently found in HER2 enriched tumours. The immunophenotype of the HER2 enriched subtype shows HER2 overexpression in the

absence of ER and PR (ER and PR -ve, HER2 +ve). Overexpression of HER2 is associated with aggressive tumours, poor response to chemotherapy and poor prognosis (38, 39). Nevertheless, following the development of anti-HER2 targeted therapy, five-year survival and overall survival rates have been improved.

Basal-like subtype

The incidence of the basal-like subtype is approximately 8-13% (27-29). The luminal/ER cluster of genes is absent in the basal-like subtype, which has a high expression of *KRT5*, *KRT17*, annexin8, *CX3CL1* and *TRIM29* (30). Immunohistochemical staining shows expression of cytokeratin (CK) 5/6, CK14, CK17, which are characteristics of basal epithelial cells, as well as epidermal growth factor receptor (EGFR), c-KIT and FOXC1 (21, 25, 40). Because this subtype usually lacks of ER, PR and HER2, it is so-called triple negative breast cancer (TNBC). Basal-like subtype cancer and TNBC, however, are not exactly the same. Basal-like subtype is a subset of TNBCs which account for 65-90% of TNBCs (40).

Basal-like subtype cancers are mostly grade 3 with aggressive behaviour (25). These cancers are more common in younger patients and African-American women (41). Patients with basal-like cancers have the worst prognosis with low disease specific survival and high locoregional recurrence rate (Figure 1.4). As a result of the lack of specific targets for treatment, there is limited therapeutic option for this group of patients. Despite the aggressive nature, this subtype is sensitive to conventional chemotherapy such as anthracycline and taxanes (41, 42).

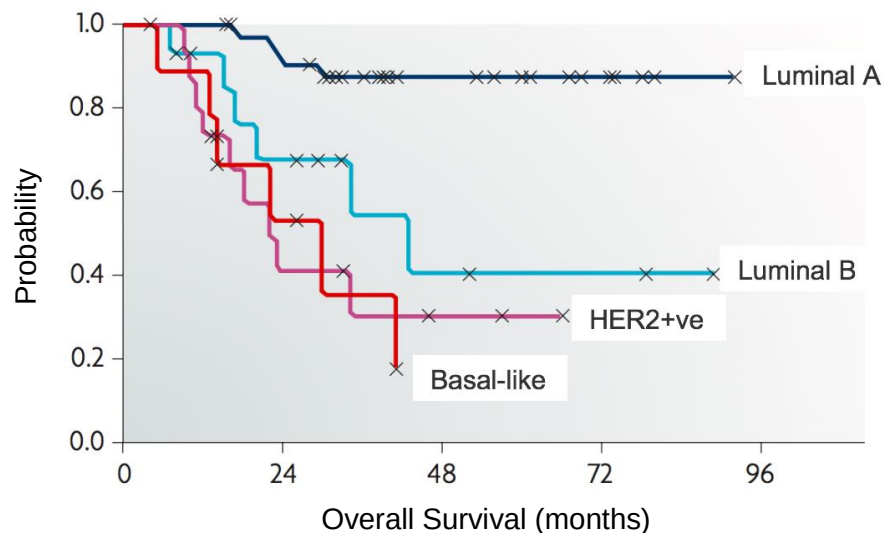


Figure 1.4 The overall survival of patients with locally advanced breast cancers (LABCs) categorised according to molecular subtype

Luminal A subtype demonstrates the best outcome, while basal-like subtype shows the worst outcome. Adapted from “Repeated observation of breast tumour subtypes in independent gene expression data sets”, by T. Sorlie and R. Tibshirani, 2003, Proceedings of the National Academy of Sciences of the United States of America, 100(14), 8418-8423. Copyright 2003 by National Academy of Sciences (43).

1.1.3 Cancer staging

Cancer staging is an important step for patient management in clinical practice. It determines the extent of disease, guides the treatment decision, provides the prognostic information for the individual patient and allows the comparison of outcomes across institutions and clinical trials (44, 45). Moreover, staging also has a pivotal role in a public health perspective where it demonstrates overall disease burden. This information is crucial for planning cancer control policies and evaluating outcomes of the system (44).

The AJCC staging system is an internationally accepted staging system used for invasive (infiltrating) carcinoma and ductal carcinoma *in situ* of the breast (46). Traditionally, it is based on the extent of the disease as defined by tumour size (T), lymph node status (N) and the absence or presence of distant metastasis (M). For

the last two decades, the increased understanding of breast cancer biology has led to identification and validation of a number of breast cancer prognostic and predictive biomarkers. Therefore, the latest edition (8th edition) of the AJCC staging system published in 2017 has incorporated biological markers including tumour grade, ER, PR, HER2 and genomic assays into the traditional TNM anatomic system (Appendix 1). These markers have a significant impact on prognosis, treatment options and outcomes (46).

According to the AJCC 8th edition, the anatomic and prognostic staging are two principal categories for staging breast cancer (46). Anatomic staging based on the traditional TNM staging remains the cornerstone for evaluation and management of breast cancer in low- and middle-income countries where molecular assays are not available. Prognostic staging is the modified anatomic staging by integrating with the biomarkers. It is divided into clinical prognostic staging and pathological prognostic staging (46).

Clinical prognostic staging is based on initial evaluation using clinical information (history and physical examination), imaging studies and relevant biopsies before any treatment (46). This staging is comprised of T, N, M, tumour grade, ER, PR and HER2. Clinical prognostic staging is used to guide initial treatment and is a basis for comparing all patients regardless of following treatment. In contrast, pathological prognostic staging is evaluated after surgical treatment using all the information from clinical staging, surgical findings and pathological findings from surgical specimens. It is not applicable to patients who receive NAC. Genomic assays (Oncotype Dx[®]) are applied to this staging for the T1-2, N0, M0, ER +ve, HER2 -ve patients to determine the benefits of adjuvant chemotherapy. Pathological prognostic staging indicates the necessity for adjuvant treatment after surgery (46).

1.1.4 Treatment

The multimodality approach combines various therapies in the course of treatment for breast cancer. These therapies include locoregional treatment (surgery, radiotherapy) and systemic treatment (chemotherapy, endocrine therapy, targeted therapy). Individual treatment options are based on disease staging (clinical and pathological staging), tumour histologic type, biological (molecular) subtypes, patient comorbidity, patient age, menopausal status, presence or absence of metastatic disease, and patient preference. An optimal choice of combined treatment from a specialist multidisciplinary team, including surgical, medical and radiation oncologists, has been demonstrated to correlate with an improvement of treatment outcome (47).

Generally, patients with early stage and operable locally advanced breast cancer (LABC) are initially treated with surgical removal of breast tumour (breast conserving surgery [BCS, lumpectomy] or mastectomy) and axillary lymph nodes (ALNs) staging (sentinel lymph node biopsy [SLNB] or axillary lymph node dissection [ALND]). Following surgical treatment, locoregional irradiation and/or systemic therapy are given as adjuvant treatment. In contrast, primary treatment of inoperable LABCs is NAC and/or radiotherapy followed by surgery. NAC is aimed to downstage the tumour and facilitate subsequent surgical removal. Moreover, NAC may be used to downsize the large tumours or operable LABCs in order to increase the likelihood of BCS. For advanced stage breast cancers with distant metastases, systemic therapy for palliation is the major treatment modality.

1.1.4.1 Locoregional treatment

Surgery

Curative surgery for breast cancer comprises of removal of the breast tumour and tumour draining ALNs.

Breast surgery

The removal of the breast tumour can be accomplished either by BCS (lumpectomy) or mastectomy. In BCS, the breast tumour with surrounding normal breast tissue is removed, with pathological confirmation of clear margins. The aim of BCS is to preserve the breast with a good cosmetic result without detracting from oncological outcome. Whole breast irradiation after BCS is necessary to reduce the risk of local recurrence by half and breast cancer related death by about one-sixth (48). Therefore, adjuvant radiotherapy is strongly recommended after BCS (49). BCS is contraindicated in early pregnancy due to an unacceptable foetal risk of radiotherapy during pregnancy. In addition, patients with diffuse suspicious microcalcification on mammography, widespread disease which cannot be removed with satisfactory cosmetic outcome and diffuse positive pathological resection margins are definitely not candidates for BCS. Relative contraindications for BCS are previous chest wall irradiation, active connective tissue disease with skin involvement, known or suspected genetic predisposition to breast cancer and tumour size more than 5 cm (49).

Mastectomy is the choice for removal of breast tumours in patients with a contraindication for BCS and for those who prefer this option (49). There is no difference in survival outcome for patients with breast cancer receiving either mastectomy or BCS (50, 51).

Axillary surgery

Axillary nodal spread is an important prognostic factor in breast cancer. ALND has traditionally been used to evaluate nodal metastasis, as well as surgical clearance of nodal disease. Nevertheless, ALND is associated with significant postoperative morbidities such as lymphoedema, paraesthesia, seroma and wound infections and

is considered excessive for those without axillary disease. Therefore, SLNB has been substituted (52, 53). The sentinel lymph nodes (SLN) are the first group of ALNs which receive lymphatic drainage from the breast and tumour. Identification of SLN either by radioisotopes and/or blue dyes facilitates removal during surgery. Histologically proven metastatic spread to the SLN usually indicates the need for ALND.

Preoperative image-guided needle biopsy of suspicious nodes is recommended for patients with clinically positive ALNs (49, 54). If the pathological result from biopsy is positive for tumour deposits, ALND is obligatory for these patients. In contrast, SLNB is recommended for patients without clinically positive ALNs and those whose preoperative nodal biopsy result is negative. Following SLNB, ALND is recommended for proven axillary nodal macrometastases (metastatic deposit more than 2 mm) (49, 55). Recent advice is that ALND can be omitted after positive SLNB in selected patients who meet all the following criteria, which are T1 or T2 tumours (tumours less than or equal to 5 cm), only 1-2 positive SLNs, treated with BCS with adjuvant whole breast radiotherapy and no preoperative systemic therapy (49, 56-58). When the SLN cannot be identified intraoperatively, ALND is mandatory.

Radiotherapy

For all patients who undergo BCS, whole breast irradiation is strongly recommended to minimise the risk of local recurrence (48, 49). Following BCS, regional lymph node irradiation including infraclavicular and supraclavicular nodal areas, internal mammary nodes and the axillary bed is recommended if nodal metastasis of more than or equal to four nodes are identified (59, 60). Following mastectomy, chest wall irradiation and regional lymph node irradiation are indicated in patients with tumours greater than 5 cm, positive surgical margins or ALN metastasis (49).

1.1.4.2 Systemic treatment

Systemic treatment refers to treatment using substances that travel through the bloodstream to treat the cancer cells at primary and distant sites. Neoadjuvant systemic therapy (preoperative systemic treatment) is given before surgical treatment to improve surgical outcome. It can be used to convert inoperable to operable tumours and to downsize operable tumours to increase the chance of BCS. Adjuvant systemic therapy is given after surgery to eradicate occult micrometastases and decrease the risk of recurrence. Palliative systemic treatment aims to relieve symptoms, improve quality of life and possibly prolong survival in patients with distant metastases. Chemotherapy, endocrine therapy and targeted therapy are systemic treatment options for patients with breast cancers.

Chemotherapeutic agents

The various chemotherapeutic agents used for cancer treatment are generally classified by their mode of action (Table 1.4). Chemotherapy may be used as a single agent or combination of two or more agents. The principle of combination therapy is to combine chemotherapeutic agents that have different modes of action. The combined drugs should be synergistic, not antagonistic, and have different toxicity profiles (61). This can enhance the efficacy of combination chemotherapy by allowing the use of the optimal dose of each drug and decreasing the likelihood of drug resistance and toxicity.

Table 1.4 Chemotherapeutic agents classified according to their mode of action

Chemotherapeutic agents	Modes of action	Examples
Alkylating agents	Transfer alkyl groups to DNA results in abnormal nucleotide sequence, miscoding of mRNA, cross-linked DNA strands, breakage of DNA strands and other damage to the transcription and translation of genetic material	Busulfan, Melphalan, Chlorambucil, Cyclophosphamide, Ifosfamide, Carmustin, Streptozocin, Dacarbazine, Temozolomide
Topoisomerase interactive agents	Inhibit the topoisomerase enzymes which function by cutting the DNA backbone and failing to solve DNA topologic problems during replication	Topoisomerase I inhibitors: Irinotecan, Topotecan, Camptothecin
		Topoisomerase II inhibitors: Etoposide, Teniposide, Doxorubicin, Epirubicin, Idarubicin, Daunorubicin, Mitoxantrone, Amsacrine, Ellipticines, Aurintricarboxylic acid
Microtubule targeting agents	Microtubule-destabilising agents: inhibit microtubule polymerisation	Vinca alkaloids (Vinblastine, Vincristine, Vinorelbine, Vindesine and Vinflunine), Cryptophycins, Halichondrins, Estramustine, Colchicine, Combretastatins

Chemotherapeutic agents	Modes of action	Examples
	Microtubule-stabilising agents: inhibit microtubule de-polymerisation	Paclitaxel, Docetaxel, the Epothilones, Discodermolide
Antimetabolites	Inhibit essential biosynthetic processes or being incorporated into macromolecules, such as DNA and RNA, and inhibiting their normal function	5-Fluorouracil, 6-Mercaptopurine, Capecitabine, Cytarabine, Gemcitabine, Methotrexate, Pemetrexed
Platinum analogues	Cause inter-strand and intra-strand DNA crosslinks which inhibit DNA repair and synthesis	Cisplatin, Carboplatin, Oxaliplatin

Anthracyclines

Anthracyclines are anti-tumour antibiotics, extracted from the *Streptomyces* bacterium. They are amongst the most effective anticancer treatments and damage or destroy many types of cancer cells. Anthracyclines act by intercalating between DNA or RNA base pairs. As a result, DNA or RNA synthesis is inhibited, interfering with the replication of cancer cells (62). Anthracyclines also inhibit topoisomerase II enzyme in relaxing the supercoiled DNA, thus preventing DNA transcription (63). After the topoisomerase II has broken the DNA chain for replication, anthracyclines can stabilise the topoisomerase II complex which is necessary to reseal the DNA chain, thereby stopping the process of DNA replication (62). Moreover, anthracyclines can enhance reactive oxygen species generation in cancer cells which leads to cytotoxicity (64).

Taxanes

Taxanes are classified as microtubule stabilising drugs which disrupt microtubule function. Taxanes stabilise guanosine diphosphate (GDP)-bound tubulin in the microtubule, thus de-polymerisation is prevented. As a result, the microtubules cannot shrink back to separate the chromatids, resulting in mitotic blocking and inhibition of cell division resulting in the triggering of apoptosis. The taxanes, paclitaxel and docetaxel, are commonly used to treat breast cancer in the adjuvant and neoadjuvant settings and also in metastatic disease. Moreover, taxanes have been demonstrated to be effective chemotherapeutic agents in many other cancers such as ovarian, lung and oesophageal cancers (65).

Cyclophosphamide

Cyclophosphamide is one of the most widely used alkylating agents. It is categorised as belonging to the nitrogen mustard group (66). It is used as a chemotherapeutic agent which is active in all phases of the cell cycle (cell cycle nonspecific). Due to its immunosuppressive property, cyclophosphamide is also used as an immunosuppressive agent to treat autoimmune disease and prevent organ transplant rejection. Cyclophosphamide is a prodrug metabolised by the liver cytochrome P-450 microsomal system to form active metabolite. 4-hydroxycyclophosphamide which is the primary active metabolite exists in equilibrium with its tautomer, aldophosphamide. Both these intermediate metabolites are taken up by cancer cells and normal cells after release into the circulation from hepatic cells. Aldophosphamide is transformed into a phosphamide mustard which forms irreversible interstrand DNA cross-links resulting in DNA replication inhibition and triggering cell death. Normal cells which are susceptible are affected and are responsible for the side effects of this drug. There is a selective toxicity in tumour cells, however, because tumour cells have more enzyme than normal cells, which converts aldophosphamide to toxic phosphamide

mustard (66, 67). Furthermore, aldehyde dehydrogenases which convert aldophosphamide to a non-cytotoxic compound carboxy-phosphamide are abundant in normal rapidly proliferating cells, thus protecting normal cells from the toxic effect of the phosphamide mustard (67, 68).

Capecitabine

Capecitabine is an oral fluoro-pyrimidine carbamate which is converted to 5-fluorouracil (5-FU) in steps by three enzymes, carboxylesterase, cytidine deaminase and thymidine phosphorylase, respectively (69). Carboxylesterase is found only in liver and hepatocellular carcinoma. Cytidine deaminase is found in liver and many types of solid cancer. Thymidine phosphorylase is found in various cancers in much higher levels than in normal tissues. Because of the location of these enzymes, capecitabine is selectively converted to 5-FU and activated in cancer cells (69). 5-FU is an antimetabolite drug which works as a thymidylate synthase inhibitor. It inhibits the synthesis of thymidine monophosphate (active form of thymidine) which is a nucleoside required for DNA synthesis, resulting in lethal DNA damage.

1.2 Large and locally advanced breast cancer (LLABC)

There are various techniques used to establish the diagnosis of breast cancer and breast cancer screening programmes are implemented in many countries worldwide. Large and locally advanced breast cancers (LLABCs), however, continue to be a significant problem, especially in developing countries. In the UK, around 20% of patients with breast cancer present with LLABC and the five-year survival rate is approximately 50-60% (5). LLABC includes T2 tumours >3 cm (large breast cancers) and T3/T4 tumours and/or N2/N3 disease (LABC). Due to the increased rate of locoregional recurrence and risk of systemic metastases after treatment, this group

of patients poses a clinical challenge in terms of treatment and subsequent monitoring.

1.3 Neoadjuvant chemotherapy (NAC)

Neoadjuvant chemotherapy (NAC, also known as induction or primary chemotherapy) is administered before definitive locoregional treatment of the primary tumour (70). Initially, it was used in advanced disease to downstage the tumour in order to facilitate surgical treatment. The first prospective NAC trial was carried out by the Milan Cancer Institute in 1973. Two hundred and twenty seven patients with stage III breast cancer received Adriamycin and vincristine prior to locoregional treatment; 62% of patients achieved a complete or partial pathological response (71). Based on the evidence that overall survival with breast conserving therapy does not differ from mastectomy (51), NAC was considered for use in large, operable breast cancers to reduce tumour size and increase the likelihood of BCS. Mamtani *et al.* (72) demonstrated that NAC may reduce the likelihood of ALND in patients with clinical stage 2 and 3 breast cancers who had pathologically confirmed involved ALNs at presentation. SLNB was technically feasible for the patients who were clinically ALN negative after NAC. Long-term follow-up was required, however, to determine locoregional disease control rate (72). Apart from down-staging the tumour and increasing the BCS rate, NAC provides an early opportunity to treat distant putative micrometastases, which should be considered in all patients with invasive breast cancer, as well as observing the response of the breast cancer to chemotherapy in individual patients and predicting their likely long-term outcome.

NAC, on the other hand, has a number of disadvantages, namely chemotherapy-induced morbidity for no clinical gain in non-responder patients, and the progression of the breast cancer to an unresectable tumour during treatment (73). This risk, nevertheless, can be significantly reduced by careful monitoring (clinical, imaging)

during NAC. The chance of metastasis formation may increase due to delayed surgical treatment of the primary tumour in those patients whose cancer is not responding.

1.4 Evaluation of tumour status in patients with LLABC treated with NAC

1.4.1 Prior to NAC

Before commencing NAC, accurate clinical staging and primary breast cancer status is determined. For clinical staging, blood tests including a full blood count and differential, liver function tests including alkaline phosphatase should be ascertained (49). Imaging studies such as a computerised tomography (CT) or magnetic resonance imaging (MRI) with contrast, of the chest, abdomen and pelvis and bone scan should be carried out to establish absence of metastatic lesions. Positron emission tomography (PET) may be performed where standard imaging is problematic. For evaluation of the breast cancer, mammography and ultrasonography are the most commonly used in clinical practice to evaluate the site, size and multifocality of the tumour(s) in the breast as well as ultrasonography of ALN status, before commencing NAC (74, 75). Breast MRI is an optional consideration for mammographically occult tumours. Ultrasound-guided core cut biopsies of any suspicious breast lesions and suspected ALN metastatic deposits should be carried out. Radio-opaque markers should be inserted into tumours under ultrasound guidance to ensure that satisfactory removal of residual breast tumours occurs following NAC (76).

1.4.2 During and post NAC

With NAC, accurate clinical evaluation of the locoregional treatment response is crucial to subsequent further treatment for each patient. The response evaluation

criteria which are widely used are the WHO criteria (77) and the Response Evaluation Criteria in Solid Tumours (RECIST) (version 1.1, revised in 2009) (78). These criteria classify clinical responses as a complete response, partial response, stable disease or progressive disease.

During NAC, patients are closely monitored by regular clinical examination and imaging, in particular ultrasonography. Clinical breast examination, however, may be unreliable in small tumours, in those with ill-defined borders or irregular shape and where there has been necrosis, fibrosis or fragmentation after NAC (79). Clinical evaluation is, therefore, not an accurate assessment of the pathological response. Fisher *et al.* (80) reported that 62% of patients who achieved a clinical complete response still had residual pathological disease. On the other hand, 7% of patients with clinical residual disease actually achieve a pathological complete response (pCR). Thus, breast imaging modalities are used as adjuncts to increase the accuracy of assessment. Although mammography and ultrasonography are commonly used to evaluate primary tumours prior to initiating NAC, they are not always reliable in determining residual tumours after NAC (81, 82). Digital breast tomosynthesis can ameliorate some of the limitation of mammography by decreasing tissue overlap with better tumour characterisation (83, 84). Unfortunately, tumour characteristics change during NAC due to necrosis, fibrosis or alteration of density making it difficult to evaluate treatment response by these conventional tools (74).

Functional and molecular imaging modalities are demonstrated to be useful and more sensitive tools to evaluate cancer response to NAC (85). They reveal physiological activity in viable cancers by detecting or measuring changes in vascular supply and cellular metabolism, which may occur before there is obvious reduction in tumour volume. Many studies report the value of MRI for monitoring NAC responses. With high sensitivity and relative high specificity, MRI, however, can overestimate or underestimate the residual cancer volume (74, 86). Furthermore, the accuracy of MRI

depends on the tumour subtypes present. Its accuracy is poor in assessing the size of some luminal subtypes, the best being with TNBC and HER2 +ve subtypes (87, 88). Molecular imaging using technetium-99m sestamibi and PET has been used to determine tumour responses during NAC, but they all have limitations (74, 82, 89, 90). At present, there is no ideal or standard imaging modality for evaluating treatment response in the NAC setting.

1.5 Clinical outcome with NAC

1.5.1 Response rate and long-term outcome

The clinical outcome in patients who receive NAC does not differ from those receiving adjuvant chemotherapy. Results from the largest randomised NAC trial in operable breast cancer, National Surgical Adjuvant Breast and Bowel Project (NSABP) Protocol B-18, indicated an overall clinical response rate of 80% with NAC (four cycles of doxorubicin/cyclophosphamide); clinical complete response was 36% and clinical partial response 43% (91, 92). Twenty six percent of women with a clinical complete response had a pCR. There were no differences in the outcome in terms of disease free survival, distant disease free survival, or overall survival between NAC and the adjuvant chemotherapy group (92, 93). Overall survival at 5, 8, 16 years was 80%, 72% and 55%, respectively in the NAC group and 81%, 72% and 55%, respectively in the adjuvant chemotherapy group ($p = 0.94$) (94). Disease free survival at 5, 8, 16 years was 67%, 58% and 42% in the NAC group and 67%, 55% and 39% in the adjuvant chemotherapy group ($p = 0.27$) (94). Nevertheless, in 13% of patients who obtained a pCR in the breast cancer, disease free survival (75% vs. 58%, $p = 0.0005$) and overall survival (85% vs. 73%, $p = 0.00008$) were significantly improved, when compared with the patients who had residual disease after NAC (92, 94).

Mieog *et al.* (95) reviewed and performed a meta-analysis of 14 randomised trials which compared NAC and adjuvant chemotherapy involving 5,500 patients. They reported that there was no difference in overall survival in either group. Patients with a pCR in these studies, which ranged between 4 and 29.2%, however, achieved better overall survival (95). The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (96) published the meta-analysis of individual patient data from 10 randomised trials including 4,756 patients. They reported an overall clinical response rate with NAC as 69%. In the NAC group, complete clinical responders to NAC had a lower distant recurrence and a better breast cancer mortality rate than non-responders. There was, however, no significant difference in distant recurrence, breast cancer mortality and death from any cause between NAC and adjuvant chemotherapy. The association between a pCR with NAC and long-term outcome improvement has been confirmed by several other studies (97-100).

1.5.2 Responses with different combinations of NAC

The addition of taxanes to anthracyclines in NAC can improve clinical response and pCR rates. Smith *et al.* (101) studied the efficacy of neoadjuvant docetaxel in patients with LLABC. They reported that sequential administration of docetaxel in patients who did not respond to initial anthracyclines resulted subsequently in a 55% clinical response and 15% pCR. Moreover, in patients who received sequential docetaxel after initial response to anthracyclines, the overall clinical response rate (94% vs. 66%, $p = 0.001$) and pCR rate (34% vs. 16%, $p = 0.04$) were significantly increased, when compared with those receiving further anthracycline administration. At three-year follow-up, patients who received docetaxel had better survival outcomes and this was associated with higher pCR rates in this group (102). In the NSABP Protocol B-27, the clinical complete response rate was increased from 40% to 64% ($p < 0.001$) in patients assigned to receive preoperative doxorubicin/cyclophosphamide followed by

docetaxel compared with preoperative doxorubicin/cyclophosphamide alone (103, 104). The pCR rate was also increased from 13.7% to 26.1% ($p < 0.001$) (103, 104). Furthermore, the rates of pathological responses in metastatic ALNs were also significantly increased. These studies further confirmed that a pCR in the primary breast cancer improved the long-term outcome of the patients. Thus, a pCR can be regarded as a surrogate marker of a good long-term outcome with NAC.

ERBB2 (Erb-B2 receptor tyrosine kinase 2) gene amplification is a prognostic factor for aggressive cancers and a poor prognosis. *ERBB2* gene encodes HER2 which is a receptor tyrosine kinase of the ErbB family present on the cancer cell membrane. The therapeutic use of anti-HER2 agents (trastuzumab, lapatinib and pertuzumab) improves treatment outcome in the adjuvant and metastatic setting. Incorporation of trastuzumab into NAC also shows promising results. Buzda *et al.* (105) reported a pCR rate as high as 60% in HER2 +ve operable breast cancer patients who received NAC with trastuzumab, when compared with chemotherapy alone. As several anti-HER2 agents have been developed, the treatment with combination chemotherapy and dual anti-HER2 therapy was reported to achieve pCR of up to 83.3% in HER2 +ve, hormonal receptor negative breast cancers (106). Moreover, the prognosis in patients with this type of cancer was altered to a favourable status in patients whose breast tumours underwent a pCR (107, 108). Overall, however, the incidence of HER2 +ve breast cancers is low, and only up to 15% of patients benefit.

1.5.3 The breast conservation rate after NAC and locoregional recurrence

Downsizing and enabling BCS is one of the major benefits of NAC. Several randomised trials have investigated the BCS rate after NAC. NSABP B-18 trial reported that the BCS rate was higher in patients who received NAC than in those who received adjuvant chemotherapy (67% vs. 60%, $p = 0.002$) and greatest in patients with tumour sizes larger than 5 cm (91). Ipsilateral breast tumour recurrence

rate of these two groups, however, were not significantly different (13% with NAC vs. 10% with adjuvant chemotherapy, $p = 0.21$) (94). Addition of preoperative docetaxel to anthracyclines in the NAC group did not further increase the rate of BCS (103).

A meta-analysis of 14 randomised trials by Mieog *et al.* (95) which compared NAC and adjuvant chemotherapy in operable breast cancer showed a significant decrease in the mastectomy rate but an increase in locoregional recurrence rate in the NAC group. There were three studies, however, in which more than one-third of patients received only radiotherapy with no surgery for local treatment after NAC. The recurrence rate for this group was reported as high as 30%. After exclusion of these trials from the analysis, locoregional recurrence rates with NAC and adjuvant chemotherapy were no different. A meta-analysis carried out by EBCTCG (96) reported that breast conserving therapy after NAC was 65% compared with 49% in the group receiving adjuvant chemotherapy. Local recurrence rate in the NAC group was higher than in the adjuvant chemotherapy group and persisted for 10 years. This was partly explained by an increased incidence of breast conserving therapy in the responder group. Moreover, there were two trials which avoided surgical treatment in patients with complete clinical response and these showed the highest local recurrence rate. In general, a high locoregional recurrence rate was reported in patients with locally advanced or inflammatory disease at presentation and in patients who did not undergo surgery as local treatment.

1.5.4 Pathological complete response (pCR) with NAC

A pCR is used as a primary end-point in most NAC trials due to its association with long-term outcome (109). The disease free survival and overall survival in patients whose cancers have a pCR are significantly better than in patients with residual cancer after NAC (110). There is no consensus, however, as regards the precise definition of a pCR. Different definitions of pCR are used in daily practice and clinical

trials. As regards pathological assessment, a pCR has been defined both as the absence of invasive cancer or absence of invasive and non-invasive cancer. As regards distribution of the residual disease, a pCR has been defined as the absence in the breast only or in both the breast and in ALNs. These various definitions of pCR have led to difficulties with interpretation and comparison of results across trials and also the prognostic benefit of pCR (110, 111). The most widely used definition of a pCR, supported by the US Food and Drug Administration (FDA), is the absence of residual invasive cancer in the breast and ipsilateral ALNs in the surgical specimens, regardless of the residual carcinoma *in situ* status in the breast (110, 112). Residual carcinoma *in situ* does not affect long-term outcome as regards overall survival and local recurrence rate (113, 114). On the other hand, residual invasive cancer in ALNs, even where there is a breast pCR, is associated with a poor outcome (115-117). A pCR in both the breast and ALNs provides the best prognosis (76, 113, 114). Eremin *et al.* (76) found that patients whose tumours had a pCR in the breast with no evidence of cancer in the ipsilateral ALNs or a pCR in both the breast and ALNs had a 100% disease free survival at six years of follow-up. Patients whose tumours had a partial response in the breast and ALNs had a 66% disease free survival at six years of follow-up, while those patients whose tumours showed no response to NAC in the breast and ALNs had a 20% disease free survival (personal communication).

Between 40-80% of patients do not have a pCR with NAC, after having undergone a prolonged course of drug therapy with significant side effects and little benefit (76, 118). Predicting the patients who are likely to achieve a pCR before commencing NAC, therefore, is important and clinically beneficial. There is, unfortunately, still a lack of reliable and accurate markers to predict a pCR in clinical practice. Establishing the predictive markers for response to NAC is an area of extensive research and continuing clinical and scientific interest.

1.6 Predictive markers of response with NAC

1.6.1 Clinical markers

Clinical factors are generally used to predict the likelihood of achieving a pCR. Young patients tend to have aggressive cancers and poor survival outcome. The pCR rate in the cancers, however, is high in this group (114). Loibl *et al.* (119) report tumour pCR rates in women aged <40, 40-49, ≥ 50 years as 20.9%, 17.7% and 13.7%, respectively. Small tumours have a higher response rate to NAC (114, 120, 121). Tumours ≤ 4 cm achieved pCR in 28.3% of cases, compared with 23.5% in larger tumours ($p = 0.024$) (120). In addition, nodal status is also a clinical predictor of pCR. Patients without nodal metastases have a higher pCR rate when compared with patients with nodal metastases (41.2% vs. 20.7%, $p = 0.002$) (120).

1.6.2 Molecular biomarkers

Due to the advancement of knowledge in the molecular biology of breast cancer, a number of molecular biomarkers have been studied and evaluated in clinical practice. ER status is one of the most widely investigated markers. ER -ve status predicted a higher pCR rate (40.0% vs. 22.1%, $p = 0.002$) but poorer survival (114, 120, 122). Androgen receptor (AR), a member of the nuclear steroid hormone receptor family, has also been shown to be a predictor of a pCR. As with ER -ve patients, those with AR negative (AR -ve) cancers have better clinical responses and pCR rates compared with AR positive (AR +ve) cancers (25.4% vs. 12.8%, $p = 0.0001$) (123, 124). Patients with AR +ve cancers, however, have a significantly better disease free survival and overall survival (123, 124).

Proliferative markers have become important predictors of pCR in breast cancer. Histological grading of tumours is a scoring system derived from the assessment of tubule formation, nuclear pleomorphism and mitotic counts (proliferative activity)

(125). Many studies have demonstrated a correlation between tumour grading and pCR rate, with high grades being related to higher pCR rates (114, 121). Lips *et al.* (126) reported that high tumour grading is the best predictor for pCR and recurrence free survival in patients with ER +ve/HER2 -ve breast cancers who received NAC. Ki-67 is a nuclear antigen expressed in every phase of the cell cycle except the G0 phase (resting stage) (127). Ki-67, therefore, represents the proliferative activity of cancer cells. Several NAC trials have reported that high Ki-67 expression was related to higher pCR rates (128). There is no consensus, however, as regards the cutoff value of Ki-67 able to predict pCR. Different cutoff values have been reported to range between 1-40% (129-133).

1.6.3 Tumour subtypes

In general, different breast cancer subtypes respond differently to NAC (134). Eremin *et al.* (76) reported that pCR rates were highest in the TNBC subtype (50%), whilst luminal A tumour subtypes had the lowest rates (17%). Von Minckwitz *et al.* (114) analysed 7 randomised NAC trials including 6,377 patients and found that the luminal A tumour subtypes had the lowest pCR rate, whilst the HER2 +ve and TNBC subtypes achieved the highest pCR rates. They reported the pCR rates in luminal A, luminal B, HER2 +ve and triple negative tumour subtypes as 6.4%, 11.2-22.2%, 27.6-32.9% and 31.7%, respectively. In addition, the patients with luminal B/HER2 -ve, HER2 +ve and triple negative tumour subtypes who achieved a pCR had a significantly improved disease free survival. The authors concluded that pCR was a suitable surrogate endpoint for these patients (114). Gentile *et al.* (135) studied 1,522 patients with LABCs to determine the pCR rate and predictive factors for pCR. They reported that the overall pCR rate was 25% (breast pCR 27%, nodal pCR 38%). The highest pCR rate was 48% in the HER2 +ve subtype. ER +ve/HER2 -ve and triple negative subtypes achieved a pCR of 7% and 23%, respectively (135). For TNBC, which was classified

into 6 subtypes by gene expression analysis, there was a heterogeneous response to NAC (136, 137). Basal-like 1 subtype achieved the highest pCR rate (52%), whereas basal-like 2 subtype and luminal androgen receptor subtype achieved the lowest pCR rate (0%, 10%) (137).

1.6.4 Gene expression signature

Gene expression signature consists of a group of genes which express specific phenotypes, such as different cancer subtypes and responses to treatment. Genomic grade index (GGI) is based on a panel of 97 genes which classify breast cancers into low and high risk categories in place of grades 1, 2 or 3 by histological grading (118). Breast cancers with high GGI, whether ER +ve or ER -ve, are more sensitive to NAC but have a worse survival outcome (138). Recurrence score (RS) is a 21 gene signature which determines recurrence risk in low-risk patients (ER +ve, node negative) and is currently used to decide adjuvant treatment for these patients. In the NAC setting, RS was demonstrated to correlate with the pCR rate, to the extent that high RS cancers were more likely to achieve a pCR (139). Prat *et al.* (140) studied the association of the risk of the relapse (ROR) score which is based on the prediction analysis of microarray 50 (PAM50) and pCR in HER2 +ve tumours. They reported that tumours with high ROR score have a higher likelihood of a pCR. The 70 gene signature (MammaPrint) was demonstrated to correlate with the pCR rate. Straver *et al.* (141) reported that patients with a poor prognosis-signature, classified by MammaPrint, achieved a pCR of 20% whereas none of the good-prognosis signature groups did. Moreover, there are other studies which have shown correlations between pCR and different gene expression signatures (142, 143). Routine usage of these commercial assays, however, is limited by their high cost, access and time required for assay completion.

Single gene markers may be more relevant to clinical practice. Only a few studies, however, have explored their roles in predicting a pCR. Increased *SPAG5* transcript and protein concentration were shown to be related to high pCR rates after combination NAC (144). Pereira *et al.* (145) concluded that *MYC* amplification was a predictive factor for pCR in patients who received docetaxel-based NAC. High levels of *BIRC5* (survivin) was demonstrated to correlate with a high pCR rate but shorter survival in patients with no pCR (146). In contrast, high *Gli1* expression was significantly correlated with a low pCR rate and poor prognosis in patients with HER2 +ve cancers (147). Patients with HER2 +ve/hormonal receptor positive cancers with *PIK3CA* mutation also had pCR rates significantly less than the wild type tumours (148).

Genes for hereditary breast cancer have been studied due to the increasing evidence that hereditary breast cancers may have different drug sensitivity from sporadic cases. Breast cancer susceptibility gene 1 (*BRCA1*) mutation carriers have been shown to achieve a pCR more often than non-carriers (31.6% vs. 11.9%, $p = 0.024$) in patients who received anthracycline-based NAC without taxanes (149). In TNBC patients, this effect was still found and extended to the patients who received anthracyclines/taxanes NAC (150). Young breast cancer patients who carried the *BRCA1* mutation had the highest rate of pCR after platinum-based treatment (151). Five-year disease free survival and overall survival were also better in *BRCA1* carrier patients who achieved pCR than in those who did not (152). In contrast to *BRCA1*, *CHECK2* mutation carriers were demonstrated to have poor response to NAC and rarely achieved a pCR (149). Other hereditary breast cancer genes such as *PALB2*, *RAD51C*, *BRIP1*, *NBS1* and *BLM* have been studied but there is little published evidence of their predictive abilities (149, 150).

1.6.5 Immune parameters

The immune system plays a pivotal role in cancer development, invasion and metastasis. It also is involved in breast cancer responses to chemotherapy. Thus, immune parameters are another areas of research interest regarding the prediction of a pCR (118). High levels of tumour-infiltrating lymphocytes (TILs) have been shown to be associated with improved outcome in patients with breast cancer and with pCR rates in the tumours with NAC (153-155). Denkert *et al.* (155) investigated TILs in 1,058 pre-NAC breast cancers and found an association between the percentage of TILs and pCR rate with NAC. The tumour specimens with lymphocyte predominance had pCR rates of 40-42%, whilst tumours with few TILs had pCR rates of 3-7% (155). Systematic review and meta-analysis of 13 published studies including 3,251 patients reported that higher TIL numbers correlated with better pCR rates (156). These correlations, however, were demonstrated in triple negative and HER2 +ve breast cancers but not in the ER +ve subtype. The review also showed that high levels of cluster of differentiation (CD) 8+ and forkhead box protein 3 (FOXP3)+ T lymphocytes in breast tumours predicted high pCR rates (156). In a number of studies, high levels of CD4+, CD8+, cytotoxic T lymphocyte antigen 4 (CTLA-4)+ (stromal) T lymphocytes and high CD8+: FOXP3+ T cell ratios were demonstrated to be associated with pCR rates (153, 157, 158).

Apart from the adaptive immune system, as described previously, the innate immune system has also been investigated. High levels of peritumoural natural killer (NK) cells and tumour-associated macrophages (TAMs) were shown to be associated with a pCR in breast cancer following NAC (159, 160). Moreover, the relation of the immune system and pCR rates in metastatic ALNs was also documented. An increasing number of innate immune cells (NK cells and macrophages) and adaptive immune cells (TILs, CD4+ and CD8+ T cells) in metastatic ALNs were demonstrated to be predictive markers for pCR (160, 161).

Interaction of programmed cell death protein 1 (PD-1)/programmed death ligand 1 (PD-L1) act as immune check-points. It downregulates the generation of T effector cells (CD4+/CD8+), enhances the activity of T regulatory cells (Tregs), suppresses T cell function, inducing T cell exhaustion and promotion of cancer cell progression. Wimberly *et al.* (162) reported that 30% of breast cancer tissues expressed PD-L1. PD-L1 expression levels were documented in the hormonal receptor negative cancer subtypes and were highest in the triple negative subtype (162, 163). The expression of PD-L1 as well as PD-1 was associated with high TIL levels and predicted high pCR rates (162, 163).

To further explore the immunological status in breast cancer, Denkert *et al.* (155) studied the expression of immune genes, their protein and mRNA levels and demonstrated their relationship with TILs by histopathological assessment. CD3D and CXCL9, which are T-cell-related markers, were shown to be significantly associated with pCR (155). Gianni *et al.* (139) studied the gene expression profile in paraffin-embedded tissue biopsies from LLABC, using reverse transcriptase polymerase chain reaction (RT-PCR) to determine mRNA levels. They reported that an immune-related gene cluster (including *MCP1*, *CD68*, *CTSB*, *CD18*, *ILT-2*, *CD3z*, *FasL*, *HLA.DPB1* and *GBP1*) correlated with a higher likelihood of pCR.

1.6.6 MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small non-coding RNA molecules, composed of 19-25 nucleotides (164). MiRNAs modulate gene expression post-transcriptionally by silencing translation or inducing mRNA degradation (165). In some specific conditions, they can up-regulate transcription (166, 167). MiRNAs dysregulation results in development, differentiation, proliferation, progression and apoptosis in many cancers including breast cancer (165, 166, 168). Several miRNAs have been demonstrated to initiate invasion and metastasis in various types of cancers by regulating the

proteins involved in these processes (169, 170). Moreover, they have a potential role as markers for diagnosis, prognosis and treatment (171, 172). Currently, miRNA-based treatments are also being investigated (171).

MiRNAs are related to post-NAC staging in many types of cancer, especially colorectal cancer. They are prognostic markers for disease free survival and overall survival (173, 174). In breast cancer, several studies have reported the role of miRNAs as biomarkers for pCR. Xue *et al.* (175) studied the expression of miR-612 in breast cancer tissue from patients who received NAC (paclitaxel plus carboplatin). They found that high levels of expression of miR-612 correlated with high pCR rates and improved disease free survival. The combination profile of intratumoural high miR-7 and low miR-340 expression can predict non-responders to NAC, with a negative predictive value of 96% and specificity of 86% (176). In TNBC, high miR200b-3p, high miR-190a and low miR-512-5p expression correlated with high rates of pCR and BCS (177). García-Vazquez *et al.* (178) proposed that miR-30a, miR-9-3p, miR-770 and miR-143-5p could be used as predictive markers for pCR in TNBC. Several genes which are the targets of these miRNAs are involved in cellular processes and signalling pathways in cancer cells, such as focal adhesion, mammalian target of rapamycin (mTOR) pathway, p53 signalling, transforming growth factor beta (TGF)- β signalling and phosphoinositide 3-kinase (PI3K) signalling pathways (178).

MiRNAs are stable in bio-fluids. Circulating miRNAs have been studied as novel non-invasive biomarkers due to their stability, reproducibility and consistency (179). Wu *et al.* (180) studied circulating miRNAs in LLABC by deep sequencing; miR-375 and miR-122 were shown to be potential serum biomarkers for pCR with NAC. These miRNAs can also predict metastatic disease relapse rate with a 100% specificity and 80% sensitivity (180). MiR-122 was demonstrated to be induced by chemotherapy (181). Increasing tumour tissue and serum miR-122 levels were significantly

associated with anthracycline-based NAC responses (181). In contrast, miR-125b levels were demonstrated to correlate with chemo-resistance (182). High serum miR-125b was significantly associated with NAC non-responsive cancers (182).

1.7 Previous pilot study

In a previous pilot study carried out in the department, it was ascertained that the expression of a specific cohort of genes in breast cancers from patients with LLABC was an important predictor of response to NAC.

Fresh-frozen breast core biopsy specimens from 10 patients with LLABC treated with NAC in a trial (XINACT: ISRCTN 00407556) were studied. Pathological responses in primary breast tumours were categorised as a pathological complete response (pCR: no residual invasive cancer cells present), pathological partial response (pPR: <100% loss of invasive cancer cells) and no pathological response (pNR: no loss of cancer cells). In metastatic ALNs, the pathological responses were categorised as pathological complete response (pCR: no residual invasive cancer cells identified, with or without fibrosis), pathological partial response (pPR: residual invasive cancer cells identified with replacement fibrosis) and no pathological response (pNR: invasive cancer cells identified with no fibrosis).

Using next generation RNA sequencing, initial breast-specific analyses compared the gene expression between patients with a pCR in the breast (group A, n = 5) and no pCR (pPR or pNR) in the breast (group B, n = 5). After filtering out the genes with discordant expression patterns (little or no transcript expression in one or more specimens) and non-coding pseudogenes, 12 genes were demonstrated to show significant upregulation in group A (*PIGR*, *LBP*, *GPRC5C*, *ALDH3B2*, *CDR2L*, *IF127*, *RARRES3*, *TMC5*, *SPDEF*, *DUSP4*, *CFD* and *CRIP2*) and four genes were upregulated in group B (*SEPT3*, *AARD*, *ACTG2*, and *PCBP3*).

As the response in ALNs is the most important prognostic factor for patients, two comparative analyses were performed based on ALN responses. The difference in gene expression between patients with a pCR in both the breast and ALNs (group C, n = 2) and patients with pNR in both the breast and ALNs (group D, n = 2) was analysed. A comparison was also carried out between patients with a pCR in both the breast and ALNs (group E, n = 2) with patients with no pCR (pPR or pNR) in both breast and ALNs (group F, n = 4). After filtering out genes with discordant expression patterns and non-coding pseudogenes, six genes were significantly upregulated in group E (*PIGR*, *MUC4*, *CEACAM6*, *PDZK1IP1*, *STARD3* and *HRASLS2*) and two genes upregulated in group F (*SEPT3* and *CRHR1*).

There were only two genes, *PIGR* (polymeric immunoglobulin receptor) and *SEPT3* (septin 3), which showed statistically significant differences in their expression in all three comparative analyses. The *PIGR* gene showed significantly higher levels of expression in the pCR group, patients with good prognosis, compared with the non-pCR group (5.96-8.39 fold). In contrast, *SEPT3* gene expression was significantly increased in the non-pCR group, poor prognosis patients, compared with the pCR group (3.72-5.88 fold).

Real-time quantitative polymerase chain reaction (real-time qPCR) was performed to validate the result of RNA sequencing. *PIGR* mRNA expression showed the highest level (median and interquartile range 0.79, 0.13-4.50 arbitrary unit [A.U.]) in five tumour specimens with pCR in breast and ALN pCR or normal node with no evidence of tumour involvement (pNN). In contrast, the lowest level (0.08, 0.02-0.17) was reported in tumours showing breast pNR/pPR and ALN pNR/pPR ($p = 0.032$). *SEPT3* mRNA expression was the reverse of *PIGR* mRNA expression. It was highest in tumours with breast pNR/pPR and ALN pNR/pPR (0.44, 0.07-4.00) and lowest in specimens with breast pCR and ALN pCR (0.09, 0.05-0.09). These differences,

however, were not statistically significant ($p = 0.73$) which may be due to the small number of samples analysed.

Western blot analysis with densitometry also demonstrated high levels of PIGR protein expression in tumour specimens showing pCR after NAC (mean $\pm$ standard error: arbitrary unit 113.39 ± 19.61). In contrast, low levels of PIGR protein expression was demonstrated in specimens with pNR/pPR (20.91 ± 9.08 , $p = 0.008$). There was a significant linear correlation between PIGR mRNA and protein level in the same sample. Due to lack of a reliable antibody, SEPT3 protein expression assessment by western blot was not obtained at the same time.

Overall, PIGR mRNA and protein expression was increased in breast cancers with a pCR in breast and ALNs after NAC, patients with a good prognosis. SEPT3 mRNA expression was elevated in tumours with partial or no response to NAC. Due to the statistically significant different result of PIGR mRNA expression between pCR and non-pCR group, PIGR was selected for further expansion in this thesis with regard to its biological function, interaction with surrounding tumour microenvironment and ability to predict pCR, using breast cancer cell lines and breast cancer core biopsy specimens.

1.8 Polymeric immunoglobulin receptor (PIGR)

The human *PIGR* gene is a member of the immunoglobulin (Ig) superfamily. It is located in the q31-q41 region of chromosome 1 and comprises 11 exons and 10 introns spanning 17,944 base pairs (183, 184). The *PIGR* gene encodes the PIGR protein which modulates transcytosis of polymeric Ig molecules (dimeric IgA and a lesser extent pentameric IgM) produced from plasma cells in the lamina propria (183). PIGR, previously identified as transmembrane secretory component (SC), is a transmembrane protein with a molecular weight of approximately 120 kilodaltons

(kDa), consisting of three portions (extracellular, transmembrane and cytoplasmic) composed of 629, 23 and 103 amino acids, respectively (185, 186). The extracellular portion of PIGR is a ligand binding region. This portion consists of five domains which homology to variable regions of Ig and a sixth non-homologous domain that joins to transmembrane portion. The first domain is important for binding with polymeric Ig whereas the sixth domain encompasses proteolytic cleavage sites (186) (Figure 1.5).

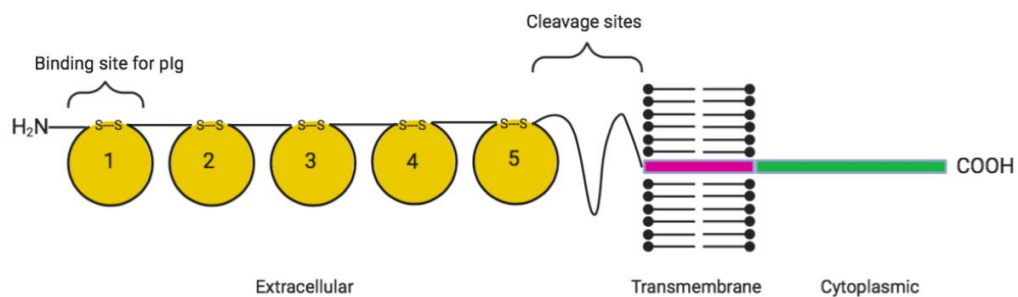


Figure 1.5 The schematic structure of the human PIGR

The figure was created with BioRender.com.

1.8.1 Pathway of transcytosis of PIGR and polymeric immunoglobulin (Ig) transportation

PIGR is synthesised in the rough endoplasmic reticulum and transferred to the Golgi apparatus (183, 186, 187). During passage through the trans-Golgi network, PIGR is compartmentalised into vesicles and then carried to the basolateral surface of epithelial cells where it binds to its ligands, polymeric Ig (Figure 1.6). Polymeric Ig is a product of plasma cells which is located in the lamina propria. Following this, PIGR, with or without polymeric Ig, is endocytosed and passes through the basolateral early endosomes (BEE), then the common endosomal compartment (CE) and apical recycling endosomes (ARE), respectively, to be expressed on the apical surface. At the apical surface, proteolytic enzymes cleave the extracellular ligand-binding portion

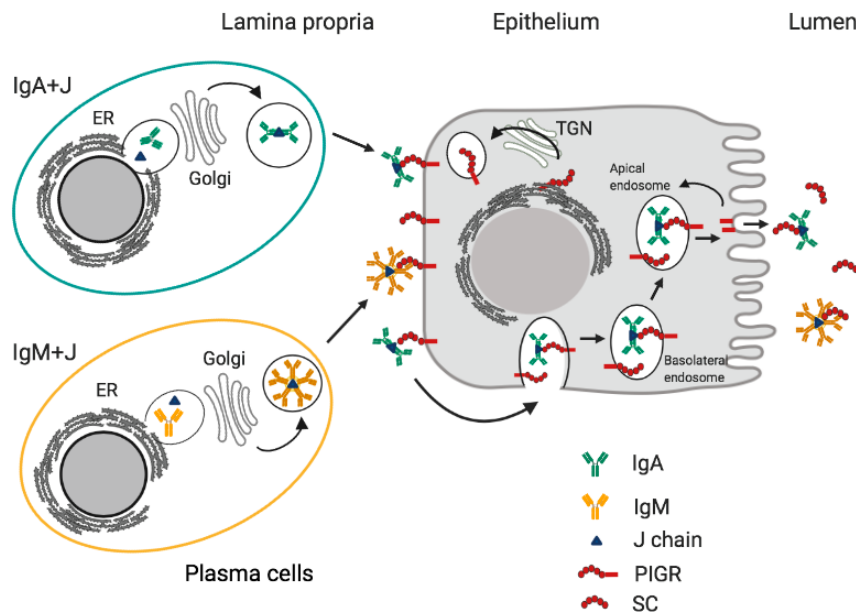


Figure 1.6 Polymeric Ig synthesis and transportation via PIGR transcytosis

Plasma cells in lamina propria produce J chain, light chain and α or μ chain in the endoplasmic reticulum. After processing in Golgi apparatus, dimeric IgA and pentameric IgM with incorporated J chain are secreted to extracellular space. PIGR is synthesised in the rough endoplasmic reticulum of secretory epithelial cell and matures in the Golgi apparatus before delivered through trans-Golgi network (TGN) to basolateral surface of epithelial cells where PIGR binds to polymeric Ig. PIGR with or without polymeric Ig is endocytosed and moved through a series of endocytotic and transcytotic vesicles to be expressed on apical plasma membrane. Then, extracellular portion of PIGR is cleaved and SIgA, SIgM and free SC are released. Some recycling of PIGR may take place. Modified with permission from "Role of J Chain and Secretory Component in Receptor-Mediated Glandular and Hepatic Transport of Immunoglobulins in Man", by P. Brandtzaeg, 1985, Scandinavian journal of immunology, 22, 111-146. Copyright 2006 by John Wiley and Sons (188). The figure was created with BioRender.com.

of PIGR to release the SC in free-form (free SC) or as part of secretory immunoglobulin (SIg). PIGR can be recycled back to the apical surface by internalisation into apical early endosomes (AEE) and back through the ARE. It can be reused for further rounds of transport by passing from BEE and CE to the basolateral surface (183, 186, 187).

Plasma cells localised in the lamina propria of secretory epithelium or exocrine glands differ from those in lymph node, bone marrow and spleen. Ig producing plasma cells in the lamina propria express joining (J) chain polypeptide which modulates polymeric formation of IgA and IgM (189). J chains incorporate into dimeric IgA and pentameric

IgM and allow selective transport across epithelial cells by PIGR (190). There is a substantially lower proportion of IgM producing plasma cells than IgA producing cells (191). Any Ig producing plasma cells in the lamina propria can produce J chain except IgE producing cells; however, J chains are degraded intracellularly in IgG and IgD producing cells (189, 192).

1.8.2 Biological function of PIGR

PIGR ensures efficient secretion of polymeric IgA at the mucosal surface. The polymeric IgA-bound or unbound extracellular portion of PIGR is cleaved at the apical surface of the epithelial cell, and released as SIgA or free SC, respectively (Figure 1.7). SIgA is the major type of antibody in human mucosal secretion which dominates humoral immunity at the mucosal surface (193). It has a role in preventing commensal bacteria from invading the lamina propria and regulates the composition and function of the microbiota (194). SIgA acts as the first line of antigen specific immunological defence in protecting the mucosal surface against pathogens, toxic substances, and antigens that could harm the human body (193). By specific binding to pathogens in the mucous, SIgA inhibits pathogen colonisation and invasion of the epithelium (195). J chains enhance antigen/polymeric IgA affinity which facilitate microbe agglutination in the secretion (194). These facilitate physical mechanisms such as peristalsis and muco-ciliary transport to eliminate immune complexes.

SC protects SIgA from proteolytic degradation which improves the stability and effector functions of polymeric IgA (183, 186). Free SC on its own has innate anti-microbial properties by performing as a non-specific microbial scavenger (183). Free SC derived from human milk was demonstrated to inhibit adhesion and invasion by enterotoxigenic *Escherichia coli* (196, 197). Furthermore, free SC was shown to interact with *Clostridium difficile* toxin A and significantly inhibit toxin binding to hamster intestinal brush border membranes resulting in a protective effect against

Clostridium difficile associated disease (198). Free SC can form a high molecular weight complex with interleukin (IL)-8 and inhibit the neutrophil chemoattractant property of IL-8 which may help to protect the mucosal surface by reducing the inflammatory response (199).

PIGR also has a role in eradicating pathogens in the lamina propria (195). Antigen missed by SIgA in mucosal surface and passing through the epithelium to the lamina propria is encountered by polymeric IgA. To remove the antigen from infected tissue, the immune complex is processed by PIGR and transcytoses to be excreted into the lumen. Polymeric IgA can also neutralise intracellular pathogens during the transcytosis process (intracellular neutralisation) (200). PIGR, therefore, plays a major role in the mucosal immune system and links innate and adaptive immunity (183).

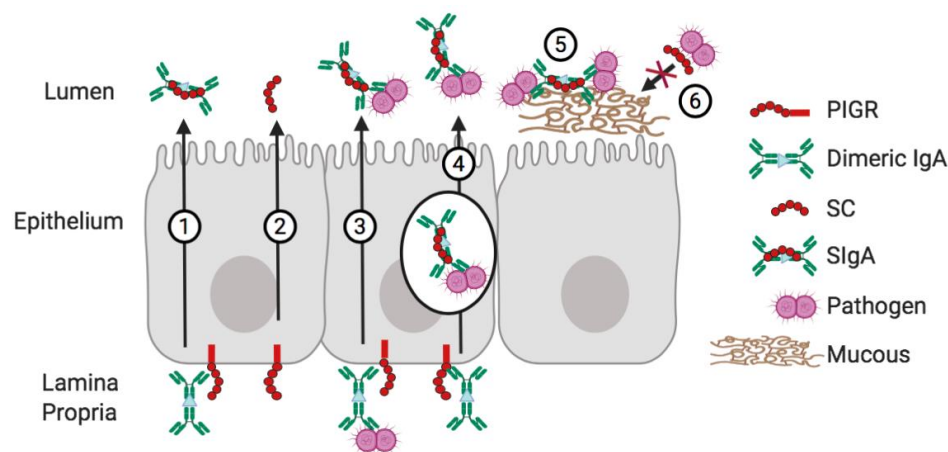


Figure 1.7 Functional roles of PIGR and SC

PIGR carries dimeric IgA from basolateral surface of epithelial cell and then transcytoses across the cell to apical surface. At apical surface, proteolytic enzymes cleave the extracellular portion of PIGR leading to the secretion of SIgA (route 1). Extracellular part of unbound PIGR is cleaved and released as free SC (route 2). PIGR can facilitate pathogen eradication from lamina propria by carries dimeric IgA-pathogen complex across the cell and excretes into the lumen at apical surface as SIgA-pathogen complex (route 3). Intracellular pathogen can be neutralised by PIGR during transcytosis to apical surface (route 4). SIgA anchored in the mucous at apical surface can neutralise pathogen in the lumen (immune exclusion, route 5). Free SC has innate anti-microbial properties by performing as a non-specific microbial scavenger (route 6). Modified with permission from "Novel functions of the polymeric Ig receptor: well beyond transport of immunoglobulins", by A. Phalipon and B. Corthesy, 2003, Trends in immunology, 24(2), 55-58. Copyright 2003 by Elsevier (195). The figure was created with BioRender.com.

1.8.3 Tissue expression of PIGR

PIGR was reported to be present in many types of epithelial cells, though the level of expression varies among anatomical sites (201). Immunohistological investigation of normal human tissues demonstrated the presence of PIGR in epithelial cells in nasal mucosa, trachea, tracheobronchial glands and bronchi, submaxillary and parotid salivary glands, epithelial lining of small intestine and colon, gall bladder and biliary epithelium, pancreatic glandular and ductal epithelial cells, renal tubule and secretory units of sweat glands (201-204).

The expression of PIGR is compartmentalised and strongly associated with hormonal changes in menstrual cycle-related female reproductive tissue (205, 206). The columnar epithelial cells of the fallopian tubes, endometrial glands and endocervix showed high expression of PIGR, while squamous epithelium of the ectocervix and vagina expressed a low level. PIGR was not present in ovaries and myometrium (207).

Polymeric IgA can be locally synthesised and transported into external secretions by PIGR expressing exocrine glands. In breast ductal epithelium, the prominent example among these, PIGR was shown to be present by the immunofluorescence technique (208, 209). Colostrum and milk contain a large amount of IgA produced by plasma cells in the mammary tissue in the form of SIgA. SIgA and free SC can be transferred from mother to the neonate and contribute to passive immunity (210). In animal studies, the expression of PIGR in mammary glands is influenced by hormones during pregnancy and lactation (211).

PIGR has been demonstrated to be expressed in various type of cancers, including breast cancer. Furthermore, many clinical studies in different cancers revealed the relation of the alteration of PIGR expression and patient outcome.

1.9 Hypotheses and objectives

Hypotheses

1. The expression of PIGR varies among different subtypes of breast cancer and the overexpression of PIGR can affect chemo-sensitivity and cellular behaviour of breast cancer cells.
2. PIGR has an effect on inflammatory cells in the tumour microenvironment leading to alteration in the chemotherapeutic response of cancer cells.
3. Tumour microenvironment plays a role in regulating PIGR expression in breast cancer cells.
4. The expression of the *PIGR* gene in breast cancers varies and can predict the response to NAC of breast cancer patients.

Objectives

1. To evaluate the basal expression of PIGR and the effect of PIGR overexpression in breast cancer cell lines and its importance regarding the response to chemotherapy and the effect on cellular behaviour.
2. To determine the effect of PIGR on macrophage polarisation.
3. To determine the effect of macrophages and cytokines relating to the alteration of PIGR expression in breast cancer cell lines.
4. To ascertain the expression of the *PIGR* gene in breast tumours in a larger cohort of women with breast cancer and the association with the response to NAC and outcome.

Chapter 2 Materials and Methods

Chapter 2 Materials and Methods

This thesis consists of two parts: preclinical and clinical studies.

2.1 Preclinical study

2.1.1 *In vitro* cell culture

Breast cancer cell lines: MCF7 (ATCC® HTB-22™) and MDA-MB-468 (ATCC® HTB-132™) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A Human embryonic kidney (HEK) 293FT cell line was supplied by the FRAME laboratory. Bone marrow derived macrophages (BMDMs) were kindly provided by Dr Luisa Martinez-Pomares, Faculty of Medicine & Health Sciences, University of Nottingham. All cell culture procedures were performed in a microbiological safety cabinet using aseptic techniques for sterility. Mycoplasma contamination testing was performed after cell thawing and before cryopreservation of the cells. Cell lines and media used in the studies are demonstrated in Table 2.1.

2.1.1.1 Handling frozen cells

Frozen cells were removed from liquid nitrogen storage and thawed in a 37 °C water bath by gentle agitation (approximately 1 min). The O-ring and cap were kept out of the water to diminish the chance of contamination. As soon as the content was thawed, the vial was removed from the water bath and decontaminated by wiping with 70% ethanol. In a microbiological safety cabinet, the cells and cryoprotective agent were transferred to a new sterile tube containing 10 ml of warm fresh media and gently resuspended by pipetting vertically. The cell suspension was centrifuged at 125 *g* for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml of fresh culture medium before being transferred to a T75 cell culture flask containing 14 ml of warm fresh media. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C.

Table 2.1 Cell lines and media used in the studies

Cell lines	Organism	Tissue	Media
MCF7 (ATCC® HTB-22)	Human	Mammary gland, breast; derived from metastatic site: pleural effusion	Eagle's Minimum Essential Medium (EMEM) (M2279; Sigma-Aldrich, St Louise, MO, USA) supplemented with 10% foetal bovine serum (FBS), 0.01 mg/ml human recombinant insulin, 2 mM L-glutamine, 1 mM sodium pyruvate and 1% Minimum Essential Medium Non-Essential Amino Acids
MDA-MB468 (ATCC® HTB-132)	Human	Mammary gland, breast; derived from metastatic site: pleural effusion	Dulbecco's Modified Eagle Medium (DMEM) (D6046; Sigma-Aldrich, St Louise, MO, USA) supplemented with 10% FBS
Human embryonic kidney (HEK) 293FT	Human	Embryonic kidney cells	EMEM (M4526; Sigma-Aldrich, St Louise, MO, USA) supplemented with 10% FBS and 2 mM L-glutamine
Bone marrow derived macrophage (BMDM)	Mouse	Macrophage	RPMI 1640 (R0883; Sigma-Aldrich, St Louise, MO, USA) supplement with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulphate

2.1.1.2 Cell line maintenance and sub-culturing

All cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C. The medium was renewed every 2-3 days and cells were passaged when they reached approximately 80% confluence. Cells were used for the experiments for up to 20

passages. For sub-culturing of the cells, cell culture medium was removed from the flask and the cells were washed twice with 1× phosphate buffered saline (PBS) (BR0014G; Oxoid, Cheshire, UK). An appropriate volume of trypsin ethylene-diamine-tetraacetic acid (EDTA) solution (T3924; Sigma-Aldrich, St Louise, MO, USA) (2 ml for T75 flask and 3 ml for T175 flask) was added to cover the entire surface area of the flask. Cells were incubated at 37 °C until complete detachment occurred (5-7 min). Serum containing culture medium (at least double the volume of trypsin) was added to inactivate trypsin. Cells were aspirated by gently pipetting and transferred into a sterile tube. The cell suspension was centrifuged at 125 g for 5 min. Thereafter, the supernatant was discarded and the cell pellet was resuspended in an appropriate amount of fresh culture medium. The appropriate aliquot of the cell suspension was transferred to a new cell culture flask containing fresh media. Sub-cultivation ratios for MCF7, MDA-MB468, HEK293FT and BMDM cells are 1:3, 1:3, 1:10 and 1:10, respectively.

2.1.1.3 Cell counting

It is important to determine the concentration and viability of a cell sample to ascertain reproducibility in the experiment, sub-culturing, monitoring growth rates and for cryopreservation. Cell concentration was determined by cell counting using a haemocytometer. Trypan blue was used to exclude dead cells which stained blue. 20 µl of cell suspension sample was diluted with 20 µl of trypan blue and mixed well. 10 µl of the mixture was loaded on to the haemocytometer under a coverslip. The cells inside the four large corner squares of the haemocytometer were counted under an inverted light microscope at 10× magnification. The cell concentration was calculated from the following formula:

$$Total\ cells\ (cells/ml) = \frac{(Total\ cell\ count \times Dilution\ factor)}{Number\ of\ squares\ counted} \times 10^4$$

2.1.1.4 Cryopreservation of cells

Cells were cryopreserved to maintain their long-term viability, minimise genetic alteration and avoid loss through contamination. Cells were harvested during the exponential phase using trypsin as described previously. After centrifugation, cells were resuspended in cryopreservation medium consisting of 5% dimethyl sulfoxide (DMSO) in complete growth medium to a concentration approximately 3×10^6 viable cells/ml. 1 ml of cell suspension was dispensed into a 1 ml labelled cryopreservation vial. Filled cryopreservation vials were placed into an insulated freezing container and cooled overnight at -80 °C before transferring to a liquid nitrogen container for long-term storage in vapor phase liquid nitrogen (between -140 °C and -180 °C).

2.1.2 Development of stable cell lines

Stable cell lines have homogeneous stable genetic and protein expression characteristics. The stable genetic characteristics are passed to and presented in the next generation of the cells. These properties increase the reproducibility of repeated experiments. Generally, the development of stable cell lines is a two-step process. First, plasmid DNA containing the gene of interest is transduced into the genome of the host cell by using the lentiviral vector system. Second, selective antibiotic is applied to screen for successfully transduced cells. Once the cells are adequately selected, colonies are chosen and the cells are expanded.

2.1.2.1 Construction of inducible PIGR expression plasmid vector

Inducible PIGR expression plasmid vectors were constructed by using a molecular cloning technique. Gateway® Technology with Clonase® II (11789-020 and 11791-020; Thermo Fisher Scientific, Waltham, MA, USA) was used to clone *PIGR* genes into pINDUCER20 plasmids. Gateway cloning consists of the BP recombination reaction to create the entry clone containing gene-specific sequences followed by the LR recombination reaction which transfers gene-specific sequences from the entry clone into a destination vector (pINDUCER20) thereby creating an expression clone. Recombination occurs between specific attachment (*att*) sites on the interacting vector by switching the DNA segment.

Designing attB-polymerase chain reaction (PCR) primers

PIGR DNA sequences were acquired from the National Center for Biotechnology Information (NCBI) database. Forward and reverse primers were designed using the Primer-BLAST open-source program that is publicly available (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>). To enable efficient Gateway® cloning, four guanine residues at the 5' end followed by 25 base pairs *attB1* site and Kozak sequence were inserted in front of the *PIGR* gene sequence, which was at least 18-25 base pairs, to construct the forward primer. Four guanine residues at the 5' end followed by 25 base pairs *attB2* site were inserted as a reverse primer (Table 2.2).

Primers were purchased from Eurofins Scientific (Luxembourg). Sterile DNase RNase free water was added to reconstitute the primers and create 100 µM of stock concentration, based on the manufacturer's guidance. The stock was diluted 1:10 to a concentration of 10 µM before use.

Table 2.2 PIGR primer sequences for Gateway® cloning

Primer	Sequence (5' → 3')
PIGR forward primer	GGGG <u>ACAAGTTTGTACAAAAAAGCAGGCT</u> TC GCCACC TCAACGGGAGAGAAGGAAGTGGCT
PIGR reverse primer	GGGG <u>ACCACTTTGTACAAGAAAGCTGGGT</u> T AGGGCCCCAGGATCGACATGA

*Underline sequences are *attB* sites. Bold sequences are *PIGR* gene.

Producing attB-PCR products

DNA templates used for amplification were cDNA synthesised from MDA-MB468 RNA. Phusion® High Fidelity DNA polymerase (M0530L; New England Biolabs, Ipswich, MA, USA) was used for PCR to produce *attB*-PCR products. The PCR was performed using a thermal cycler (T100™ Thermal Cycler; Bio-Rad, Hercules, CA, USA). Reaction conditions and thermocycling conditions are shown in Table 2.3 and 2.4, respectively. 10 µl of *attB*-PCR products were then mixed with loading buffer and separated via 1% agarose gel electrophoresis. The *attB*-PCR products were visualised using ultraviolet light (GeneGenius Imaging System; Syngene, India).

Table 2.3 Reaction conditions for producing attB PCR products

Component	Volume (µl)
5× Phusion HF Buffer	10
10 mM dNTPs	2
10 µM forward primer	2
10 µM reverse primer	2
Template DNA	2
Phusion DNA Polymerase	0.5
Nuclease-free water	31.5 (Final volume up to 50 µl)

Table 2.4 Thermocycling conditions for PCR

Step	Temperature	Time	Cycle(s)
Initial denaturation	98 °C	1 min	1
Denaturation	98 °C	30 sec	30
Annealing	60 °C	30 sec	
Extension	72 °C	2 min	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	-

Agarose gel electrophoresis

Agarose gel electrophoresis was used to verify the quality and quantity of PCR products. By applying an electrical field, negatively charged DNA is moved through an agarose matrix toward a positive electrode. Short DNA fragments migrate through the gel faster than long fragments, thus DNA fragments are separated by length.

To prepare 1% agarose gel, 1 gm of agarose powder (A9539; Sigma-Aldrich, St Louise, MO, USA) was dispersed in 100 ml of 1× Tris-acetate-ethylene-diamine-tetraacetic acid (TAE) buffer (pH 8.3). The agarose was heated in a microwave oven using medium power for 2-3 min until completely dissolved. The melted agarose was cooled to 50 °C. Ethidium bromide (46067; Sigma-Aldrich, St Louise, MO, USA) was added to the agarose to a final concentration 1 µg/ml before slowly pouring it into a gel tray with a comb in place to create wells for loading samples. The agarose was left at RT for 15 min until completely solidified. Once solidified, the agarose gel was placed into a gel tank. 1× TAE buffer was added until the agarose gel was covered, then the comb was removed. 10 µl of PCR product was mixed with 2 µl of 6× loading buffer (B7024S; New England Biolabs, Ipswich, MA, USA) before loading into the well alongside a 1 kilobase (kb) DNA ladder (N3232S; New England Biolabs, Ipswich, MA,

USA). The gel was run at 100 volts (V) for 1 hr. PCR products were visualised under ultraviolet light (GeneGenius Imaging System; Syngene, India).

Purifying attB-PCR products

Purification of *attB*-PCR products is recommended to eliminate *attB* primers and any *attB* primer-dimers. Primers and primer-dimers can recombine with the donor vectors in the BP reaction and increase the number of background colonies after transformation into *Escherichia coli*.

150 µl of Tris-ethylene-diamine-tetraacetic acid (TE; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer was added to a 50 µl of amplification reaction containing *attB*-PCR products followed by 100 µl of 30% polyethylene glycol (PEG) 8000/30 mM MgCl₂. The mixture was vortexed thoroughly and centrifuged immediately at 10,000 *g* for 15 min at RT. Supernatant was removed and the pellet was dissolved in 30 µl of TE buffer (pH 8.0) to a concentration of more than 10 ng/µl. The concentration of purified products was determined using a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany). 5 µl of purified *attB*-PCR product was mixed with loading buffer and separated via 1% agarose gel electrophoresis to evaluate the quality.

Creating entry clones using the BP recombination reaction

By using BP clonase II enzyme, the BP recombination reaction facilitates the insertion of the *attB*-PCR product into the *attP*-containing donor vector (pDONRTM221 [35-1687; Invitrogen, Carlsbad, CA, USA]) to create an entry clone (Figure 2.1). The reaction mixture is transformed into a suitable *E. coli* host (One Shot Top10 Chemically Competent *E. coli* [44-0301; Invitrogen, Carlsbad, CA, USA]). Then entry clones are selected by appropriate antibiotics.

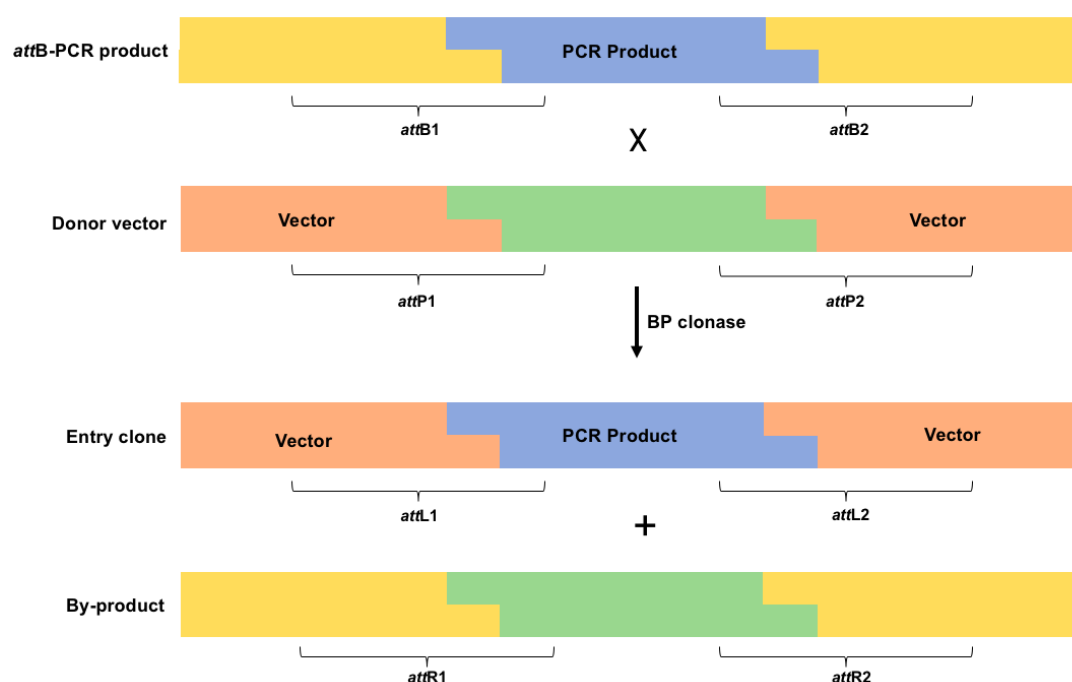


Figure 2.1 BP recombination reaction

The diagram depicts a BP recombination reaction between an *attB*-PCR product and a donor vector to create an entry clone and a by-product. The PCR product is transferred from the *attB*-PCR product into the entry clone following recombination.

Performing the BP recombination reaction

Fifty femtomoles (fmol) of *attB*-PCR products were mixed with 150 ng of pDONRTM221 at RT. TE buffer (pH 8.0) was added to a final volume of 8 μ l. Gateway[®] BP clonase[®] II enzyme mix was thawed on ice for 2 min, then vortexed 2 sec twice. 2 μ l of the enzyme mix was added to the sample and mixed well by vortexing twice at 2 sec each time. 100 ng of pEXP7-tet vector was used as a positive control. A negative control was set up by omitting the enzyme mix. The reactions were incubated at 25 °C overnight. 1 μ l of Proteinase K solution was added to each sample to terminate the reaction before incubating at 37 °C for 10 min.

Transforming competent *E.coli* and selecting entry clones

A vial of One Shot Top10 Chemically Competent *E.coli* was thawed on ice for each transformation. 1 µl of BP recombination reaction was added to a vial of Top10 *E.coli* and mixed gently. 10 pg of pUC19 was used as a positive control for transformation. The vials were incubated on ice for 30 min. The heat-shock was performed at 42 °C for 30 sec followed by incubation on ice for 2 min. 250 µl of S.O.C medium (0.5% Yeast Extract, 2% Tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM Glucose) was added to each vial. The vials were incubated at 37 °C for 1 hr on the horizontal shaker at 225 revolutions per minute (rpm). After the transformation mixture was diluted 1:10 in lysogeny broth (LB) medium (1% trypton, 0.5% yeast extract and 1% NaCl, pH 7.0), 50 µl was spread on the pre-warm agar plate (1.5% agar in LB medium) with appropriate antibiotic and incubated at 37 °C overnight.

Colony PCR

To verify that the *PIGR* gene presented as an entry clone, a colony PCR was performed using M13 sequencing primers (Table 2.5). Reaction mixture was prepared using BIOTAQ™ DNA Polymerase (BIO-21040; Bioline, London, UK) (Table 2.6). The colony was labelled and half was scraped up using a pipette tip then resuspended in the reaction mixture. The PCR was performed with a thermal cycler (Table 2.7). After PCR was complete, the PCR product was separated via 1% agarose gel electrophoresis at 120 V for 15-20 min and then visualised under ultraviolet light.

Table 2.5 M13 sequencing primers

Primer	Sequence (5' → 3')
M13 forward primer	GTAAACGACGGCCAGT
M13 reverse primer	GGAAACAGCTATGACCATG

Table 2.6 Reaction condition for colony PCR

Component	Volume (μl)
10× NH ₄ Reaction Buffer	2
50 mM MgCl ₂ Solution	0.6
10 mM dNTPs	0.4
10 μM forward primer	1
10 μM reverse primer	1
BIOTAQ DNA Polymerase	0.25
Nuclease-free water	14.75 (Final volume up to 20 μl)

Table 2.7 Thermocycling conditions for colony PCR

Step	Temperature (°C)	Time	Cycle(s)
Initial denaturation	96 °C	5 min	1
Denaturation	96 °C	15 sec	30
Annealing	50 °C	20 sec	
Extension	72 °C	70 sec	
Final extension	72 °C	5 min	1
Hold	4 °C	∞	-

The single colony demonstrating *PIGR* gene insertion was selected and transferred into a tube containing 5 ml LB medium with kanamycin 50 μg/ml. The bacterial culture was incubated at 37 °C overnight in a horizontal shaker at 225 rpm. 500 μl of bacterial culture was mixed with 500 μl of 50% glycerol to create bacterial glycerol stock stored at -80 °C. The entry clone was recovered from 4 ml of bacterial culture using QIAprep

Spin Miniprep Kit (27104; Qiagen, Hilden, Germany) according to the manufacturer's instructions. The entry clone was quantified by a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany) and submitted for Sanger sequencing (Source Bioscience, Nottingham, UK) to verify the insertion sequence. Sequences were analysed using A plasmid Editor (ApE) software.

Creating expression clones using the LR recombination

The LR recombination reaction was performed after the BP recombination reaction in order to transfer the gene of interest from the entry clone into an *attR*-containing destination vector (pINDUCER20 [44012; Addgene, Watertown, MA, USA]) (Figure 2.2), creating an *attB*-containing expression clone (Figure 2.3). The reaction mixture was then transformed into a suitable *E.coli* host (One Shot Stbl3 Chemically Competent *E. coli* [C7373-03; Invitrogen, Carlsbad, CA, USA]). Finally, entry clones were selected using appropriate antibiotics.

Performing the LR recombination reaction

The reaction was set up at RT by mixing 150 ng of entry clone with 150 ng of pINDUCER20. TE buffer, pH 8.0, was added to a final volume of 8 µl. Gateway® LR clonase® II enzyme mix was thawed on ice for 2 min, then vortexed twice for 2 sec. 2 µl of the enzyme mix was added to the sample and mixed well by vortexing twice for 2 sec. 100 ng of pENTR-gus vector was used as a positive control. A negative control was set up by omitting the enzyme mix. The reactions were incubated at 25 °C overnight. 1 µl of Proteinase K solution was added to each sample to terminate the reaction before incubating at 37 °C for 10 min.

Transforming competent *E.coli* and selecting expression clones

The LR recombination reaction mixture was transformed into One Shot Stbl3 Chemically Competent *E.coli* using the protocol described previously and pUC19 was used as a positive control for transformation. The heat-shock step was performed at 42 °C for 45 sec.

Colony PCR

To select the expression clones, a colony PCR was performed as described previously. LNCX and PIND20 were used as forward and reverse primers, respectively (Table 2.8). The annealing temperature in thermocycling condition was 55 °C.

Table 2.8 LNCX and PIND20 primers

Primer	Sequence (5' → 3')
LNCX forward primer	AGCTCGTTTAGTGAACCGTCAGATC
PIND20 reverse primer	GGTTACTCCAGACTGCCTTGG

The colony which carried the *PIGR* gene was selected and inoculated into a 5 ml LB medium containing ampicillin 50 µg/ml. The bacterial culture was incubated at 37 °C for 8 hr in a horizontal shaker at 225 rpm. 400 µl of bacterial culture was then mixed with 200 ml LB medium containing ampicillin 50 µg/ml and incubated at 37 °C overnight on the horizontal shaker at 225 rpm. Bacterial glycerol stock was created and stored at -80 °C. The expression clone was recovered from bacterial culture using NucleoBond Xtra Midi (740410.50; Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The expression clone was quantified by a spectrophotometer (FLUOstar Omega microplate reader; BMG

Labtech, Offenburg, Germany) and submitted for Sanger sequencing (Source Bioscience, Nottingham, UK) to verify the insertion sequence. Sequences were analysed using ApE (A plasmid Editor) Version 2.0 software (available at <https://jorgensen.biology.utah.edu/wayned/apel/>).

After finishing construction of the human PIGR expression plasmid vector (pINDUCER20-hPIGR), lentivirus containing pINDUCER20-hPIGR plasmid was produced and prepared to be transduced into breast cancer cells.

2.1.2.2 Production and concentration of lentiviral vector

Lentivirus is a genus of retroviruses commonly used in research because of its ability to introduce gene products. Lentiviruses used in research are based on the human immunodeficiency virus (HIV) -1 and classified as Biosafety Level 2 (BSL-2) organisms. The components for viral production are separated into multiple plasmids to increase the safety of working with this virus. Moreover, many HIV proteins are deleted which prevents the generation of replication-capable virus. All steps involved with lentivirus were performed in a biological safety cabinet class 2. Lentivirus particles were transported or stored out of the safety cabinet using double containment. All equipment was decontaminated with 10% Chemgene or 2% Distel for 24 hr prior to disposal.

HEK 293FT cells were used to generate the lentiviral vector because they are fast growing and highly transfectable. HEK 293FT cells were plated at a density of 1.4×10^6 cells on a 10 cm culture dish containing 10 ml EMEM supplemented with 10% FBS and 0.292 g/L L-Glutamine (2mM). The cells were incubated at 37 °C in 5% CO₂ humidified atmosphere overnight to reach confluency of approximately 80% before transfection.

Next day, 2 µg of lentiviral transfer plasmid (expression clone [pINDUCER20-hPIGR] or pINDUCER20-green fluorescent protein [pINDUCER20-GFP] used as positive control and for optimisation), 2 µg of packaging plasmid (psPAX2 [12260; Addgene, Watertown, MA, USA]) and 2 µg of envelope plasmid (pMD2.G [12259; Addgene, Watertown, MA, USA]) were diluted in 600 µl of Opti-MEM™ I media (11058021; Thermo Fisher Scientific, Waltham, MA, USA). 18 µl of the X-tremeGENE HP DNA Transfection Reagent (06366236001; Roche, Penzberg, Germany) was added directly to diluted plasmid (3:1 ratio of transfection reagent [µl] to plasmid [µg]). The transfection complex was mixed gently by pipetting and incubated at RT for 30 min. HEK 293FT cell media was gently removed and replaced with 7 ml Opti-MEM™ I media. After 30 min of incubation, the transfection complex was added to the cells in a dropwise manner. The culture dish was swirled gently to distribute the transfection complex over the entire plate before incubating at 37 °C in 5% CO₂ humidified atmosphere for 6 hr. After that, Opti-MEM™ I media was removed and replaced with 12 ml fresh complete EMEM medium. Transfected cells were then incubated at 37 °C in 5% CO₂ humidified atmosphere for 48 hr. After incubation was complete, virus containing supernatant was harvested using sterile syringes and filtered through a 0.45 µm syringe filter. The filtered lentiviral supernatant can be stored at 4 °C for up to 14 days. Morphology of HEK293FT cells during the viral production process is demonstrated in Figure 2.4.

To concentrate the virus, the lentiviral supernatant was gradually overlaid over 1 ml of 10% sucrose solution to create a density gradient in a 10 ml thin wall ultracentrifuge tube. The lentiviral supernatant was centrifuged at 100,000 g (28,000 rpm) for 2 hr at 4 °C in SW 41 Ti rotor. In the safety cabinet, supernatant was poured out. The tube was inverted on an absorbent paper towel to allow drainage of residual liquid for 10 min. The viral pellet was resuspended in 300 µl of 1× PBS and stored at 4 °C overnight to complete resuspension. The next morning, viruses in 1× PBS were mixed by gentle

pipetting. 300 μ l of 50% glycerol was added and mixed well. The virus prep was aliquoted into screw cap tubes and stored at -80 $^{\circ}$ C.

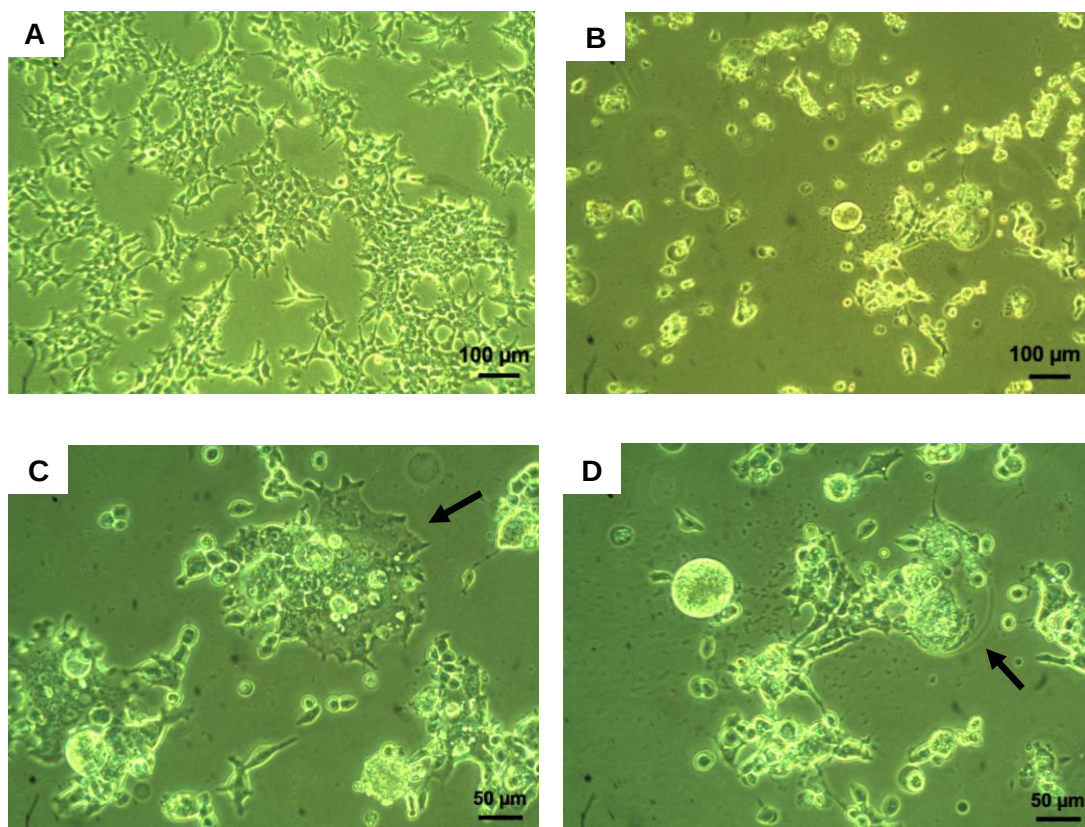


Figure 2.4 Morphology of HEK293FT cells during viral production process

A: HEK293FT cells were transfected with lentiviral plasmid for 6 hr. The cell morphology did not change from the morphology prior to transfection. **B:** After 48 hr of transfection, many cells died. The cells showed cytopathic effects of viral infection such as rounding of the infected cells, syncytial formation (fusion with surrounding cells) (**C, arrow**), appearance of nuclear or cytoplasmic inclusion bodies and cellular swelling (**D, arrow**). The images were collected with an inverted light microscope.

2.1.2.3 Lentiviral titration

Lentiviral titrations were performed to determine the number of the viruses and ensure viral viability. By using QuickTiter™ Lentivirus Titer Kit (VPK-107; Cell Biolabs, San Diego, CA, USA), enzyme immunoassay, lentivirus associated HIV-1 p24 core protein

was detected and quantified. The lentiviral sample was prepared by diluting 10 µl of lentiviral supernatant in 990 µl of fresh medium (complete EMEM medium). Fresh medium without lentiviral sample was included as a negative control. 10 µl of ViraBind™ Lentivirus Reagent A was added to the lentiviral sample and mixed by inverting. Immediately, 10 µl of ViraBind™ Lentivirus Reagent B was added and mixed by inverting. The sample was incubated at 37 °C for 30 min. After completion of incubation, the sample was centrifuged at 12,000 rpm for 5 min at RT. Supernatant was carefully removed. The pellet was dissolved in 250 µl of sample diluent by pipetting and vortexing before incubating at 37 °C for 30 min to inactivate the viruses. At this time, a standard curve was prepared by utilising serial 2-fold dilutions of the recombinant HIV-1 p24 antigen standard in sample diluent in the concentration range of 100 ng/ml to 1 ng/ml. All standard curve samples were vortexed and incubated at 37 °C for 30 min.

The lentiviral sample, negative control sample, standard curve and blank samples were assayed in duplicate. 100 µl of sample was added to the well of an anti-p24 antibody coated plate. The plate was covered and incubated at 4 °C overnight.

After emptying, the wells were washed three times with 250 µl of 1× wash buffer per well. After the last wash, the plate was turned upside down and tapped on an absorbent paper towel to remove any excess wash buffer. 100 µl of diluted fluorescein isothiocyanate (FITC)-Conjugated Anti-p24 Monoclonal Antibody was added to each well. The plate was covered and incubated at RT for 1 hr on an orbital shaker at 100 rpm. The plate cover was removed and the wells were emptied and washed three times as described previously. 100 µl of diluted Horseradish peroxidase (HRP)-Conjugated Anti-FITC Monoclonal Antibody was added to each well. The plate was covered and incubated at RT for 1 hr on an orbital shaker at 100 rpm. The wells were emptied and washed three times as described previously. 100 µl of substrate solution was immediately added to each well. The plate was incubated at RT on an orbital

shaker at 100 rpm for 10-15 min until the development of the colour reaction was stable. The enzyme reaction was stopped by adding 100 µl of stop solution into each well. Using a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany), the absorbance level was immediately read in each well at 450 nm.

Based on p24 standard curve, the lentivirus associated p24 amount in the lentiviral samples were determined using the formula:

$$\text{Lentivirus associated p24 (ng/ml)} = \text{p24} \times \text{Dilution factor} \times 0.25$$

Lentiviral particle (LP) concentrations (LP/ml) were calculated based on that 1 ng of p24 = 1.25×10^7 LPs, therefore,

$$\text{Lentiviral particles (LP/ml)} = \text{Lentivirus associated p24 (ng/ml)} \times 1.25 \times 10^7$$

The concentrations of transformation unit (TU) which is the viral infectious titre (TU/ml) were then calculated based on 1 TU is 100 to 1,000 LP, therefore,

$$\text{Lower limit of transformation unit (TU/ml)} = \frac{\text{Lentiviral particle (LP/ml)}}{1,000}$$

The viral infectious titre was applied to calculate the volume of the viral stock used to transduce breast cancer cells.

2.1.2.4 Lentiviral transduction

MCF7 or MDA-MB468 cells were plated at a density of 2×10^5 cells/well in 12-well cell culture plates containing 1 ml complete medium in each well. The cells were incubated at 37 °C in 5% CO₂ humidified atmosphere overnight to reach confluency of approximately 50-60%. Cell culture media was gently removed and replaced with 1 ml fresh media containing 10 µg/ml hexadimethrine bromide (polybrene [9268; Sigma-Aldrich, St Louise, MO, USA]). Hexadimethrine bromide is a polycation which neutralises a charge repulsion between virus and target cell surface and thus helps viral integration into the cell. It may be toxic to the cells and thus needs to be optimised. Lentiviral aliquot was thawed on ice and then added to the cells in a dropwise manner. A couple of wells were left without viral addition to serve as un-transduction control. The cell culture plate was swirled gently to ensure even distribution of virus over the entire plate before incubation at 37 °C in 5% CO₂ humidified atmosphere for 4 hr. Then the culture media was changed to fresh complete medium and further incubated for 48 hr. Lentiviral transduction conditions varied greatly across cell types and needed to be optimised prior to an experiment to ensure the success of transduction.

2.1.2.5 Stable cell line generation

At 48 hr post viral transduction, the cells in each well were transferred to a 10 cm culture dish containing fresh complete medium and geneticin (G418 [61-234-RG; Corning, Corning, NY, USA]) for selection. Culture medium was changed every 2-3 days. After seven days of selection, when isolated colonies appeared and un-transduction control cells all died, colony picking was performed.

The cells were rinsed with 1× PBS and then 5% trypsin in warm 1× PBS was added. Under an inverted light microscope, a pipette with sterile tip was used to scrape the

colony of interest and pick the cells. Five to ten colonies were chosen and the cells were transferred to a 96-well plate. The cells were maintained in selection medium for an additional seven days. Then the cells were expanded and maintained in the media containing geneticin at half concentration used for selection. The stable cell lines generation process is summarised in Figure 2.5.

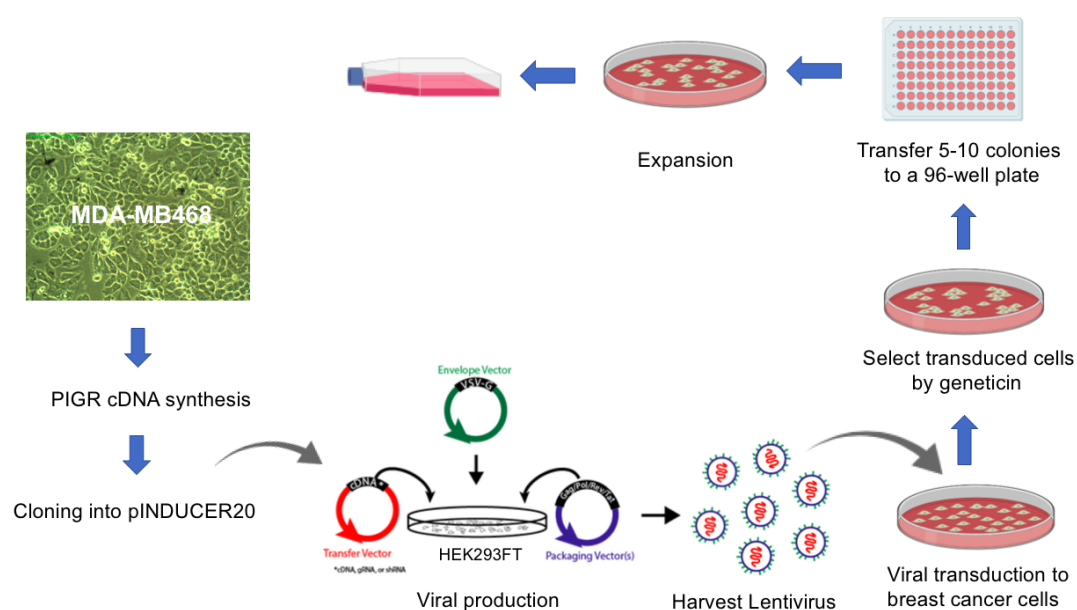


Figure 2.5 MCF7/PIGR and MDA-MB468/PIGR stable cell lines generation

PIGR cDNA was synthesised from MDA-MB468 RNA and then cloned into pINDUCER20 transfer plasmid vector. Lentiviruses were produced by transfection of viral transfer plasmids, packaging plasmids and envelope plasmids to HEK293FT cells. Lentiviruses were harvested from cell culture media and concentrated. Breast cancer cells were transduced by concentrated lentiviruses. Successful transduced cells were selected by geneticin and 5-10 colonies were selected to be expanded. The figure was created with BioRender.com.

2.1.2.6 Determining the optimal geneticin concentration for stable cell line selection

Stable cell line selection is performed using positive selection markers. The geneticin resistant gene is present in the pINDUCER20 backbone. Therefore, geneticin was used as a selection marker for successfully transduced cells that express the PIGR

gene. The optimal concentration of selection marker is cell type dependent. To determine the optimal geneticin concentration, a dose-response experiment for each cell type was performed. By increasing the amounts of geneticin, the minimum concentration needed to kill all the cells over the course of one week was determined.

The cells were plated at a density of 1.5×10^5 cells in a 24-well cell culture plate containing 1 ml complete medium in each well. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C overnight to reach confluency of approximately 70-80%. Cell culture media was gently removed and replaced with 1 ml fresh media containing geneticin at increasing concentrations including non-geneticin control (0, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1,000 µg/ml, with duplicate wells for each concentration). Geneticin containing media was changed every 2-3 days for up to a week. The cells were examined every day to assess the evidence of cell toxicity. The optimal dose of geneticin for selection is the lowest concentration at which all cells in the well die after seven days.

2.1.3 RNA isolation and quantification

Total RNA was isolated from the cells using TRI Reagent® (T9424; Sigma-Aldrich, St Louise, MO, USA) (1 ml per 10 cm² of culture surface area). The cells were lysed using cold TRI Reagent®, then the samples were placed at RT for 5 min to permit complete dissociation of nucleoprotein complexes. 200 µl of 1-bromo-3-chloropropane (BCP) per 1 ml of TRI Reagent® lysate was added. Samples were vortexed for 10 sec and placed at RT for 15 min. Centrifugation of the samples at 12,000 *g* for 15 min at 4 °C was performed to separate the lysate into three phases. The clear upper aqueous phase was transferred to a sterile nuclease-free micro-centrifuge tube and mixed with 500 µl of 2-propanol per 1 ml of TRI Reagent® lysate to precipitate the RNA. Samples were incubated at RT for 10 min and then at -20 °C overnight. The samples were then centrifuged at 12,000 *g* for 10 min at 4 °C followed by 21,130 *g* for 10 min at 4 °C.

The supernatant was aspirated and discarded. The RNA pellets were washed twice in 0.5 ml of 70% ethanol per 1 ml of TRI Reagent® lysate and centrifuged at 21,130 *g* for 10 min at 4 °C. This was followed by removal of the ethanol and brief air drying of the RNA pellet for 5 min. It is important to ensure that the RNA pellet is not completely dry, otherwise its solubility will decrease. Diethyl pyro-carbonate (DEPC)-treated high-performance liquid chromatography (HPLC) grade water, 87.5 µl, was added to the samples before heating at 65 °C for 5 min. Deoxy-ribonuclease I (DNase I) 2.5 µl and RDD buffer 10 µl (79254; Qiagen, Hilden, Germany) were added to each sample for DNA digestion and RNA purification. Samples were placed at RT for 10 min. 300 µl of DEPC-treated HPLC grade water and 400 µl of phenol/chloroform/iso-amyl alcohol were added and mixed with each sample. The samples were separated into two phases by centrifugation at 10,000 *g* for 10 min at 4 °C. The upper aqueous phase was transferred to a sterile nuclease-free micro-centrifuge tube. 40 µl of 3M sodium acetate (pH 5.2) and 800 µl of absolute alcohol were added and mixed with each sample to precipitate the pure RNA. The samples were then incubated at -20 °C overnight. The RNA was pelleted by centrifugation at 21,130 *g* for 10 min at 4 °C. The supernatant was removed and the RNA pellets were washed twice in 0.5 ml of 70% ethanol per 1 ml of TRI Reagent® lysate and centrifuged at 21,130 *g* for 10 min at 4 °C. The ethanol was removed and the RNA pellets were briefly air dried for 5 min. The RNA was dissolved in 30-40 µl of DEPC-treated HPLC grade water. The concentration and purity of the RNA were estimated by measuring the absorbance at 260 and 280 nm. The 260/280 ratio of 1.8-2.2 was indicative that the RNA was free from contamination.

2.1.4 CDNA synthesis via reverse transcription

The Affinity-Script Multiple Temperature cDNA Synthesis Kit (200436; Agilent Technologies, Santa Clara, CA, USA) was used for the first-strand cDNA synthesis using 500 ng of total RNA. DEPC-treated HPLC grade water was added to the calculated RNA volume for a total volume of 10.7 µl and followed by adding random primers 3 µl (0.1 µg/µl). The samples were incubated at 65 °C for 5 min and then cooled down to RT for 10 min to allow the primers to anneal to the RNA. The following components were added: 2 µl of 10× Affinity-Script RT Buffer, 2 µl of 100 mM dithiothreitol (DTT), 0.5 µl of dNTP mix (25 mM each dNTP), 0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl), 1 µl of Affinity-Script Multiple Temperature RT. The samples were placed in a temperature-controlled thermal block at 25 °C for 10 min to extend the primers. The temperature was then increased to 50 °C for 60 min to synthesise cDNA. The reaction was terminated by incubating the sample at 70 °C for 15 min. Samples were stored at 4 °C for subsequent real-time qPCR. Non-template and non-enzyme controls were included in every experiment.

2.1.5 Real-time quantitative polymerase chain reaction (real-time qPCR)

Real-time qPCR was performed to measure a cDNA target during each amplification cycle of the PCR. The gene expression was quantified using a relative standard curve method. Primers and probes sequences were designed using Primer Express® Software Version 3.0 (Applied Biosystems Inc., Foster City, CA USA) (Table 2.9, 2.10). Primers and probes were purchased from Eurofins Scientific (Luxembourg). Sterile DNase RNase free water was added according to manufacturer's instruction to reconstitute the primers and create 100 µM stock concentration. The stock was diluted 1:10 to a concentration of 10 µM before use.

Table 2.9 Primers and probes sequences for real-time qPCR, Human gene

Human Gene	Sequence (5'-3')
<i>PIGR</i>	Forward primer: CAAGATTATCGAAGGAGAACCAAAC Reverse primer: CCCGTGTTATTCCACTTGCA Probe: CAAGGTCCCCTGTCACTTTCCATGCA
<i>GAPDH</i>	Forward primer: CAACAGCCTCAAGATCATCAGC Reverse primer: TGGCATGGACTGTGGTCATGAG Probe: CCTGGCCAAGGTCATCCATGACAA

Table 2.10 Primers and probes sequences for real-time qPCR, Mouse gene

Mouse Gene	Sequence (5'-3')
<i>IL-1β</i>	Forward primer: TGACAGTGATGAGAATGACCTGTTC Reverse primer: GGACAGCCCAGGTCAAAGG Probe: ACCCCAAAAGATGAAGGGCTGCTTCC
<i>iNOS</i>	Forward primer: AGCGAGGAGCAGGTGGAA Reverse primer: GGAAAAGACTGCACCGAAGATATC Probe: CCAGCTCAAGAGCCAGAAACGTTATCATG
<i>IL-10</i>	Forward primer: TTTGAATTCCCTGGGTGAGAA Reverse primer: CTCCACTGCCTTGCTCTTATTTTC Probe: AGGCGCTGTCATCGATTTCTCCCC
<i>ARG1</i>	Forward primer: GCAGAGGTCCAGAAGAATGGAA Reverse primer: GCATCCACCCAAATGACACA Probe: ACCACAGTCTGGCAGTTGGAAGCATCTC

Mouse Gene	Sequence (5'-3')
<i>IFN-γ</i>	Forward primer: ACAATGAACGCTACACACTGCAT Reverse primer: TGGCAGTAACAGCCAGAAACA Probe: TTGGCTTTGCAGCTCTTCCTCATGG
<i>β-actin</i>	Forward primer: GCTTCTTTGCAGCTCCTTCGT Reverse primer: GCGCAGCGATATCGTCATC Probe: CACCCGCCACCAGTTCGCCAT

The neat standard sample was produced by pooling an equal amount of each sample together. For a standard curve, two-fold serial dilutions of the neat standard sample were performed at specific dilution ranges: 1:2, 1:4, 1:8, 1:16 and 1:32 for MCF7 and MDA-MB468 cells samples and 1:4, 1:8, 1:16, 1:32 and 1:64 for BMDMs samples. Each sample was diluted with DEPC-treated HPLC grade water to 1:8 for MCF7 and MDA-MB468 cells samples and 1:16 for BMDMs samples. The reaction mixture for each sample was prepared as shown in Table 2.11. 10 μ l of the reaction mixture was transferred into each well of a 96-well PCR plate and 3 μ l of the diluted neat standard samples or first-strand cDNA samples were added and gently mixed without creating bubbles. The plate was sealed with transparent film and centrifuged for 30 sec before being placed into the real-time qPCR machine (G8830A [AriaMx Real-Time PCR instrument]; Agilent Technologies, Santa Clara, CA, USA)]. The real-time qPCR cycling programme was set up as recommended by the qPCR master mix manufacturer (Table 2.12). Each sample was examined in triplicate including non-template and non-enzyme control samples. A standard curve was included in each plate cycle. The target gene expressions were normalised to the reference gene expression. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β -actin* were used as reference genes for human and mouse genes, respectively.

Table 2.11 Reaction mixture for real-time qPCR

Component	Volume (μl)
Precision® FAST qPCR Master Mix (Z-PFAST-LR; Primerdesign, Camberley, UK)	6.5
10 μM forward primer	0.4
10 μM reverse primer	0.4
10 μM probe	0.25
DEPC-treated HPLC grade water	2.45

Table 2.12 Thermocycling conditions for real-time qPCR

Step	Temperature	Time	Cycle(s)
Enzyme Activation - Hot Start	95 °C	2 min	1
Denaturation	95 °C	5 sec	40
Data collection	60 °C	20 sec	

2.1.6 Protein isolation

After careful removal of cell culture media, cells were gently washed with ice cold 1× PBS three times. Approximately 100-150 μl/10 cm² culture surface area of cold radioimmunoprecipitation assay (RIPA) buffer was added to the cells. Then the cell lysate was mixed and gathered to one side by scraping. The lysate was transferred to a clean microcentrifuge tube and sonicated on ice using the following setting: amplitude 3%, process time 30 sec, pulse on 10 sec, pulse off 15 sec. The sonicated sample was incubated in the cold room on end over end rotator for 45 min. Centrifugation at 21,130 g for 15 min at 4 °C was performed to pellet the debris. The supernatant was collected for further analysis.

2.1.7 Determination of protein concentration

Protein was quantified by the Pierce™ BCA Protein Assay Kit (23227; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The standard curve was prepared by serial dilutions of the albumin standard (bovine serum albumin [BSA]) in diluent in the concentration range of 2,000 µg/ml to 25 µg/ml as dilution scheme for standard test tube protocol and microplate procedure. Samples were diluted in HPLC water at 1:10. Bicinchoninic acid (BCA) working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). 10 µl of each standard or diluted sample was pipetted into each well of a 96 well plate in triplicate. 200 µl of BCA working reagent was added to each well. The plate was placed on a plate shaker for 30 sec for mixing and incubated at 37 °C for 30 min. The plate was then cooled at RT for 10 min before measuring the absorbance at 562 nm by a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany).

2.1.8 Western blotting

Western blotting is an analytical technique to identify target protein in a complex sample. Target protein can be identified by antibody-based probes after electrophoretic separation of the protein in the sample.

The protein sample was mixed with Laemmli buffer and heated at 95 °C for 5 min for denaturation. The denatured protein samples were separated on a 10% polyacrylamide gel which was submerged in running buffer. The gel was run at 100 V through a stacking gel (approximately 15 min) followed by 200 V through a resolving gel (approximately 45 min). The gel was removed from the electrophoresis tank and the stacking gel was cut off. A cassette sandwich was used for transferring the protein on to the nitrocellulose membrane by placing the membrane next to the gel and

sandwiching it between the sponges and filter papers. The cassette was placed in a transfer tank filled with transfer buffer and an ice pack. The transfer cassette was run at 200 milliamperes (mA) for 90 min for one gel or 300 mA for 120 min for two gels. After completing the protein transfer, the membrane was stained with diluted Ponceau S solution (P7170; Sigma-Aldrich, St Louis, MO, USA) to demonstrate the protein bands on the membrane. The membrane was destained by rinsing with deionized water and washing in Tris-buffered saline and 0.1% Tween 20 (TBST) on an orbital shaker. The membrane was blocked to prevent non-specific background binding of primary and secondary antibodies by incubation with 5% skimmed milk in TBST on an orbital shaker for 60 min at RT. The primary antibody was prepared in a 50 ml Falcon tube by dilution with 5% skimmed milk in TBST (Table 2.13). The membrane was incubated with the primary antibody in a Falcon tube overnight on a roller in the cold room. Subsequently, the membrane was washed with TBST three times, 10 min per wash on an orbital shaker to remove residual primary antibody and then incubated with a peroxidase conjugated secondary antibody diluted with 5% skimmed milk in TBST (Table 2.14) at RT for 60 min on a roller. The membrane was washed with TBST three times for 5 min per wash on an orbital shaker. Enhanced chemiluminescent detection was performed using Immobilon™ Western Chemiluminescent HRP substrate (WBKL0500; Millipore, Burlington, MA, USA) according to the manufacturer's instructions. Working HRP substrate was prepared by mixing equal volumes of Luminol Reagent and Peroxide Solution in a clean tube and allowed to reach RT for 10 min. Approximately 0.1 ml of working HRP substrate is required per 1 cm² membrane area. The membrane was placed side up on a clean smooth container. Working HRP substrate was added onto the blot, then incubated for 5 min at RT. The membrane was removed from HRP substrate and covered with clean plastic wrap. The chemiluminescent signal was visualised using Luminescent Image Analyzer (LAS-3000; Fujifilm Life Science, Cambridge, MA, USA).

Table 2.13 Primary antibodies for Western blotting

Primary antibodies	Dilution (Concentration)	Product code; Supplier
Goat anti-Human PIGR antibody	1:5,000 (0.04 µg/ml) (unless otherwise specified)	AF2717; R&D Systems, Minneapolis, MN, USA
Mouse anti-Human α -tubulin antibody	1:500 (5 µg/ml)	T9026; Sigma-Aldrich, St Louise, MO, USA

Table 2.14 Secondary antibodies for Western blotting

Secondary antibodies	Dilution (Concentration)	Product code; Supplier
Rabbit anti-Goat IgG HRP conjugated Antibody	1:1,000 (0.1 µg/ml)	HAF017; R&D systems, Minneapolis, MN, USA
Goat anti-Mouse IgG HRP conjugated Antibody	1:70,000 (0.1 µg/ml)	A9917; Sigma-Aldrich, St Louise, MO, USA

2.1.9 Chemo-sensitivity test

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was used to evaluate the chemo-sensitivity *in vitro* by determining half maximal effective concentration (EC₅₀, concentration of drugs required to induce cell death to 50% of maximum cell death) of chemotherapeutic agents for breast cancer cell lines. MTS assay is a colorimetric method which determines the number of viable cells. Nicotinamide adenine dinucleotide phosphate (NADPH or NADH) generated in metabolically active cells converts MTS tetrazolium compound to a coloured formazan product which is soluble in cell culture medium. The intensity of the dye is proportional to the number of living cells.

The cells were plated at a density of 5×10^3 cells in each well of 96-well cell culture plates with 100 µl complete medium. Wells having only complete medium without

cells were used as background controls. The cells were incubated at 37 °C in 5% CO₂ humidified atmosphere overnight. Cell culture media was gently removed and replaced with 100 µl of fresh medium with varied concentrations of chemotherapeutic agent (Doxorubicin [S1208; Selleckchem, Houston, TX, USA] or Docetaxel [S11480; Selleckchem, Houston, TX, USA], concentration of 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50 µM) and with or without doxycycline. Control wells were added with fresh medium without chemotherapeutic agent. Then the cells were incubated at 37°C in 5% CO₂ humidified atmosphere for 48 hr. 20 µl of CellTiter 96® AQueous One Solution Reagent (G3581; Promega, Madison, WI, USA) which contains tetrazolium compound was added to each well. After 2 hr of incubation at 37 °C in 5% CO₂ humidified atmosphere, the absorbance at 490 nm was recorded using a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany). Each treatment was assayed at least in quadruplicate. The results were analysed and EC₅₀ values were determined using GraphPad Prism version 8.1.2 for macOS, GraphPad Software (San Diego, CA, USA).

2.1.10 Cell viability test

An MTS assay was used to determine cell viability of breast cancer cell lines. The method was as described in the chemo-sensitivity test but did not include the chemotherapeutic agent for cell treatment.

2.1.11 Cell proliferation assay

The bromodeoxyuridine (BrdU) assay (2752; Millipore, Burlington, MA, USA) was performed to determine proliferation of breast cancer cells. BrdU which is a thymidine analogue is incorporated into newly synthesised DNA strands of active proliferating

cells. BrdU is detected immunochemically when double stranded DNA is denatured. Therefore, the population of cells which are synthesising DNA can be assessed.

The cells were plated at a density of 5×10^3 cells in each well of 96-well cell culture plates with 100 μ l complete medium. Wells having complete medium without cells were used as blank control wells. The cells were incubated at 37 °C in 5% CO₂ humidified atmosphere overnight. 100 μ l of fresh medium with or without doxycycline was gently added into each well. Fresh medium was added to control wells. After incubation for 24 hr, 20 μ l of diluted BrdU Reagent was added to each well and cells were incubated for a 24 hr period.

The cell media was removed and 200 μ l of Fixing Solution was placed in each well. The cells were incubated at RT for 30 min. After aspiration of the Fixing Solution, the wells were washed three times with 200 μ l of 1 $\times$ wash buffer per well. After the last wash, the plate was blotted dry on an absorbent paper towel to remove excess wash buffer. 100 μ l of diluted anti-BrdU monoclonal antibody was added and incubated for 1 hr at RT. The wells were emptied and washed three times as described previously. Then 100 μ l of diluted Goat anti-Mouse IgG, Peroxidase Conjugate was added and incubated for 30 min at RT. The wells were emptied and washed three times as described previously. After a final wash, the entire plate was flooded with deionized water and patted dry on an absorbent paper towel. 100 μ l of Peroxidase Substrate Solution was added to each well. The plate was incubated at RT for 30 min in the dark. 100 μ l of stop solution was added to each well to stop enzyme reaction. Using a spectrophotometer (FLUOstar Omega microplate reader; BMG Labtech, Offenburg, Germany), the absorbance level was immediately read in each well at 450 nm.

2.1.12 Macrophage polarisation

Macrophages were polarised as M1 or M2 subtypes by treatment with lipopolysaccharide (LPS) or IL-4, respectively. These polarised macrophages were used to evaluate the effect on PIGR expression in breast cancer cells.

BMDM cells were plated at a density of 8×10^4 cells/well in 12-well plates and incubated in a humidified atmosphere containing 5% CO₂ at 37°C for 5 hr. The cell culture media was changed to fresh media containing LPS (L2630; Sigma-Aldrich, St Louise, MO, USA) 50 ng/ml or recombinant murine IL-4 (214-14; Peprotech, Rocky Hill, NJ, USA) 20 ng/ml and then incubated for 24 or 48 hr before harvesting for further assays.

2.1.13 Conditioned medium (CM) preparation

CM is spent media collected from cultured cells. It contains secreted protein from the cells including growth factors, enzyme, cytokines and hormones. MDA-MB468/PIGR CM and macrophage CM were prepared for further experiments.

MDA-MB468/PIGR cells were used to prepare enriched free secretory component (SC) CM by inducing PIGR expression in these cells. MDA-MB468/PIGR cells were plated in T175 flasks at a cell density of 7×10^6 cells/flask in 35 ml complete medium and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C overnight. Doxycycline 250 ng/ml was applied to obtain enriched free SC CM. A flask without doxycycline was prepared for CM without free SC for comparison. The cells were then further incubated for 48 hr before cell culture media collection. CM was filtered through a 0.22 µm syringe filter and stored at -80 °C until used.

For M1 and M2 macrophage CM, BMDMs were plated in T175 flasks at a cell density of 4×10^6 cells/flask in 35 ml complete medium and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C for 5 hr. A flask without cells was prepared for control CM. After the incubation period, LPS 50 ng/ml or IL-4 20 ng/ml was applied. Next, the

cells were further incubated for 24 (for LPS treatment) or 48 hr (for IL-4 treatment). Cell culture media was aspirated and the cells were gently washed with warm 1× PBS for three times. 35 ml of fresh complete medium was added. The cells were incubated for 24 hr. Finally, CM was collected, filtered through a 0.22 µm syringe filter and stored at -80 °C until used.

2.1.14 Concentration of protein in cell culture media

MCF7 and MDA-MB468 cells were plated on 60 mm culture dishes at a density of 9×10^5 cells/dish in 5 ml complete medium. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37 °C overnight. Cell media was gently aspirated and replaced with 5 ml fresh serum free media with cytokine treatment at a desired concentration. Then the cells were incubated for 48 hr. Cell culture media was collected into a clean 15 ml conical tube and centrifuged at 5,000 rpm for 5 min to remove cell debris. The supernatant was transferred to a clean tube. 2 ml of the supernatant was filled into a Vivaspın® 2, 10,000 molecular weight cutoff (MWCO), polyethersulfone (PES) membrane (VS0201; Sartorius, Göttingen, Germany) and centrifuged in a swing bucket at 3,901 g at 20 °C for approximately 10 min each time until the concentrated sample was less than 45 µl.

2.1.15 Induction of PIGR expression by recombinant cytokines

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well in 1 ml complete medium and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C overnight. Cell culture media was removed and replaced with fresh complete media containing recombinant human (rHu) interferon (IFN)-γ (300-02; Peprotech, Rocky Hill, NJ, USA), tumour necrosis factor (TNF)-α (300-01; Peprotech, Rocky Hill, NJ, USA), IL-10 (200-10; Peprotech, Rocky Hill, NJ, USA),

transforming growth factor (TGF)- β (240-B; R&D, Minneapolis, MN, USA) or IL-1 β (AF200-01B; Peprotech, Rocky Hill, NJ, USA). The cells were incubated for 24 or 48 hr before harvesting for further assays.

2.1.16 IL-1 receptor blockade assay

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well in 1 ml complete medium and incubated in a humidified atmosphere containing 5% CO₂ at 37 °C overnight. The cells were treated first with rHu IL-1 receptor antagonist (IL-1RA) (200-01RA; Peprotech, Rocky Hill, NJ, USA) 500 ng/ml for 30 min before treating with M1 CM together with rHu IL-1RA 500 ng/ml. The cells were incubated for 48 hr before harvesting for further assays.

2.1.17 Statistical analyses

Statistical analyses were performed with the Graph Pad Prism version 8.1.2 for macOS, GraphPad Software (San Diego, CA, USA). Unpaired t-test was used to compare the data between two groups and one-way analysis of variance (ANOVA) was used to compare three or more groups. A probability value (*p* value) of less than 0.05 (*p* < 0.05, 2-tailed) was considered statistically significant. Multiple testing correction was performed using Bonferroni post-test correction to adjust a significant *p* value.

2.2 Clinical study

2.2.1 Clinical specimens

The clinical study initially aimed to evaluate the expression of the *PIGR* gene and its association with the response to NAC and the clinical outcome in LLABC patients undergoing NAC. However, due to the limited availability of the samples, small breast cancers, size less than 3 cm, were also included. These patients had lymph node metastases and/or HER2 +ve breast cancers and were clinically judged by a multidisciplinary team to be suitable for NAC. The majority of the patients in the cohort had LLABCs (>3 cm, T3-4, N0-2 and M0). This clinical study utilised pre-treatment breast cancer core biopsies from 46 patients. Formalin-fixed paraffin-embedded (FFPE) tissue specimens and related clinical and pathological information were obtained from the University of Birmingham Human Biomaterials Resource Centre (project reference: 18-330) under the Research Tissue Bank ethical approval (NRES Committee North West – Haydock; Ref 15/NW/0079). RNA *in situ* hybridisation (ISH) assays were used to demonstrate mRNA expression in these tissue samples. Positive controls were carried out with normal human colon tissue which is known to express a high level of *PIGR*. They were simultaneously assessed with every batch of assay. Normal human colon tissues were provided by Dr Abed M. Zaitoun, Department of Cellular Pathology, Queen's Medical Centre, Nottingham University Hospital NHS Trust. Human breast cancer and normal adjacent tissue FFPE blocks used for optimisation were purchased from AMS Biotechnology (AMS-ERN.HER2N-NA; AMS Biotechnology, Abingdon, UK). All tissue specimens were sectioned and mounted on a positive charged glass slide and allowed to dry overnight.

2.2.2 Haematoxylin and eosin (H&E) staining

H&E staining was used to demonstrate tissue structure and confirm that tissue was correct and appropriately processed. Staining involves electrostatic attraction between oppositely charged ions in the dye and component of the cells. Haematoxylin combines with aluminium salts to form a cationic complex which is positively charged and acts as a basic dye. It stains nucleic acid in nuclei, which are basophilic negatively charged structures, deep blue-purple. Conversely, eosin is anionic and negatively charged and acts as an acidic dye. It reacts with acidophilic positively charged structures such as protein in cytoplasm or extracellular matrix to give different shades of pink.

The slide was placed in a slide rack and submerged in two xylene containing dishes for 5 min each for deparaffinisation. The slide was rehydrated in graded ethanol by dipping it up and down in 100%, 90%, 70%, 50% ethanol and tap water for 30 sec each, respectively. The slide then was sequentially incubated in Harris haematoxylin for 5 min, acid alcohol for 10 sec, Scott's tap water for 1 min and 1% eosin for 5 min. Each incubation was performed with occasional lifting of the slide up and down and followed by washing the slide in tap water. After eosin incubation, the slide was rapidly dipped in graded ethanol: 50%, 70%, 90%, 100% ethanol and xylene, respectively. The slide was mounted with a coverslip using dibutylphthalate polystyrene xylene (DPX) and left to dry overnight before being examined under an upright light microscope (Zeiss Axioplan; Carl Zeiss AG, Thornwood, NY, USA).

2.2.3 RNA *in situ* hybridisation (ISH)

RNA ISH is a technique used to detect and localise target RNA in fixed tissue samples or intact cells. RNAscope®2.5 HD Assay-Red (322350; Advanced Cell Diagnostics

[ACD], Newark, CA, USA) was used in this study according to the manufacturer's instructions.

All FFPE tissues mounted on slides need pre-treatment before being assayed. To ensure the adherence of the tissue section, the slide was heated at 60 °C for 1 hr in a dry oven. Then the tissue was deparaffinised in a fume hood by submerging in two xylene containing dishes for 5 min each followed by submersion in two dishes of 100% ethanol for 1 min, and finally air drying for 5 min. Endogenous peroxidase activity was blocked by incubating tissue with hydrogen peroxide at RT for 10 min followed by washing twice in dishes of deionized water. Target retrieval was performed by boiling the slides in 1× target retrieval solution maintained at 98-104 °C. Duration of the target retrieval depends on the type of tissue sample (Table 2.15). After finishing target retrieval, the slide was cooled in deionized water, washed in 100% ethanol and then allowed to dry at RT. When completely dry, the tissue was incubated with protease for cellular permeabilisation at 40 °C in a humidified oven (HybEZ hybridisation oven [321720; ACD, Newark, CA, USA]). The incubation time depends on type of tissue (Table 2.15). Then, the slide was washed in deionized water on a shaker and proceeded to RNA ISH assay.

Table 2.15 Tissue specific pre-treatment conditions

Tissue type	Target retrieval time	Protease incubation time
HeLa cell pellet (control slide) (310045; ACD, Newark, CA, USA)	10 min	30 min
Human colon tissue (normal)	15 min	30 min
Human breast tissue (normal and cancerous)	15 min	30 min

The tissue was incubated with an RNA specific probe (*Homo sapiens* peptidylprolyl isomerase B [cyclophilin B, PPIB] positive control probe [313901; ACD, Newark, CA, USA], *Bacillus subtilis* strain dihydrodipicolinate reductase [DapB] negative control probe [310043, ACD, Newark, CA, USA] or *Homo sapiens* PIGR probe [472681; ACD, Newark, CA, USA]) for 2 hr at 40 °C in the humidified oven. The probe was hybridised to RNA molecules in cells. After the incubation was completed, the slide was removed from the oven and washed for 2 min in 1× wash buffer on a shaker. Washing was repeated once with fresh 1× wash buffer. The signal was amplified using a set of amplifiers (Amp 1-6). The incubation condition for each amplifier is demonstrated in Table 2.16. After each incubation, the slide was washed twice in 1× wash buffer for 2 min each cycle on a shaker.

Table 2.16 Incubation condition of Amp 1-6 for signal amplification

Amplifier	Temperature	Time
Amp 1	40 °C (in humidified oven)	30 min
Amp 2	40 °C (in humidified oven)	15 min
Amp 3	40 °C (in humidified oven)	30 min
Amp 4	40 °C (in humidified oven)	15 min
Amp 5	RT	30 min
Amp 6	RT	15 min

RED working solution was prepared by mixing Fast Red-B with Fast Red-A in a ratio of 1:60. After the last wash, the slide was incubated with RED working solution for 10 min at RT in the dark. The slide was tapped to remove the RED working solution and immediately washed in deionized water twice. For the chromogenic technique, the slide was counterstained with 50% Gill's Haematoxylin I (GHS132; Sigma-Aldrich, St Louise, MO, USA) for 2 min at RT in the dark and then washed in deionized water until

the slide was clear while the tissue section remained purple. The slide was washed in 0.02% ammonia water for 10 sec followed by washing in deionized water 3-5 times and allowed to dry at RT before mounting. For the fluorescence technique, the slide was counterstained with 4',6-diamidino-2-phenylindole (DAPI) 1mg/ml for 5 min at RT to demonstrate the nuclei. DAPI was removed by physically tapping the slide. Mounting medium was dropped on the slide and a coverslip was placed to cover the tissue section. The signal, which represents RNA expression, was evaluated under an upright light microscope (Zeiss Axioplan; Carl Zeiss AG, Thornwood, NY, USA) or a wide field inverted fluorescence microscope (Zeiss 200M; Carl Zeiss AG, Thornwood, NY, USA).

2.2.4 Immunofluorescence (IF) staining

IF staining uses an antigen-antibody interaction to detect and localise proteins in the intact tissue samples. The protein was visualised by fluorescent dye using a fluorescence microscope.

The FFPE tissue section was deparaffinised in two xylene containing dishes for 5 min each followed by rehydration in graded ethanol: 100%, 70%, 50% ethanol for 5 min each. The slide was then washed under cold tap water for 5 min. Antigen retrieval was carried out by boiling the slide in a jar containing 10 mM sodium citrate buffer, pH 6.0 at 95 °C in a water bath for 20 min. The jar was then removed from the water bath and cooled down at RT for 30 min. The slide was washed in deionized water twice followed by phosphate buffered saline with Triton X-100 (PBST) three times, 5 min each. The tissue was incubated with 4% horse serum in PBST for 1 hr at RT to block unspecific binding of the antibodies. After removing the serum, the tissue was incubated with primary antibody (rabbit anti-human PIGR antibody [HPA012012; Sigma-Aldrich, St Louise, MO, USA]) in PBST at 4 °C overnight in a humidified chamber. The slide was washed three times in PBST for 5 min each. The secondary

antibody (Alexa Fluor™ 568 Donkey anti-rabbit IgG [H+L] [A10042; Invitrogen, Carlsbad, CA, USA] or Alexa Fluor™ 488 Donkey anti-rabbit IgG [H+L] [A21206; Invitrogen, Carlsbad, CA, USA]) in PBST was dropped on to the tissue and incubated for 1 hr at RT in the dark followed by washing the slide in 0.1 M phosphate buffer (PB) by moving it up and down 3-5 times. The tissue was incubated with DAPI (1 mg/ml) for 5 min and then washed three times in PBST for 5 min each in the dark. The slide was allowed to dry overnight before mounting a coverslip with a drop of mounting medium. The coverslip was sealed and fixed to the slide with nail polish. The tissue was examined under a wide field inverted fluorescence microscope (Zeiss 200M; Carl Zeiss AG, Thornwood, NY, USA).

2.2.5 RNA ISH combined with IF staining

The tissue specimen was pre-treated and assayed as described in the RNA ISH procedure. However, the protease incubation time had to be optimised to balance the RNA and protein signal presented after staining. After the tissue was incubated with RED working solution for 10 min, the slide was washed with tap water twice followed by two washes in deionized water and three washes in PBST for 5 min each. After that, the tissue was blocked with horse serum, incubated with primary and secondary antibodies, counterstained with DAPI and mounted with a coverslip as described in the IF staining procedure.

2.2.6 Quantification of PIGR mRNA expression

RNA ISH assays result in signals in which mRNA molecules are represented by punctate dots. Each dot represents a single mRNA molecule. The whole section was evaluated for the presence of PIGR mRNA expression in the breast cancer and adjacent normal breast tissue. The level of PIGR mRNA expression was semi-

quantified using the histo (H) scoring system (213). The H score is calculated by multiplying the percentage of cells that express PIGR mRNA by an average expression level following ACD scoring system (Table 2.17), giving a range of 0-400. PIGR mRNA expression were semi-quantified by both a consultant pathologist (AMZ) and myself. All sections were blinded to the patients' clinical and pathological parameters. Where there was disagreement on the score, the average score was considered.

Table 2.17 The semi-quantitative ACD scoring system for the RNAscope® assay

ACD Score	Scoring Criteria
0	No staining or <1 dot/ 10 cells
1	1-3 dots/cell
2	4-9 dots/cell and none or very few dot clusters
3	10-15 dots/cell and/or <10% dots are in clusters
4	>15 dots/cell and/or >10% dots are in clusters

Whole slide imaging was performed by a digital slide scanner (NanoZoomer C9600-02; Hamamatsu, Hamamatsu-city, Japan) at 40× magnification for image presentation.

2.2.7 Statistical analyses and sample size calculation

Statistical analysis was performed with IBM SPSS software, version 24 (IBM®, SPSS®, Armonk, NY, USA). Where the data did not follow a normal distribution, the Mann-Whitney U test and Wilcoxon signed rank test were used to compare two groups of continuous variables for unpaired and paired data, respectively. Pearson Chi-Squared test and Fisher's exact test were used to compare two groups of categorical variables. Kaplan-Meier method and the log-rank test were applied for survival analyses depending on presence or absence of PIGR expression. Spearman rank

correlation coefficient analysis was carried out to determine the correlation between H score and overall survival in patients with PIGR positive breast cancers. Overall survival was defined as an interval, in months, between the date of diagnosis to either the date of death or the last date of contact for those alive at the time of analysis. A probability value (p value) of less than 0.05 ($p < 0.05$, 2-tailed) was considered statistically significant.

A sample size of 22 patients is required for a 90% chance of detection, as significant at the 5% level, of an increase in the PIGR expression level from 0.08 in the samples from patients who did not achieve a pCR to 0.79 in the samples from patients who achieved a pCR. This is based on the median level of PIGR expression in the samples of the pCR and non-pCR groups of patients by real-time qPCR from the pilot study. The sample size calculation was carried out with online Power (Sample Size) Calculators (214).

**Chapter 3 Expression of PIGR and
the Effect of PIGR Overexpression on
Breast Cancer Cell Lines**

Chapter 3 Expression of PIGR and the Effect of PIGR Overexpression on Breast Cancer Cell Lines

3.1 Introduction

PIGR has a major role in mucosal immunity as a transporter of polymeric Ig across the epithelial cells. PIGR has been demonstrated and described as a biomarker in many cancers. The level of PIGR expression varies among cancer types and is associated with different patient outcomes. PIGR expression in hepatocellular cancer, colon cancer, pancreatic cancer, osteosarcoma and glioma has been demonstrated to correlate with poor prognosis (215-219). In contrast, many studies have reported favourable outcomes with PIGR expression in patients with other cancers, including upper gastrointestinal tract adenocarcinoma, lung, endometrial, ovarian and breast cancer (220-226).

The role of PIGR in cancer and related molecular mechanisms were explored in several studies and showed different outcomes. Pro-oncogenic function of PIGR in hepatocellular carcinoma has been demonstrated. By activating the proto-oncogene tyrosine-protein kinase Yes, which is a member of the Src family tyrosine kinase, and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signalling, PIGR was found to promote cellular transformation and proliferation (227). Moreover, increasing the expression of PIGR in hepatocellular carcinoma cells can activate small mothers against decapentaplegic (Smad) signalling and increase the expression of zinc finger protein SNAI1 (Snail), zinc finger protein SNAI2 (Slug) and zinc finger E-box binding homeobox (ZEB) 1, which induce epithelial mesenchymal transition (EMT) of cancer cells and lead to tumour metastasis (215). Thus, the overexpression of PIGR in hepatocellular carcinoma not only induces malignant transformation and cancer growth, but also increases the potential for metastasis.

Downregulation of PIGR in pancreatic cancer cells markedly changed cellular morphology and reduced cellular proliferation, adhesion, migration and invasion (228). In contrast, downregulation of PIGR in an endometrial adenocarcinoma cell line promoted migration of the cells although proliferation was not affected (229). In a lung cancer cell line, overexpression of PIGR was shown to inhibit proliferation over time and in a dose-dependent manner and had a tendency to increase apoptosis and necrosis (224). The expression of downstream target genes was further investigated by gene expression array. Notch receptor (NOTCH) 3 expression, which contributes to the inhibition of cellular differentiation, was revealed to have a reverse correlation with PIGR expression in lung cancer cells (224). However, the functional role of PIGR in breast cancer cells has not been elucidated.

Breast cancer cell lines have been widely used in translational breast cancer research. Substantial knowledge about breast cancer has been acquired from *in vitro* and *in vivo* studies using breast cancer cell lines as experimental models. Cell lines provide an infinite self-replicating source of material, which is easily multiplied in standard cell culture medium and is cost effective. Furthermore, they present a homogeneity without contamination by stromal cells, which allows consistency of the samples and reproducibility of the results (230, 231). However, caution is necessary when using cell lines. Cell lines are susceptible to genetic drift during serial passages for continuous culture. Rapidly growing clones in the population are selected over time resulting in phenotypic changes of the cells. These can cause heterogeneity of the cells at a single point (231, 232). Additionally, a cell line may be contaminated with other cell lines or mycoplasma (232).

The first breast cancer cell line, BT-20, was established in 1958 (233). Subsequently, other breast cancer cell lines were established and routinely used as breast cancer models, including MCF7, which is the most commonly used (231). Many studies have characterised breast cancer cell lines according to their phenotypic and molecular

profiling and have related them to breast tumour subtypes (Table 3.1). Most of these studies revealed that genes which correlated with the ER +ve phenotype breast cancer cell lines usually correlated with ER +ve tumours and genes which correlated with the ER -ve breast cancer cell lines usually correlated with ER -ve tumours. Moreover, no gene which correlated with ER +ve breast cancer cell lines also correlated with ER -ve breast tumours, and no gene which correlated with ER -ve breast cancer cell lines also correlated with ER +ve breast tumours. Therefore, the molecular markers used to differentiate breast cancer cell line subtypes may also differentiate breast tumours (230).

Breast cancer is a heterogeneous disease divided into several subtypes. Each subtype has different clinical characteristics, treatment responses and prognosis. Breast cancer cell lines are feasible models for tumours of the same subtype (234). The diversity of breast cancer cell lines provides a valuable insight into the different biological and molecular events in each subtype of breast tumours. Thus, selecting an accurate breast cancer cell line to be the experimental model is of paramount importance.

Table 3.1 Molecular subtypes and immunoprofiles of commonly used breast cancer cell lines

Molecular subtype	Immunoprofile			Example cell lines
	ER	PR	HER2	
Luminal A	+	±	-	MCF7, T47D, SUM185
Luminal B	+	±	±	BT474, ZR-75
HER2 enriched (HER2 +ve)	-	-	+	SKBR3, MDA-MB453
Basal-like	-	-	-	MDA-MB231, MDA-MB468, SUM190

The aim of this chapter was to evaluate the basal expression of *PIGR* gene in two representative breast cancer cell lines, MCF7 and MDA-MB468, and the relationship with chemo-sensitivity. The effect of PIGR overexpression on chemo-sensitivity and cellular behaviour of these cell lines was also investigated.

3.2 Results

3.2.1 Morphological characteristics of MCF7 and MDA-MB468 cells

MCF7 and MDA-MB468 cell lines were selected as breast cancer *in vitro* models due to their differences in phenotypical characteristics and molecular subtypes (235). The MCF7 cell line is classified as a luminal A subtype and is representative of chemo-resistant breast cancer cells. The MDA-MB468 cell line is classified as a basal-like subtype and is representative of chemo-sensitive breast cancer cells. The difference in morphology between MCF7 and MDA-MB468 cell lines is demonstrated by the microscopic images in Figure 3.1. MCF7 cells have an epithelial-like polygonal morphology with tight cell to cell junction. MCF7 cells grow as a monolayer and form a dome-like structure due to fluid accumulation between the cells and culture vessel (Figure 3.1A). MDA-MB468 cells also have an epithelial-like morphology and grow as a monolayer but, unlike MCF7 cells, they do not form a dome. MDA-MB468 cells normally produce large amounts of floating cells and debris (Figure 3.1B).

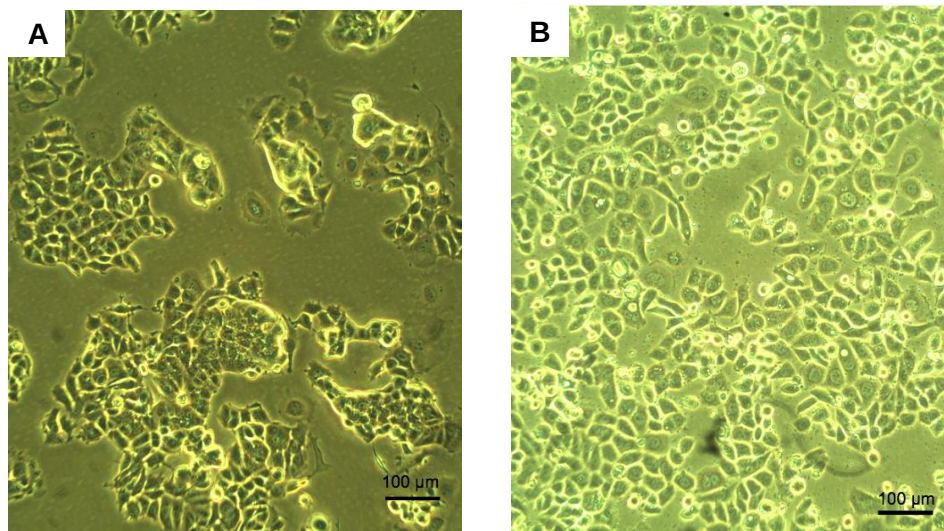


Figure 3.1 Morphology of MCF7 and MDA-MB468 cells

MCF7 **(A)** and MDA-MB468 cells **(B)** were cultured in complete media for four days. The images were collected with an inverted light microscope.

3.2.2 Cellular growth curves and cellular doubling times of MCF7 and MDA-MB468 cells

It is important to observe cell growth characteristics during cellular culture in an experiment. An alteration in cellular growth may indicate a significant problem in a cell line and can have deleterious effects on experimental results. Thus, the growth characteristics of cell lines must be evaluated before commencing the experiments. Generally, there are four phases of cellular growth, which are lag, log (exponential), stationary and decline (death) (Figure 3.2).

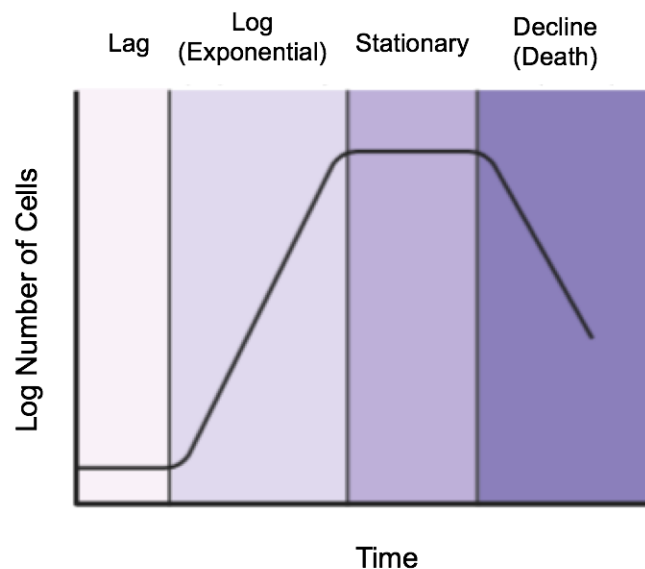


Figure 3.2 Phases of cell growth

A typical growth curve for a cell line has a characteristic “S”-shaped curve or sigmoid curve. There are four phases of cellular growth: lag, log (exponential), stationary and decline (death). The figure was created with BioRender.com.

Immediately after plating, cells turn to the lag phase which can last from a few hours to several days. The duration depends on the growth phase of the cells at the time of sub-culturing and on seeding density. During this phase, the cells recover from trypsinization, adapt to culture conditions and are not able to divide. Subsequently, the cells enter the log (exponential) phase in which they actively proliferate and grow

exponentially. The cell population is most viable in this phase and increases at double the usual rate. It is the optimal phase in which to assess cellular function or study the effect of drugs and chemical agents on the cells. When the cell population becomes confluent, cellular proliferation slows down and the cells enter the stationary phase. Due to exhaustion of the nutrients and enrichment of the wastes in culture media, the number of cells in the active cell cycle drops to 0-10% in this phase. Finally, with the natural progression of the cellular cycle, the cells enter the decline (death) phase in which cell death predominates and the number of viable cells decreases.

To define the growth characteristics of MCF7 and MDA-MB468 cells, the cellular growth curve of each cell line was established and cellular doubling time was determined. The cellular growth curves were created by using the data from MTS assays performed at 2 hr and then every 24 hr for five days after cellular plating. The MCF7 cell growth curve manifested three phases of growth (Figure 3.3A). The duration of lag phase of MCF7 cells was shorter than 24 hr and is not evident in their growth curve. The MCF7 cells log phase constantly increased for 72 hr, as shown in the growth curve. Cellular growth was then stable in the stationary phase for 24 hr before entering the decline phase at 96 hr after plating. Cellular doubling time of MCF7 cells calculated from the log phase of the cellular growth curve was 40.3 hr. The growth of MDA-MB468 cells was only seen in the log phase (Figure 3.3B). The lag phase of MDA-MB468 cells was also of short duration and was not evident in the growth curve. The cells remained in the log phase for 120 hr without reaching a stationary phase. Cellular doubling time of MDA-MB468 cells was 56.8 hr.

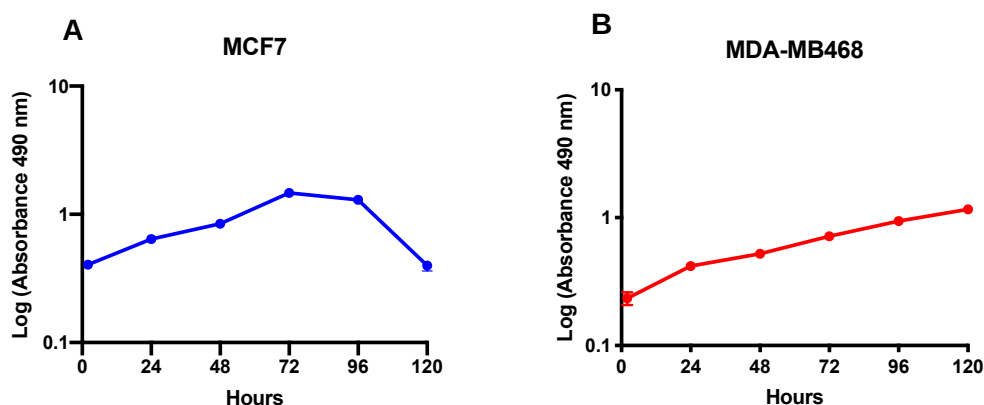


Figure 3.3 MCF7 and MDA-MB468 cellular growth curves

MCF7 and MDA-MB468 cells were plated in 96-well plates at a density of 5×10^3 cells/well. The MTS assays were performed at 2 hr and then every 24 hr after plating for five days to determine cellular viability. The results were plotted on a log-linear scale to demonstrate cellular growth curves of MCF7 **(A)** and MDA-MB468 **(B)**. Data are presented as mean $\pm$ standard error of mean (SEM) of triplicate samples.

3.2.3 MDA-MB468 cells expressed a higher level of PIGR than MCF7 cells

Real-time qPCR was performed to evaluate the basal expression of PIGR mRNA in MCF7 and MDA-MB468 cells. GAPDH was used as an endogenous control for normalisation. The results revealed that PIGR mRNA expression level in MDA-MB468 cells was approximately 380-fold higher than in MCF7 cells, which expressed very low levels (Figure 3.4). Using an unpaired t-test, PIGR mRNA expression in MDA-MB468 cells was significantly higher than in MCF7 cells ($p < 0.0001$).

Western blotting was carried out to demonstrate PIGR protein expression in these two cell lines. Alpha-tubulin (α -tubulin) was used as a reference protein for loading control. 300 μ g of total protein extracted from cell lysate was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:500. The result showed that PIGR protein expression in MDA-MB468 cells was higher than in MCF7 cells (Figure 3.5). The protein expression levels correlated with the levels of mRNA expression.

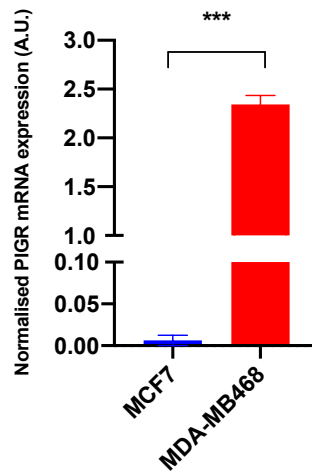


Figure 3.4 Basal expression of PIGR mRNA in MCF7 and MDA-MB468 cells

MCF7 and MDA-MB468 cells were plated in 6-well plates at a density of 3×10^5 cells/well. The cells were allowed to grow and were harvested when they reached 70-80% confluence. The expression of PIGR mRNA in MCF7 and MDA-MB468 cells was determined by real-time qPCR and normalised with GAPDH mRNA expression. Data are presented as mean $\pm$ SEM of three independent experiments ($***p < 0.0001$, unpaired t-test).

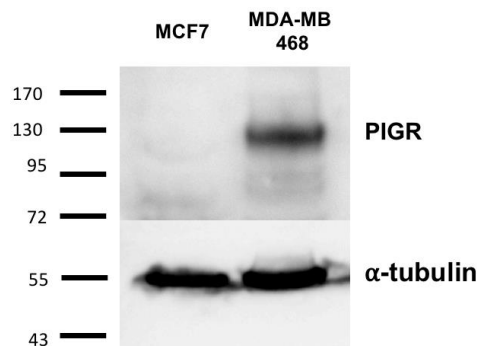


Figure 3.5 Basal expression of PIGR protein in MCF7 and MDA-MB468 cells

Representative Western blotting of three independent experiments for PIGR protein expression in MCF7 and MDA-MB468 cell lysates. MCF7 and MDA-MB468 cells were plated in 6-well plates at a density of 3×10^5 cells/well. The cells were allowed to grow and were harvested when they reached 70-80% confluence. 300 μ g of total protein from cell lysate was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:500. α -tubulin was used as a loading control.

3.2.4 Chemo-sensitivity of MCF7 and MDA-MB468 cells

Doxorubicin and docetaxel, which are the most commonly used neoadjuvant chemotherapeutic agents in breast cancer patients, were selected for *in vitro* study with MCF7 and MDA-MB468 cells. To evaluate the chemo-sensitivity *in vitro*, MCF7 and MDA-MB468 cells were treated with doxorubicin or docetaxel at a concentration of 0, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50 μM for 48 hr. Then the MTS assay was performed to determine EC_{50} (Table 3.2).

Table 3.2 EC_{50} of doxorubicin and docetaxel for MCF7 and MDA-MB468 cells

	EC_{50} MCF7 (Mean $\pm$ SEM [μM])	EC_{50} MDA-MB468 (Mean $\pm$ SEM [μM])	<i>p</i> value
Doxorubicin	0.354 $\pm$ 0.145	0.246 $\pm$ 0.096	0.57
Docetaxel	0.056 $\pm$ 0.033	0.035 $\pm$ 0.015	0.58

MCF7 and MDA-MB468 cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were treated with different concentrations of doxorubicin or docetaxel (0-50 μM) for 48 hr. MTS assay was performed to determine EC_{50} . Data are presented as mean and SEM of the EC_{50} of doxorubicin and docetaxel for MCF7 and MDA-MB468 from three independent experiments (no statistically significant difference, unpaired t-test).

MDA-MB468 cells showed lower EC_{50} for both doxorubicin and docetaxel compared with MCF7 cells. There was no statistically significant difference, although, MDA-MB468 cells tended to be more sensitive to these chemotherapeutic agents.

3.2.5 MCF7/PIGR stable cell line generation

Considering the low basal PIGR expression and tendency to be more chemo-resistant, the MCF7 cell line was selected to evaluate the effect of PIGR overexpression on chemo-sensitivity and cellular behaviour. An MCF7/PIGR stable cell line, with overexpression of the *PIGR* gene, was generated using the lentiviral

vector with tetracycline dependent transactivation system. First, PIGR cDNA was synthesised and cloned into pINDUCER20. pINDUCER20 is an inducible lentiviral vector in which the expression of an inserted gene can be induced by doxycycline. A lentiviral vector system with pINDUCER20-hPIGR was constructed, quantified and transduced into MCF7 cells. Transduced cells were selected by geneticin. Once the cells were growing in a stable and reproducible manner, five to ten colonies were selected and expanded.

3.2.6 Optimal multiplicity of infection (MOI) determination

MOI is a ratio of the number of viral particles to the number of the target cells during transduction. Optimal MOI is required to obtain high efficiency of transduction and optimal gene expression of the transduced cells. It varies depending on several factors, such as transduction efficiency, nature of transduced cell line and gene of interest. Therefore, optimal MOI should be determined before performing transduction.

To optimise the MOI, pINDUCER20-GFP lentiviral vectors were used as reporter lentiviruses to transduce MCF7 cells in five different MOIs. After the transduction process, doxycycline 1 µg/ml was applied for 48 hr to induce GFP expression (Figure 3.6). GFP expression of the successfully transduced cells was evaluated under the fluorescence microscope. The results from these preliminary experiments were used to determine an optimal MOI, which is the minimum MOI at which more than 85% of the cells express the target protein. The optimal MOI for MCF7 cells was seven, which was used for transduction of the pINDUCER20-hPIGR lentiviral vectors to create an MCF7/PIGR stable cell line.

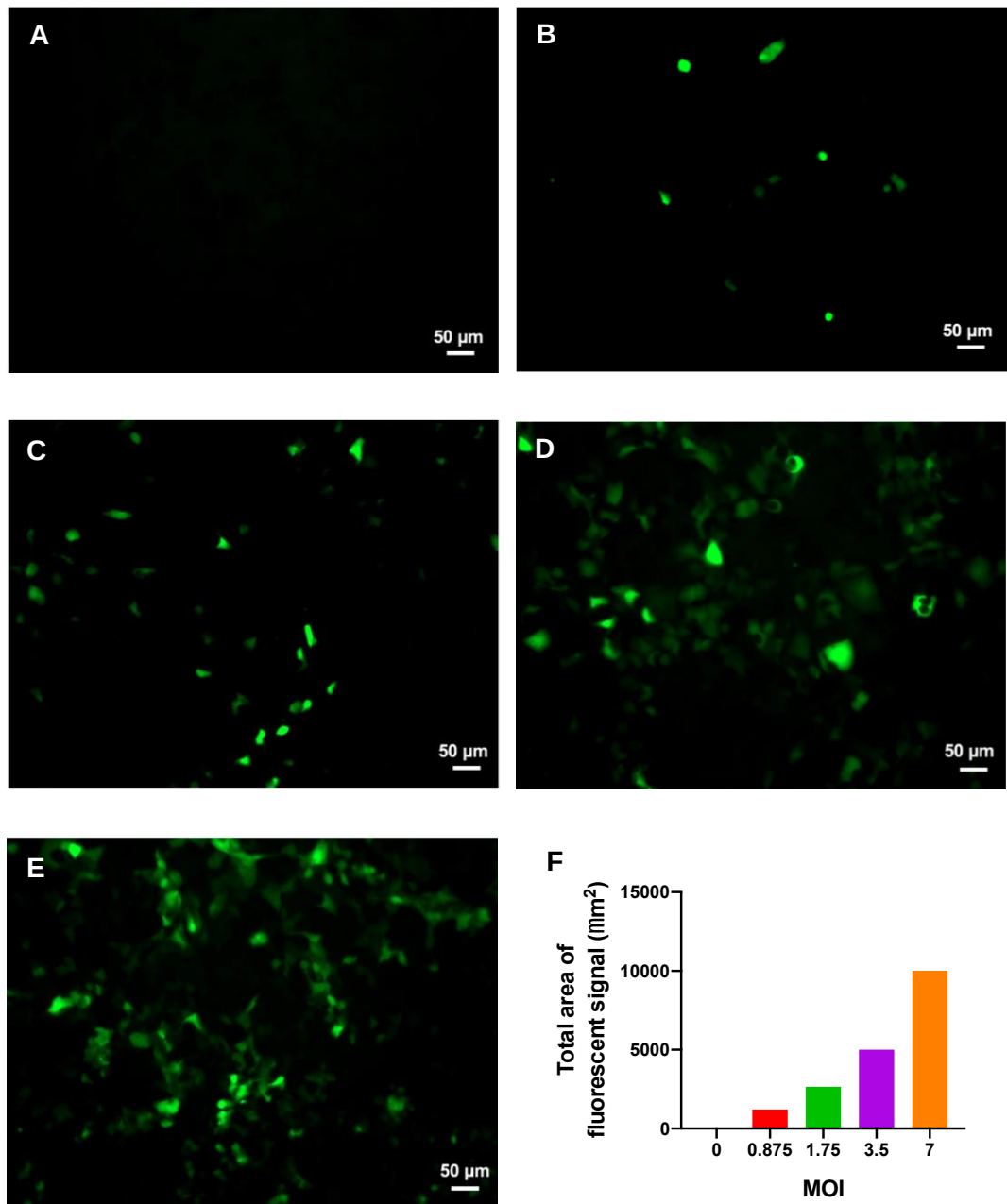


Figure 3.6 Optimal MOI determination of MCF7 cells

MCF7 cells were transduced with pINDUCER20-GFP lentiviral vectors at MOI 0 (**A**), 0.875 (**B**), 1.75 (**C**), 3.5 (**D**), 7 (**E**) and induced GFP expression with doxycycline 1 µg/ml for 48 hr. GFP expression was absent in figure **A** which was the control well (MOI 0, no viral transduction). The expression of GFP increased with increasing MOI (**B**, **C**, **D** and **E**). The images were collected with a widefield inverted fluorescence microscope. Figure **F** shows the total area of fluorescent signal (µm²) for different MOI as indicated.

3.2.7 Optimal geneticin concentration for stable cell line selection

Geneticin was used as a selection marker for successfully transduced cells. Optimal concentration of geneticin for selecting stable cells was determined for MCF7 cells by dose response experiments. 800 $\mu\text{g/ml}$ of geneticin was the minimum concentration that killed all the cells over the course of one week. Thus, 800 $\mu\text{g/ml}$ of geneticin was used as a selection dose and 400 $\mu\text{g/ml}$ (half the concentration used for selection) was used for maintenance of stable cells.

3.2.8 Overexpression of PIGR did not change morphological characteristics of MCF7 cells

The morphology of MCF7 and doxycycline-induced MCF7/PIGR cells is demonstrated by the microscopic images in Figure 3.7. Overexpression of PIGR did not affect the morphology of the cells. MCF7/PIGR cells had an epithelial-like polygonal morphology, tight cell to cell junction and dome structure formation as appeared in MCF7 cellular morphology.

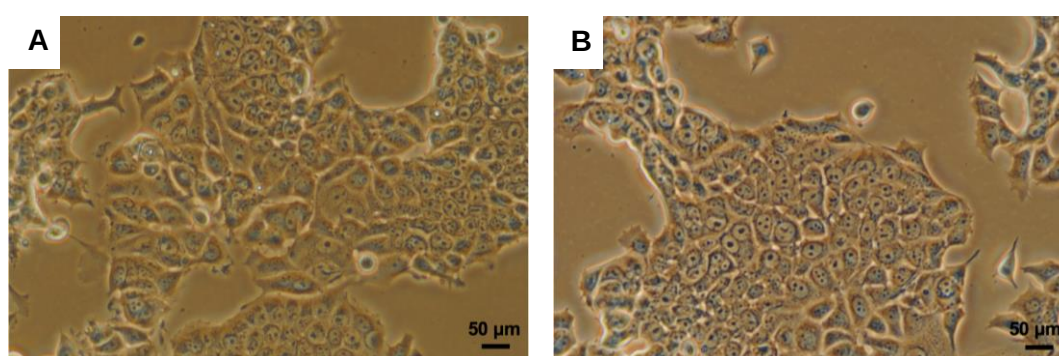


Figure 3.7 Morphology of MCF7 and MCF7/PIGR cells

A: MCF7 cells were cultured in complete EMEM media. **B:** MCF7/PIGR cells were treated with doxycycline 1 $\mu\text{g/ml}$ for 48 hr. The images were collected with an inverted light microscope.

3.2.9 MCF7/PIGR cells were stable and overexpressed PIGR

After an MCF7/PIGR stable cell line was generated, stable PIGR overexpression was verified by Western blotting. PIGR expression was induced in the cells with doxycycline 1 µg/ml for 48 hr before harvesting. Protein extracted from cell lysate at 4th, 8th, 12th, 16th and 20th cell passage was evaluated for PIGR expression. The Western blotting demonstrated PIGR overexpression in MCF7/PIGR cell lysate through 20 passages (Figure 3.8).

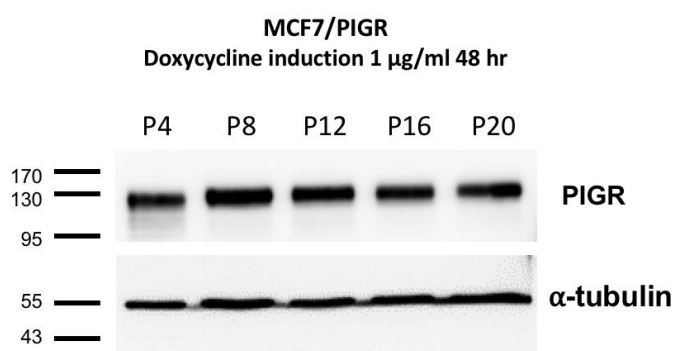


Figure 3.8 Stable PIGR overexpression in MCF7/PIGR cell line

Western blotting demonstrates PIGR protein expression in MCF7/PIGR cell lysate at 4th, 8th, 12th, 16th and 20th cell passage after doxycycline treatment. MCF7/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with doxycycline 1 µg/ml for 48 hr before harvesting. 5 µg of total protein from cell lysate was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:5,000. α-tubulin was used as a loading control.

To determine the kinetics of PIGR inducible expression, MCF7/PIGR cells were treated with doxycycline for 0, 8, 16, 24 and 48 hr. Protein from cell lysate was assessed for PIGR expression by Western blotting. The result showed that PIGR protein expression was detectable by 8 hr after doxycycline treatment and continuously accumulated over 48 hr (Figure 3.9).

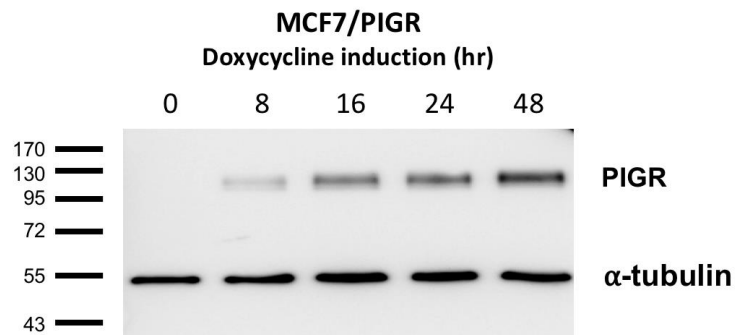


Figure 3.9 Expression of PIGR in MCF7/PIGR cells after doxycycline induction over the time

Western blotting demonstrates PIGR protein expression in MCF7/PIGR cell lysate after inducing the expression with doxycycline for the indicated duration. MCF7/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with doxycycline 1 $\mu\text{g/ml}$ for 0, 8, 16, 24 and 48 hr before harvesting. 5 μg of total protein from cell lysate was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:5,000. α -tubulin was used as a loading control.

Plasmid pINDUCER20 is an inducible lentiviral vector in which an inserted gene can be induced by doxycycline. Moreover, the expression can be titrated with increasing doxycycline concentrations. This effect was demonstrated in MCF7/PIGR cells and the results were applied to determine the optimal dose of doxycycline to induce PIGR expression in further experiments. MCF7/PIGR cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr. PIGR mRNA and protein expression was determined by real-time qPCR and Western blotting, respectively.

Doxycycline increased the PIGR mRNA expression level in MCF7/PIGR cells in a dose-dependent manner (Figure 3.10) which correlated with the level of protein expression. Protein expression in MCF7/PIGR cells was detectable with doxycycline 20 ng/ml (Figure 3.11). The expression was increased with increasing concentration of doxycycline and reached a plateau at doxycycline concentration of 100 ng/ml. Therefore, doxycycline concentrations of 20 and 100 ng/ml were selected as the low and high doses used to induce PIGR expression in further experiments.

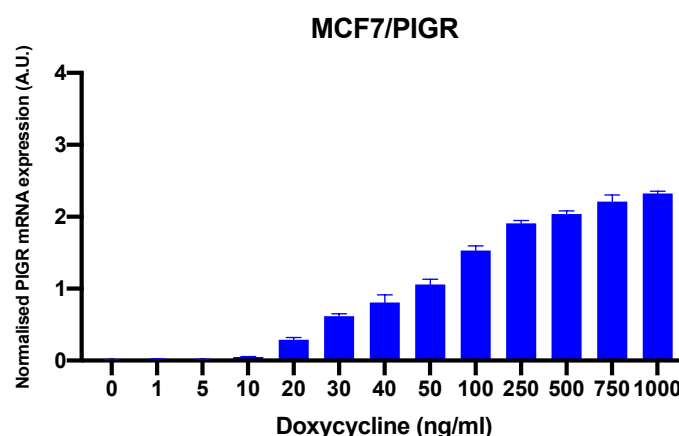


Figure 3.10 Doxycycline dose-dependent effect on PIGR mRNA expression in MCF7/PIGR cells

MCF7/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with doxycycline at the indicated concentration for 48 hr before harvesting. The expression of PIGR mRNA in MCF7/PIGR cells was determined by real-time qPCR. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation.

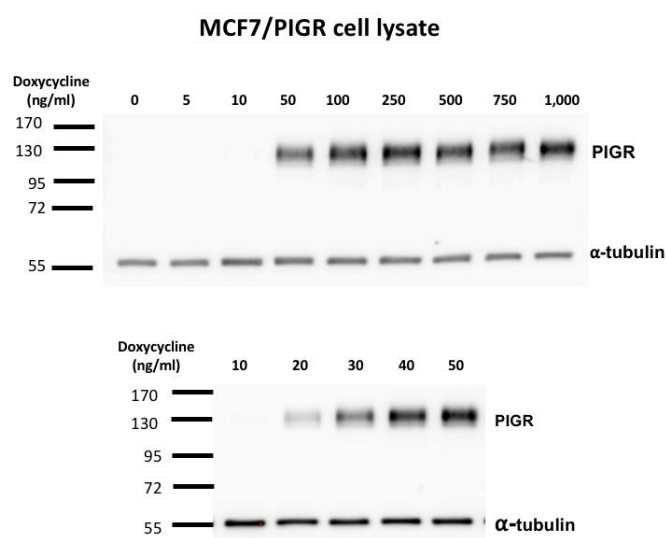


Figure 3.11 Doxycycline dose-dependent effect on PIGR protein expression in MCF7/PIGR cells

Representative Western blotting of three independent experiments for PIGR protein expression in MCF7/PIGR cell lysate after treatment with doxycycline at the indicated concentration. MCF7/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr before harvesting. 5 μ g of total protein from cell lysate was loaded into each well. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000. α -tubulin was used as a loading control.

In order to confirm that doxycycline-induced PIGR in MCF7/PIGR was functional, free secretory component (SC) in cell culture media was evaluated by Western blotting. Free SC is an extracellular part of PIGR which is cleaved and released from the cell. MCF7/PIGR cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr before collecting the cell culture media. 45 µl of cell culture media was loaded into each well. Anti-PIGR antibody used to detect PIGR protein can be used to detect free SC, as the immunogen of this antibody is the extracellular portion of PIGR.

Doxycycline increased free SC secretion from MCF7/PIGR cells in a dose-dependent manner which correlated with PIGR protein expression in the cells (Figure 3.12). The Western blotting demonstrated free SC bands at a molecular weight of 100 kDa which was less than full PIGR protein (120 kDa).

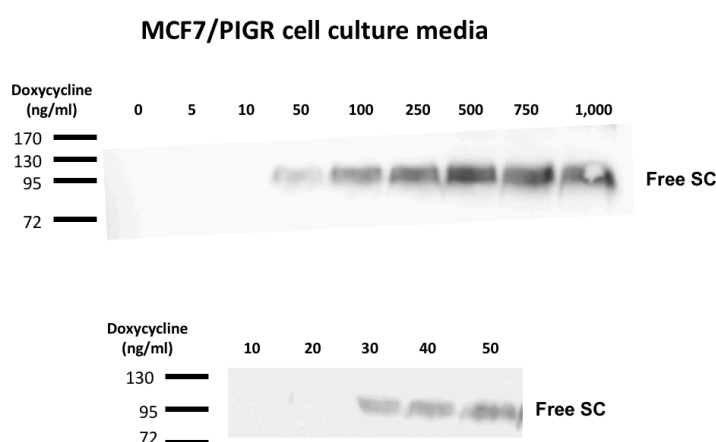


Figure 3.12 Doxycycline dose-dependent effect on free SC secretion from MCF7/PIGR cells

Western blotting demonstrates free SC secreted from MCF7/PIGR cells after treatment with doxycycline at the indicated concentration. MCF7/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well in 2 ml complete media and incubated overnight. The cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr before cell culture media collection. Cell culture media was centrifuged at 5,000 rpm for 5 min to remove cell debris. 45 µl of the supernatant was loaded into each well. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000.

3.2.10 PIGR overexpression did not significantly change chemo-sensitivity of MCF7/PIGR cells

The EC_{50} values of doxorubicin and docetaxel were determined for MCF7 and MCF7/PIGR cells in which PIGR transcription had been induced with doxycycline 20 and 100 ng/ml. The values were compared to demonstrate the effect of PIGR overexpression on chemo-sensitivity of the cells (Table 3.3, 3.4).

Compared with MCF7 cells, MCF7/PIGR cells showed a lower EC_{50} for doxorubicin, but a higher EC_{50} for docetaxel irrespective of whether induction was carried out with doxycycline 20 or 100 ng/ml. These results indicated that MCF7/PIGR cells tended to be more sensitive to doxorubicin but more resistant to docetaxel when compared with MCF7 cells. Greater differences of EC_{50} for both chemotherapeutic agents appeared when a higher dose of doxycycline was used to induce gene transcription. However, the differences were not statistically significant.

Table 3.3 EC_{50} of doxorubicin and docetaxel for MCF7 and MCF7/PIGR cells after treatment with doxycycline 20 ng/ml

	EC_{50} MCF7 (doxycycline 20 ng/ml) (Mean $\pm$ SEM [μ M])	EC_{50} MCF7/PIGR (doxycycline 20 ng/ml) (Mean $\pm$ SEM [μ M])	<i>p</i> value
Doxorubicin	0.989 $\pm$ 0.355	0.840 $\pm$ 0.324	0.77
Docetaxel	0.061 $\pm$ 0.015	0.069 $\pm$ 0.028	0.81

MCF7 and MCF7/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were treated with different concentrations of doxorubicin or docetaxel (0-50 μ M) with doxycycline 20 ng/ml for 48 hr. MTS assay was performed to determine EC_{50} . Data are presented as mean and SEM of the EC_{50} of doxorubicin and docetaxel for MCF7 and MCF7/PIGR from three independent experiments (no statistically significant difference, unpaired t-test).

Table 3.4 EC₅₀ of doxorubicin and docetaxel for MCF7 and MCF7/PIGR cells after treatment with doxycycline 100 ng/ml

	EC₅₀ MCF7 (doxycycline 100 ng/ml) (Mean ± SEM [μM])	EC₅₀ MCF7/PIGR (doxycycline 100 ng/ml) (Mean ± SEM [μM])	p value
Doxorubicin	0.876 ± 0.161	0.498 ± 0.128	0.14
Docetaxel	0.046 ± 0.005	0.063 ± 0.003	0.0514

MCF7 and MCF7/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were treated with different concentrations of doxorubicin or docetaxel (0-50 μM) with doxycycline 100 ng/ml for 48 hr. MTS assay was performed to determine EC₅₀. Data are presented as mean and SEM of the EC₅₀ of doxorubicin and docetaxel for MCF7 and MCF7/PIGR from three independent experiments (no statistically significant difference, unpaired t-test).

3.2.11 PIGR overexpression did not significantly change cellular behaviour of MCF7/PIGR cells

The effects of PIGR overexpression on MCF7/PIGR cellular behaviour was investigated. Cell viability and cell proliferation were evaluated because these two properties relate to the chemo-sensitivity of cancer cells.

Cell viability of MCF7 and MCF7/PIGR cells was determined by MTS assay. Each cell line was evaluated in three conditions: without treatment, with treatment with doxycycline 20 ng/ml for 48 hr, and with doxycycline 100 ng/ml for 48 hr. However, there were no statistically significant differences between cell viability of MCF7 and MCF7/PIGR cells whether treated or untreated (Figure 3.13). Moreover, doxycycline treatment at 20 and 100 ng/ml did not change cell viability of MCF7 cells from that of untreated MCF7 cells. This result implied that doxycycline itself in the dosage used in the experiments did not cause cellular toxicity to MCF7 cells.

BrdU assay was performed to determine cell proliferation of MCF7 and MCF7/PIGR cells in three conditions as in the cell viability experiment. The results also showed no statistically significant differences between cell proliferation of MCF7 and MCF7/PIGR

cells in all three conditions (Figure 3.14). Doxycycline treatment at 20 and 100 ng/ml did not affect cell proliferation of MCF7 cells.

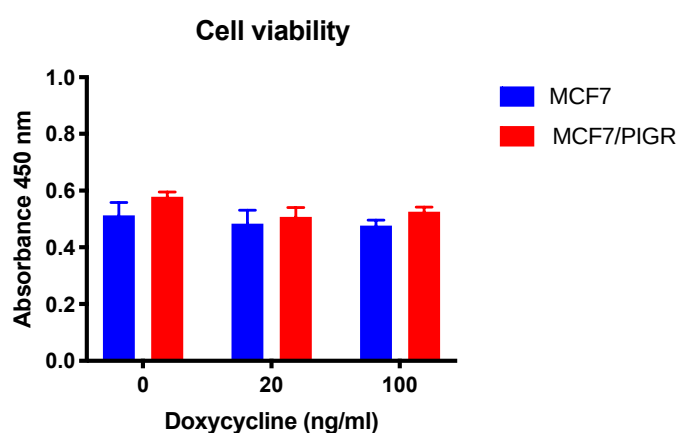


Figure 3.13 Cell viability of MCF7 and MCF7/PIGR cells after 48 hr of doxycycline induction

MCF7 and MCF7/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were cultured either in the absence or presence of doxycycline 20 or 100 ng/ml to induce PIGR transcription for 48 hr. MTS assay was performed to determine cell viability. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, one-way ANOVA with Bonferroni post-test correction).

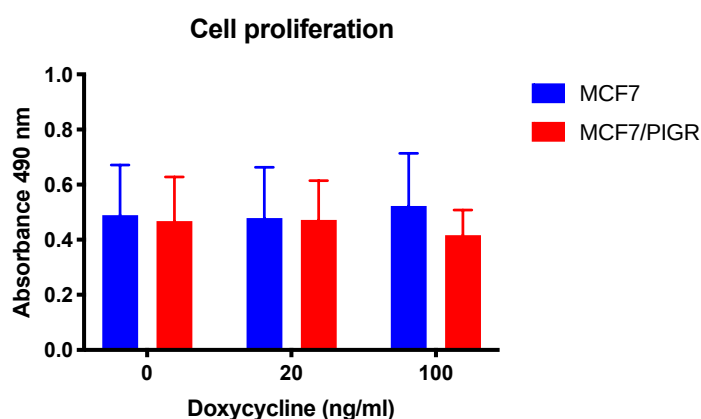


Figure 3.14 Cell proliferation of MCF7 and MCF7/PIGR cells after 48 hr of doxycycline induction

MCF7 and MCF7/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were cultured either in the absence or presence of doxycycline 20 or 100 ng/ml to induce PIGR transcription for 48 hr. BrdU assay was performed to determine cell proliferation. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, one-way ANOVA with Bonferroni post-test correction).

Overall, PIGR overexpression did not have a significant effect on chemo-sensitivity, cell viability and cell proliferation in MCF7/PIGR cells. Therefore, the effect of PIGR expression in the MDA-MB468 cell line, which is regarded as a different breast cancer subtype from the MCF7 cell line, was subsequently determined.

3.2.12 MDA-MB468/PIGR stable cell line generation

An MDA-MB468/PIGR stable cell line was generated using the lentiviral vector with tetracycline dependent transactivation system as describe previously. After the optimisation of MOI and geneticin concentration, pINDUCER20-hPIGR lentiviral vectors were transduced to MDA-MB468 cells at an MOI of seven to create MDA-MB468/PIGR cells. Transduced cells were selected by geneticin 800 µg/ml for one week, then five to ten colonies were selected and expanded.

3.2.13 Overexpression of PIGR did not change morphological characteristics of MDA-MB468 cells

The morphology of MDA-MB468 and doxycycline-induced MDA-MB468/PIGR cells is demonstrated by the microscopic images in Figure 3.15. MDA-MB468/PIGR cellular morphology did not differ from MDA-MB468 cells. They had an epithelial-like morphology, grew as a monolayer and produced large amounts of floating cells and debris.

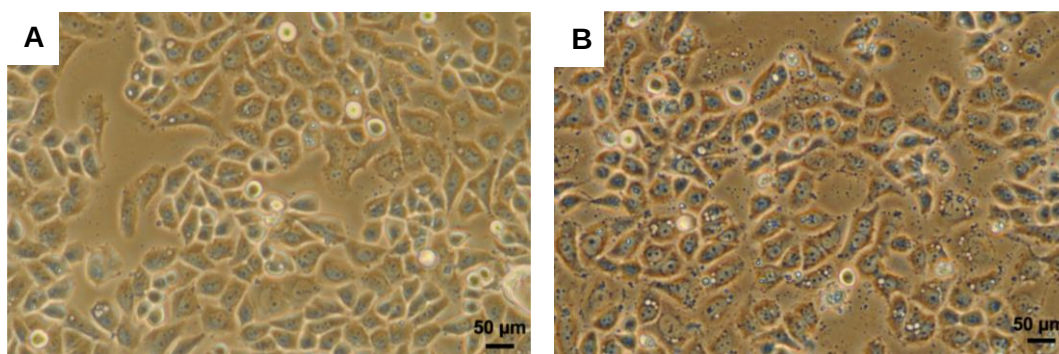


Figure 3.15 Morphology of MDA-MB468 and MDA-MB468/PIGR cells

A: MDA-MB468 cells were cultured in complete DMEM media. **B:** MDA-MB468/PIGR cells were treated with doxycycline 1 µg/ml for 48 hr. The images were collected with an inverted light microscope.

3.2.14 MDA-MB468/PIGR cells were stable and overexpressed PIGR

MDA-MB468/PIGR cells were stable and overexpressed PIGR through 20 passages as shown by Western blotting in Figure 3.16. PIGR mRNA expression of MDA-MB468/PIGR cells responded to doxycycline induction in a dose-dependent manner (Figure 3.17). Doxycycline dose-dependent effect was also demonstrated in PIGR protein expression (Figure 3.18) and free SC secretion from the cells (Figure 3.19) as was observed in MCF7/PIGR cells.

PIGR protein expression in MDA-MB468/PIGR cells was detectable after induction with doxycycline 20 ng/ml. The expression gradually increased with increasing concentration of doxycycline to 250 ng/ml. Doxycycline concentrations higher than 250 ng/ml slightly increased PIGR protein expression. Therefore, doxycycline at concentrations of 20 and 250 ng/ml were used as low and high doses to induce PIGR expression in MDA-MB468 cells in subsequent experiments.

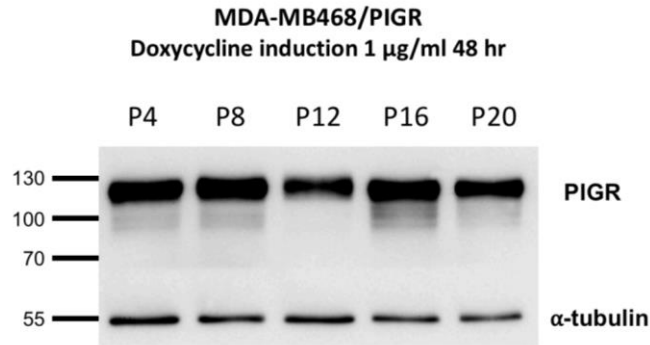


Figure 3.16 Stable PIGR overexpression in MDA-MB468/PIGR cell line

Western blotting demonstrates PIGR protein expression in MDA-MB468/PIGR cell lysate at 4th, 8th, 12th, 16th and 20th cell passage after doxycycline treatment. MDA-MB468/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with doxycycline 1 µg/ml for 48 hr before harvesting. 5 µg of total protein from cell lysate was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:5,000. α-tubulin was used as a loading control.

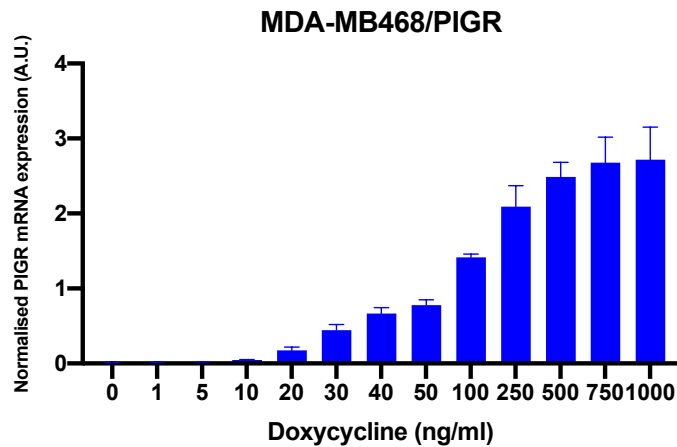


Figure 3.17 Doxycycline dose-dependent effect on PIGR mRNA expression in MDA-MB468/PIGR cells

MDA-MB468/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with doxycycline at the indicated concentration for 48 hr before harvesting. The expression of PIGR mRNA in MDA-MB468/PIGR cells was determined by real-time qPCR. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation.

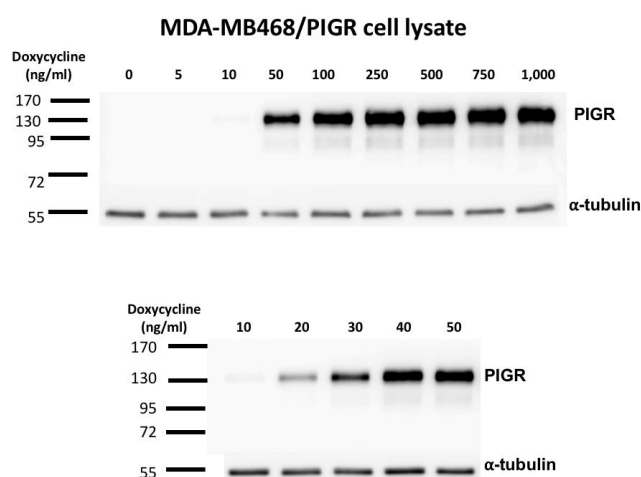


Figure 3.18 Doxycycline dose-dependent effect on PIGR protein expression in MDA-MB468/PIGR cells

Representative Western blotting of three independent experiments for PIGR protein expression in MDA-MB468/PIGR cell lysate after treatment with doxycycline at the indicated concentration. MDA-MB468/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well and incubated overnight. The cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr before harvesting. 5 μ g of total protein from cell lysate was loaded into each well. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000. α -tubulin was used as a loading control.

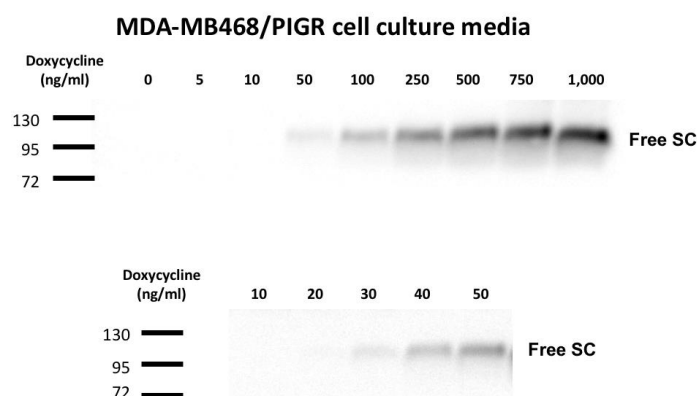


Figure 3.19 Doxycycline dose-dependent effect on free SC secretion from MDA-MB468/PIGR cells

Western blotting demonstrates free SC secreted from MDA-MB468/PIGR cells after treatment with doxycycline at the indicated concentration. MDA-MB468/PIGR cells were plated in 6-well plates at a density of 3×10^5 cells/well in 2 ml complete media and incubated overnight. The cells were treated with varied concentrations of doxycycline (0-1,000 ng/ml) for 48 hr before cell culture media collection. Cell culture media was centrifuged at 5,000 rpm for 5 min to remove cell debris. 45 μ l of the supernatant was loaded into each well. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000.

3.2.15 PIGR overexpression did not significantly change chemo-sensitivity of MDA-MB468/PIGR cells

To demonstrate the effect of PIGR overexpression on chemo-sensitivity of MDA-MB468/PIGR cells, MTS assay was performed to determine EC₅₀ of doxorubicin and docetaxel for both MDA-MB468 and MDA-MB468/PIGR cells. PIGR transcription was induced with doxycycline 20 and 250 ng/ml (Table 3.5 and 3.6).

EC₅₀ of docetaxel for MDA-MB468/PIGR cells was lower than for MDA-MB468 cells. In contrast, MDA-MB468/PIGR cells showed a higher EC₅₀ for doxorubicin than MDA-MB468, whether induction was with doxycycline 20 or 250 ng/ml. These results implied that MDA-MB468/PIGR cells tend to be more sensitive to docetaxel but more resistant to doxorubicin when compared with MDA-MB468 cells. However, the differences did not reach statistical significance.

Table 3.5 EC₅₀ of doxorubicin and docetaxel for MDA-MB468 and MDA-MB468/PIGR cells after treatment with doxycycline 20 ng/ml

	EC₅₀ MDA-MB468 (doxycycline 20 ng/ml) (Mean ± SEM [μM])	EC₅₀ MDA-MB468/PIGR (doxycycline 20 ng/ml) (Mean ± SEM [μM])	<i>p</i> value
Doxorubicin	0.095 ± 0.028	0.098 ± 0.024	0.934
Docetaxel	0.037 ± 0.001	0.033 ± 0.001	0.065

MDA-MB468 and MDA-MB468/PIGR cells were plated in 96-well plates at a density of 5×10³ cells/well and incubated overnight. The cells were treated with different concentrations of doxorubicin or docetaxel (0-50 μM) with doxycycline 20 ng/ml for 48 hr. MTS assay was performed to determine EC₅₀. Data are presented as mean and SEM of the EC₅₀ of doxorubicin and docetaxel for MDA-MB468 and MDA-MB468/PIGR from three independent experiments (no statistically significant difference, unpaired t-test).

Table 3.6 EC₅₀ of doxorubicin and docetaxel for MDA-MB468 and MDA-MB468/PIGR cells after treatment with doxycycline 250 ng/ml

	EC₅₀ MDA-MB468 (doxycycline 250 ng/ml) (Mean ± SEM [μM])	EC₅₀ MDA-MB468/PIGR (doxycycline 250 ng/ml) (Mean ± SEM [μM])	p value
Doxorubicin	0.110 ± 0.028	0.123 ± 0.008	0.683
Docetaxel	0.036 ± 0.002	0.033 ± 0.001	0.290

MDA-MB468 and MDA-MB468/PIGR cells were plated in 96-well plates at a density of 5×10³ cells/well and incubated overnight. The cells were treated with different concentrations of doxorubicin or docetaxel (0-50 μM) with doxycycline 250 ng/ml for 48 hr. MTS assay was performed to determine EC₅₀. Data are presented as mean and SEM of the EC₅₀ of doxorubicin and docetaxel for MDA-MB468 and MDA-MB468/PIGR from three independent experiments (no statistically significant difference, unpaired t-test).

3.2.16 PIGR overexpression did not significantly change cellular behaviour of MDA-MB468/PIGR cells

Cell viability and cell proliferation of MDA-MB468/PIGR cells were evaluated and compared with MDA-MB468 cells to demonstrate the effect of PIGR overexpression on MDA-MB468/PIGR cellular behaviour. Cell viability and cell proliferation were determined by MTS and BrdU assays, respectively. Both cell lines were evaluated in three conditions: without any treatment and treatment with doxycycline 20 and 250 ng/ml for 48 hr. The results demonstrated that there were no statistically significant differences between MDA-MB468 and MDA-MB468/PIGR cells in cell viability and cell proliferation whether cells were treated or untreated (Figure 3.20, 3.21). Doxycycline treatment at 20 and 250 ng/ml did not affect cell viability and cell proliferation of MDA-MB468 cells.

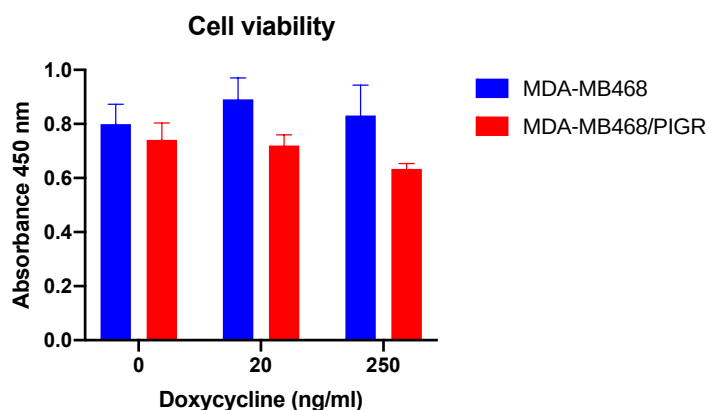


Figure 3.20 Cell viability of MDA-MB468 and MDA-MB468/PIGR cells after 48 hr of doxycycline induction

MDA-MB468 and MDA-MB468/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were cultured either in the absence or presence of doxycycline 20 or 250 ng/ml to induce PIGR transcription for 48 hr. MTS assay was performed to determine cell viability. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, one-way ANOVA with Bonferroni post-test correction).

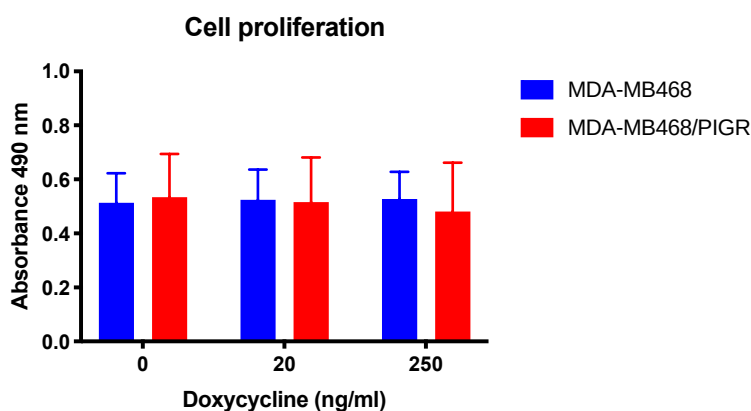


Figure 3.21 Cell proliferation of MDA-MB468 and MDA-MB468/PIGR cells after 48 hr of doxycycline induction

MDA-MB468 and MDA-MB468/PIGR cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were cultured either in the absence or presence of doxycycline 20 or 250 ng/ml to induce PIGR transcription for 48 hr. BrdU assay was performed to determine cell proliferation. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, one-way ANOVA with Bonferroni post-test correction).

3.3 Discussion

PIGR is widely expressed in epithelial cells and has a major responsibility for mucosal immunity. However, overexpression of PIGR has also been reported in many cancers. The level of PIGR expression varies among cancer types and is related to prognosis. Clinical significance of PIGR has been demonstrated in many studies. However, only a few studies have investigated its functional roles in cancers, and its role in breast cancer remains unexplored. The present study aimed to investigate the effect of PIGR expression on breast cancers as regards chemo-sensitivity, cell viability and cell proliferation. MCF7 and MDA-MB468 cell lines used in *in vitro* studies represented the genotypic and phenotypic disparities of breast cancers.

The MCF7 cell line was established in 1973 by Herbert Soule and colleagues at Michigan Cancer Foundation (MCF) (236). MCF7 cells were isolated from the pleural effusion of a 69-year-old Caucasian woman who was diagnosed with metastatic breast adenocarcinoma. This cell line expresses ER and PR but does not express HER2 (234). It is classified as a luminal A subtype of breast cancer which is of low aggression and low metastatic potential. *In vitro* chemo-sensitivity studies found that the MCF7 cell line was resistant to anthracyclines and taxanes (237, 238). These characteristics were also found in the luminal A subtype of breast cancers *in vivo*, with the lowest pCR rate with NAC (76, 114). MCF7, therefore, is representative of a chemo-resistant breast cancer cell line that may mimic the non-responsive or partially responsive cancers in patients.

The MDA-MB468 cell line was established in 1977 by Relda Cailleau and colleagues at M.D. Anderson Hospital and Tumour Institute (239). MDA-MB468 cells were isolated from the pleural effusion of a 51-year-old black female patient with metastatic adenocarcinoma of the breast. MDA-MB468 cells lack ER, PR and HER2 expression and are classified as an aggressive basal-like subtype with poor prognosis (234). The cells were found to be sensitive to anthracyclines, taxanes and 5-FU (42, 237, 240).

These characteristics correspond to triple negative primary breast cancer which has the highest pCR rate with NAC (76, 114). MDA-MB468, hence, is representative of a chemo-sensitive breast cancer cell line that may mimic responsive cancers in patients.

The levels of PIGR mRNA expression were demonstrated to be significantly higher in MDA-MB468 cells when compared with MCF7 cells. This parallels the findings previously documented in the Human Protein Atlas showing a very low level of PIGR mRNA expression in MCF7 cell lines (241). The result is also in agreement with the pilot study previously carried out in the department, in which the level of PIGR mRNA expression was high in breast cancers which subsequently achieved a pCR after NAC, but low in non-responders or partial responders. Furthermore, PIGR protein expression in these two cell lines was demonstrated by Western blotting using cell lysates. The results are in agreement with the mRNA expression data showing that PIGR protein expression was higher in MDA-MB468 than in MCF7 cells. However, the present study required a large amount of total protein (300 µg) and high concentration of primary antibody (1:500) to demonstrate PIGR expression in MDA-MB468 cells by Western blotting, while PIGR expression in MCF7 cells was not detected. This indicated that both the studied breast cancer cell lines naturally possess a low level of PIGR expression. Park *et al.* (242) previously reported a similar finding. They used Western blotting to examine PIGR protein expression in five breast cancer cell lines, MCF7, MDA-MB231, Hs578T, SK-BR-3 and ZR-75-1, and in a normal breast cell line (Hs578Bst) and found that PIGR protein was barely detected in any of the cell lines tested. The findings from the present study demonstrated different levels of PIGR expression in distinct subtypes of breast cancer and suggested that higher level of PIGR expression appeared to be associated with a beneficial response to chemotherapy. This is the first time, to the best of our

knowledge, that the expression of PIGR mRNA and PIGR protein in MDA-MB468 cells (TNBC cell line) has been described.

Chemo-sensitivity tests were performed to confirm the relation between PIGR expression and chemo-sensitivity of MCF7 and MDA-MB468 cells. EC_{50} was determined from dose-response curves generated over 10 logs of concentrations (ranging from 0-50 μ M) for doxorubicin and docetaxel. The EC_{50} with doxorubicin for MCF7 cells was slightly higher than for MDA-MB468 cells; comparable findings were seen for the EC_{50} with docetaxel. Thus, there was a tendency for MDA-MB468 cells to be more sensitive than MCF7 cells to both chemotherapeutic agents. However, there was no statistically significant difference, although the sensitivity tends to parallel the levels of PIGR expression.

Doxorubicin intercalates between DNA or RNA base pairs and inhibits topoisomerase II enzyme activity, leading to the inhibition of DNA or RNA synthesis. Docetaxel inhibits microtubule de-polymerisation resulting in mitotic blockage and cellular apoptosis. Doxorubicin and docetaxel are commonly used as neoadjuvant chemotherapeutic agents for breast cancers. Thus, they were chosen for the experiments. EC_{50} values of doxorubicin and docetaxel in MCF7 and MDA-MB468 cells have been determined by many studies with different treatment durations and assays applied (Table 3.7). Overall, MDA-MB468 cells are more sensitive to doxorubicin and docetaxel than MCF7 cells, which is in agreement with the results from the present study.

Table 3.7 EC₅₀ of doxorubicin and docetaxel for MCF7 and MDA-MB468 cell lines from literatures

Cell line	Drug (EC ₅₀ of the present study)	Treatment duration	Assay; EC ₅₀
MCF7	Doxorubicin (0.354 ± 0.145 µM)	4 h	MTT; 10 µM (243)
		24 h	MTT; 0.004471µM (244)
		48 h	MTT; 0.004195 µM (244), 1.525 µM (245), 1-5 µM (246), 0.25 µM (247)
			Alamar blue assay; 22 µM (248)
		72 h	MTT; 0.003827 µM (244)
			MTS; 17 µM (249)
			FMCA; 29 µM (250)
			Alamar blue assay; 0.18 µM (251)
		7 Day	MTS; 0.001 µM (252)
			Coulter counter; 0.0011 µM (253)
		NA	NA; 0.00000147 µM (254)
	Docetaxel (0.056 ± 0.033 µM)	24 h	MTT; 0.02 µM (255)
			CCK-8; 13.368 µM (256)
		48 h	MTT; 0.037 µM (245)
		72 h	ATP assay; >10 µM (238)
			FMCA; 25 µM (250)
MDA-MB468	Doxorubicin (0.246 ± 0.096 µM)	4 h	MTT; 0.1 µM (243)
		72 h	MTT; 0.085 ± 0.029 µM (257)
			SRB assay; 0.007 µM (258)
			Alamar blue assay; 0.1 µM (251)
		7 Day	MTS; 0.005 µM (252)
	Docetaxel (0.035 ± 0.015 µM)	48 h	MTT; 0.0285 µM (259)

NA: not applicable, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, FMCA: fluorometric microculture cytotoxicity assay, CCK-8: cell counting kit, ATP: adenosine triphosphate, SRB: sulforhodamine B

To evaluate the effect of PIGR expression on chemo-sensitivity and cellular behaviour, *PIGR* gene transduction was initially performed in MCF7 cells. MCF7 cells have a lower level of PIGR expression and tend to be more chemo-resistant than MDA-MB468 cells. A lentiviral vector system was used, as it can integrate into the genome of both dividing and non-dividing cells, which results in potent transduction and stable expression of the inserted gene. A stable MCF7/PIGR cell line was generated for the experiment. The advantage of a stable cell line is genetic homogeneity, which increases reproducibility.

Experiments were carried out to compare un-transduced cells with PIGR transduced cells as regards chemo-sensitivity and cellular behaviour. In the chemo-sensitivity assay, MCF7/PIGR cells showed a tendency to be more sensitive to doxorubicin when compared with MCF7 cells; by contrast, they were more resistant to docetaxel. Therefore, the overexpression of PIGR may confer sensitivity to doxorubicin in MCF7 cells, while on the other hand it appears to confer resistance to docetaxel. There was no statistically significant difference, however, between the EC_{50} of these two cell types.

Assessment of cellular behaviour, cell viability and cell proliferation, showed that there was no statistically significant difference between MCF7 and MCF7/PIGR cell lines. These results imply that increasing the expression of PIGR to the levels seen in these experiments did not affect cell viability and cell proliferation and could not be translated to significant chemo-sensitivity of MCF7 cells.

Breast cancer is a heterogeneous disease which is classified into different subtypes. Each subtype is different in clinical, molecular and genetic characteristics. Therefore, overexpression of PIGR may affect different breast cancer subtypes differently. For this reason, the effect of PIGR expression was further explored in MDA-MB468 cells which are classified as a basal-like subtype. The results showed that MDA-MB468/PIGR cells tend to be more sensitive to docetaxel but more resistant to

doxorubicin when compared with MDA-MB468 cells. Thus, the overexpression of PIGR in MDA-MB468 may confer sensitivity to docetaxel but it appears to confer resistance to doxorubicin. This implication contrasts to the finding in MCF7 cells and may indicate a cell type specific role of PIGR. However, there was no statistically significant difference between the EC₅₀ of MDA-MB468 and MDA-MB468/PIGR.

PIGR overexpression did not affect cell viability and cell proliferation of MDA-MB468 cells, which is consistent with the finding in MCF7 cells. The role of PIGR in cell proliferation in these breast cancer cell lines is in agreement with its role in endometrial cancer cells, in which alteration of PIGR does not affect cell proliferation (229). Nevertheless, the role of PIGR in cell proliferation varies in other cancer cell types. Increasing PIGR expression can promote cell proliferation in hepatocellular carcinoma cells, but can inhibit cell proliferation in lung cancer (224, 227). Reducing PIGR expression markedly decreases cell proliferation in pancreatic cancer cells (228).

The limitation of the studies in this chapter is the usage of two-dimension (2D) monolayer cultures of breast cancer cell lines as an *in vitro* model of experiment. Most cancer biology research is based on 2D cell culture experiments. However, 2D cell cultures do not contain relevant components of the tumour microenvironment (TME) such as surrounding extracellular matrix, immune inflammatory cells, cytokines, oxygen and nutrient. Cancer cells in 2D culture lose their interactions between both cancer cells and non-cancerous host cells within the TME that they would have *in vivo*. These interactions affect cancer cell behaviour including cell viability and proliferation, response to chemotherapy, RNA and protein expression and other cellular functions (260, 261). Therefore, the studies in 2D cancer cell culture may not accurately predict responses *in vivo*. Improvements in cell culture condition provide culture environments which closely resemble the *in vivo* environment, such as three-dimensional (3D) matrices and coculture with normal cell counterparts such as

myofibroblasts and immune cells. Despite its disadvantages, 2D cell culture is still used for cell culture research because of its simplicity and familiarity to the researchers. There is a great deal of literature with which to compare experimental results. Moreover, cell observation and measurement are uncomplicated thus making the analysis easier.

Another limitation of the studies is a small sample size due to a time constraints. A small sample size decreases statistical power and increases margin of error. Low statistical power affects statistical comparisons which may result in failure to reach statistical significance, even though the differences exists.

In conclusion, the present studies demonstrated that MDA-MB468 cells expressed significantly higher levels of PIGR mRNA and protein expression than MCF7 cells. However, the differential expression of PIGR in these two cell lines did not lead to significant differences in chemo-sensitivity. Viral overexpression of PIGR was also not found to change chemo-sensitivity, cell viability and cell proliferation in either cell line. The assumption is that PIGR *per se* does not affect cellular behaviour and chemo-sensitivity of these breast cancer cell lines. It may be a marker affected by other factors rather than being a direct contributor to the favourable outcome in patients. Many studies have documented that apart from the factors in the tumour cell itself, TME interactions may impact on biological characteristics of tumour cells such as cell growth, invasion, metastasis and treatment response (262, 263). Inflammatory cells in the TME secrete various cytokines which affect tumour cells. In this regard, the interactions between breast cancer cells and inflammatory cells in TME were subsequently explored.

Chapter 4 Effect of PIGR in Breast Cancer Cells on Macrophages and *Vice Versa*

Chapter 4 Effect of PIGR in Breast Cancer Cells on Macrophages and *Vice Versa*

4.1 Introduction

4.1.1 Tumour microenvironment (TME)

Tumours are heterogeneous masses which consist of tumour cells encompassed by TME. TME is an ecological niche composed of immune inflammatory cells, myofibroblasts, fibroblasts, neuroendocrine cells, adipose cells, blood and lymphatic vessels and extracellular matrix which contains cytokines, growth factors and hormones secreted by stromal and cancer cells (264). Cancer cells grow within the surrounding microenvironment and have a complex interaction with it.

Immune inflammatory cells are important elements in the TME. Cancer cells produce cytokines which can induce immune cell infiltration. Diversity, density, location and functional orientation of tumour-infiltrating immune cells, along with tumour heterogeneity, contribute to different effects on tumour progression and patient clinical outcome, which may be positive or negative (265-267). These effects provide the information which is relevant to prognostic and predictive factors in cancer treatment. Immune cells in the TME originate from lymphoid lineage (T lymphocytes, B lymphocytes, natural killer [NK] cells) and myeloid lineage (macrophages, myeloid-derived suppressor cells [MDSCs], dendritic cells [DCs], neutrophils). These cells may be intratumoural (in the core/centre of tumour), peritumoural (in the invasive margin/surrounding stroma) or in the tertiary lymphoid structures. Previous studies have demonstrated the relationship between the level of various immune cells in primary breast cancers and their association with pCR. For example, high levels of tumour-infiltrating lymphocytes (TILs), T lymphocyte subsets (CD4+, CD8+, FOXP3+, CTLA-4+), NK cells and tumour-associated macrophages (TAMs) were demonstrated to be associated with high pCR rates (153-160). The possible underlying mechanisms, however, require further investigation.

Macrophages are the major component of immune infiltrating cells in the TME which comprises up to 50% of the tumour mass (268). TAMs are mainly derived from circulating monocytes which extravasate and differentiate into macrophages (269). Macrophage phenotypes and functions encompass a wide spectrum from proinflammatory phenotypes (M1 or classically activated macrophages) to anti-inflammatory phenotypes (M2 or alternatively activated macrophages) (270, 271). Tumour cells and surrounding stromal cells in the TME produce various cytokines, chemokines and growth factors which are responsible for macrophage differentiation. IFN- γ , LPS and bacterial molecules can polarise macrophages to the M1 macrophage, while IL-4, IL-10, IL-13 and glucocorticoid are responsible for M2 polarisation (272, 273). M1 macrophages produce proinflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-23 and toxic mediators such as nitric oxide (NO) and reactive oxygen intermediates. They promote T helper cell type 1 responses against pathogens causing tumour cells destruction (274). M1 macrophages have a role as efficient antigen presenting cells which enhance microbicidal and tumouricidal activity (270, 275). M2 macrophages have weak antigen presenting ability. They produce anti-inflammatory cytokines such as IL-10 and TGF- β which have mainly immunosuppressive effects (276). These cytokines can promote healing of tissues and stimulate blood vessel formation and thus enhance the tumour promoting effect of M2 macrophages (275). M2 macrophages express high levels of scavenging molecules, mannose and galactose receptors. They also produce ornithine and polyamines by the arginase (ARG) pathway using ARG1 enzyme which supports fibrosis and tissue remodelling functions (277).

The functional heterogeneity of macrophages is dynamic and depends on differential regulation by environmental factors. On the other hand, macrophages themselves have a broad range of effects on the TME. The effect of macrophages on tumour cells has been demonstrated in many cancers such as colon, pancreas, prostate and

breast cancer (278-281). It is well recognised that TAMs closely resemble M2 macrophages which can promote tumour growth and metastasis (282). However, not all TAMs are M2 macrophages, as they are also composed of M1 macrophages which have anti-neoplastic properties (283). Under certain circumstances, TAMs display properties of M1 macrophages promoting inflammation, tissue damage and tumouricidal responses.

4.1.2 PIGR transcription

PIGR transcription factor binding sites are located in the 5' regulatory region, exon 1 and intron 1 of the *PIGR* gene (183, 186, 284). The PIGR promoter TATA box (TTTAA sequence, a DNA sequence in the promoter region that indicates the site of transcription initiation in eukaryotes) is located approximately 30 base pairs upstream of the transcription initiation site in exon 1 (Figure 4.1) (207). PIGR basal promoter activity requires enhancer box (E-box) motif at 71 base pairs upstream of the transcription initiation site (285). Transcription factors, including upstream stimulation factor (USF) 1 and USF 2, can bind to E-box and regulate basal promoter activity. The activator protein-2 (AP-2) binding site at 46 base pairs upstream of the transcription initiation site is another potential regulatory element. Key regulatory factors of PIGR expression are microbes and their products, cytokines and hormones which modulate PIGR transcription via different signal transduction pathways (183, 186, 284).

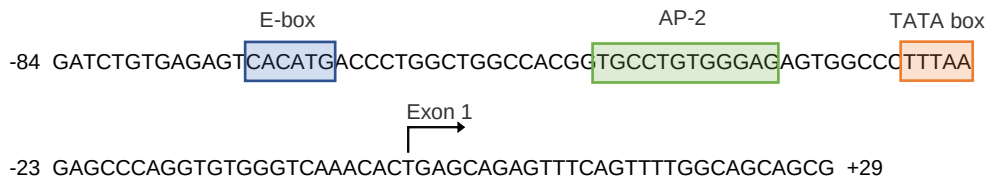


Figure 4.1 Sequence alignment of the promoter regions of the human *PIGR* gene

The transcription start site is indicated with the bent arrow. Numbering is relative to the transcription start site of the *PIGR* gene. Modified with permission from "Transcriptional Regulation of the Human Polymeric Ig Receptor Gene: Analysis of Basal Promoter Element" by P. Hampen and K Phillips, 2002, The Journal of Immunology, 169, 1912-1921. Copyright 2002 by The American Association of Immunologists (285).

4.1.2.1 Regulation of PIGR expression by microbes and their products

Pathogen-associated molecular patterns (PAMPs) are small molecules shared by groups of related microbes and essential for their survival. They can be detected by host cells and activate the host immune response to protect against infection. PAMPs can stimulate PIGR expression by activating toll-like receptors (TLR) on the cell membrane (Figure 4.2). Following activation, adaptor molecule myeloid differentiation primary response 88 (MyD88) is recruited to propagate the signal, then nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling pathway is initiated (286-288). NF- κ B is a family of transcription factors having an important role in modulating the immune response to infection (289). Moreover, NF- κ B can modulate the expression of the genes which regulate cell survival, proliferation and differentiation. There are five subfamilies of NF- κ B, including RelA (p65), Relb, c-Rel, NF- κ B1 (p50) and NF- κ B2 (p52), which can form 15 homo and heterodimers (289). The induction of PIGR transcription via the TLR signalling pathway requires RelA dependent NF- κ B pathway and κ B element (NF- κ B binding site) in intron 1 of *PIGR* gene (290). Studies of the HT-29 human intestinal epithelial cell line have demonstrated that PIGR

expression is increased by stimulation with LPS, double stranded RNA, bacteria of the family *Enterobacteriaceae* and reovirus (287, 290-292).

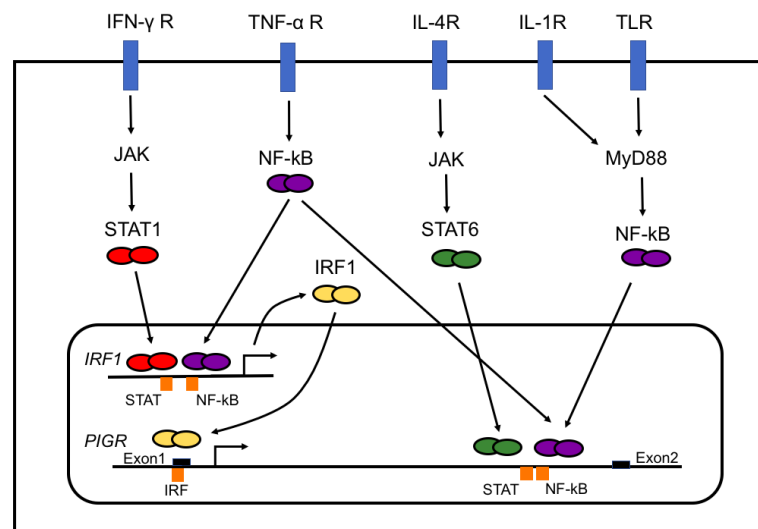


Figure 4.2 Regulation of PIGR transcription by microbial products and host cytokines

A binding of PAMPs and cytokines to their receptors activates NF-κB and JAK-STAT signalling pathways. Activated transcription factors translocate into the nucleus. STAT1 and NF-κB induce de novo synthesis of IRF1 which is an additional transcription factor. IRF1, STAT6 and NF-κB bind to their regulatory elements in the *PIGR* gene and regulate PIGR transcription. Adapted with permission from “Regulation of the polymeric immunoglobulin receptor and IgA transport: New advances in environmental factors that stimulate plgR expression and its role in mucosal immunity” by F. Johansen and C. Kaetzel, 2011, *Mucosal Immunology*, 4(6), 598-602. Copyright 2011 by Springer Nature (284).

4.1.2.2 Regulation of PIGR expression by cytokines

PIGR expression can be modulated directly by cytokines. The key cytokines in PIGR regulation are IFN-γ, TNF-α, IL-1β and IL-4 (183, 284). IFN-γ initiates signal transduction by binding to the IFN-γ receptor on the cell surface (284, 293). The signal is transduced by Janus kinase (JAK), signal transducer and activator of transcription 1 (STAT1) pathway, resulting in tyrosine phosphorylation, dimerization and nuclear translocation of STAT1. Phosphorylated STAT1 dimers induce transcription of interferon regulatory factor 1 (IRF1), the transcription factor that binds to the interferon

stimulated response element (ISRE) in exon 1 of *PIGR* gene to activate *PIGR* transcription (284, 293, 294).

TNF- α binds to receptors on the cell membrane and activates the NF- κ B signalling pathway. The NF- κ B transcription factor is activated and translocates to the nucleus where it binds to the κ B element in intron 1 to induce *PIGR* transcription (183, 290, 295). This κ B element can be induced by the TLR signalling pathway, thus suggesting the cooperation in *PIGR* regulation by microbes and the host cytokine (284). TNF- α induced activation of *PIGR* transcription requires the RelA dependent NF- κ B pathway and may be assisted by RelB (290). TNF- α stimulated NF- κ B also induces *IRF1* gene transcription. IRF1 binds to ISRE in exon 1 and stimulates *PIGR* transcription similar to the IFN- γ signal (296, 297).

The signalling pathway of IL-1 β to induce *PIGR* transcription has not been clearly identified. However, it is likely that IL-1 β activates the NF- κ B element in intron 1 of the *PIGR* gene using an MyD88 dependent signalling pathway that shares many elements with the TLR signalling pathway (284). Binding of IL-4 to its receptor activates the JAK signalling pathway followed by phosphorylation and dimerization of STAT6. After translocation to the nucleus, STAT6 binds to its regulatory element in intron 1 of the *PIGR* gene to activate *PIGR* transcription (298). Other cytokines reported to modulate *PIGR* expression are IL-17 and TGF- β . IL-17 was demonstrated to regulate *PIGR* expression in the HT-29 cell line and in small and large airway epithelium in mice (299, 300). TGF- β was reported to upregulate *PIGR* expression in the Calu-3 cell line (301).

The HT-29 human colon carcinoma cell line and Calu-3 lung adenocarcinoma cell line have generally been used to model studies of *PIGR* expression regulated by cytokines. However, studies in other cell types demonstrated different *PIGR* modulation effects suggesting a cell type specific effect of the cytokines. In mammary epithelial cells from the pregnant ewe, IFN- γ significantly increased *PIGR* expression

via STAT1 and IRF1 regulation as found in HT-29 cells (302). In contrast, IFN- γ did not affect PIGR expression in HEC-1-A and RL95-22 endometrial adenocarcinoma cell lines (303). In normal endometrial epithelial cells, IFN- γ and IL-4 did not significantly increase PIGR expression in an oestradiol free environment. However, with oestradiol present, IFN- γ , IL-4 and TNF- α enhanced PIGR expression in these cells (303). Arumugam *et al.* (228) studied PIGR mRNA expression in a pancreatic ductal carcinoma cell line (AsPC1) and a normal pancreatic cell line (DEChTERT) over time courses of treatment with IL-1, IL-4 and TNF- α . The results did not demonstrate upregulation of PIGR expression. Nevertheless, there is no data concerning breast cancer regarding the regulation of PIGR expression by these cytokines.

4.1.2.3 Regulation of PIGR expression by hormones

PIGR expression is regulated by hormones in a cell specific manner. The expression of PIGR in human endometrium varies during the menstrual cycle suggesting the effect of hormonal changes (206). Oestradiol increased PIGR expression in human endometrial cells and in rat uterus (303, 304). In contrast, oestrogen and progesterone inhibited PIGR expression in early to mid-pregnancy in rabbit mammary gland (305). In the late pregnancy and lactational stages, prolactin and glucocorticoids increased the level of PIGR expression in rabbit and sheep mammary glands (211, 305). In male rats, androgen could upregulate PIGR transcription in prostate, seminal vesicles and lacrimal glands (306, 307).

This chapter aims to investigate the effect of PIGR expression in breast cancer cells on macrophage polarisation and the role of macrophages and cytokines on PIGR expression in breast cancer cell lines.

4.2 Results

4.2.1 Murine bone marrow derived macrophages (BMDMs) can be polarised to M1 or M2 macrophages

BMDMs were selected for *in vitro* models for macrophage polarisation. Treatment with LPS or IL-4 polarises macrophages to form M1 or M2 subtypes, respectively. To demonstrate the efficiency of polarisation after treatment with LPS 50 ng/ml or IL-4 20 ng/ml for 24 and 48 hr, real-time qPCR was used to evaluate the expression of IL-1 β , inducible nitric oxide synthase (iNOS), IL-10 and ARG1 mRNA in BMDM cells. Expression of IL-1 β and iNOS are markers for M1 polarisation, while expression of IL-10 and ARG1 are markers for M2 polarisation (273, 308, 309). Untreated BMDM cells served as the control.

The results demonstrated that in response to LPS treatment, IL-1 β and iNOS mRNA expression in BMDM cells was significantly increased at 24 hr and then declined considerably at 48 hr (Figure 4.3A). In contrast, IL-4 treatment had a minor effect, whether at 24 or 48 hr of treatment. These results corresponded with M1 polarisation of BMDM cells by LPS, which had a greater effect at 24 hr. IL-10 mRNA expression had the same response as IL-1 β and iNOS after LPS and IL-4 treatment, which was inconsistent with its role as an M2 marker. However, the 100-fold change of IL-10 mRNA expression after LPS treatment for 24 hr compared with untreated cells was markedly lower than the fold changes of IL-1 β (340-fold) and iNOS (860-fold) mRNA expression (Figure 4.3B). This demonstrated a spectrum of polarisation of macrophages which tended more to M1 than M2 polarisation by LPS.

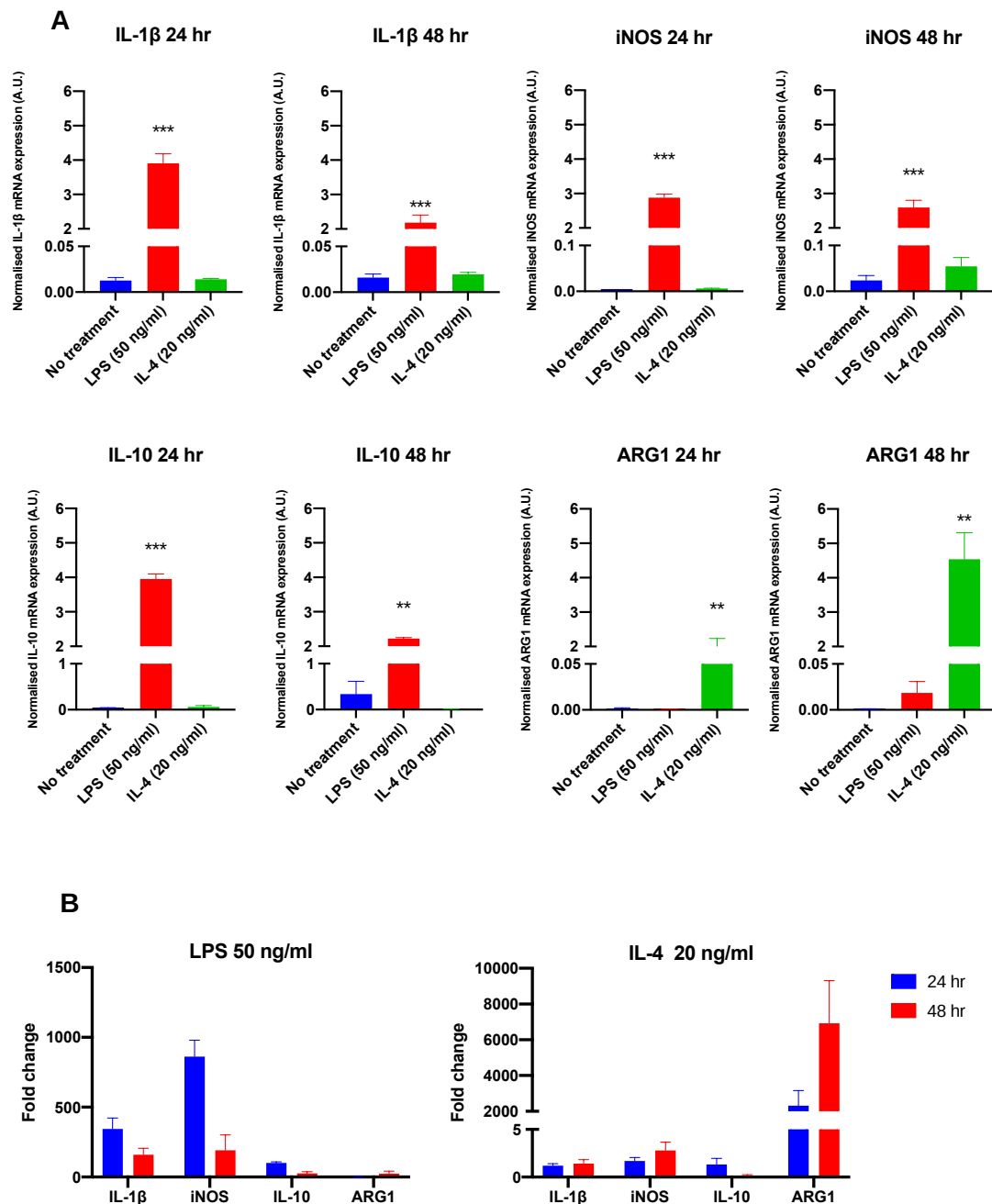


Figure 4.3 Expression of IL-1 β , iNOS, IL-10 and ARG1 mRNA in BMDM cells as markers of their polarisation after LPS and IL-4 treatment

BMDM cells were plated at a density of 8×10^4 cells/well in 12-well plates and incubated for 5 hr. The cells were treated with LPS 50 ng/ml or IL-4 20 ng/ml for 24 and 48 hr as indicated. The expression of IL-1 β , iNOS, IL-10 and ARG1 mRNA in BMDM cells was determined by real-time qPCR. Figure **A** shows normalised IL-1 β , iNOS, IL-10 and ARG1 mRNA expression levels in BMDM cells after treatment with LPS 50 ng/ml or IL-4 20 ng/ml for 24 and 48 hr. Data are presented as mean $\pm$ SEM of three independent experiments. β -actin was used as an endogenous control for normalisation. Statistically significant differences from control (No treatment) were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (one-way ANOVA with Bonferroni post-test correction). Figure **B** shows fold changes in IL-1 β , iNOS, IL-10 and ARG1 mRNA expression levels of BMDM cells after treatment with LPS 50 ng/ml or IL-4 20 ng/ml for 24 and 48 hr compared with untreated cells.

ARG1 mRNA expression, another established M2 macrophage marker, was significantly elevated after IL-4 treatment at 24 hr and further increased at 48 hr. The level of ARG1 expression was very low after LPS treatment, validating polarisation. Thus, ARG1 expression responding to LPS and IL-4 treatment was in agreement with M2 polarisation. All these gene expression markers did not increase in the untreated macrophages, which demonstrated the M0 status of these BMDM cells.

These data demonstrated that, based on gene expression markers, untreated BMDM cells were M0 macrophages and were able to be polarised to M1 or M2 macrophages by treatment with LPS for 24 hr and IL-4 for 48 hr, respectively. These untreated and polarised macrophages were used in subsequent studies with breast cancer cells.

4.2.2 Morphological characteristics of polarised macrophages

M1 and M2 macrophages were obtained by treatment BMDM cells with LPS and IL-4, respectively. The morphology of untreated and polarised BMDM cells was examined under an inverted light microscope. Untreated BMDM cells (M0 macrophages) appeared as a mixture of small roundish and elongated cells with cytoplasmic extensions on the apical ends of the cell bodies (Figure 4.4). M1 macrophages demonstrated enlarged irregular shaped cells with abundant vesicles and were multinucleated (Figure 4.5). M2 macrophages consisted of small round and elongated shaped cells similar to untreated cells (Figure 4.6).

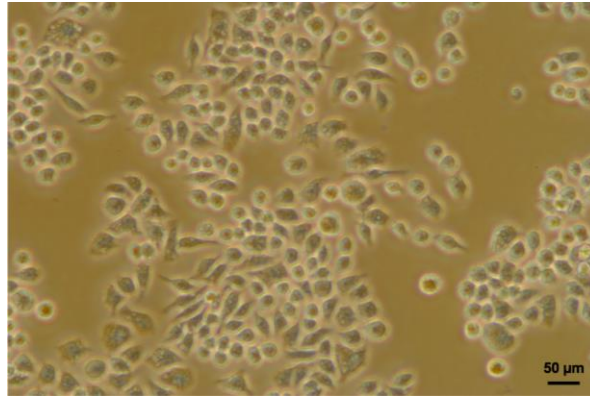


Figure 4.4 Morphology of untreated BMDM cells

The image was collected with an inverted light microscope.

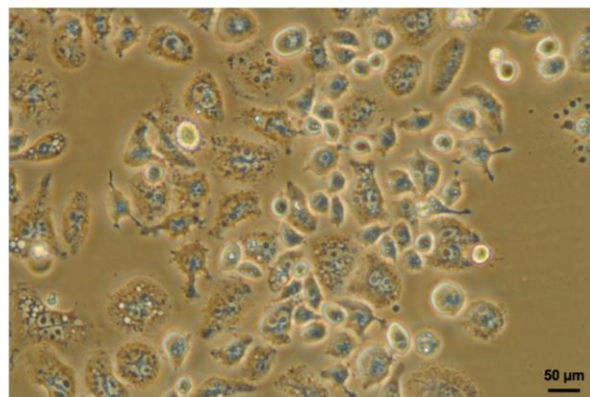


Figure 4.5 Morphology of M1 macrophages

BMDM cells were treated with LPS 50 ng/ml for 24 hr. The image was collected with an inverted light microscope.

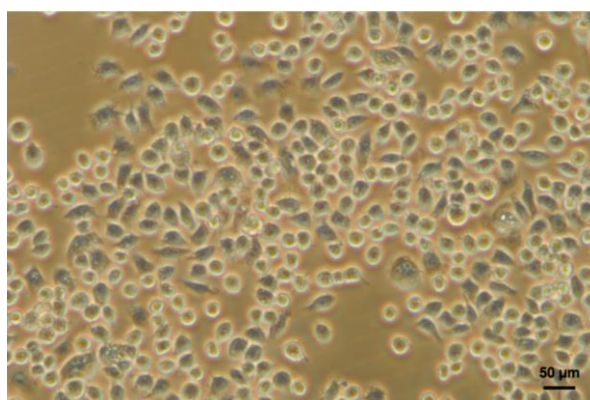


Figure 4.6 Morphology of M2 macrophages

BMDM cells were treated with IL-4 20 ng/ml for 48 hr. The image was collected with an inverted light microscope.

4.2.3 Free secretory component (SC) did not polarise macrophages

The effect of PIGR on macrophage polarisation was investigated. Conditioned media (CM) of MDA-MB468/PIGR cells with or without doxycycline (250 ng/ml) induction were prepared. The presence or absence of free SC (extracellular portion of PIGR cleaved out of the cells) in the CM was demonstrated by Western blotting (Figure 4.7). BMDMs were treated with these CM for 24 and 48 hr before harvesting. Real-time qPCR was performed to evaluate the expression of M1 (IL-1 β and iNOS) and M2 markers (IL-10 and ARG1) which demonstrated polarisation of macrophages. The results showed that there was no significant difference in M1 and M2 markers between BMDMs treated with CM of MDA-MB468/PIGR with or without doxycycline induction whether at 24 or 48 hr (Figure 4.8). These indicated that free SC did not polarise macrophages.

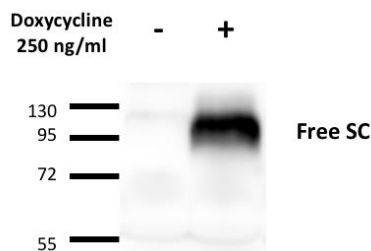


Figure 4.7 Free SC in MDA-MB468/PIGR CM with or without doxycycline induction

Western blotting demonstrates free SC in CM of MDA-MB468/PIGR cells with or without doxycycline treatment. MDA-MB468/PIGR cells were seeded in T175 flasks at a density of 7×10^6 cells/flask and incubated overnight. Cell culture media was changed to fresh complete media with or without doxycycline 250 ng/ml. The cells were further incubated for 48 hr. CM was collected, filtered through a 0.22 μ m syringe filter and stored at -80°C until used. 45 μ l of CM was loaded into each well. The blot was incubated with anti-PIGR antibody at a dilution of 1:5,000.

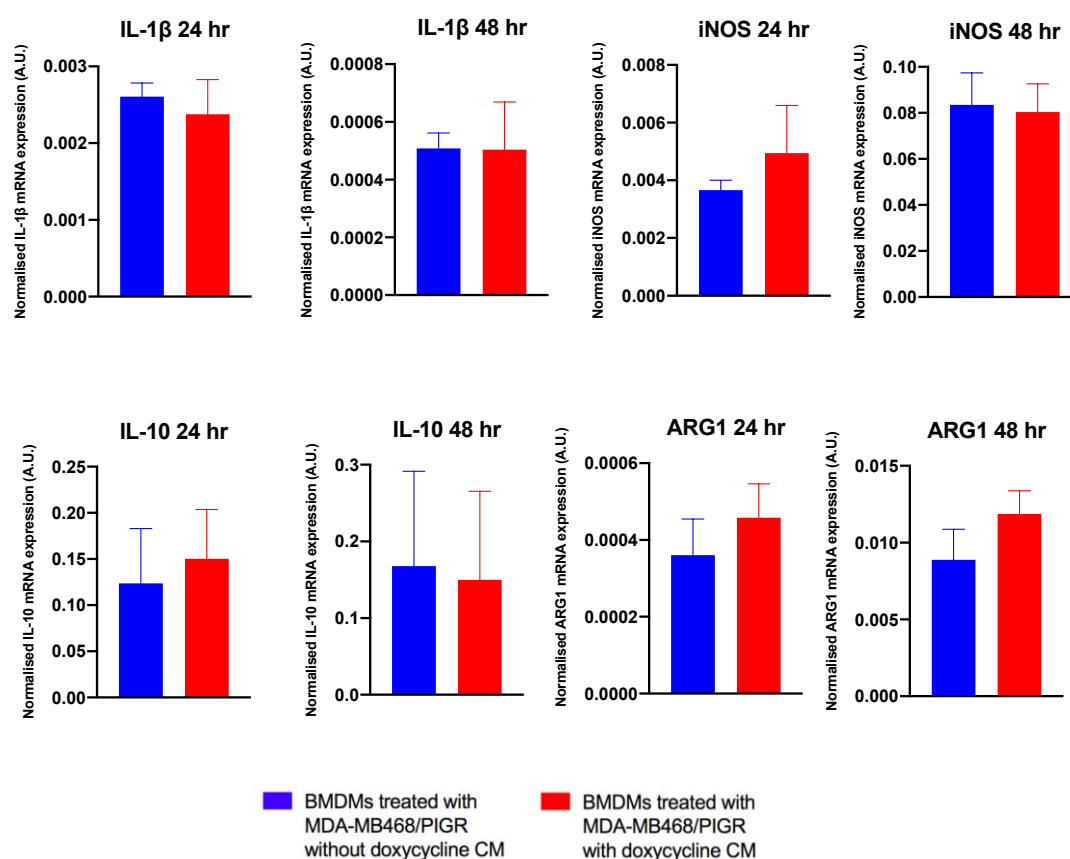


Figure 4.8 Expression of IL-1 β , iNOS, IL-10 and ARG1 mRNA in BMDMs after MDA-MB468/PIGR CM treatment

The figure shows normalised IL-1 β , iNOS, IL-10 and ARG1 mRNA expression levels of BMDMs after treatment with MDA-MB468/PIGR with or without doxycycline 250 ng/ml treatment CM for 24 and 48 hr. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, unpaired t-test). β -actin was used as an endogenous control for normalisation.

4.2.4 M1 macrophages induced PIGR expression in MDA-MB468 cells

To evaluate the effect of polarised macrophages on breast cancer cells, CM of untreated (M0 CM), M1 (M1 CM) and M2 (M2 CM) macrophages were prepared. M1 CM and M2 CM were prepared from BMDM cells after polarisation to M1 or M2 macrophages by treatment with LPS 50 ng/ml for 24 hr or IL-4 20 ng/ml for 48 hr, respectively (Figure 4.9). After washing with warm PBS, the cells were further incubated in fresh media for 24 hr before conditioned media collection. CM prepared from untreated BMDM cells was M0 CM. To serve as the control, media was prepared using the same process as for M1 CM or M2 CM but without cells in the flasks (No

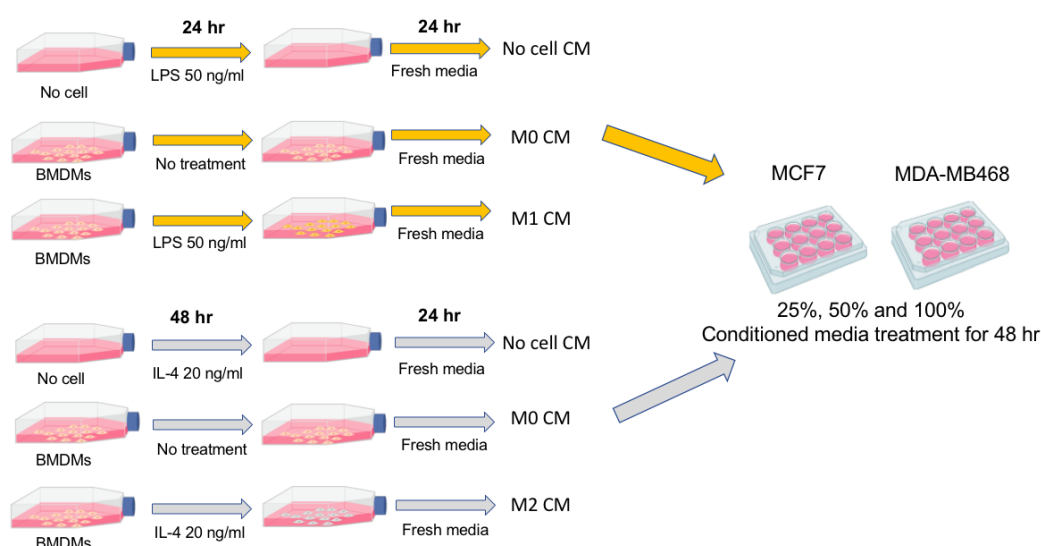


Figure 4.9 Schematic of experimental protocol for macrophage conditioned media preparation and breast cancer cells treatment

BMDM cells were plated in T175 flasks at a cell density of 4×10^6 cells/flask in 35 ml complete medium and incubated for 5 hr. Flasks without cells were prepared for control CM. After the incubation period, LPS 50 ng/ml or IL-4 20 ng/ml was applied as indicated to polarise macrophages to M1 and M2 macrophages, respectively. Untreated cells served as M0 macrophages. The cells were further incubated for 24 (for LPS treatment) or 48 hr (for IL-4 treatment). Cell culture media was discarded and the cells were gently washed with warm $1 \times$ PBS 3 times. 35 ml of fresh complete medium was replaced and the cells were further incubated for 24 hr. Finally, CM was collected, filtered through a $0.22 \mu\text{m}$ syringe filter and stored at -80°C until used. MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well in 1 ml complete medium and incubated overnight. Cell culture media was removed and replaced with 25%, 50% or 100% CM in fresh complete media. PIGR mRNA expression in MCF7 and MDA-MB468 cells was evaluated after treatment with CM for 48 hr. The figure was created with BioRender.com.

cell CM). PIGR mRNA expression in MCF7 and MDA-MB468 cells after treatment with M0, M1 and M2 CM for 48 hr was examined by real-time qPCR. CM treatment was performed in three different concentrations: 25%, 50% and 100%.

Macrophage CM, whether M1 or M2, did not significantly change the PIGR mRNA expression level in MCF7 cells compared with M0 CM (Figure 4.10). Nevertheless, the level of PIGR expression in MCF7 cells was very low.

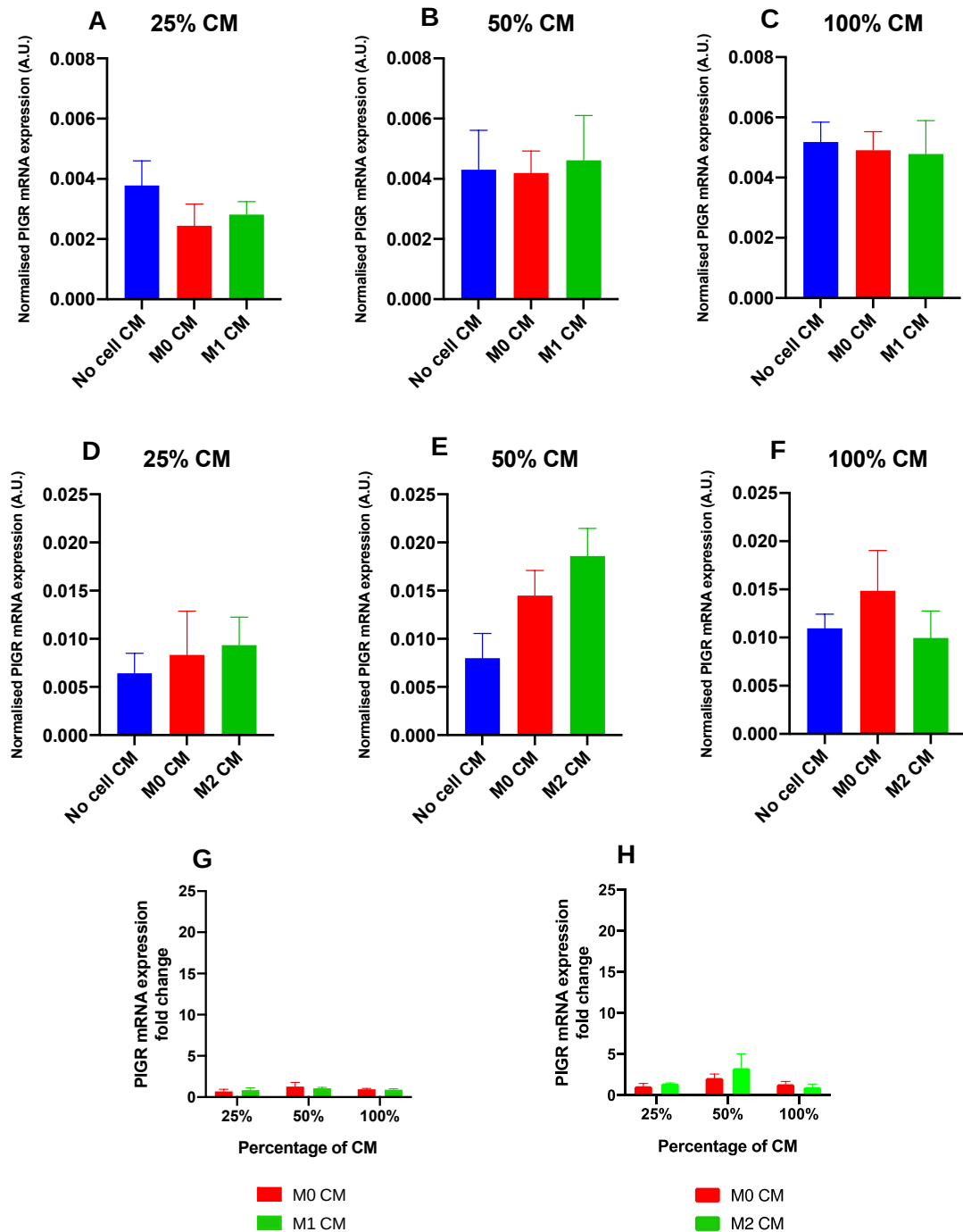


Figure 4.10 Expression of PIGR mRNA in MCF7 cells after macrophage CM treatment

Figure **A-F** show normalised PIGR mRNA expression of MCF7 cells after treatment with 25%, 50% or 100% of No cell, M0 and M1 CM (**A-C**) or M2 CM (**D-F**) for 48 hr. Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, one-way ANOVA with Bonferroni post-test correction). GAPDH was used as an endogenous control for normalisation. Figure **G** and **H** show fold changes of PIGR mRNA expression of MCF7 cells after treatment with 25%, 50% or 100% of M0 and M1 CM (**G**) or M2 CM (**H**) for 48 hr from control (No cell CM). Data are presented as mean $\pm$ SEM of three independent experiments (no statistically significant difference, multiple t-test)

PIGR mRNA expression in MDA-MB468 cells was slightly increased after treatment with M0 CM (Figure 4.11). M1 CM treatment, however, resulted in a significant elevation of PIGR expression in MDA-MB468 cells (Figure 4.11A-C) and the fold change from control was significantly higher than M0 CM treatment (Figure 4.11G). Moreover, M1 CM treatment resulted in a striking dose-dependent increase in PIGR mRNA expression in MDA-MB468 cells, up to 20-fold in 100% CM. M2 CM treatment significantly increased PIGR mRNA expression in MDA-MB468 but on a smaller scale (Figure 4.11D-F). Nevertheless, the fold change did not further increase when compared with M0 CM effect (Figure 4.11H). No cell CM did not affect PIGR expression in MDA-MB468 cells, demonstrating that residual LPS or IL-4, if present, were unlikely to contribute to the induction of PIGR expression by macrophage CM. Therefore, the results revealed an effect of M1 macrophages, which have proinflammatory function as well as a high tumouricidal capacity, on PIGR transcription in MDA-MB468 cells.

4.2.5 IFN- γ not TNF- α proinflammatory cytokine was the regulator of PIGR expression in breast cancer cells

The effect of recombinant human proinflammatory and anti-inflammatory cytokines on PIGR expression was evaluated. MCF7 and MDA-MB468 cells were treated with recombinant human IFN- γ 10 ng/ml, TNF- α 10 ng/ml, IL-10 20 ng/ml or TGF- β 5 ng/ml for 24 and 48 hr. IFN- γ and TNF- α are proinflammatory cytokines, whereas IL-10 and TGF- β are anti-inflammatory cytokines. The levels of PIGR mRNA expression in the breast cancer cells were evaluated by real-time qPCR. The intracellular PIGR protein expression and the secretory component in cell culture media were demonstrated by Western blotting.

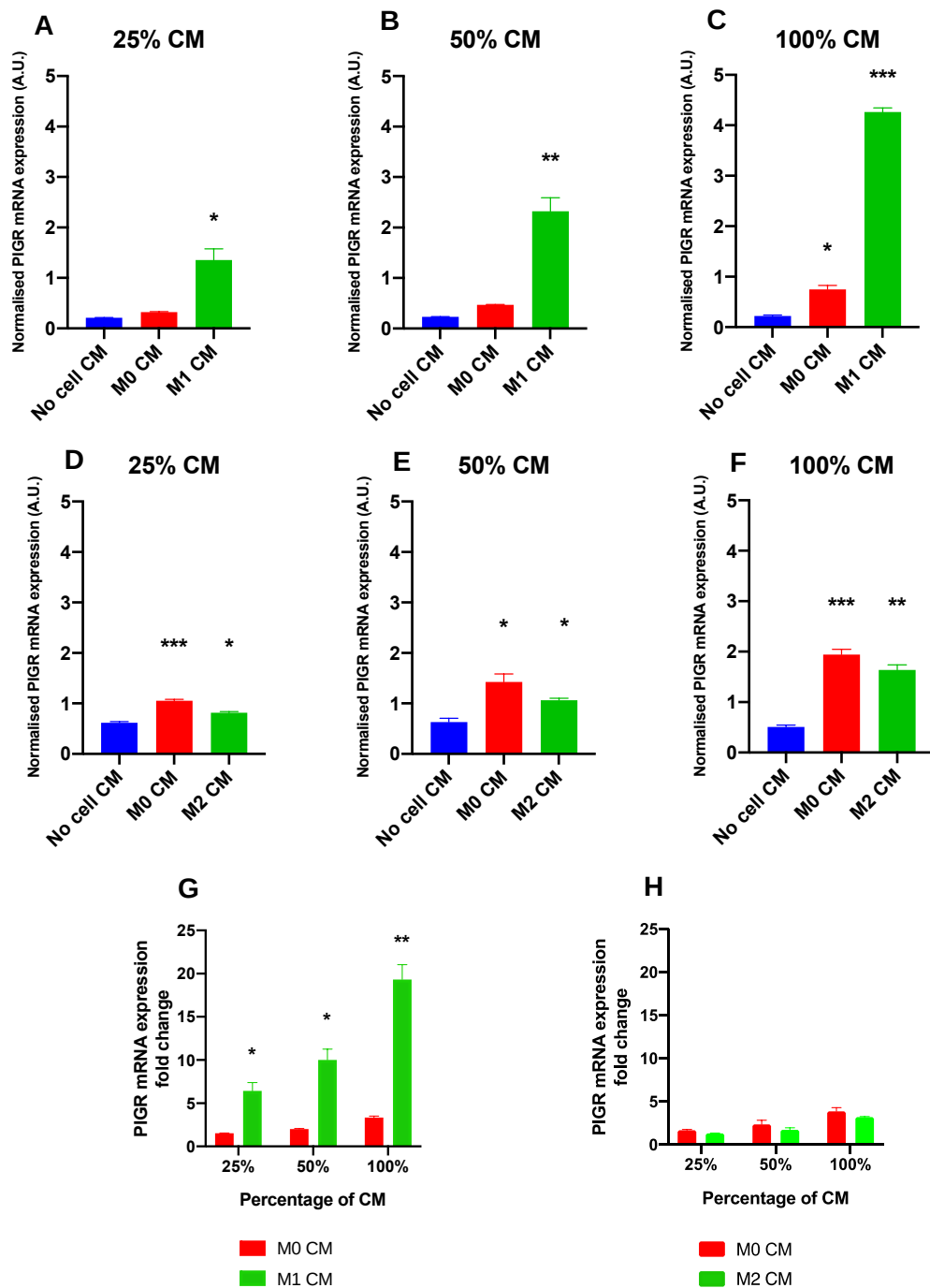


Figure 4.11 Expression of PIGR mRNA in MDA-MB468 cells after macrophage CM treatment

Figure **A-F** show normalised PIGR mRNA expression of MDA-MB468 cells after treatment with 25%, 50% or 100% of No cell, M0 and M1 CM (**A-C**) or M2 CM (**D-F**) for 48 hr. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (No cell CM) were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (one-way ANOVA with Bonferroni post-test correction). Figure **G** and **H** show fold changes of PIGR mRNA expression of MDA-MB468 cells after treatment with 25%, 50% or 100% of M0 and M1 CM (**G**) or M2 CM (**H**) for 48 hr from control (No cell CM). Data are presented as mean $\pm$ SEM of three independent experiments. Statistically significant differences were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (multiple t-test).

IFN- γ treatment was demonstrated to upregulate PIGR mRNA expression in MCF7 cells at 24 hr and significantly at 48 hr, whereas other cytokines had no significant effect (Figure 4.12A, B). The level of PIGR expression in MCF7 cells, however, was very low. The effect of IFN- γ treatment on MDA-MB468 cells was more profound and greater over time (Figure 4.12C, D). While other cytokines were ineffective, the level of PIGR expression in MDA-MB468 cells was significantly increased after IFN- γ treatment at both 24 and 48 hr.

Moreover, by treating MCF7 and MDA-MB468 cells with varied concentrations of IFN- γ (0, 1, 10, 100 ng/ml), PIGR mRNA expression was increased in a dose-dependent manner which reached a plateau at 10 ng/ml (Figure 4.13). In MDA-MB468 cells, IFN- γ at a low concentration (1 ng/ml) significantly increased PIGR mRNA expression, whereas MCF7 cells required at least 10 ng/ml to react. MCF7 cells showed a paucity of PIGR mRNA expression even when stimulated with a high concentration of IFN- γ (100 ng/ml). PIGR protein expression in the cells and free SC in cell culture media demonstrated the same pattern as mRNA expression (Figure 4.14). Intracellular PIGR protein and free SC in culture media were unable to be identified in MCF7 cells. In MDA-MB468 cells, PIGR protein expression and free SC were present with IFN- γ treatment at 1 ng/ml and the expression was increased with 10 ng/ml treatment and then stable at 100 ng/ml. These results suggest an important role of IFN- γ as a proinflammatory cytokine in the TME to modulate PIGR expression in breast cancer cells.

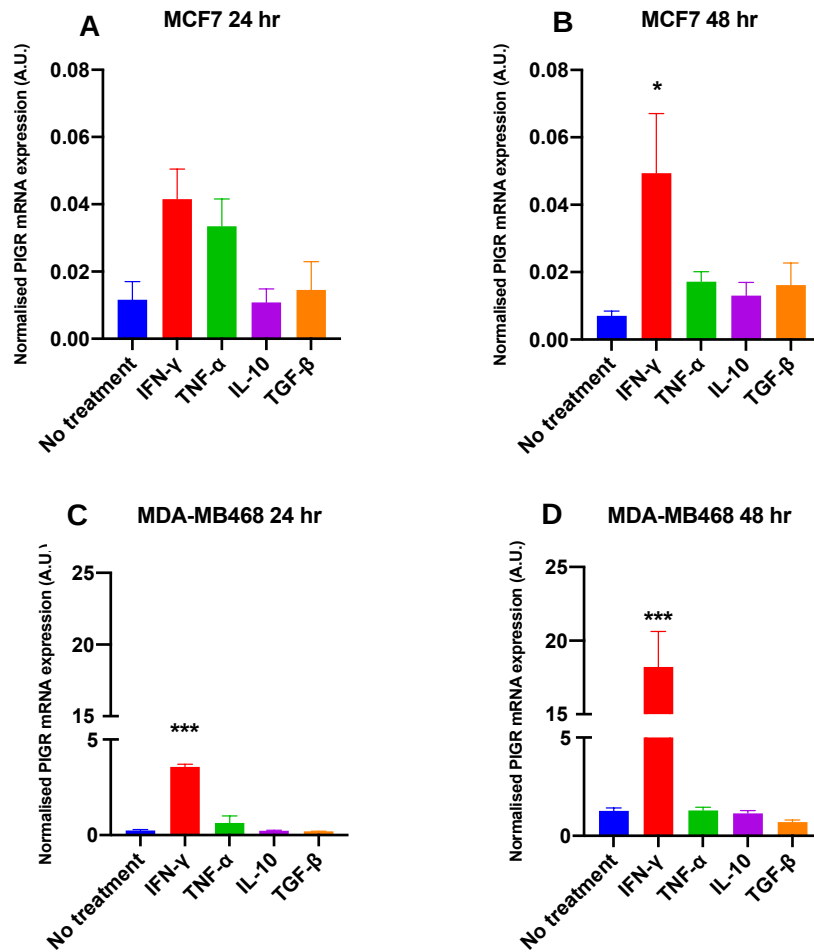


Figure 4.12 Expression of PIGR mRNA in MCF7 and MDA-MB468 cells after different recombinant cytokine treatment

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well and incubated overnight. The cells were treated with IFN- γ 10 ng/ml, TNF- α 10 ng/ml, IL-10 20 ng/ml or TGF- β 5 ng/ml for 24 and 48 hr as indicated. The expression of PIGR mRNA in MCF7 and MDA-MB468 cells was determined by real-time qPCR. Normalised PIGR mRNA expression levels in MCF7 (**A, B**) and MDA-MB468 (**C, D**) cells after different cytokine treatments for 24 (**A, C**) and 48 hr (**B, D**) are shown. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (No treatment) were: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (one-way ANOVA with Bonferroni post-test correction).

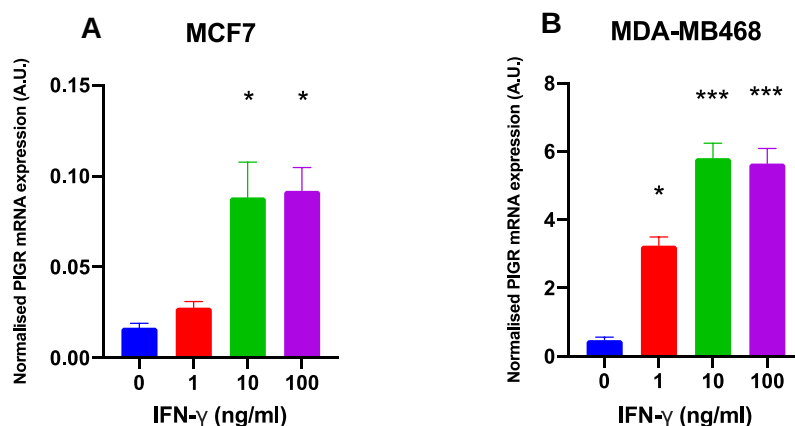


Figure 4.13 IFN- γ dose-dependent effect on PIGR mRNA expression in MCF7 and MDA-MB468 cells

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well and incubated overnight. The cells were treated with IFN- γ 0, 1, 10 and 100 ng/ml for 48 hr as indicated. The expressions of PIGR mRNA in MCF7 and MDA-MB468 cells were determined by real-time qPCR. Normalised PIGR mRNA expression levels in MCF7 (**A**) and MDA-MB468 (**B**) cells after different concentration of IFN- γ treatment for 48 hr are shown. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (IFN- γ 0 ng/ml) were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (one-way ANOVA with Bonferroni post-test correction).

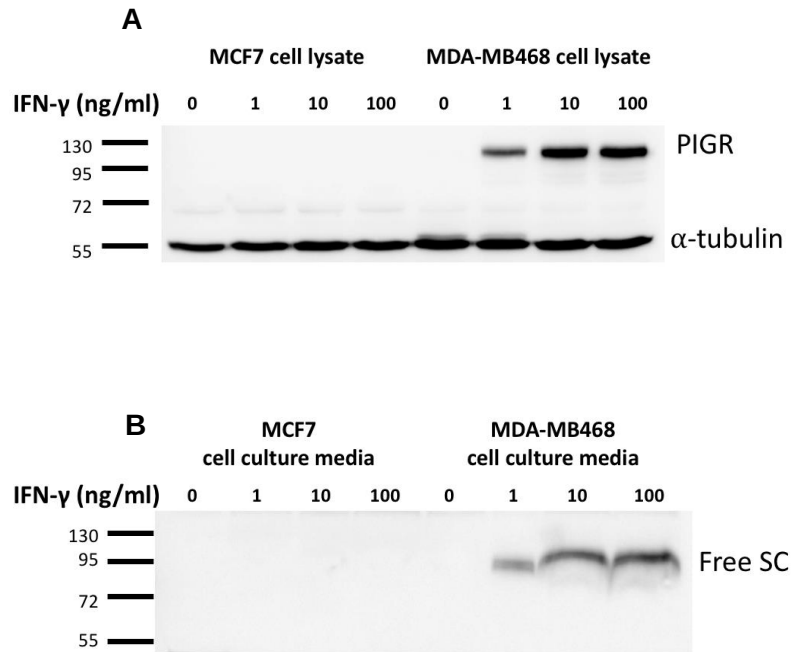


Figure 4.14 Expression of intracellular PIGR protein and free SC in culture media of MCF7 and MDA-MB468 cells after varied concentrations of IFN-γ treatment

Representative Western blotting of two independent experiments for PIGR protein expression in MCF7 and MDA-MB468 cell lysates (**A**) and free SC in concentrated cell culture media (**B**) are shown. MCF7 and MDA-MB468 cells were plated on 60 mm culture dishes at a cell density of 9×10^5 cells/dish in 5 ml complete media and incubated overnight. The cells were treated with IFN-γ 0, 1, 10 and 100 ng/ml in 5 ml serum free media for 48 hr as indicated before harvesting. 100 μg of total protein from cell lysate was loaded into each well. α-tubulin was used as a loading control for cell lysate. All of the concentrated cell culture media was loaded into each well to demonstrate free SC. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000.

4.2.6 IFN-γ was not produced by M1 macrophages

To determine whether IFN-γ which was able to regulate PIGR transcription in breast cancer cells was produced by M1 macrophages, IFN-γ mRNA expression in BMDM cells after polarisation to M1 macrophages with LPS for 24 hr was evaluated by real-time qPCR. However, IFN-γ expression could not be detected. Shorter time periods of LPS treatment were subsequently assessed because IFN-γ mRNA expression may increase early after LPS treatment. Nevertheless, IFN-γ expression still could not be detected at 1, 2, 4, 8 and 12 hr of LPS treatment.

4.2.7 M1 macrophages induced PIGR expression in breast cancer cells through IL-1 β

As regards the increasing level of IL-1 β mRNA expression in BMDM cells after LPS treatment in the macrophage polarisation study as described previously, it is possible that IL-1 β may be the M1 macrophage cytokine regulating PIGR transcription in breast cancer cells. Therefore, the effect of IL-1 β on PIGR expression in breast cancer cell lines was evaluated. This aimed to identify the M1 macrophage secreted cytokine which was able to regulate PIGR expression in breast cancer cells. MCF7 and MDA-MB468 cells were treated with recombinant human IL-1 β 10 ng/ml for 24 and 48 hr. IFN- γ 10 ng/ml treated cells were used as a positive control. Untreated cells served as a negative control group. PIGR mRNA expression levels were determined by real-time qPCR.

The response of PIGR mRNA expression in MCF7 and MDA-MB468 cells to IL-1 β was similar to the response to IFN- γ (Figure 4.15). IL-1 β and IFN- γ significantly increased PIGR mRNA expression in MCF7 cells at 24 and 48 hr but the level of expression was very low (Figure 4.15A, B). In MDA-MB468 cells, PIGR mRNA expression significantly increased at 24 hr and the level was higher at 48 hr after IL-1 β and IFN- γ treatment (Figure 4.15C, D).

The results demonstrated that IL-1 β was able to induce PIGR mRNA expression in MCF7 and MDA-MB468 cells. Together with the finding of the macrophage polarisation study, in which IL-1 β was expressed in BMDM cells after LPS treatment, the results suggest that IL-1 β may be the cytokine produced from M1 macrophages, promoting PIGR mRNA expression in breast cancer cells.

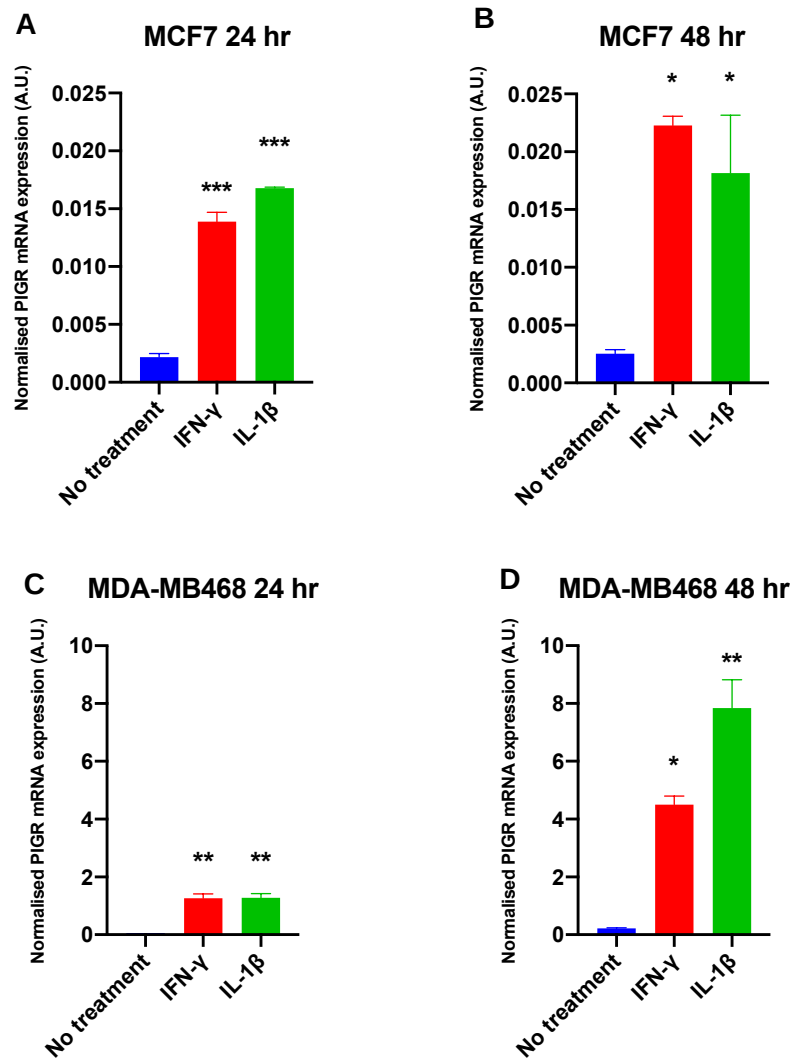


Figure 4.15 Expression of PIGR mRNA in MCF7 and MDA-MB468 cells after IFN-γ and IL-1β treatment

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well and incubated overnight. The cells were treated with IFN-γ 10 ng/ml or IL-1β 10 ng/ml for 24 and 48 hr as indicated. The expression of PIGR mRNA in MCF7 and MDA-MB468 cells was determined by real-time qPCR. Normalised PIGR mRNA expression levels in MCF7 (**A, B**) and MDA-MB468 (**C, D**) cell lines after IFN-γ 10 ng/ml and IL-1β 10 ng/ml treatment for 24 (**A, C**) and 48 hr (**B, D**) are shown. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (No treatment) were: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (one-way ANOVA with Bonferroni post-test correction).

To confirm the action of IL-1 β secreted from M1 macrophages on the expression of PIGR in breast cancer cells, an IL-1 receptor blockade assay using IL-1 receptor antagonist (IL-1RA) was performed. MCF7 and MDA-MB468 cells were treated first with IL-1RA 500 ng/ml for 30 min and then with M1 CM together with IL-1RA 500 ng/ml. Results were compared with treatment with M1 CM alone, with no IL-1RA. Cells treated with M0 CM served as the control. The results showed that PIGR mRNA expression in MDA-MB468 cells treated with M1 CM decreased significantly with IL-1RA co-treatment. This implies that IL-1RA can reverse the effect of M1 macrophages on increasing the PIGR expression in MDA-MB468 cells, confirming the role of IL-1 β as an M1 macrophage cytokine increasing PIGR expression in breast cancer cells. However, there was no significant difference of PIGR expression in MCF7 cells (Figure 4.16).

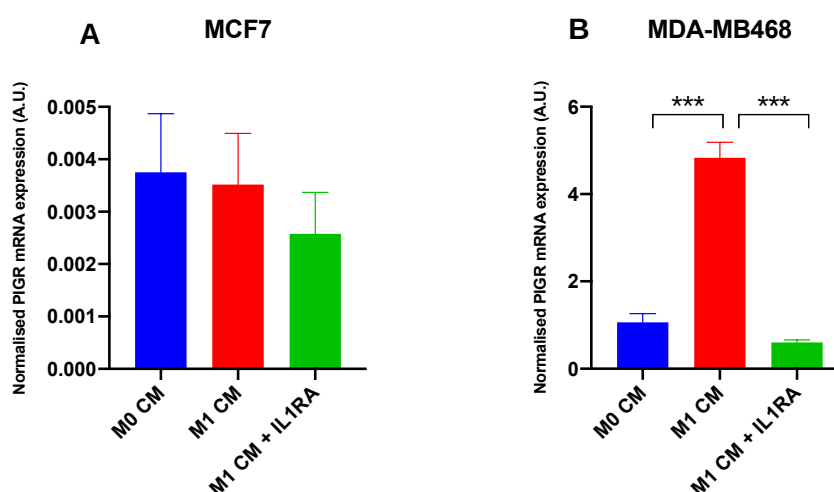


Figure 4.16 Expression of PIGR mRNA in MCF7 and MDA-MB468 cells after M1 CM with or without IL-1RA treatment

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well and incubated overnight. The cells were treated first with IL-1RA 500 ng/ml for 30 min and then with M1 CM together with IL-1RA 500 ng/ml. These were compared with treatment with M1 CM alone, with no IL-1RA. The cells treated with M0 CM served as the control. The expression of PIGR mRNA in MCF7 and MDA-MB468 cells was determined by real-time qPCR. Normalised PIGR mRNA expressions of MCF7 (**A**) and MDA-MB468 (**B**) cells after treatment with M1 CM with or without IL-1RA for 48 hr are shown. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (No cell CM) were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (one-way ANOVA with Bonferroni post-test correction).

4.2.8 IL-1 β increased PIGR expression and the shedding of free SC in a dose-dependent manner in MDA-MB468 cells

The effect of increasing the concentration of IL-1 β on PIGR expression and secretory component secretion in breast cancer cells was further investigated. MCF7 and MDA-MB468 cells were treated with IL-1 β 0, 1, 10, 100 ng/ml for 48 hr. PIGR mRNA expression was evaluated by real-time qPCR. PIGR protein expression and free SC secretion were evaluated by Western blotting.

IL-1 β induced a dose-dependent rise in PIGR mRNA expression in MDA-MB468 cells (Figure 4.17). IL-1 β at a low concentration (1 ng/ml) significantly increased PIGR mRNA expression and the expression was higher with increasing IL-1 β concentration. However, MCF7 cells showed a paucity of PIGR mRNA expression even when stimulated with a high concentration of IL-1 β (100 ng/ml). PIGR protein expression and free SC demonstrated similar patterns to mRNA expression. Intracellular PIGR protein and free SC could not be demonstrated in MCF7 cells (Figure 4.18). In MDA-MB468 cells, PIGR protein expression and free SC were present with IL-1 β treatment at 1 ng/ml and the expression was increased with 10 and 100 ng/ml treatment.

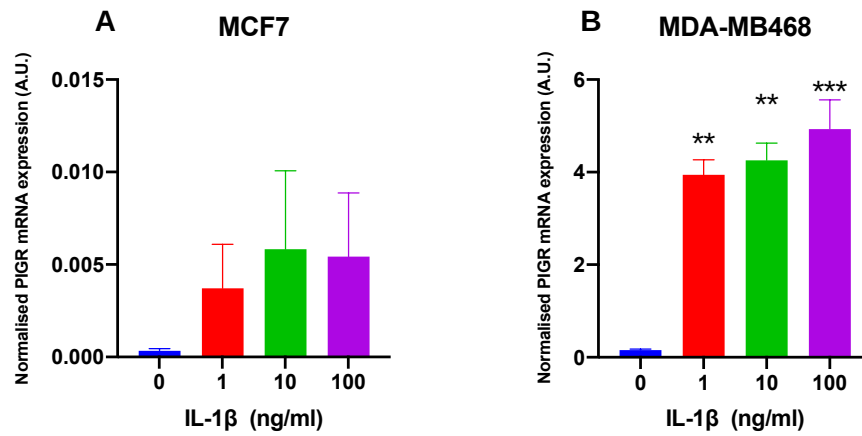


Figure 4.17 IL-1 β dose-dependent effect on PIGR mRNA expression in MCF7 and MDA-MB468 cells

MCF7 and MDA-MB468 cells were plated in 12-well plates at a cell density of 2×10^5 cells/well and incubated overnight. The cells were treated with IL-1 β 0, 1, 10 and 100 ng/ml for 48 hr as indicated. The expression of PIGR mRNA in MCF7 and MDA-MB468 cells was determined by real-time qPCR. Normalised PIGR mRNA expression levels in MCF7 (**A**) and MDA-MB468 (**B**) cells after different concentrations of IL-1 β treatment for 48 hr are shown. Data are presented as mean $\pm$ SEM of three independent experiments. GAPDH was used as an endogenous control for normalisation. Statistically significant differences from control (IL-1 β 0 ng/ml) were: * p < 0.05, ** p < 0.001, *** p < 0.0001 (one-way ANOVA with Bonferroni post-test correction).

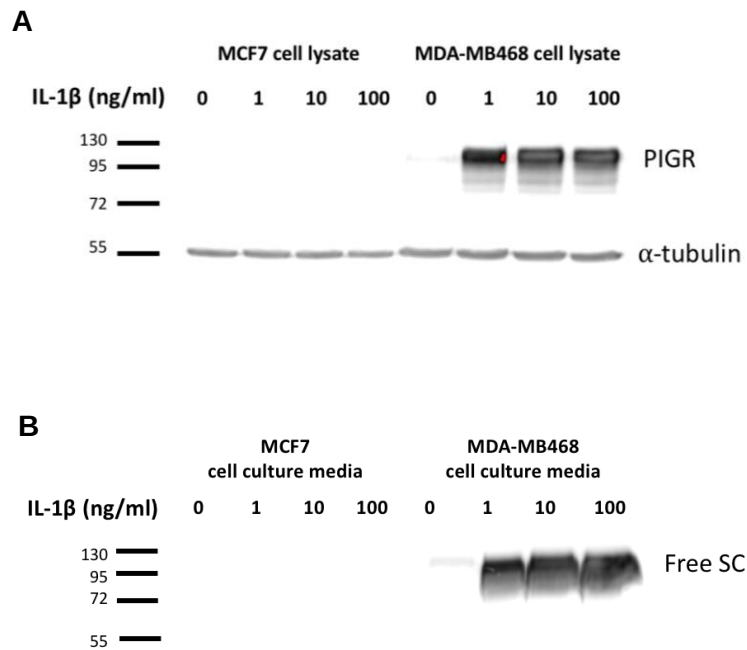


Figure 4.18 Expression of intracellular PIGR protein and free SC in culture media of MCF7 and MDA-MB468 cells after varied concentrations of IL-1 β treatment

Representative Western blotting of two independent experiments for PIGR protein expression in MCF7 and MDA-MB468 cell lysates **(A)** and free SC in concentrated cell culture media **(B)** are shown. MCF7 and MDA-MB468 cells were plated on 60 mm culture dishes at a cell density of 9×10^5 cells/dish in 5 ml complete media and incubated overnight. The cells were treated with IL-1 β 0, 1, 10 and 100 ng/ml in 5 ml serum free media for 48 hr as indicated before harvesting. 100 μ g of total protein from cell lysate was loaded into each well. α -tubulin was used as a loading control for cell lysate. All of the concentrated cell culture media was loaded into each well to demonstrate free SC. The blots were incubated with anti-PIGR antibody at a dilution of 1:5,000.

4.3 Discussion

Although breast cancer has been recognised as a relatively non-immunogenic cancer; it is, however, characterised by a highly inflammatory microenvironment which has many immune infiltrating cells with distinct functions (310). Immune infiltrating cells provide necessary signals that activate transcription factors in cancer cells, resulting in tumour cell growth, progression, and metastasis. They also have predictive and prognostic values in terms of response to treatment and patient outcome (311). Macrophages are the crucial component of the immune infiltrating cells in the TME. They have remarkable plasticity in their phenotype which responds to environmental stimuli. Macrophages can be polarised into the spectrum between M1 (proinflammatory phenotype) and M2 (anti-inflammatory phenotype) and present mixed characters in their protein expressions and cytokine secretions depending on the balance between M1 and M2 (312). In the TME, macrophages are involved in the behaviour of the cancer, such as tumour growth, angiogenesis, tumour metastasis and response to hormonal and chemotherapeutic agents (313).

PIGR expression level varies between cancers and is related to prognosis. In breast cancer, an increased PIGR expression is associated with a favourable outcome. In addition, the pilot study carried out in the department demonstrated that PIGR expression was increased in breast cancers achieving a pCR after NAC and was associated with a good prognosis. However, the underlying mechanism is unknown. The previous chapter demonstrated that PIGR did not directly affect cell viability, cell proliferation and chemo-sensitivity of breast cancer cells. Therefore, this chapter aimed to investigate effects of PIGR in breast cancer cells on macrophages and vice versa, which may be a surrogate indicator of treatment outcome and prognosis in patients with breast cancer.

An *in vitro* model of macrophage polarisation was generated and characterised using BMDM cells. BMDMs and peritoneal macrophages are the most common primary

macrophages used for *in vitro* studies. Zajd *et al.* (312) directly compared primary BMDMs to peritoneal macrophages in terms of their functions, including polarisation. The results showed that alterations of polarisation markers, whether surface molecular markers, gene expression or cytokine/chemokine release of BMDM cells, were greater than those in peritoneal macrophages after cytokines stimulation. Thus, primary BMDM is the preferred cell type for studying macrophage polarisation. BMDM used in this present study was an immortalised macrophage cell line derived from primary BMDM cells of mice. This macrophage cell line has the ability to be polarised to M1 or M2 macrophages and can be used as a model for macrophage polarisation in the place of primary macrophages (314).

LPS and IL-4 were used to polarise macrophages to form M1 and M2 macrophages, respectively. LPS treatment increased IL-1 β and iNOS mRNA expression in BMDM cells which demonstrated M1 macrophage polarisation, whilst IL-4 treatment induced the expression of ARG1 which is an M2 macrophage marker. Interestingly, IL-10 mRNA expression increased after LPS treatment but did not increase after IL-4 treatment which is in disagreement with its role as an M2 marker. However, the fold change of IL-10 mRNA expression in response to LPS was considerably lower than the fold change of IL-1 β and iNOS mRNA expression. This can be explained by the spectrum of macrophage polarisation that can contribute to mixed characters of M1 and M2 macrophages as described previously. Another possible explanation is that IL-10 upregulation in response to IL-4 treatment may require longer stimulation or more time for full expression. The results are in line with findings in a previous study investigating the responses of BMDMs to polarising cytokines conducted by Zajd *et al.* (312). The differences are that Zajd *et al.* (312) used IFN- γ for M1 polarisation and a combination of IL-4 and IL-13 for M2 polarisation. BMDMs were treated for 24 hr. However, the results also showed that IL-10 mRNA and protein secretion was increased after M1 polarisation but decreased after M2 polarisation. In addition,

Chanteux *et al.* (315) demonstrated an increasing level of IL-10 production in human alveolar macrophages after LPS treatment.

The effect of PIGR on macrophage polarisation was examined. However, free SC which is an extracellular portion of PIGR cleaved out of the cells, did not have a substantial effect on macrophage polarisation. Turula *et al.* (316) demonstrated the effect of PIGR on macrophages. PIGR-deficient mice (PIGR KO mice) were generated to study the role of PIGR and SIg in mucosal homeostasis. They found that PIGR KO mice had higher numbers of macrophages and dendritic cells in Peyer's patches in the intestine than wild type control mice. Moreover, IFN- γ and iNOS mRNA levels in the small intestine increased in PIGR KO mice compared with wild type control mice, possibly because of the increase in certain immune cell subsets. However, the effect of PIGR or free SC on macrophage polarisation has not been documented.

It has been demonstrated that PIGR expression is regulated by various factors including microbes and their products, cytokines and hormones (183, 186, 284). Thus, in the TME, inflammatory cells and secreted cytokines may participate in regulation of PIGR expression in cancer cells. Although many studies have demonstrated the effect of cytokines on PIGR expression in several cell types, it has not been addressed in breast cancer cells.

The CM from polarised macrophages was used to perform the experiments with MCF7 and MDA-MB468 breast cancer cell lines, which differ in their phenotypical characteristics and molecular subtypes. The MCF7 cell line is classified as a low aggressive luminal A subtype, while the MDA-MB468 cell line is classified as an aggressive basal-like subtype (234). The results revealed that M1 CM increased PIGR expression in MDA-MB468 cells in a dose-dependent manner, while M2 CM had no effect. These results demonstrated the effect of M1 macrophages on MDA-MB468 cells, in which the soluble products of M1 macrophages can regulate the expression of PIGR in this cell line. In the host defence mechanism, M1 macrophages

release proinflammatory cytokines and possess microbicidal capacity. In the TME, M1 macrophages exert tumouricidal activity and elicit tumour destruction (270, 271). They can release lytic factors which cause tumour cell lysis directly and also secrete factors/cytokines which indirectly activate anti-tumour effects of other cell types (317). Multiple clinical studies have reported the association between TAMs and poor prognosis in breast cancer patients but most studies did not evaluate the effect of different subsets of TAMs (318). Jeong *et al.* (274) assessed the prognostic value of TAMs in invasive breast cancer by immunohistochemistry (IHC) of tissue microarrays. CD68, CD11c and CD163 were used as markers of pan-macrophages, M1 and M2 macrophages, respectively. The results showed that high numbers of CD68+ TAMs (pan-macrophages) infiltration related with unfavourable clinical and pathological parameters and determined poor prognosis. CD163+ TAMs (M2 macrophages) in the tumour nest related with poor prognosis as regards overall survival and disease free survival. In contrast, infiltration by CD11c+ TAMs (M1 macrophages) in the tumour stroma led to a good prognosis with greater overall survival and disease free survival. Heys *et al.* (319) evaluated the role of TAMs in large and locally advanced breast cancer patients who received NAC. CD68, a suppressor of cytokine signalling (SOCS)3 and SOCS1 were used as markers of pan-macrophage, M1 and M2 macrophages, respectively. SOCS3 inhibits STAT3 signalling which is responsible for the macrophage anti-inflammatory effect, while SOCS1 inhibits proinflammatory signalling pathways downstream of IFN- γ and toll-like receptors 4. The results demonstrated that a high number of SOCS3+ TAMs (M1 macrophages) were associated with a pCR after NAC. On the other hand, there was no association between SOCS1+ TAMs (M2 macrophages) and response to NAC.

Therefore, the findings from the present study (M1 CM increased PIGR expression in MDA-MB468 cells) can supplement and support previous clinical data, which showed the relationship of high PIGR expression and good prognosis in breast cancer

patients. They also support the pilot study finding that the PIGR expression level was high in breast cancers which subsequently achieved a pCR after NAC. These favourable outcomes related to increasing PIGR expression may be explained by a beneficial anti-tumour effect of M1 macrophages in the TME. The level of PIGR mRNA expression in MCF7 cells was not affected by polarised macrophages whether M1 or M2. However, the level of PIGR expression in MCF7 cells was very low and may not have a physiological role in this breast cancer cell line.

The ability of recombinant cytokines to increase the expression of PIGR in breast cancer cells was examined. IFN- γ and IL-1 β were shown to significantly increase PIGR mRNA and protein expression in MDA-MB468 cells in a dose-dependent manner. The free SC was also documented in cell culture media from these cells in the same pattern, which clearly demonstrated the presence of functional protein. However, IFN- γ and IL-1 β minimally increased PIGR mRNA expression in MCF7 cells which correlated with undetectable protein expression, both intracellular and in secretion. The responses of breast cancer cell lines to IFN- γ and IL-1 β are in keeping with previous reports that IFN- γ and IL-1 β increased PIGR expression in human colon carcinoma cell line (HT-29) and lung adenocarcinoma cell line (Calu-3) (293, 320-325). Nevertheless, a different response was observed for TNF- α , which is another proinflammatory cytokine. TNF- α was demonstrated to increase PIGR expression in the colon carcinoma cell line, while there was no effect on breast cancer cells in this study (326).

Previous studies in a colon carcinoma cell line (HT-29) showed that TNF- α , IL-1 β and TLR ligands regulate PIGR transcription via activation of NF- κ B signalling pathway (Figure 4.19) (290, 295). NF- κ B is a proinflammatory transcription factor family expressed in almost all cell types. It modulates more than 500 genes with different functions such as inflammation, cellular transformation, proliferation, invasion and metastasis (327). Generally, NF- κ B dimers bind to an inhibitory κ B protein (I κ B) and

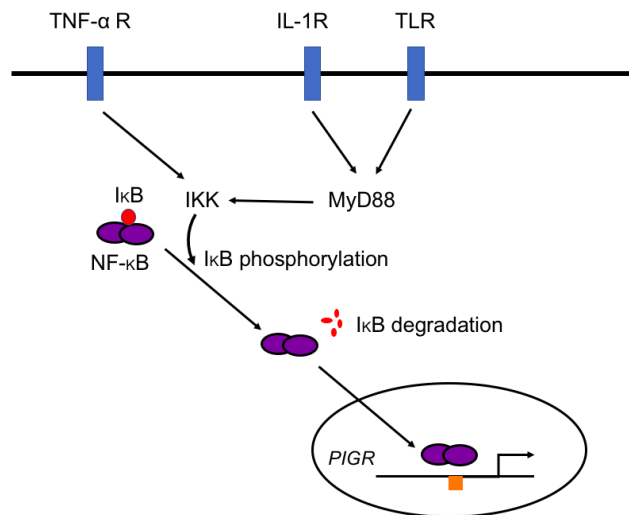


Figure 4.19 Regulation of PIGR transcription by TNF- α , IL-1 β and TLR ligands in HT-29 colon carcinoma cell line

The binding of TNF- α to its receptors on the cell surface activates IKK, while IL-1 β and TLR ligands activate IKK via the MyD88 dependent signalling pathway. I κ B which binds to NF- κ B is phosphorylated by IKK leading to NF- κ B releasing. Then, NF- κ B translocates into the nucleus to activate PIGR transcription.

present in an inactive form in the cytoplasm. TNF- α stimulates cells through their receptors on the cell surface resulting in the activation of I κ B kinase (IKK), while TLR and IL-1 β signals pass through the MyD88 dependent signalling pathway before activating IKK. The activated IKK phosphorylates I κ B, leading to I κ B degradation. After I κ B degradation, NF- κ B is released and then translocates into the nucleus where it binds to the κ B element in intron 1 to activate PIGR transcription. In the present study, TNF- α did not increase PIGR expression in breast cancer cells. This suggests that the described NF- κ B signalling pathway activated by TNF- α in colon carcinoma cells is not completed or activated in breast cancer cells. These different cellular responses to the same extracellular signal may be caused by the specific receptors and intracellular signalling molecules that are active in different cell types.

Another point of interest from this present study is that while TNF- α did not increase PIGR expression in breast cancer cells, IL-1 β , which also works through the NF- κ B

signalling pathway, did enhance PIGR transcription. This result parallels the findings of Bruno *et al.* (290) which examined the PIGR expression in RelA knockdown HT-29 cells. RelA is the major subunit of NF- κ B which is necessary for PIGR induction in a colon carcinoma cell line. They found that RelA knockdown inhibited the upregulation of PIGR mRNA by TNF- α but not by LPS which is the TLR ligand. The suggested explanation was that TLR activation might induce additional signalling pathways that compensate for the loss of RelA and lead to PIGR induction (290). Since IL-1 β induces PIGR expression through MyD88 and NF- κ B signalling pathways in the same way as TLR ligand, this may explain the findings from the present study.

While IFN- γ and IL-1 β , which are proinflammatory cytokines, could increase PIGR transcription, anti-inflammatory cytokines, whether IL-10 or TGF- β , did not have any effect on PIGR transcription in either MCF7 or MDA-MB468 cells. These results indicate the important effect of proinflammatory cytokines over anti-inflammatory cytokines on PIGR expression in breast cancer cells. This is consistent with the finding for macrophage CM that M1 CM upregulated PIGR expression, while M2 CM did not. Although the expressions of PIGR in MDA-MB468 cells and MCF7 cells differed, with very low expression in MCF7 cells, PIGR expression in MCF7 cells showed the same response as MDA-MB468 cells to these cytokine treatments. However, the effect on MDA-MB468 was greater.

The effective cytokine secreted from M1 macrophages was determined. IL-1 β mRNA expression increased in M1 macrophages, while IFN- γ mRNA expression was not detected. The role of IL-1 β secreted by M1 macrophages in increasing expression of PIGR was confirmed by IL-1 receptor blockade, indicating that IL-1 β is the M1 macrophage cytokine that enhances PIGR expression in breast cancer cells. IL-1 β is a potent proinflammatory cytokine which has multiple effects not only on immune cells, regarding host defence mechanisms against infection and injury, but also on

cancer cells. The association of macrophages, IL-1 β and breast cancers has been reported in several studies. Jin *et al.* (328) studied the level of IL-1 β in breast cancer tissues. They found that IL-1 β level in invasive carcinomas was significantly higher than in ductal carcinoma *in situ* and benign lesions (328). Moreover, a high level of IL-1 β in breast cancer tissues correlated with CD68 positivity, which is a macrophage marker, and was associated with pathologic features of an aggressive tumour. Macrophage-derived IL-1 β was also shown to enhance migration and lymphatic adhesion in MCF7 and MDA-MB231 breast cancer cell lines (329). Additionally, IL-1 β has been reported to be associated with angiogenesis and invasiveness of breast cancer cells (330-332). However, contrary effects of IL-1 β have been demonstrated, including inhibition of cell proliferation and growth of breast cancer cells (333, 334). The contradictory results may be caused by the differences in cell line model, concentration of IL-1 β used and condition of the experiment (335). IL-1 β has been reported to support the growth of oestrogen-dependent tumours and its concentration determined the stimulatory or inhibitory effect on cell growth. In MCF7 cells, a high level of IL-1 β caused inhibition of cell growth compared with a moderate level which stimulated the growth of cells (335). The effect of IL-1 β on chemo-sensitivity of breast cancer cells was demonstrated *in vitro* and *in vivo* by Greco *et al.* (336). They reported that IL-1 β secreted by mesenchymal stem cells increased sensitivity to carboplatin in MDA-MB231 breast cancer cells. However, there was no correlation between the expression *in situ* of IL-1 and pCR following NAC in the study using LLABC tissue from patients (153).

IFN- γ is mainly produced by T cells and NK cells in response to pathogens or immune stimuli. In the TME, TILs are the major source of IFN- γ . However, some myeloid cells have also been demonstrated to have the potential to produce IFN- γ (337-339). In various cancers, including breast, colorectal and hepatocellular cancer, IFN- γ inhibits proliferation of tumour cells by increasing the expression of cell cycle inhibition

proteins and inducing apoptosis (340-342). Moreover, IFN- γ can suppress the differentiation of monocyte-derived TAMs in the TME and polarise TAMs from M2 to M1 macrophages, which promotes their tumouricidal capacity (343). Therefore, IFN- γ performs anti-tumoural functions through an effect on both the tumour cells and the immune cells in the TME. On the other hand, IFN- γ has also been reported to promote tumourigenesis, angiogenesis, migration, invasion and epithelial mesenchymal transition of cancer cells, which contribute to subsequent cancer evasion and an adverse outcome in certain cancers such as papillary thyroid and ovarian cancer (343-345). The effect of IFN- γ on chemo-sensitivity in breast cancer was demonstrated by Rogers *et al.* (346). They found that IFN- γ can enhance the apoptotic effect of doxorubicin, etoposide, 5-FU and SN38 on breast cancer cells. Nevertheless, there was no significant association between the expression *in situ* of IFN- γ and pCR following NAC in the study using LLABC tissue from patients (153).

The limitation of the studies in this chapter are that they were conducted with murine BMDM. Murine BMDM is one of the most common *in vitro* models of macrophages, apart from human monocyte-derived macrophages from peripheral blood and human leukemic cell lines THP-1 (347). There are many conflicting reports regarding macrophage behaviour such as macrophage polarisation, the roles in response to injury or infection and interaction with other cells. One possible factor which causes the discrepancy of the findings is that a different source of macrophages used as a model for the study. Spiller *et al.* (347) compared gene expression profiles of macrophages from different sources after *in vitro* polarisation. They found several differences in gene expression trends among macrophages from different sources, especially between murine BMDMs and human monocyte-derived macrophages, according to genes commonly used as phenotype markers, genes associated with angiogenesis and tissue regeneration (347). The differences in gene expression between murine and human macrophages suggest substantial differences in their

function. Additionally, the *in vitro* conditions of the experiment lost the effect of surrounding tissue microenvironment, which has a major effect on macrophage behaviour *in vivo*. These suggest that the results from murine macrophages *in vitro* studies need to be confirmed in human macrophages *in vivo*.

In conclusion, this present study demonstrated a previously unknown interplay between macrophages and breast cancer cells, regarding M1 macrophages enhancing PIGR expression in breast cancer cells through IL-1 β , while free SC secreted from breast cancer cells did not affect macrophage polarisation. The effect of M1 macrophages on PIGR expression was clearly demonstrated in MDA-MB468 cells, which are a basal-like subtype of breast cancer. In contrast, MCF7 cells, which are a luminal A subtype, expressed a very low level of PIGR which may not be physiologically relevant. This study also provided evidence that IFN- γ has a similar effect to IL-1 β on increasing PIGR expression in breast cancer cells. Although, in a TME, IL-1 β is secreted primarily by myeloid cells, it also can be produced and secreted by a variety of cell types such as innate immune cells, fibroblasts and cancer cells (335, 348, 349). IFN- γ is predominantly produced from T lymphocytes and NK cells (350, 351). These imply that the complexity of PIGR regulation *in vivo* is likely to involve the coordination of various immunomodulatory factors and that the elevation of PIGR expression in breast cancer *in vivo* may reflect the polarisation state of tumour-associated immune cells.

**Chapter 5 Expression of PIGR and Ability
to Predict a pCR in Breast Cancer in
Women Undergoing NAC**

Chapter 5 Expression of PIGR and Ability to Predict a pCR in Breast Cancer in Women Undergoing NAC

5.1 Introduction

Many tumours, such as colon, breast, endometrial, bladder and hepatocellular cancer show overexpression of PIGR (217). Alteration of PIGR expression, increased or decreased, has been shown in various malignant and premalignant lesions and to be related to cancer outcome. Ai *et al.* (215) studied the role of PIGR in hepatocellular carcinoma and reported that overexpression of PIGR was associated with early recurrence and metastasis. In colon cancer, PIGR expression was associated with liver metastases and a poor outcome in these patients after hepatic resection (216). Sixty-eight percent of osteosarcoma tissues expressed PIGR, which was associated with poor prognosis (217). PIGR expression was documented in 69.2% of paraffin-embedded glioma tissue samples by immunohistochemical staining and was associated with poor prognosis (218). Recently, Ohkuma *et al.* (219) investigated the correlation of PIGR with pancreatic cancer using archived FFPE tumour tissues and pancreatic cancer patient-derived xenografts. They reported that a high level of PIGR protein expression on immunohistochemical staining was associated with poor survival rates of the patients and increasing expression of PIGR mRNA in patient-derived xenografts correlated with chemo-resistance.

In contrast, other studies have reported favourable outcomes in patients with cancers with increased PIGR expression. Fristedt *et al.* (220) studied PIGR expression in adenocarcinoma of the upper gastrointestinal tract by IHC. They reported that intestinal metaplasia expressed higher levels of PIGR than normal tissue and cancer cells. In cancer, however, high PIGR expression was related to low recurrence rate and prolonged survival. Gologan *et al.* (221) demonstrated more frequent lymph node metastasis in patients with oesophageal and gastro-oesophageal junction adenocarcinomas that did not express PIGR. Overexpression of PIGR in epithelial

ovarian cancer, using immunohistochemical staining of tissue microarrays, related with favourable features of the tumour and improved overall survival of the patient (223). Fristedt *et al.* (352) examined the prognostic significance of PIGR in tissue microarrays of pancreatic and peri-ampullary adenocarcinoma from patients treated with pancreaticoduodenectomy. The result demonstrated that a low PIGR expression level significantly correlated with an impaired five-year survival and early recurrence of the cancer. In endometrial cancer, overexpression of PIGR correlated with a less aggressive type cancer (222). Down regulation of PIGR was demonstrated in pre-invasive bronchial lesions, in the majority of lung cancers and during lung cancer progression (224, 225).

Breast cancer was one of the earliest cancers to be shown to express PIGR. Harris *et al.* (208, 209) demonstrated intense IF staining of secretory component (SC) in invasive breast cancer cells and metastatic cancer cells in ALNs. Plasma concentration of SC in patients with metastatic breast cancer was higher than in normal women and was related to the status of the disease (353). The SC was later known as PIGR (354, 355). Nevertheless, a study comparing PIGR protein expression in normal breast tissue with breast cancer tissue demonstrated lower PIGR expression in the cancer than in normal tissue (1.6-fold) (242). In breast cancer, high expression of PIGR mRNA is a favourable prognostic factor (226). There is, however, no documentation regarding its association with response to NAC in breast cancer patients.

Following the pilot study carried out in the department which revealed an association between PIGR overexpression and pCR after NAC in LLABCs, this chapter initially aimed to examine the expression of PIGR, and the predictive and prognostic roles of PIGR in a larger cohort of women with LLABCs who received NAC. However, due to the limited availability of the samples, patients with smaller breast cancers with a tumour size less than 3 cm and suitable for NAC were also included. This present

study utilised FFPE tissue specimens of pre-treatment breast cancer core biopsies from 46 patients who received NAC.

5.2 Technical optimisation

Due to the limitation of the specimens obtained, RNA ISH combined with IF staining was planned to evaluate PIGR mRNA and protein expression in the same tissue section. The advantage of the fluorescence technique over the chromogenic technique is that the expression of both mRNA and protein can be demonstrated separately by the fluorescence microscope using different wavelengths. This facilitates the evaluation of levels of both mRNA and protein expression.

5.2.1 RNA ISH probes assessment and assay optimisation

A positive control probe (PPIB) and a negative control probe (DapB) for RNA ISH were assessed by performing the RNA ISH assay using control slides containing an FFPE cultured human HeLa cell pellet. This assay also served as an assay control check. Target retrieval was performed at 10 min and protease incubation time was 30 min, according to the manufacturer's recommendation for the HeLa cell pellet. Nuclei were counterstained with DAPI.

The assay, performed with a PPIB positive control probe, which is a house keeping gene, showed a strong signal in HeLa cells. This signal demonstrated the expression of PPIB mRNA (Figure 5.1). On the other hand, the assay performed with a DapB negative control probe, which is a bacterial gene, did not show any signal. These results were as predicted and confirmed that the control probes worked well and the assay was successful.

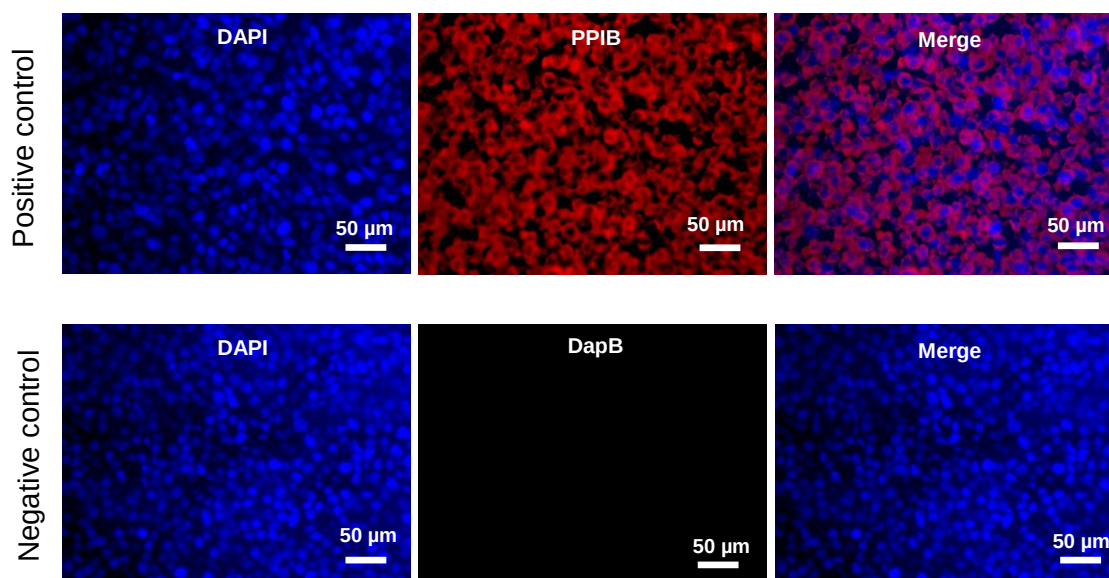


Figure 5.1 PPIB and DapB mRNA expressions in human HeLa cell pellet control slides by RNA ISH assay (fluorescence technique)

RNA ISH assay was performed using control slides containing an FFPE cultured human HeLa cell pellet with a PPIB positive control probe (red) and a DapB negative control probe. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

The PIGR probe was then assessed using normal human colon tissue. Normal colonic epithelial cells are known to express a high level of PIGR. Therefore, normal colonic tissue was used as a positive control for the present study. RNA ISH assay was performed with a PIGR probe, a PPIB positive control probe and a DapB negative control probe. Target retrieval time was 15 min and protease incubation time was 30 min, according to the manufacturer's recommendation for a human colon tissue samples. The results showed a distinct red signal of PIGR and PPIB mRNA expression in colonic epithelial cells which conglomerated into clusters, representing high expression (Figure 5.2). No signal was observed with the DapB negative control probe. This verified that the PIGR probe also worked well.

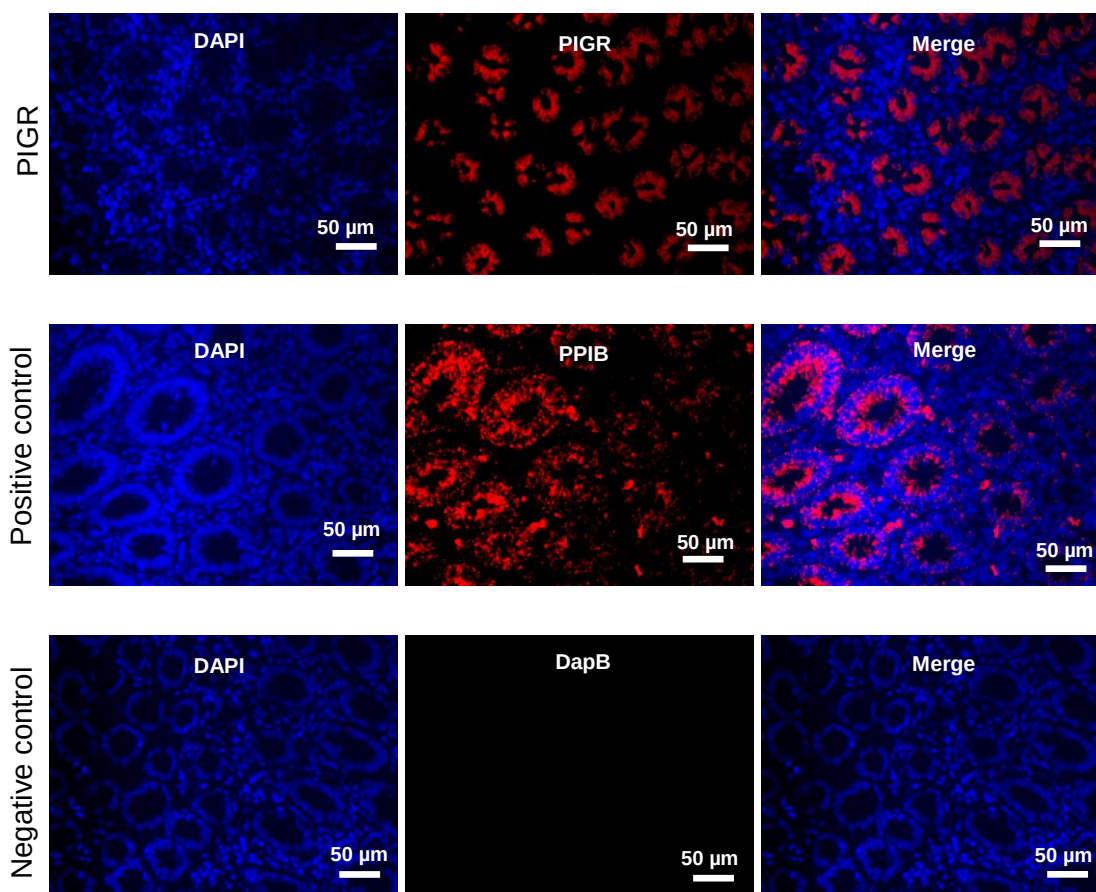


Figure 5.2 PIGR, PPIB and DapB mRNA expressions in normal human colon tissue by RNA ISH assay (fluorescence technique)

RNA ISH assay was performed using normal human colon tissue specimens with a PIGR probe (red), a PPIB positive control probe (red) and a DapB negative control probe. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

5.2.2 Optimisation of PIGR antibody for IF staining

To achieve high specificity with low non-specific background immunostaining, antibody concentration, incubation time and temperature must be optimised. These factors affect the quality of the staining. Primary antibody concentration was optimised by staining tissue with different concentrations to evaluate specificity and background staining. The antibody concentration for optimisation included a concentration recommended by the supplier in a datasheet, at one higher concentration and one lower concentration than recommended. In general, tissue

may be incubated with primary antibody at RT for 1 hr or at 4 °C overnight depending on the affinity and specificity of an antibody.

Normal human colon tissue specimens were used for antibody optimisation due to the high expression of PIGR. The recommended PIGR antibody concentration for immunostaining is a dilution of 1:500 (0.13 µg/ml). Therefore, PIGR antibody diluted 1:800 (0.08 µg/ml), 1:500 and 1:200 (0.31 µg/ml) was applied for optimisation of staining. Primary antibody incubation was performed in two conditions which were RT for 1 hr and 4 °C overnight. Alexa Fluor™ 568 at a dilution of 1:400 (5 µg/ml) was used as a secondary antibody. Staining without primary antibody served as a negative control.

Primary antibody at a dilution of 1:200 incubated at RT for 1 hr showed a faint signal at the cell membrane and cytoplasm of colonic epithelial cells, whilst increasing dilution of antibody did not demonstrate any signal in the cells (Figure 5.3). On the other hand, primary antibody incubation at 4 °C overnight showed a faint signal at a dilution of 1:800 and the signal increased with increasing antibody concentration (1:500 and 1:200). Background signal was low for all staining samples. Staining without primary antibody did not show any signal in colonic epithelial cells. The result showed that the appropriate incubation condition for this PIGR antibody was at 4 °C overnight. Antibody dilution of 1:500 was selected for further experiments.

5.2.3 Optimisation of the combined RNA ISH with IF staining

The combining of RNA ISH with IF staining was carried out by sequential approach. To minimise the risk of RNA degradation, RNA ISH was performed prior to IF staining. However, protease for the permeabilisation step in RNA ISH can affect the integrity of proteins. Therefore, the combined assay condition including pre-treatment condition

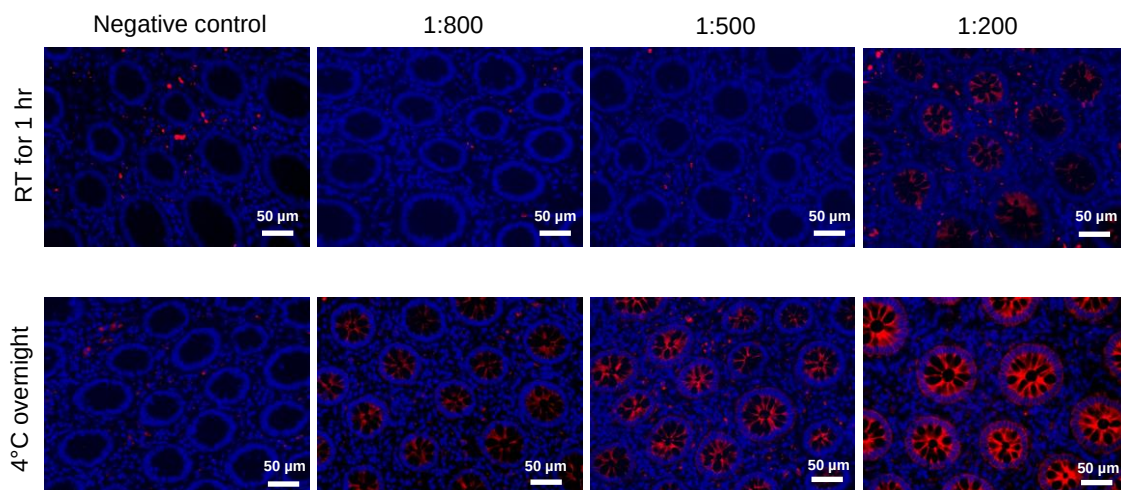


Figure 5.3 PIGR primary antibody optimisation for IF staining using normal human colon tissue

IF staining of normal human colon tissue was performed with different concentrations of PIGR antibody (antibody diluted 1:800, 1:500 and 1:200). Primary antibody incubation condition was assessed at RT for 1 hr and at 4 °C overnight. Staining without primary antibody served as a negative control. Alexa Fluor™ 568 (red) at a dilution of 1:400 was used as a secondary antibody and incubated at RT for 1 hr. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

in RNA ISH and antibody concentration for IF needs to be optimised to obtain a good RNA expression signal without affecting the integrity of proteins.

The combined assay was performed concurrently with RNA ISH alone and IF staining alone to compare the signal of PIGR expression in each assay. RNA ISH was performed under the recommended condition using normal human colon tissue. Target retrieval time was 15 min and protease incubation time was 30 min. For IF staining, PIGR antibody was diluted to 1:500 and incubated at 4 °C overnight. Alexa Fluor™ 488 at a dilution of 1:400 (5 µg/ml) was used as a secondary antibody and incubated at RT for 1 hr. However, PIGR protein expression could not be detected in the combined assay stained tissue. Therefore, primary antibody concentration was increased to a dilution of 1:100 (0.63 µg/ml). The result demonstrated a faint signal of PIGR protein expression in the combined assay while IF staining alone showed a

strong signal (Figure 5.4). PIGR mRNA expression signal in the combined assay was not reduced compared with RNA ISH alone. These results suggest that a large amount of PIGR protein was degraded by the protease pre-treatment step in the RNA ISH assay. Therefore, protease incubation time was further optimised.

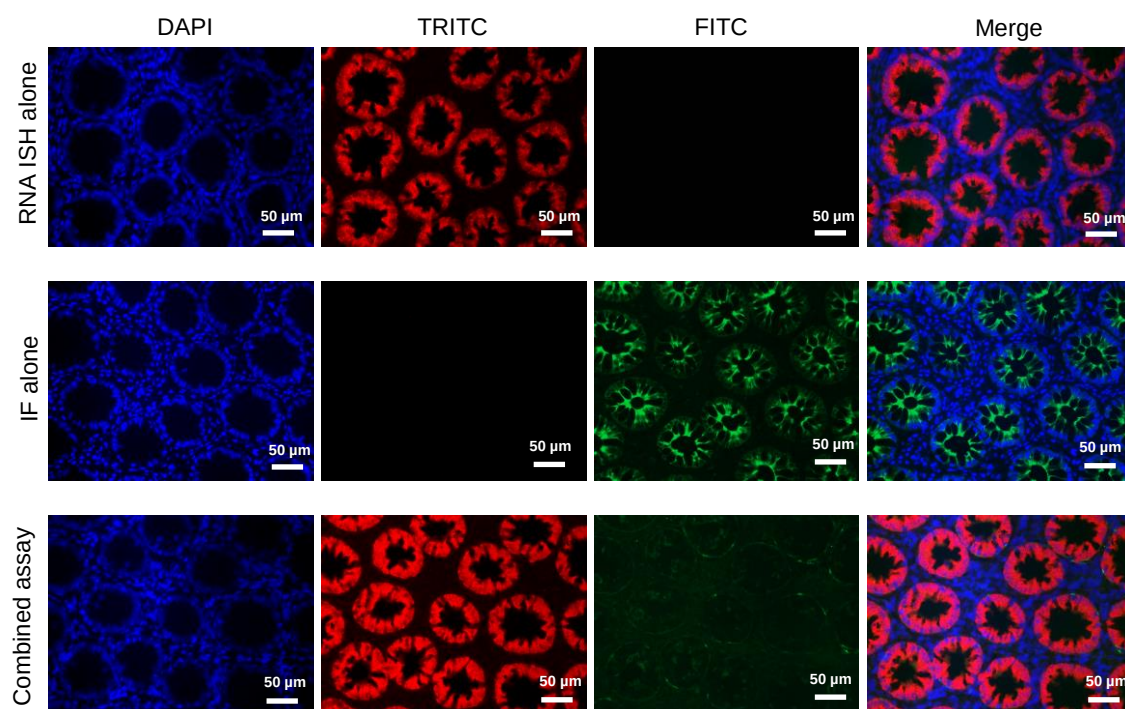


Figure 5.4 PIGR mRNA and protein expressions in normal human colon tissue by RNA ISH alone (fluorescence technique), IF alone and combined assay

RNA ISH was performed with a PIGR probe (TRITC channel, red). IF staining was performed with PIGR antibody diluted 1:100 and incubated at 4 °C overnight. Alexa Fluor™ 488 (FITC channel, green) diluted 1:400 was used as a secondary antibody and incubated at RT for 1 hr. The combined assay was performed with RNA ISH followed by IF staining. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

Combined RNA ISH with IF staining assay was performed with different protease pre-treatment incubation times (5, 10 and 20 min) for RNA ISH (Figure 5.5). The longer protease incubation time gave a stronger signal of PIGR mRNA expression which was contrary to the protein expression. The PIGR protein expression signal was stronger with a shorter protease incubation time.

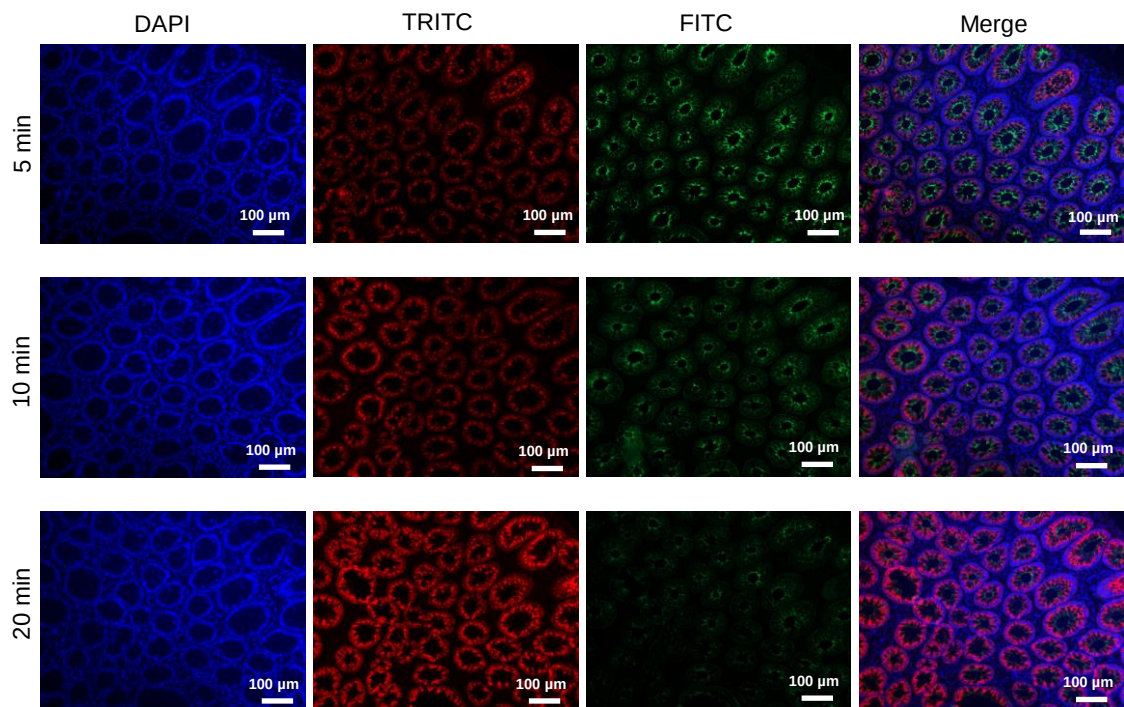


Figure 5.5 PIGR mRNA and protein expressions in normal human colon tissue by combined RNA ISH (fluorescence technique) with IF staining with different protease incubation times

RNA ISH was performed with a PIGR probe (TRITC channel, red). Specimens were incubated with protease for 5, 10 and 20 min. Subsequently, IF staining was performed with PIGR antibody diluted 1:100 and incubated at 4 °C overnight. Alexa Fluor™ 488 (FITC channel, green) diluted 1:400 was used as a secondary antibody and incubated at RT for 1 hr. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

RNA ISH and IF staining assays were further optimised using breast cancer and normal breast tissue samples. H&E staining was initially performed to confirm both cancerous and normal histology of the tissue samples (Figure 5.6).

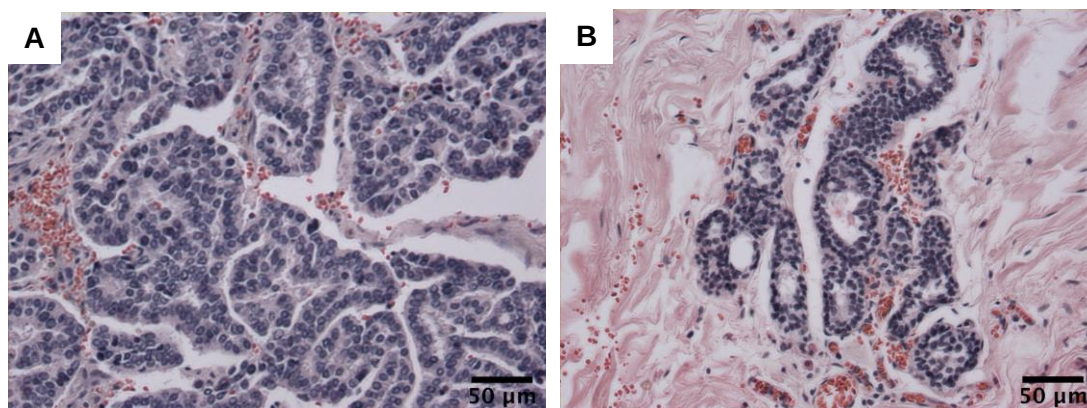


Figure 5.6 H&E stain of breast cancer and normal breast tissues

H&E stain demonstrates breast cancer tissue (papillary carcinoma) **(A)** and normal breast tissue **(B)**.

RNA ISH alone was performed with a target retrieval time of 15 min and protease incubation time of 30 min for breast cancer and normal breast tissue. The results showed very low expression of PIGR mRNA in both breast cancer cells and normal mammary epithelial cells (Figure 5.7, 5.8). Moreover, there was no signal of PIGR protein expression by IF staining alone in either tissue except in the lumen of normal mammary duct, which may represent free SC secreted by normal mammary epithelial cells (Figure 5.9). These results suggested that the level of PIGR mRNA and protein expressions in these breast cancer cells and normal mammary epithelial cells was very low. Along with the evidence from literature, which documents a low level of PIGR expression in breast cancer, it indicates that using combined RNA ISH with IF staining to evaluate mRNA and protein expressions in the same tissue sample is not suitable for the PIGR expression study (226, 242). The protease pre-treatment step in the RNA ISH assay may completely destroy the small amount of PIGR protein in

the cells. Due to the limited tissue samples obtained from the biobank and considering that next generation RNA sequencing was initially used to evaluate RNA expression in breast cancer samples in the pilot study, PIGR mRNA expression was selected rather than protein expression for evaluation in this present study. Therefore, the plan of the experiment was changed to evaluate only PIGR mRNA expression in breast cancer tissues by RNA ISH using the chromogenic technique.

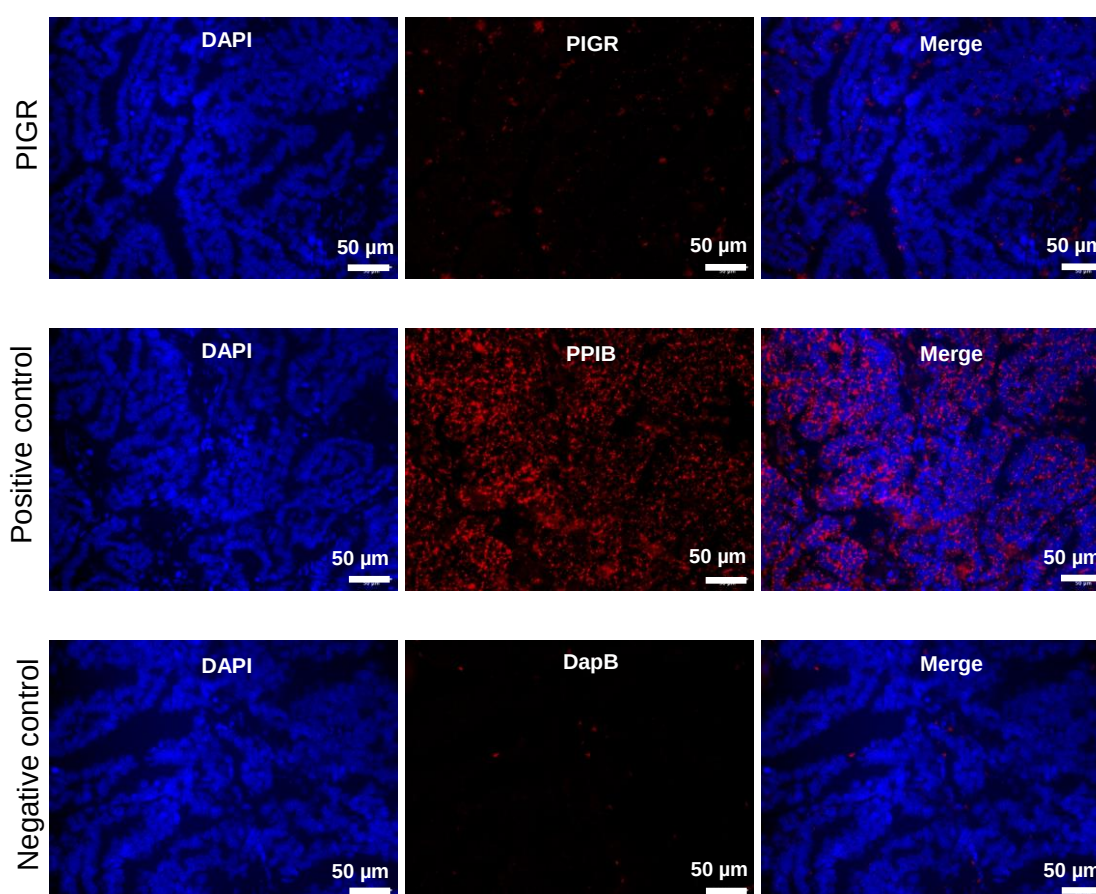


Figure 5.7 PIGR, PPIB and DapB mRNA expressions in breast cancer tissue by RNA ISH assay (fluorescence technique)

RNA ISH assay was performed using breast cancer tissue specimens with a PIGR probe (red), a PPIB positive control probe (red) and a DapB negative control probe. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

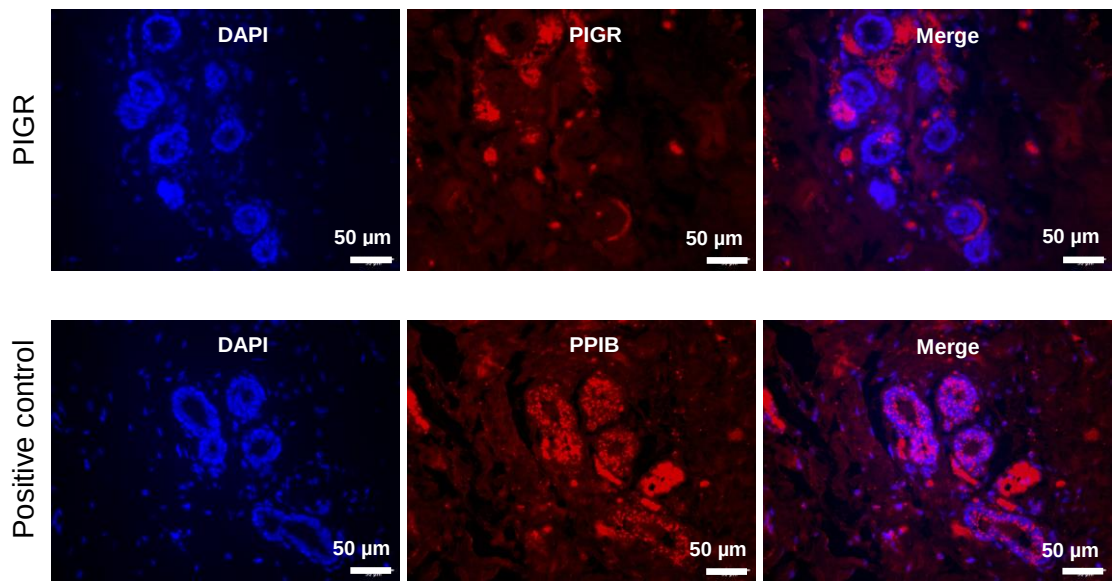


Figure 5.8 PIGR and PPIB mRNA expressions in normal breast tissue by RNA ISH assay (fluorescence technique)

RNA ISH assay was performed using normal breast tissue specimens with a PIGR probe (red) and a PPIB positive control probe (red). Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope.

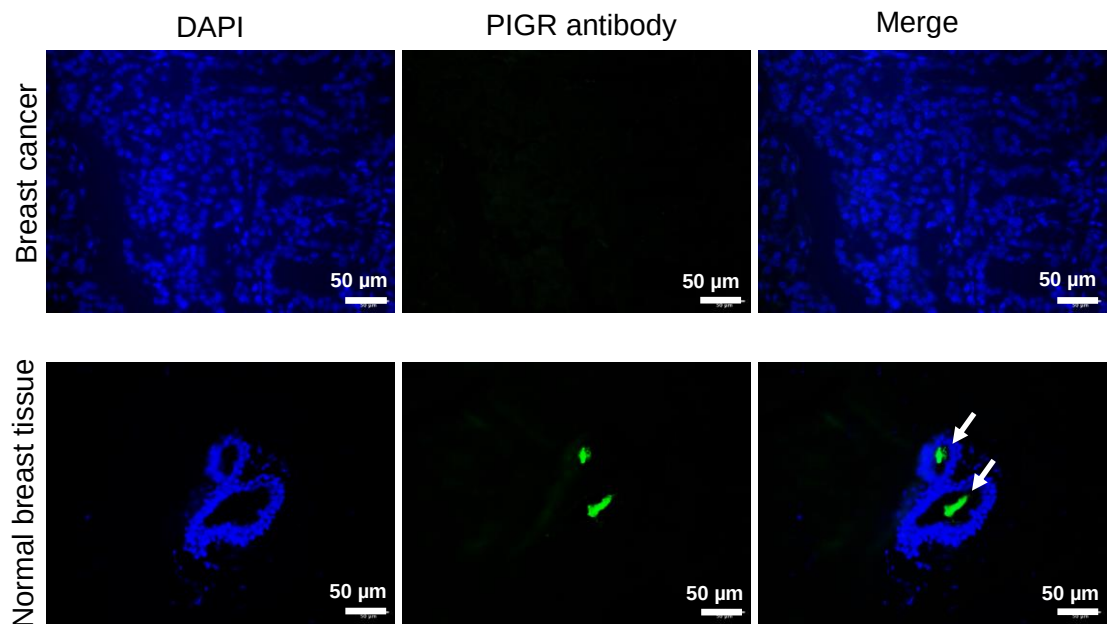


Figure 5.9 PIGR protein expressions in breast cancer and normal breast tissue by IF staining

IF staining was performed with PIGR antibody diluted 1:100 and incubated at 4 °C overnight. Alexa Fluor™ 488 (green) diluted 1:400 was used as a secondary antibody and incubated at RT for 1 hr. Nuclei were counterstained with DAPI (blue). The images were collected with a widefield inverted fluorescence microscope. The white arrows in the right bottom image demonstrates free SC (green) in normal mammary ducts.

RNA ISH using the chromogenic technique was performed with breast cancer and normal breast tissue samples for technical familiarisation. Normal colon tissue was used as a positive control tissue and showed a strong signal for PIGR mRNA expression (Figure 5.10). This confirmed that the technique was successful. However, there was no signal for PIGR expression in the breast cancer tissue sample, while a very low signal was detected in normal mammary epithelial cells. Subsequently, the study using patients' breast cancer tissues was commenced.

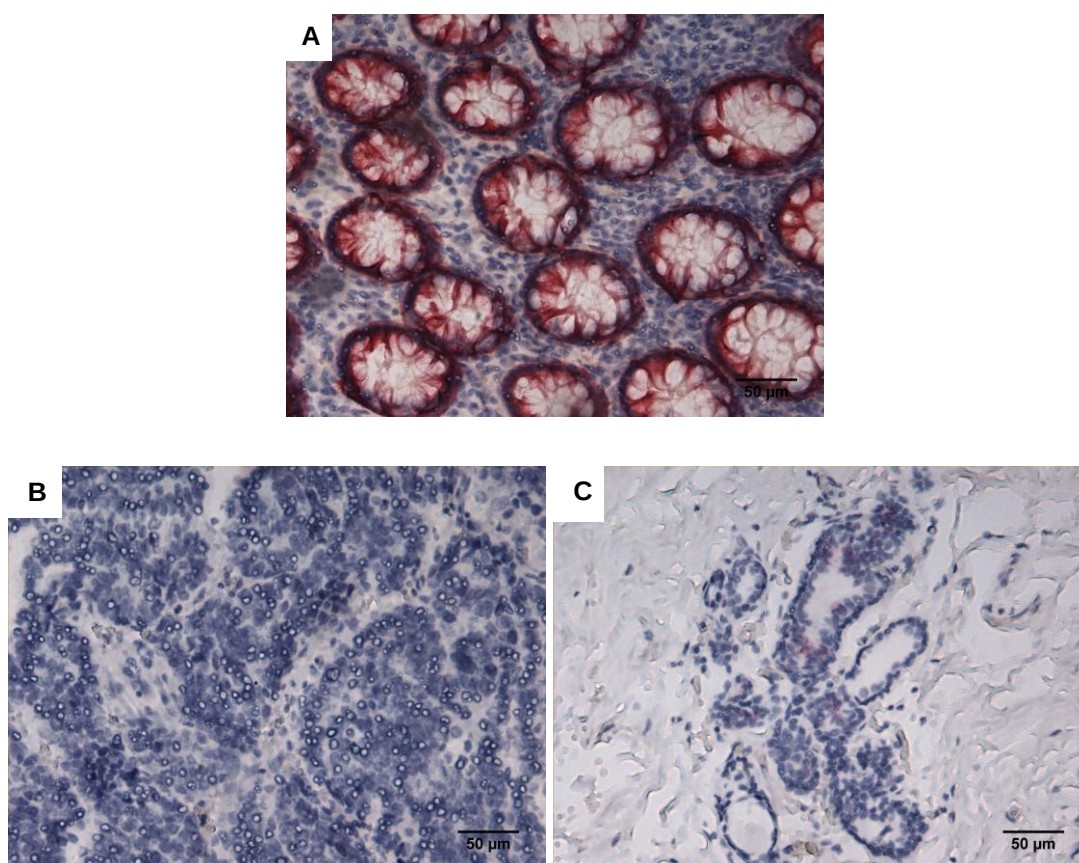


Figure 5.10 PIGR mRNA expression in normal colon tissue, breast cancer tissue, and normal breast tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed using normal colon tissue **(A)**, breast cancer tissue **(B)** and normal breast tissue **(C)** with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue).

5.3 Results

5.3.1 Clinicopathological characteristics of the cohort

Table 5.1 shows clinicopathological characteristics of the 46 patients in this cohort. All patients were women with a median age at diagnosis of 48 years (interquartile range [IQR] 42.50-60.25 years). The median tumour size was 26 mm (IQR 20.25-32.50 mm). Histologically, the majority of the tumours (80.4%) were classified as invasive ductal carcinoma of NST. Most of the tumours were grade 2 (50%) or grade 3 (47.8%). ER and PR were positive (Allred score ≥ 3) in 43.5% and 39.1% of tumours, respectively. HER2 was positive in 23.9% of tumours (IHC score 3+ and/or fluorescence *in situ* hybridisation [FISH] positive [HER2/CEP17 {centromeric region of chromosome 17} ratio ≥ 2.0]). Tumours were categorised as hormonal receptor positive/human epidermal growth factor receptor 2 negative (HR +ve/HER2 -ve) subtype (30.4%), hormonal receptor positive/human epidermal growth factor receptor 2 positive (HR +ve/HER2 +ve) subtype (15.2%), hormonal receptor negative/human epidermal growth factor receptor 2 positive (HR -ve/HER2 +ve) subtype (8.7%) and triple negative breast cancer (TNBC) subtype (45.7%). Axillary lymph node (ALN) status was evaluated prior to NAC by fine needle aspiration cytology (FNAC) and showed metastases of breast cancer in 63.0%. Surgical treatment was performed after NAC in all patients. Breast conserving surgery (73.9%) was undertaken more often than mastectomy (26.1%).

Table 5.1 Clinicopathological characteristics of the cohort

Clinicopathological characteristics	n (%)
Age (years)	
Median (IQR)	48 (42.50-60.25)
Tumour size (mm)^a	
Median (IQR)	26 (20.25-32.50)
Histological tumour type	
Invasive ductal carcinoma of NST	37 (80.4)
Invasive lobular carcinoma	2 (4.3)
NST with tubular features	1 (2.2)
NST with apocrine features	1 (2.2)
NST with basal phenotype	3 (6.5)
NST with lobular features	1 (2.2)
NST with focal micropapillary features	1 (2.2)
Tumour grade	
1	1 (2.2)
2	23 (50.0)
3	22 (47.8)
Tubular formation	
1	0 (0)
2	5 (10.9)
3	41 (89.1)
Pleomorphism	
1	0 (0)
2	13 (28.3)
3	33 (71.7)
Mitotic figures	
1	25 (54.3)
2	6 (13.0)
3	15 (32.6)
ER status	
ER -ve	26 (56.5)
ER +ve	20 (43.5)
PR status	
PR -ve	28 (60.9)
PR +ve	18 (39.1)
HER2 status	
HER2 -ve	35 (76.1)
HER2 +ve	11 (23.9)

(Table 5.1 continues in next page)

Clinicopathological characteristics	n (%)
(Continues from previous page)	
Tumour subtype	
HR +ve/HER2 -ve	14 (30.4)
HR +ve/HER2 +ve	7 (15.2)
HR -ve/HER2 +ve	4 (8.7)
TNBC	21 (45.7)
Nodal status by FNAC	
Negative	17 (37.0)
Positive	29 (63.0)
Breast surgery	
Mastectomy	12 (26.1)
Breast conserving surgery	34 (73.9)

n: Number, IQR: Interquartile range, NST: no special type, ER: Oestrogen receptor, ER -ve: Oestrogen receptor negative, ER +ve: Oestrogen receptor positive, PR: Progesterone receptor, PR -ve: Progesterone receptor negative, PR +ve: Progesterone receptor positive, HER2: Human epidermal growth factor receptor 2, HER2 -ve: Human epidermal growth factor receptor 2 negative, HER2 +ve: Human epidermal growth factor receptor 2 positive, HR +ve: Hormonal receptor positive, HR -ve: Hormonal receptor negative, TNBC: Triple negative breast cancer, FNAC: Fine needle aspiration cytology

^aTumour size was measured radiologically by ultrasound scan for 43 patients, by mammography for one patient. Tumour size was not applicable for two patients. One patient had a faint calcified lesion in the breast and another did not have a breast lesion at diagnosis. Two tumours were recorded by ultrasound scan as more than 50 mm.

5.3.2 NAC regimens used

Patients were treated with different NAC regimens (Table 5.2). FEC (fluorouracil/epirubicin/cyclophosphamide) with docetaxel was the most commonly used (65.2%) follow by docetaxel alone (15.2%). All patients who had HER2 +ve tumours (11 patients) received anti-HER2 agents. Concurrently with NAC, nine patients received trastuzumab and two patients received trastuzumab and pertuzumab.

Table 5.2 NAC regimens used in the cohort

Regimens	n (%)
FEC with T	30 (65.2)
T alone	7 (15.2)
FEC	3 (6.5)
CMF with epirubicin	3 (6.5)
TC	2 (4.3)
TE with carboplatin	1 (2.1)

NAC: Neoadjuvant chemotherapy, n: Number, FEC: fluorouracil and epirubicin and cyclophosphamide, T: docetaxel, CMF: cyclophosphamide and methotrexate and fluorouracil, TC: docetaxel and cyclophosphamide, TE: docetaxel and epirubicin

5.3.3 Response to NAC

Pathological complete response (pCR) was ascertained using previously published criteria (76, 101, 112). A pCR in a primary breast cancer was defined as no residual invasive disease. A pCR in ALNs was defined as no invasive cells identified with or without fibrosis. Overall, pCR in breast and ALN was observed in 16 patients (34.8%) and 24 patients (52.2%), respectively. Thirteen patients (28.3%) achieved pCR in both breast and ALN. On the other hand, 19 patients (41.3%) did not obtain a pCR in either breast or ALN after NAC.

5.3.4 PIGR mRNA expression was lower in breast cancer than in normal breast tissue

In this study, normal colon tissue was used as a positive control tissue and assayed with every batch of breast cancer tissue samples to confirm that the assay worked well (Figure 5.11). PPIB (positive control) mRNA expression was evaluated in consecutive sections of tissue samples to determine whether the quality of RNA in the tissue specimens was sufficient for detecting the RNA target. Due to the limited amount of tissue for each subject and all samples were prepared using the same

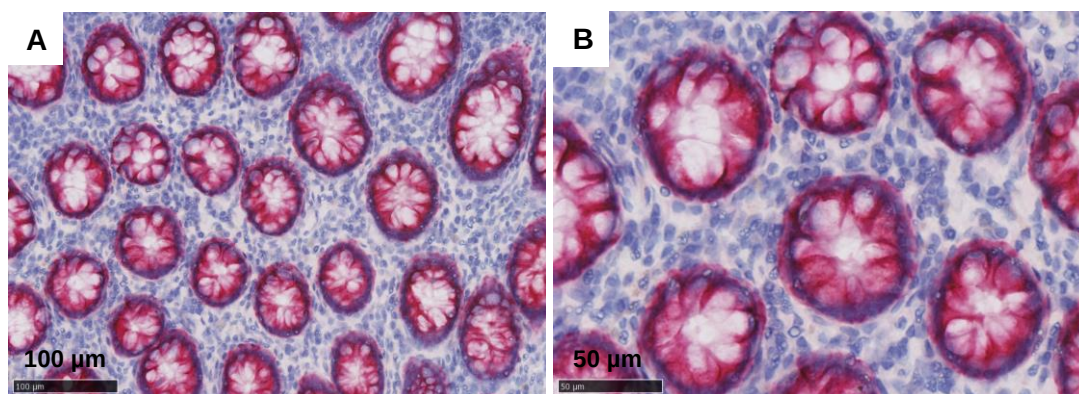


Figure 5.11 PIGR mRNA expression in normal colon tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). Normal colonic epithelial cells showed a high PIGR mRNA expression (score 4). **A:** Low magnification, **B:** High magnification

protocol, DapB (negative control) mRNA expression was evaluated in one sample of each batch of the assay. The assay performed with DapB negative control probe ensures that there is no background staining related to the assay and that the tissue sample is appropriately prepared. Every samples showed a PPIB expression score of equal or more than two and DapB was negative in all batches (Figure 5.12). Thus, the RNA quality was sufficient and the samples were appropriately prepared for RNA ISH assay.

The level of PIGR mRNA expressions in breast cancer cells and normal mammary epithelial cells varied from a score 0-4 (Figure 5.13, 5.14). The percentage of cells expressing PIGR mRNA ranged from 0-100%. Most samples, both breast cancer and adjacent normal breast tissue, showed heterogeneity of PIGR expression level in different areas, varying from negative to intense staining (Figure 5.15, 5.16, 5.17). In the present cohort, 18 out of 46 (39.1%) breast cancer samples expressed PIGR mRNA (Table 5.3). Median H score calculated by multiplying the percentage of cells expressing PIGR mRNA by an average expression level was 0 and IQR was 0-10. Interestingly, in 23 out of 25 (92%) cases, adjacent normal breast tissues expressed

PIGR mRNA with a significantly higher H score than breast cancer tissue (median H score 120 vs. 0, $p < 0.0001$) (Figure 5.18).

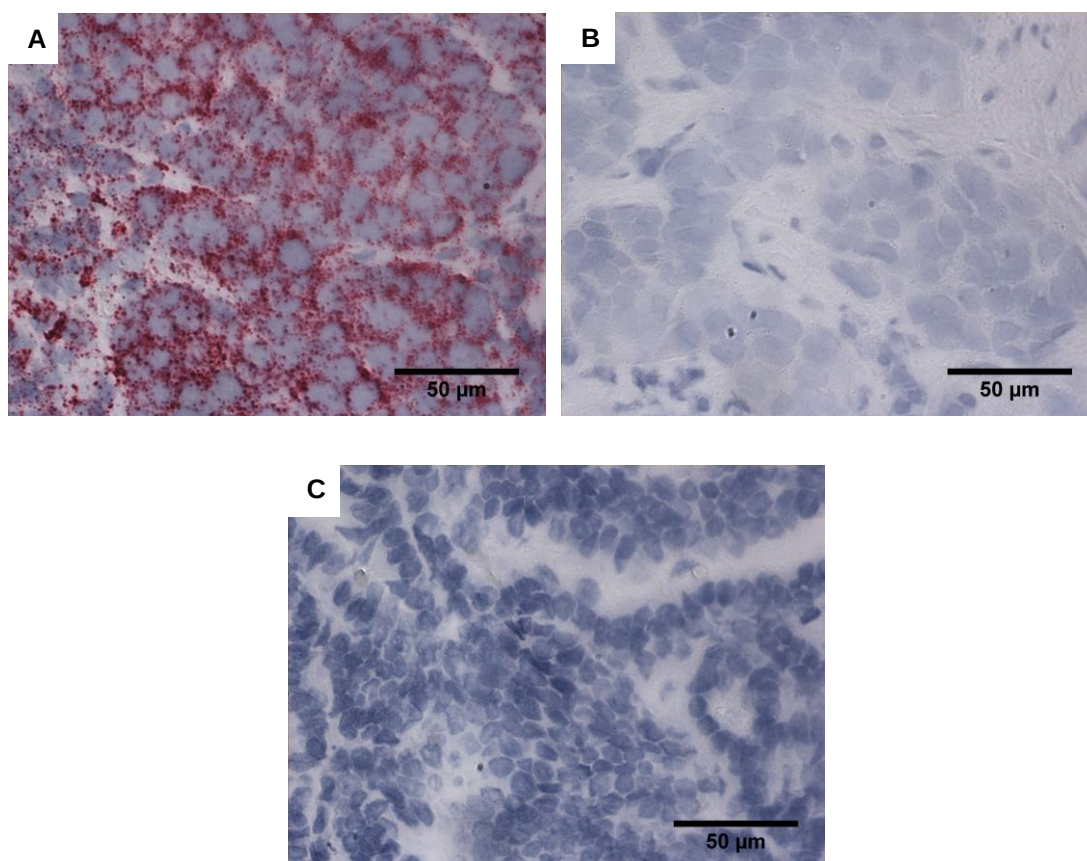


Figure 5.12 PPIB, PIGR and DapB mRNA expression in breast cancer tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with PPIB, PIGR and DapB probes. Nuclei were counterstained with haematoxylin (blue). Breast cancer tissue showed a high expression of PPIB mRNA (score 4) (red, **A**). The consecutive breast cancer tissue section did not show PIGR mRNA expression (**B**). DapB mRNA was not detect in the breast tissue assayed in the same batch (**C**).

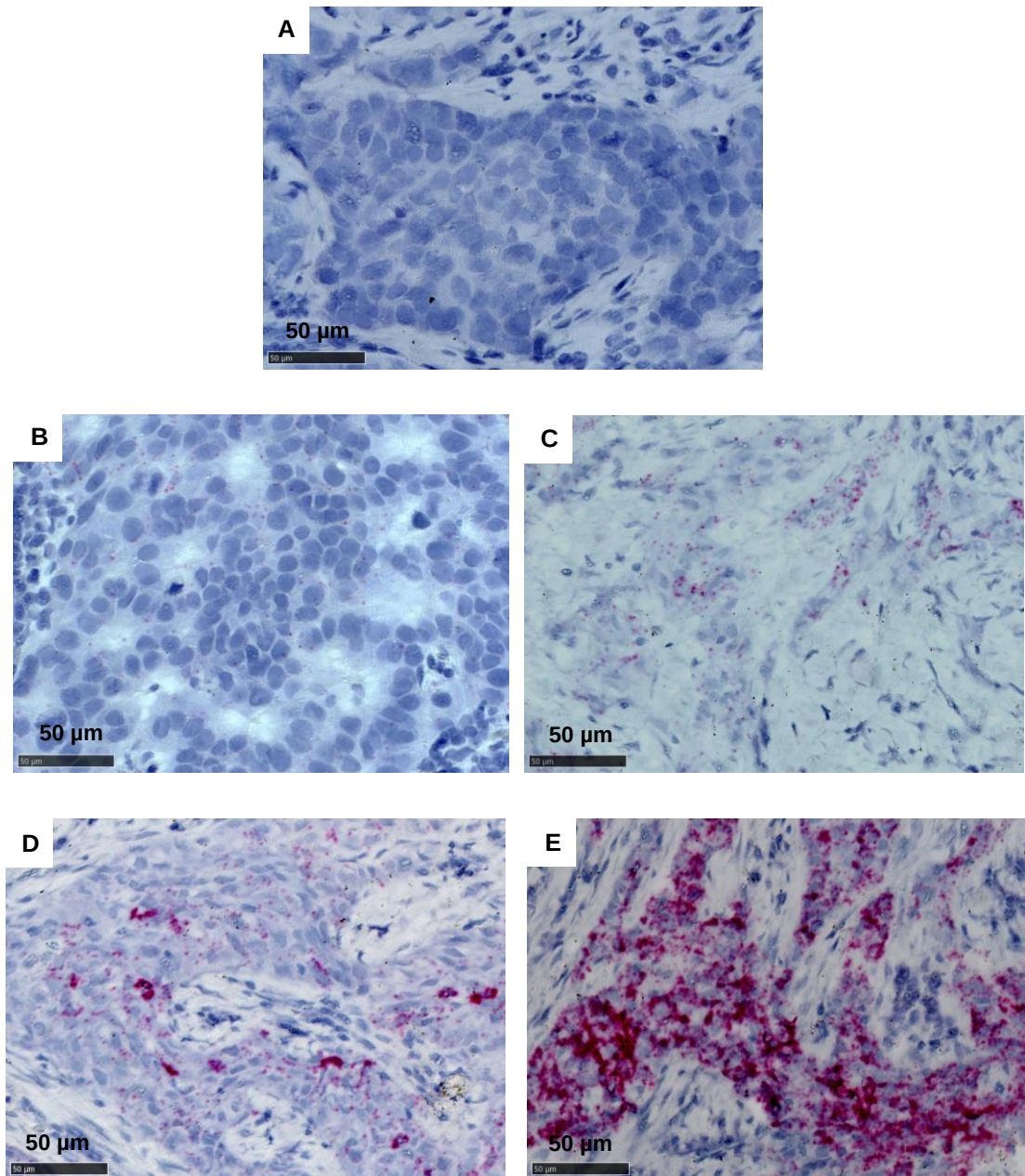


Figure 5.13 Variation of the level of PIGR mRNA expression in breast cancer tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). Breast cancer tissues showed a variation of the level of PIGR mRNA expression from a score 0-4. **A:** score 0 (no staining or <1 dot/10 cells), **B:** score 1 (1-3 dots/cell), **C:** score 2 (4-9 dots/cell and none or very few dot clusters), **D:** score 3 (10-15 dots/cell and/or <10% dots are in clusters), **E:** score 4 (>15 dots/cell and/or >10% dots are in clusters).

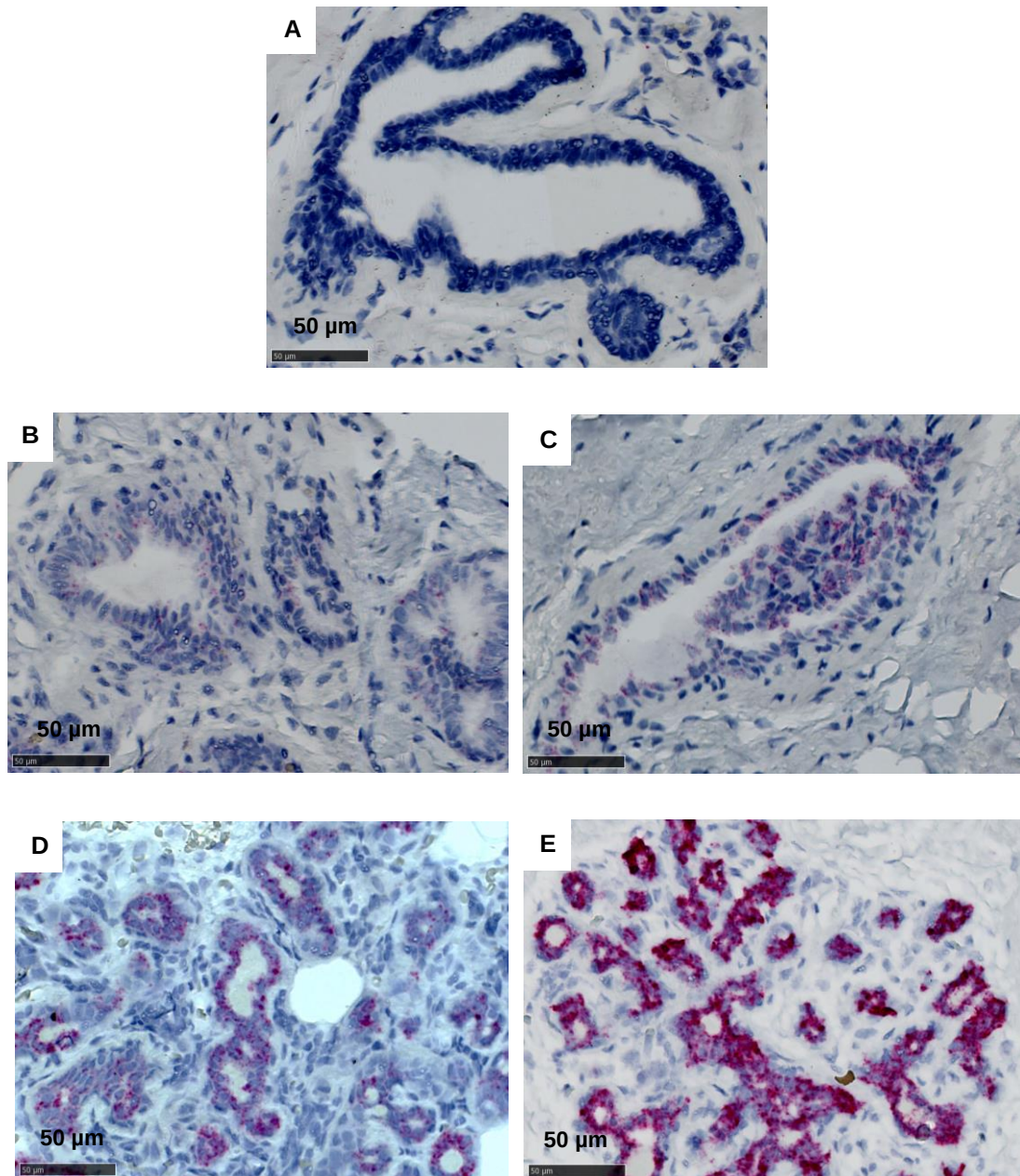


Figure 5.14 Variation of the level of PIGR mRNA expression in normal breast tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). Normal breast tissues showed a variation of the level of PIGR mRNA expression from a score 0-4. **A:** score 0 (no staining or <1 dot/10 cells), **B:** score 1 (1-3 dots/cell), **C:** score 2 (4-9 dots/cell and none or very few dot clusters), **D:** score 3 (10-15 dots/cell and/or <10% dots are in clusters), **E:** score 4 (>15 dots/cell and/or >10% dots are in clusters).

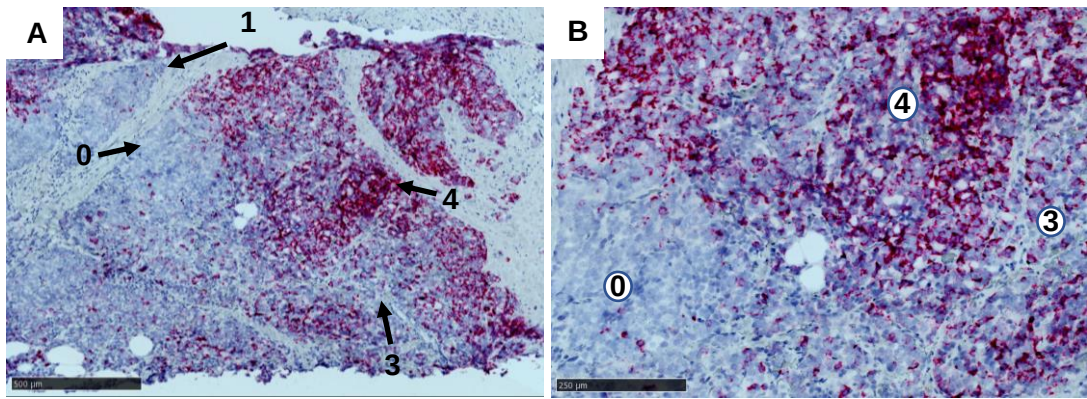


Figure 5.15 Heterogeneous PIGR mRNA expression in breast cancer tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). Breast cancer tissue showed heterogeneous PIGR mRNA expression. Numbers represent expression levels for each area. **A:** Low magnification, **B:** High magnification

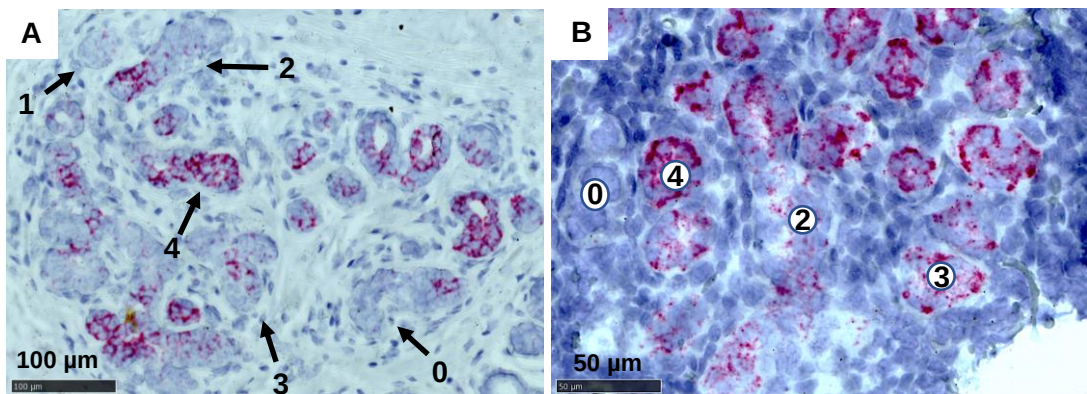


Figure 5.16 Heterogeneous PIGR mRNA expression in normal breast tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). Normal mammary duct in breast tissue showed heterogeneous PIGR mRNA expression. Numbers represent expression levels for each area. **A:** Low magnification, **B:** High magnification

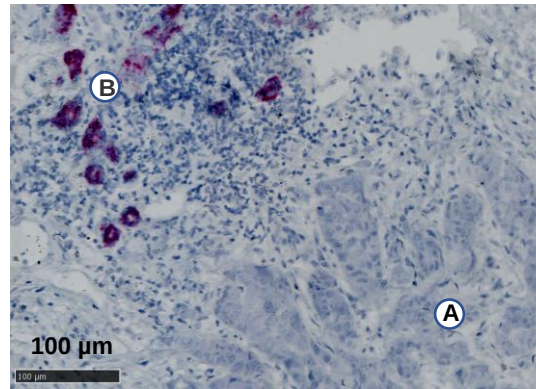


Figure 5.17 Differential PIGR mRNA expression of breast cancer and adjacent normal breast tissue by RNA ISH assay (chromogenic technique)

RNA ISH assay was performed with a PIGR probe (red). Nuclei were counterstained with haematoxylin (blue). The image demonstrates the differentiation of PIGR mRNA expression between breast cancer **(A)** which did not express PIGR mRNA (score 0) and adjacent normal breast tissue **(B)** which highly expressed PIGR mRNA (score 4).

Table 5.3 PIGR mRNA expression in invasive breast cancer and adjacent normal breast tissue

	Invasive breast cancer	Adjacent normal breast tissue
Number of samples	46	25
Present PIGR mRNA signal (n, %)	18 (39.1)	23 (92)
H score		
Median	0	120
IQR	0-10	36.25-355
Minimum	0	0
Maximum	210	400

PIGR: Polymeric immunoglobulin receptor, n: Number, H score: Histo score, IQR: Interquartile range

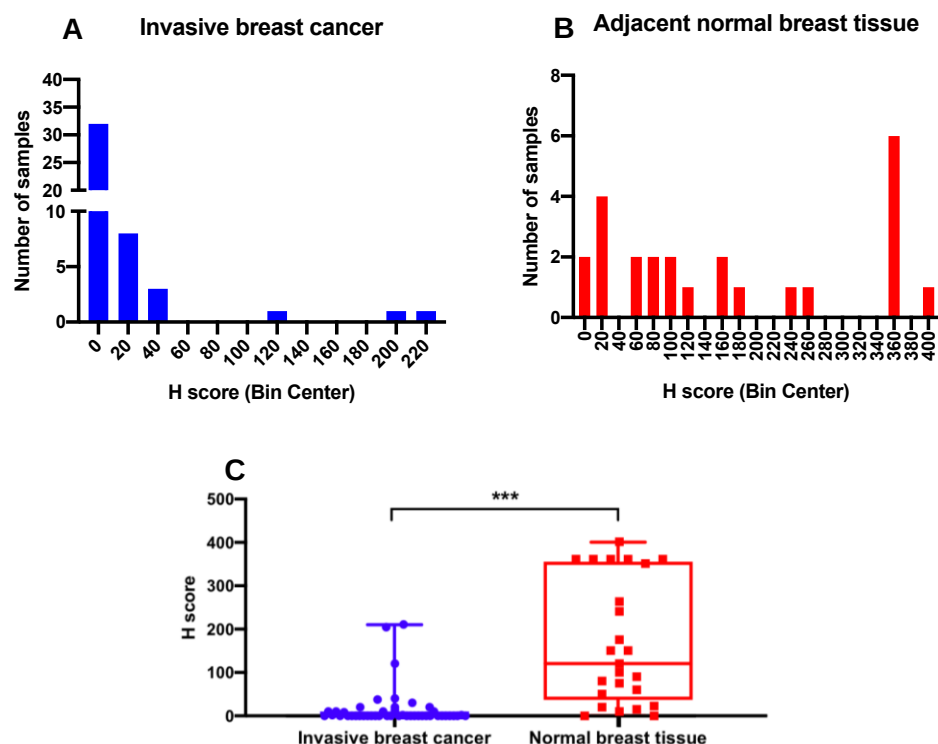


Figure 5.18 H score of PIGR mRNA expression in invasive breast cancer and adjacent normal breast tissue

Histograms demonstrate the distribution of the H score of PIGR mRNA expression in invasive breast cancer samples (**A**) and adjacent normal breast tissue samples (**B**). A box and whisker plot with individual values of the H score of invasive breast cancer samples compared with adjacent normal breast tissue samples (**C**) (** $p < 0.0001$, Wilcoxon signed rank test).

5.3.5 Positive expression of PIGR was associated with a high score of nuclear pleomorphism in breast cancer cells

The association between PIGR mRNA expression in breast cancers and the clinicopathological characteristics of the cohort was determined. Considering that as many as 60% of the breast cancer samples did not express PIGR mRNA and the median H score was 0, this was used as a cutoff point to classify PIGR mRNA expressions in breast cancer samples into PIGR positive (H score >0) and PIGR negative (H score = 0) for the analysis. According to this classification, 18 out of 46 tumours (39.1%) were classified as positive for PIGR mRNA expression. The analysis for the association with clinicopathological characteristics revealed a statistically

significant association between PIGR positivity and a high score of nuclear pleomorphism of breast cancer cells ($\chi^2 = 7.519$, degree of freedom [d.f.] = 1, $p = 0.006$) (Table 5.4). PIGR was positive in 51.5% (17 out of 33) of tumours with nuclear pleomorphism score 3 compared with only 7.7% (1 out of 13) of tumours with nuclear pleomorphism score 2. There were no significant associations between PIGR mRNA expression and other clinicopathological characteristics, including patient age, tumour size, tumour grade, tubular formation, mitotic figures, ER, PR and HER2 status, tumour subtype and nodal status.

5.3.6 PIGR mRNA expression was not associated with a pCR after NAC in the cohort studied

The association between PIGR expression and pCR in breast cancers after NAC was evaluated to assess whether PIGR could predict a pCR in breast cancers after NAC. Due to the different definitions of pCR used in clinical practice, the analyses were performed as breast-specific analysis, and breast and ALN analysis. Breast-specific analysis focussed only on the pathological response in the breast. Breast and ALN analysis focussed on the pathological response in both breast and ALN in which pCR was defined as the absence of invasive cancer in both breast and ALN. The distribution of the H score of PIGR mRNA expression in pCR and non-pCR group in breast-specific and breast and ALN analyses was demonstrated by histograms in Figure 5.19.

Table 5.4 Associations between PIGR mRNA expression and clinicopathological characteristics of the cohort

Parameter	PIGR negative n (%)	PIGR positive n (%)	Pearson Chi-Squared value (χ^2)	p value ^c
Age (years)				
≤50 years	19 (70.4)	8 (29.6)	2.477	0.116
>50 years	9 (47.4)	10 (52.6)		
Tumour size^a				
≤3 cm	19 (59.4)	13 (40.6)	0.196	0.658
>3 cm	8 (66.7)	4 (33.3)		
Tumour grade				
1 and 2	17 (70.8)	7 (29.2)	2.092	0.148
3	11 (50.0)	11 (50.0)		
Tubular formation^b				
2	4 (80.0)	1 (20.0)	0.862	0.634 ^d
3	24 (58.5)	17 (41.5)		
Pleomorphism^b				
2	12 (92.3)	1 (7.7)	7.519	0.006*
3	16 (48.5)	17 (51.5)		
Mitotic figures				
1	16 (64.0)	9 (36.0)	2.225	0.362
2	2 (33.3)	4 (66.7)		
3	10 (66.7)	5 (33.3)		
ER status				
ER -ve	14 (53.8)	12 (46.2)	1.238	0.266
ER +ve	14 (70.0)	6 (30.0)		
PR status				
PR -ve	17 (60.7)	11 (39.3)	0.001	0.979
PR +ve	11 (61.1)	7 (38.9)		
HER2 status				
HER2 -ve	23 (65.7)	12 (34.3)	1.442	0.230
HER2 +ve	5 (45.5)	6 (54.5)		
Tumour subtype				
HR +ve/HER2 -ve	11 (78.6)	3 (21.4)	3.116	0.401
HR +ve/HER2 +ve	3 (42.9)	4 (57.1)		
HR -ve/HER2 +ve	2 (50.0)	2 (50.0)		
TNBC	12 (57.1)	9 (42.9)		
Nodal status by FNAC				
Negative	12 (70.6)	5 (29.4)	1.069	0.301
Positive	16 (55.2)	13 (44.8)		

PIGR: Polymeric immunoglobulin receptor, n: Number, ER: Oestrogen receptor, ER -ve: Oestrogen receptor negative, ER +ve: Oestrogen receptor positive, PR: Progesterone receptor, PR -ve: Progesterone receptor negative, PR +ve: Progesterone receptor positive, HER2: Human epidermal growth factor receptor 2, HER2 -ve: Human epidermal growth factor receptor 2 negative, HER2 +ve: Human epidermal growth factor receptor 2 positive, HR +ve: Hormonal receptor positive, HR -ve: Hormonal receptor negative, TNBC: Triple negative breast cancer, FNAC: Fine needle aspiration cytology

^aTumour size was not applicable for two patients. One patient had a faint calcified lesion in the breast and another did not have a breast lesion at diagnosis.

^bNo tumour was classified as a score 1

^cThe *p* values were obtained from Pearson Chi-Squared test unless otherwise specified.

^dThe *p* values were obtained from Fisher's Exact test because at least one cell in 2×2 table had an expected count less than five.

*Statistically significant

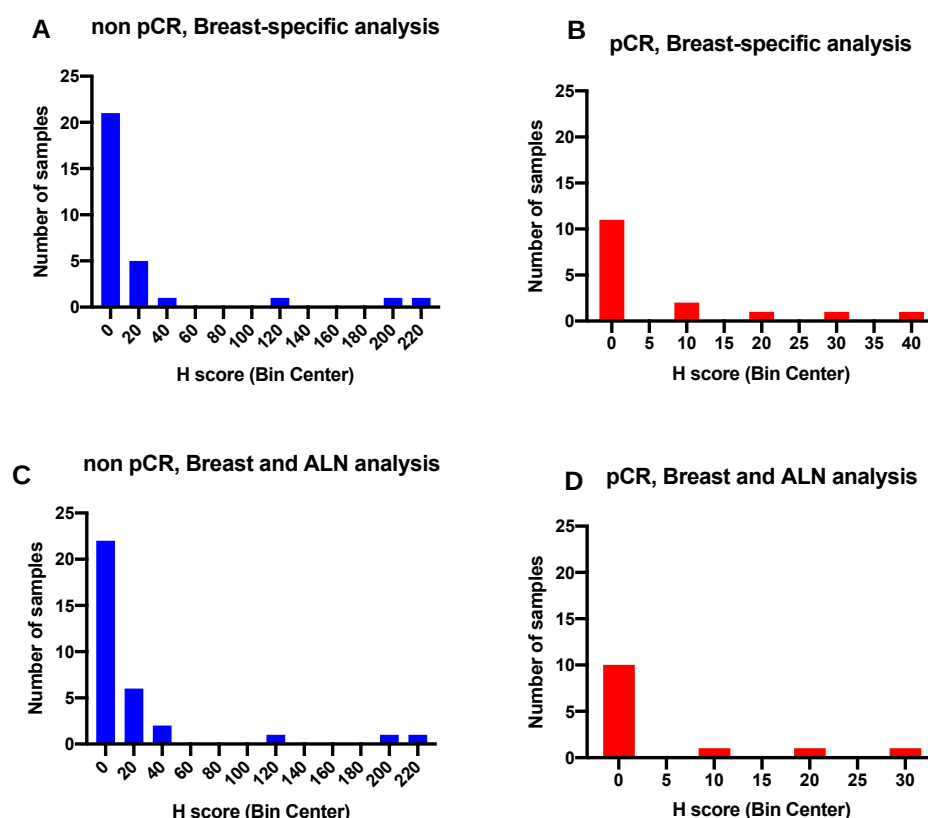


Figure 5.19 H score of PIGR mRNA expression in non-pCR and pCR group in breast-specific and breast and ALN analyses

Histograms demonstrate the distribution of the H score of PIGR mRNA expression in invasive breast cancer samples. **A** and **B** demonstrate distribution of the H score in the samples from patients who did not achieve and who did achieve a pCR after NAC respectively by breast-specific analysis. **C** and **D** demonstrate distribution of the H score in the samples from patients who did not achieve and who did achieve a pCR after NAC respectively by breast and ALN analysis.

For breast-specific analysis, 43.8% of the patients who achieved pCR after NAC had PIGR expressing tumours compared with 36.7% of the patients who did not achieve pCR (Table 5.5). However, both the pCR and the non-pCR group of patients had a median of H score at 0. There was no statistically significant difference of the H score between the groups ($p = 0.865$). For breast and ALN analysis, 30.8% of the patients who achieved a pCR after NAC had PIGR expressing tumours compared with 27.3% of the patients who did not achieve a pCR. The median H score of these two groups was 0. There was no statistically significant difference of the H score between the groups ($p = 0.398$).

Table 5.5 Associations between pCR after NAC and H score

		Present PIGR mRNA signal (n, %)	H score			<i>p</i> value
n (%)			Median	IQR	Min-max	
Breast-specific analysis						
Non pCR	30 (65.2)	11 (36.7)	0	0-11	0-210	0.865
pCR	16 (34.8)	7 (43.8)	0	0-10	0-40	
Breast and ALN analysis						
Non pCR	33 (71.7)	9 (27.3)	0	0-12	0-210	0.398
pCR	13 (28.3)	4 (30.8)	0	0-6	0-30	

H score: Histo score, pCR: Pathological complete response, NAC: Neoadjuvant chemotherapy, n: Number, PIGR: Polymeric immunoglobulin receptor, IQR: Interquartile range, Min-max: minimum-maximum, ALN: Axillary lymph node

The p values were obtained by Mann-Whitney U test.

The H score of 0 was applied as a cutoff point to classify PIGR mRNA expressions in breast cancer samples in each group into PIGR positive (H score >0) and PIGR negative (H score = 0) to determine the correlation between pCR after NAC and PIGR positivity. For breast-specific analysis, pCR rate was 38.9% in PIGR positive tumours compared with 32.1% in PIGR negative tumours (Table 5.6). For breast and ALN

Table 5.6 Associations between PIGR mRNA expression and pCR after NAC

	pCR n (%)	Non pCR n (%)	Pearson Chi-Squared value (χ^2)	p value
Breast-specific analysis				
PIGR negative	9 (32.1)	19 (67.9)	0.22	0.639
PIGR positive	7 (38.9)	11 (61.1)		
Breast and ALN analysis				
PIGR negative	9 (32.1)	19 (67.9)	0.532	0.466
PIGR positive	4 (22.2)	14 (77.8)		

PIGR: Polymeric immunoglobulin receptor, pCR: Pathological complete response, NAC: Neoadjuvant chemotherapy, n: Number, ALN: Axillary lymph node

The *p* values were obtained by Pearson Chi-Squared test.

analysis, pCR rate was 22.2% in PIGR positive tumours compared with 32.1% in PIGR negative tumours. However, there were no statistically significant differences in the proportion of pCR in PIGR positive tumours compared with PIGR negative tumours either for breast-specific analysis ($\chi^2 = 0.220$, d.f. = 1, $p = 0.639$) or for breast and ALN analysis ($\chi^2 = 0.532$, d.f. = 1, $p = 0.466$). These results suggest that there was insufficient evidence to demonstrate an association between PIGR mRNA expression and a pCR in breast cancer. Therefore, PIGR mRNA expression has not been shown to be a predictive marker of pCR in breast cancer after NAC in this cohort.

5.3.7 PIGR mRNA expression was not associated with improved overall survival

Survival analysis was performed after a median follow up time (time from diagnosis to death or last contact) of 57 months (range 12-132 months). Eight patients (17.4%) have died and 38 patients (82.6%) are still alive. Five-year cumulative survival probability was 88.9% in patients with PIGR positive tumours and 78.3% in patients with PIGR negative tumours. The log-rank test of overall survival provided a *p* value

of 0.406 indicating that the difference in survival between patients with PIGR positive and negative tumours was not statistically significant. Median survival time cannot be calculated because fewer than 50% of the patients had died. A Kaplan-Meier plot of overall survival of these patients is demonstrated in Figure 5.20.

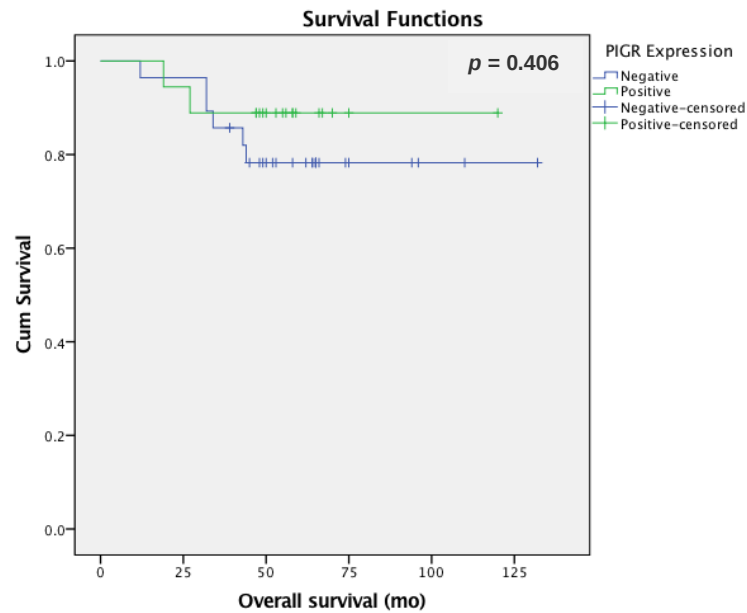


Figure 5.20 A Kaplan-Meier plot of overall survival of the patients with PIGR positive and PIGR negative breast cancers

To determine the correlation between the level of H score and overall survival of the patients with PIGR positive tumours subgroup ($n = 18$), Spearman rank correlation coefficient analysis was carried out. The result showed that there was a weakly positive correlation between H score and overall survival (Spearman correlation coefficient [r_s] = 0.261). However, the correlation was not statistically significant ($p = 0.296$). A scatterplot of the H score and overall survival of the patients with PIGR positive cancers is demonstrated in Figure 5.21.

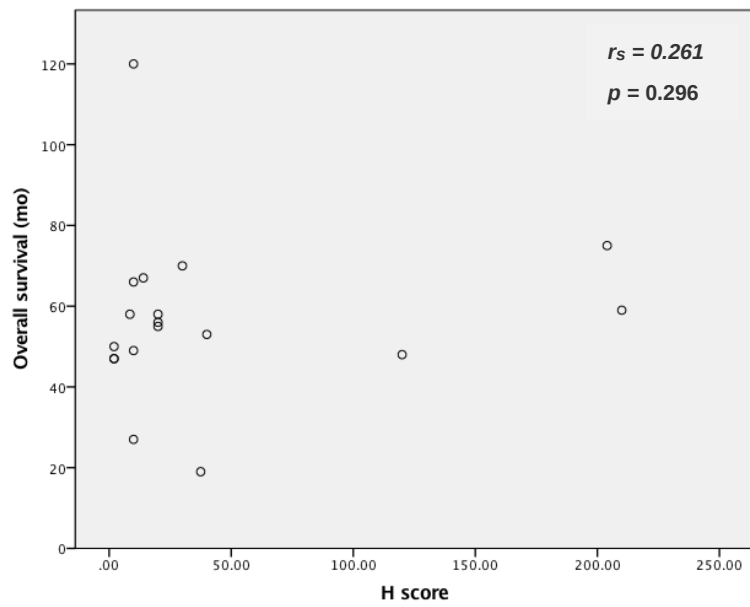


Figure 5.21 A scatterplot of the H score and overall survival of the patients with PIGR positive breast cancers

5.4 Discussion

Neoadjuvant chemotherapy (NAC) is used with increasing frequency as the primary treatment for breast cancer before definite surgery. It aims to downstage the tumour in advanced disease and reduce tumour size in large breast cancers in order to facilitate surgical treatment and increase the likelihood of breast preservation after the surgery. Only 20-30% of patients, however, achieve a pCR, which contributes to survival benefit (102-104). The remaining patients receive little clinical benefit, utilise precious medical resources and may experience significant morbidity. Predicting the patients who are likely to achieve a pCR before commencing NAC, therefore, is beneficial in many respects. The pilot study involved next generation RNA sequencing of fresh-frozen breast cancer biopsy specimens from 10 patients with LLABC prior to NAC. The results revealed that the expression of *PIGR* gene was significantly increased in breast cancers achieving a pCR following NAC. Numerous studies have reported the prognostic significance of *PIGR* in patients with different cancers. Associations between the expression of *PIGR* and cancer prognosis appear to be tissue dependent. To date, a significant predictive role for *PIGR* expression has been reported by only one study on chemo-resistance of pancreatic cancer (219). In the present study, *PIGR* mRNA expression in breast cancers was determined by RNA ISH assay to assess its predictive ability for a pCR after NAC, using FFPE breast cancer tissue specimens from the patients prior to NAC.

The level of *PIGR* mRNA expression was demonstrated to be lower in breast cancer compared with normal breast tissue. The concordance result was previously demonstrated by Park *et al.* (242). They examined *PIGR* protein expression in breast cancer and matched normal tissues by using the cleavable isotope-coded affinity tag (cICAT) labelling approach and tandem mass spectrometry. Western blotting was used to confirm the result that *PIGR* was decreased in breast cancer tissues compared with normal tissues. Downregulation of *PIGR* has been reported in other

malignant and premalignant lesions. Chang *et al.* (356) studied the expression of PIGR in nasopharyngeal cancers and non-malignant nasopharyngeal tissues by RT-PCR and immunohistochemical staining. They found that PIGR expression was detected in 9% of nasopharyngeal cancer tissue biopsies, while 90% of non-malignant nasopharyngeal tissues were PIGR positive. Ocak *et al.* (224) evaluated PIGR expression in airways by IHC. The results showed that PIGR expression was downregulated in preinvasive lesions and lung cancer compared with normal bronchial and alveolar tissues. Colorectal cancers and pre-invasive lesions were also reported to show decreased PIGR expression compared with normal colorectal mucosa (357, 358). These findings are in line with the hypothesis that PIGR expression has a protective effect against carcinogenesis. However, an underlying mechanism of this potential tumour suppressing effect of PIGR in these cancers remains to be elucidated. On the other hand, PIGR expression has been reported to be upregulated in several cancers such as pancreatic ductal adenocarcinoma and endometrial adenocarcinoma (228, 229). In gastric mucosa, PIGR expression was higher in intestinal metaplasia and gastric cancer than in normal gastric mucosa (359). These results support a tumour promoting role of PIGR which was confirmed in hepatocellular carcinoma by Yue *et al.* (227). They demonstrated that PIGR promoted transformation and proliferation of hepatic cells through YES proto-oncogene 1 and Src family tyrosine kinase and suggested that targeting PIGR may have therapeutic potential. The differential expression of PIGR in cancers compared with normal tissues indicates a different functional role of PIGR in carcinogenesis in different cancers, and may serve as a marker of cancer transformation.

Nuclear pleomorphism was the only clinicopathological characteristic significantly associated with PIGR expression in the present study. A higher score of nuclear pleomorphism was associated with a higher proportion of PIGR positive tumours. Nuclear pleomorphism is a factor, combined with tubular formation and frequency of

mitoses of tumour cells, in the Nottingham histologic grading system for breast cancer. High grade tumours were demonstrated to correlate with poorer prognosis but better response to NAC than low grade tumours. Stierer *et al.* (360) found that grading, and particularly nuclear pleomorphism, had a prognostic impact on distant recurrence free survival and overall survival in breast cancer patients stage T1a and T1b without ALN metastases. They concluded that nuclear pleomorphism was a strong prognostic factor in this group of patients. Gass *et al.* (361) reported that a high histologic grade of tumour (grade 3) was a predictor for higher pCR which related to increasing disease free survival and overall survival in TNBC. Huober *et al.* (362) assessed clinical and biological factors for predicting the response to NAC during treatment and pCR at surgery after NAC in operable and LABCs. They reported that a high grade of tumour was a predictive factor for a pCR in luminal subtype breast cancer and related with mid-course response in the TNBCs. They concluded that a high grade can identify the patients who have an increased benefit from NAC in luminal and TNBC subgroup. Spring *et al.* (363) studied the association between pCR and long-term outcomes in young women with breast cancer. They found that a high tumour grade was one of significant predictors for pCR and that pCR was associated with better disease free survival and overall survival.

The association between PIGR expression and tumour grading has been reported in several cancers. Low PIGR expression was significantly associated with a poor differentiation grade of pancreatic and periampullary adenocarcinoma (352). Moreover, it was significantly associated with an impaired five-year survival and early recurrence of the cancer in the patients. In ovarian cancer, PIGR expression was significantly associated with lower grade of cancer cells and improvement in overall survival and cancer specific survival of the patients (223). These results provide evidence of different associations between PIGR expression and tumour grading, which may have different effects on prognosis of patients with each cancer type.

However, in the present study, the association of PIGR expression and pleomorphism did not lead to the association of PIGR expression and tumour grading, which indicates the impact on tumour grading of other factors apart from nuclear pleomorphism.

Tumour subtype was shown to correlate with PIGR expression. Wang *et al.* (364) evaluated a 90-gene expression signature in breast cancer samples and revealed PIGR overexpression in TNBC compared with other breast cancer subtypes. However, an association of PIGR mRNA expression and tumour subtypes was not apparent in this present study. The different results may be caused by the difference in the assay used to evaluate PIGR expression.

The predictive value of PIGR mRNA expression for pCR after NAC was assessed. There was no significant association between PIGR mRNA expression and pCR after NAC, whether breast-specific analysis or breast and ALN analysis. As regards prognostic value, overall survival of patients with PIGR positive tumours was higher than for patients with PIGR negative tumours. However, there was no statistically significant difference. Moreover, the H score and overall survival of the patients with PIGR positive cancers showed a weakly positive correlation that was not statistically significant. These results may be caused by the relatively small sample size in the study which leads to underpower of the statistical test and type II error. Although a total sample size of 22 patients was obtained from sample size calculation to have a 90% power to detect a difference between two groups with p values of ≤ 0.05 , this calculation was based on the data from the pilot study which showed a large difference in a small number of patients in each group. The calculated sample size was probably inaccurate due to the small size of the sample in the pilot study. A larger cohort, therefore, is necessary to determine whether PIGR expression is associated with the overall survival.

These results are at variance with the pilot study which reported an association between high PIGR expression and pCR and good prognosis after NAC. Possible explanations for the discrepancy are the following.

Firstly, the format of the tissue samples used was different in the pilot study, in which fresh-frozen tissue was used, from the present study, which used FFPE tissue. Fresh or fresh-frozen tissue is the preferred sample for most molecular assays because the quality of nucleic acid is better preserved than in FFPE tissue (365, 366). Cross-linking, degradation and fragmentation of nucleic acid during fixation and archiving processes of FFPE tissue have an effect on the quality and quantity of DNA and RNA in the samples (367). Moreover, modification of RNA caused by formalin fixation can reduce base pairing necessary for the hybridisation assay (367). However, fresh-frozen tissues need a specific process and specialised equipment (ultra-low temperature freezer), and incur a high cost for harvesting and storage, which are not usually available in clinical practice (368). Most available specimens from pathological laboratories are FFPE tissue blocks which are routinely prepared and can be stored at room temperature for several years. Therefore, FFPE tissue has become the most common sample type used for molecular testing (368). Several studies have introduced fixation and tissue processing protocols for FFPE samples to minimise the effect on quality and quantity of RNA (369). Overall, studies using RNA sequencing have shown a strong correlation between gene expression of FFPE and of matched fresh-frozen samples (370-372).

Secondly, the RNA evaluation assay used in the pilot study was next generation RNA sequencing and the result was verified by real time qPCR, while the RNA ISH assay was used in the present study. Real time qPCR is more quantitative and sensitive than the ISH assay (373). Small amounts of RNA can be detected and quantified by real time qPCR. PCR itself is a very powerful and practical research tool but the efficiency of RNA extraction and reverse transcription steps may cause some

variations. Moreover, clinically relevant information about cellular and tissue context and heterogeneity of expression pattern is lost in the process. On the other hand, ISH assay preserves morphological context of a tissue which is valuable because it allows the examination of biomarker status within the histopathological context of samples. ISH provides data on individual cells rather than an average of total cellular populations and allows analysis for heterogeneity. RNA expression can be localised in the specific cell type of interest by RNA ISH. Laser microdissection can overcome this issue for PCR to some extent but it cannot provide information about single cells or the heterogeneity pattern of a gene expression. In the pilot study, PIGR mRNA expression was evaluated by real time qPCR using RNA extracted from fresh-frozen samples without prior microdissection to select the breast cancer component. Therefore, the samples may have been a mixture of cancer cells and unintended components, particularly normal breast tissue, giving a false positive result of PIGR expression in the samples.

Thirdly, the present study included patients with smaller breast cancers, of size less than 3 cm, unlike the pilot study. Therefore, these 2 studies represent different populations of breast cancer which may have different profiles of PIGR expression.

Fourthly, HER2 targeted therapy was combined with NAC in the treatment of patients with HER2 +ve cancers in the cohort of the present study but not in the pilot study. All patients with HER2 +ve cancers in the pilot study received HER2 targeted therapy as an adjuvant treatment. Anti-HER2 agents have been demonstrated to improve pCR rate when combined with NAC for patients with HER2 +ve breast cancer (374). Moreover, dual anti-HER2 agents combined with NAC significantly increases pCR rate over a single anti-HER2 agent combined with NAC (375, 376). Achieving a pCR in this group of patients has been shown to result in better long-term outcomes (377, 378).

An important limitation of the present study is the limited amount of tissue obtained for each subject from the biobank. PIGR mRNA expression was an interesting biomarker chosen to be evaluated in this present study. However, an mRNA expression level cannot accurately reflect the level of protein expression which is a final functional product of a gene (379, 380). This may be caused by various factors such as the differences in the rate of production and degradation between RNA and protein, and post-transcription and post-translation processes (379, 380). Because of the limitations of the samples obtained, PIGR protein expression could not be evaluated to confirm the findings of the pilot study and to correlate with PIGR mRNA expression in the present study.

Subjective assessment of RNA ISH assay is another limitation of this study. The interpretation of mRNA expression was performed by visual examination which may cause inconsistency and bias. The level of mRNA expression was determined by a semi-quantitative scoring system. This was multiplied by the percentage of positive cells, resulting in numerical and categorical values for statistical analyses. Digital pathology can be applied to overcome the subjectivity of manual scoring. Automated digital pathology scanners and automated image analysis tools facilitate the interpretation and quantification of biomarker expression within whole tissue sections. They enhance reproducibility and reliability of the scoring results. However, the sensitivity and specificity of an automated system remain uncertain (381). Additionally, this system needs more time for analysis. Therefore, manual semi-quantification was used in the present study.

In conclusion, the present study confirmed previous reports that PIGR mRNA expression is lower in breast cancer in comparison with normal breast tissue. This is the first study, to the best of our knowledge, on the predictive and prognostic values of PIGR mRNA expression in breast cancer. However, the expression of PIGR did

not appear to be associated with pCR after NAC and did not correlate with overall survival.

**Chapter 6 Summary of the Significant
Findings from the Studies, General
Discussion and Further Studies**

Chapter 6 Summary of the Significant Findings from the Studies, General Discussion and Further Studies

6.1 Summary of the significant findings from the studies

The significant findings from the studies in this thesis are summarised in Table 6.1.

Table 6.1 Summary of the significant findings from the studies in this thesis

New/Original findings
1. The levels of PIGR mRNA and protein expression were significantly higher in MDA-MB468 breast cancer cells than in MCF7 breast cancer cells. 2. PIGR overexpression did not lead to significant differences in cell viability, cell proliferation and chemo-sensitivity between un-transduced and PIGR transduced cells for both MCF7 and MDA-MB468 cell lines. 3. IFN-γ and IL-1β increased PIGR expression in MCF7 and MDA-MB468 cell lines. 4. TNF-α, IL-10 and TGF-β did not regulate PIGR expression in MCF7 and MDA-MB468 cell lines. 5. M1 macrophages enhanced PIGR expression in MDA-MB468 cells through IL-1β. 6. Free secretory component did not polarise macrophages. 7. PIGR mRNA expression was significantly associated with a high score of nuclear pleomorphism of breast cancer cells. 8. PIGR mRNA expression was not associated with a pathological complete response and did not correlate with overall survival in breast cancer after NAC.
Confirmed previous published findings
1. PIGR expression was down-regulated in breast cancer in comparison with normal breast tissue (Park <i>et al.</i> [2015]) (242).

6.2 General discussion

Gene expression studies have been used in many aspects of diagnosis and treatment of breast cancer, for example screening of high risk groups, classification of tumour subtypes, categorisation of patients according to risk of recurrence, and guidance for effective treatment in adjuvant therapeutic settings (21, 382, 383). Studies of gene expression as predictive and prognostic markers for breast cancer are, therefore, areas of active clinical and basic scientific research.

This thesis is based on a previous pilot study conducted in the department. Fresh-frozen breast cancer biopsy specimens from patients with LLABCs prior to NAC were studied. By using next generation RNA sequencing, real-time qPCR and Western blotting, the expression of PIGR mRNA and protein was demonstrated to be increased significantly in LLABCs achieving a pCR after NAC. In this thesis, *in vitro* cell line models were used to explore the expression and role of PIGR in breast cancer and its relation to macrophages. Predictive and prognostic values of PIGR in breast cancer were determined using FFPE breast cancer core biopsy specimens from patients prior to NAC.

The expression of PIGR in breast cancer cells was determined in MCF7 and MDA-MB468 breast cancer cell lines. PIGR mRNA and protein expression was shown to be higher in MDA-MB468 breast cancer cells than in MCF7 breast cancer cells. Breast cancer is a heterogeneous disease. Gene expression profiling studies have classified breast cancer into several subtypes with distinct clinical features and outcomes (21-23, 384-386). Breast cancer cell lines which have been widely used in breast cancer modelling are characterised according to gene expression profiling and correspond to breast cancer subtypes *in vivo* (230, 234). The MCF7 cell line is classified as Luminal A subtype, while the MDA-MB468 cell line is classified as basal-like 1 subtype (234). The most significant difference in gene expression profiling is the ER cluster of genes such as *ER1* and *LIV-1* (30). From the results of the study,

PIGR was shown to be another gene which is expressed differently in the luminal A and basal-like 1 subtype. The expression of *PIGR* was significantly higher in MDA-MB468 cells than in MCF7 cells, which indicates that the role of *PIGR* may be specific to the subtype of breast cancer. The concordance result was reported by Wang *et al.* (364) who analysed the expression profiles of 90 tumour specific genes in TNBC and compared them with those in non-TNBC. They found that *PIGR* was one of 17 genes overexpressed in TNBC compared with non-TNBC and suggested its role as a discriminatory marker of TNBC. TNBC is a highly diverse group of breast cancers characterised by the absence of ER, PR and HER2 expression. Treatment of patients with TNBC has been a challenge because of a lack of molecular targets for treatment and worse outcomes (387). Basal-like 1 subtype is classified as one of the six genomic subtypes of TNBC: basal-like 1, basal-like 2, immunomodulatory, mesenchymal-like, mesenchymal stem-like and luminal androgen receptor subtypes (136). Each subtype of TNBC has a different gene expression profile and requires different effective treatments. Basal-like 1 and 2 subtypes have increased expression of cell cycle and DNA damage-response genes and are sensitive to DNA-damaging agents such as cisplatin (388). Therefore, *PIGR* may play a part as a predictive marker to facilitate patient-tailored treatment for TNBC.

The role of *PIGR* in breast cancer was subsequently determined by evaluating cellular behaviour and chemo-sensitivity after *PIGR* gene transduction. Viral overexpression of *PIGR* in MCF7 and MDA-MB468 cells did not affect cell viability, cell proliferation and chemo-sensitivity. These results have not been documented previously, and provide a better understanding of the role of *PIGR* in breast cancer. The results imply that *PIGR* may not have a role in cell viability and cell proliferation and may not translate to chemo-sensitivity of the cells. However, there is evidence that *PIGR* expression is associated with a good prognosis in breast cancer patients (226). Therefore, *PIGR* may have a role in, or relate to, other mechanisms not studied in

this thesis, such as cellular invasion, metastasis, apoptosis and epithelial mesenchymal transition. These mechanisms are associated with the aggressiveness of the tumour cells and affect the prognosis of breast cancer patients. Evidence to support this hypothesis was demonstrated in hepatocellular carcinoma, pancreatic carcinoma, endometrial carcinoma and lung cancer (224, 227-229).

Physiologically, PIGR is expressed on mucosal epithelial cells and has a pivotal role in the mucosal immune system, protecting the mucosa from invading pathogens (183, 186). Several cytokines secreted by immune cells have been demonstrated to induce PIGR expression (183, 284, 293, 324-326). In this thesis the association between PIGR and macrophages, which are the major component of the immune inflammatory cells in the TME, was investigated (268). Furthermore, cytokines which regulate PIGR expression in breast cancer cells were identified. The results showed that free secretory component, which is the secreted part of PIGR, did not affect macrophage polarisation. On the other hand, M1 macrophages were able to enhance PIGR expression in MDA-MB468 cells through IL-1 β . IFN- γ was also demonstrated to increase PIGR expression. The level of PIGR expression increased with increasing concentration of these cytokines. These results suggest the complexity of PIGR regulation *in vivo* and imply that PIGR expression in breast cancer may reflect the immune status of the TME.

The TME has been demonstrated to have a significant role in cancer development, progression and response to therapy (262). Interactions between the TME and cancer cells can recruit, activate and reprogram the stromal and immune cells in the TME to advantage during cancer progression (389, 390). The immune status of the TME is a result of a complex interplay between many immune components (391). In breast cancer, many studies have reported the association of components in the TME with patient outcome and response to therapy. Jeong *et al.* (274) reported the prognostic value of TAMs in invasive breast cancer and that infiltration of CD11c⁺ TAMs (M1

macrophage) was associated with a good prognosis with higher disease free survival and overall survival, while CD163⁺ TAMs (M2 macrophage) were related to poor prognosis. Heys *et al.* (319) demonstrated the association between a high number of SOCS3⁺ TAMs (M1 macrophages) and a pCR after NAC. A significant correlation between TILs and specific lineage subsets and response to NAC has been reported. Kaewkangsan *et al.* (153) demonstrated that high levels of TILs and of CD4⁺, CD8⁺ and CTLA-4⁺ T cells, and high ratios of CD8⁺:FOXP3⁺ were significantly associated with a pCR after NAC. Tumour-infiltrating NK cells was demonstrated to correlate with pCR after neoadjuvant treatment with anti-HER2 agent combined with standard NAC in HER2 +ve breast cancer (392). However, there was no correlation between tumour-infiltrating neutrophils or tumour-infiltrating dendritic cells and a pCR following NAC in LLABCs (159). It is obvious that the immune status of the TME surrounding the cancer is crucial and is considered as a predictive and prognostic marker. Establishing biomarkers which represent the immune status of the TME may improve clinical decisions for optimal cancer treatment. PIGR may be one of the factors providing an overview of immune status in the TME of breast cancer and be beneficial for patient care.

A clinical study of PIGR was conducted to evaluate the expression and predictive and prognostic roles of PIGR in breast cancer. Breast cancer tissue from patients who underwent NAC was studied for PIGR mRNA expression by RNA ISH and correlated with clinical and pathological characteristics. The results showed that the expression of PIGR mRNA was lower in breast cancer tissue than in adjacent normal breast tissue and was not associated with a pCR after NAC or with overall survival. Downregulation of PIGR in breast cancer tissue compared with normal breast tissue was consistent with the observations in colorectal and lung cancer (224, 357, 358). This finding implies a broader role of PIGR in tumour suppressive functions in cancers. The functional roles of PIGR in cancer development and progression have been

determined in several cancers. Overexpression of PIGR in lung cancer cell lines was demonstrated to reduce cell proliferation significantly but did not affect cell cycle, apoptosis and necrosis of the cells (224). In contrast, PIGR was documented to promote transformation and proliferation in hepatic cells (227). In this thesis, the functional role of PIGR in breast cancer was evaluated regarding cell viability and cell proliferation; however, PIGR overexpression did not have an effect on these cellular behaviours. These diverse results provide evidence that PIGR may have different functional roles and affect different molecular mechanisms depending on cancer cell type. Further knowledge of functional roles of PIGR in breast cancer and their molecular mechanisms may improve the understanding of the molecular steps leading to breast cancer.

6.3 Further studies

In this thesis, studies of PIGR expression, effect of PIGR overexpression on chemosensitivity and cellular behaviour and the association between PIGR and TME were performed in two different cell lines categorised as luminal A (MCF7 cell line) and basal-like (MDA-MB468 cell line) subtypes. Breast cancer is a heterogeneous malignancy which varies in clinical course, response to treatment and clinical outcome. Therefore, it would be of interest to further study PIGR expression and its roles in other subtypes of breast cancer, including luminal B and HER2 enriched subtypes as well as in each subtype of TNBC, to obtain more insight into PIGR. This information will be of interest for future studies involving both basic and clinical research.

Another interesting area for further exploration is documenting the association of PIGR expression with other components in the TME such as TILs, NK cells and T cells and their relation to tumour progression and response to treatment. PIGR

expression may be an additional biomarker reflecting immune status of the TME, indicating the treatment response and prognosis of patients.

Although PIGR mRNA expression was not associated with a pCR and overall survival in breast cancer patients in the cohort studied in this thesis, a larger cohort is necessary to confirm whether PIGR expression is associated with a pCR and overall survival. It is worth considering these associations in a more specific or homogeneous group of patients such as in each subtype, early stage breast cancer or LLABCs. Moreover, further study investigating PIGR protein expression in breast cancer tissue from patients would provide more information on the predictive and prognostic roles of the PIGR.

It is interesting that PIGR expression was demonstrated to be decreased in breast cancer compared with normal breast tissue. As discussed previously, this finding implies a suppressive role of PIGR against carcinogenesis. Together with the evidence that PIGR expression is associated with a good prognosis in breast cancer patients, it is possible that PIGR may have a role in cancer development and progression. Therefore, further studies to identify the role of PIGR and related molecular mechanisms in processes such as cell cycle, transformation, apoptosis and epithelial mesenchymal transition will improve our knowledge of carcinogenesis and progression of breast cancer. This may translate to early detection and treatment of breast cancer patients.

An accurate, reliable and practical predictive profile of a pCR after NAC still needs to be identified. According to the results from the pilot study, *SEPT3* is another gene which showed different expression between a pCR and a non-pCR group by next generation RNA sequencing and has a reverse relationship with *PIGR*. Therefore, it would be worth investigating the role of *SEPT3* in breast cancer and its association with patient outcomes in a larger cohort of breast cancer patients in future studies.

TNM Classification of Breast Cancer (AJCC 8th Edition, 2017)

Primary tumour (T)

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis (DCIS)	Ductal carcinoma <i>in situ</i>
Tis (Paget)	Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma <i>in situ</i> (DCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget disease should still be noted
T1	Tumour ≤ 20 mm in greatest dimension
T1mi	Tumour ≤ 1 mm in greatest dimension
T1a	Tumour >1 mm but ≤ 5 mm in greatest dimension (round any measurement >1.0 - 1.9 mm to 2 mm)
T1b	Tumour >5 mm but ≤ 10 mm in greatest dimension
T1c	Tumour >10 mm but ≤ 20 mm in greatest dimension
T2	Tumour >20 mm but ≤ 50 mm in greatest dimension
T3	Tumour >50 mm in greatest dimension
T4	Tumour of any size with direct extension to the chest wall and/ or to the skin (ulceration or macroscopic nodules); invasion of the dermis alone does not qualify as T4

T4a	Extension to the chest wall; invasion or adherence to pectoralis muscle in the absence of invasion of chest wall structures does not qualify as T4
T4b	Ulceration and/or ipsilateral macroscopic satellite nodules and/or oedema (including peau d'orange) of the skin that does not meet the criteria for inflammatory carcinoma
T4c	Both T4a and T4b are present
T4d	Inflammatory carcinoma

Regional lymph nodes (N)

Clinical (cN)

cNX	Regional lymph nodes cannot be assessed (e.g., previously removed)
cN0	No regional lymph node metastases (by imaging or clinical examination)
cN1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
cN1mi	Micrometastases (approximately 200 cells, larger than 0.2 mm, but none larger than 2.0 mm)
cN2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases
cN2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
cN2b	Metastases only in ipsilateral internal mammary nodes in the absence of axillary lymph node metastases

cN3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in ipsilateral internal mammary lymph node(s) with level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
cN3a	Metastases in ipsilateral infraclavicular lymph node(s)
cN3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
cN3c	Metastases in ipsilateral supraclavicular lymph node(s)

Pathological (pN)

pNX	Regional lymph nodes cannot be assessed (e.g., not removed for pathological study or previously removed)
pN0	No regional lymph node metastasis identified or isolated tumour cell clusters (ITCs; malignant cells clusters no larger than 0.2 mm) only
pN0(i+)	ITCs only in regional lymph node(s)
pN0(mol+)	Positive molecular findings by reverse transcriptase polymerase chain reaction (RT-PCR); no ITCs detected
pN1	Micrometastases; or metastases in 1–3 axillary lymph nodes; and/or in clinically negative internal mammary nodes with micrometastases or macrometastases by sentinel lymph node biopsy
pN1mi	Micrometastases (approximately 200 cells, larger than 0.2 mm, but none larger than 2.0 mm)
pN1a	Metastases in 1–3 axillary lymph nodes, at least one metastasis larger than 2.0 mm

pN1b	Metastases in ipsilateral internal mammary sentinel nodes, excluding ITCs
pN1c	pN1a and pN1b combined
pN2	Metastases in 4–9 axillary lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the absence of axillary lymph node metastases
pN2a	Metastases in 4–9 axillary lymph nodes (at least one tumour deposit larger than 2.0 mm)
pN2b	Metastases in clinically detected internal mammary lymph nodes with or without microscopic confirmation; with pathologically negative axillary nodes
pN3	Metastases in 10 or more axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the presence of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and micrometastases or macrometastases by sentinel lymph node biopsy in clinically negative ipsilateral internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in 10 or more axillary lymph nodes (at least one tumour deposit larger than 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	pN1a or pN2a in the presence of cN2b (positive internal mammary nodes by imaging); or pN2a in the presence of pN1b
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

Distant metastasis (M)

M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases in the presence of tumour cells or deposits no larger than 0.2 mm detected microscopically or by molecular techniques in circulating blood, bone marrow, or other nonregional nodal tissue in a patient without symptoms or signs of metastases
cM1	Distant metastases detected by clinical and radiographic means
pM1	Any histologically proven metastases in distant organs; or if in non-regional nodes, metastases greater than 0.2 mm

Histologic grade (G)**Invasive Cancer (Scarff-Bloom-Richardson [SBR] Grading System, Nottingham Modification)**

GX	Grade cannot be assessed
G1	Low combined histologic grade (favourable); SBR score of 3-5 points
G2	Intermediate combined histologic grade (moderately favourable); SBR score of 6-7 points
G3	High combined histologic grade (unfavourable); SBR score of 8-9 points

Ductal Carcinoma *in situ*: Nuclear Grade

The grade that should be used for ductal carcinoma *in situ* is nuclear grade

GX Grade cannot be assessed

G1 Low nuclear grade

G2 Intermediate nuclear grade

G3 High nuclear grade

Anatomic stage groups

	T	N	M
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0	N1mi	M0
	T1	N1mi	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Clinical prognostic stage

TNM	Grade	HER2	ER	PR	Stage
Tis N0 M0	Any	Any	Any	Any	0
T1 N0 M0 T0 N1mi M0 T1 N1mi M0	G1	Positive	Positive	Positive	IA
				Negative	
		Negative	Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
		Negative	Negative	Positive	IB
				Negative	
	G2	Positive	Positive	Positive	IA
				Negative	
		Negative	Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
		Negative	Negative	Positive	IB
				Negative	
	G3	Positive	Positive	Positive	IA
				Negative	
		Negative	Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
		Negative	Negative	Positive	IB
				Negative	

TNM	Grade	HER2	ER	PR	Stage
T0 N1 M0 T1 N1 M0 T2 N0 M0	G1	Positive	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	
	G2	Positive	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	
	G3	Positive	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IIB
				Negative	
			Negative	Positive	
				Negative	

TNM	Grade	HER2	ER	PR	Stage
T2 N1 M0 T3 N0 M0	G1	Positive	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	IIB
		Negative	Positive	Positive	IIA
				Negative	IIB
			Negative	Positive	
				Negative	
	G2	Positive	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	IIB
		Negative	Positive	Positive	IIA
				Negative	IIB
			Negative	Positive	
				Negative	IIIB
	G3	Positive	Positive	Positive	IB
				Negative	IIB
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IIA
				Negative	
			Negative	Positive	IIB
				Negative	

TNM	Grade	HER2	ER	PR	Stage	
T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0	G1	Positive	Positive	Positive	IIA	
				Negative	IIIA	
			Negative	Positive		
				Negative		
		Negative	Positive	Positive		IIA
				Negative	IIIA	
			Negative	Positive		
				Negative	IIIB	
	G2	Positive	Positive	Positive	IIA	
				Negative	IIIA	
			Negative	Positive		
				Negative		
		Negative	Positive	Positive		IIA
				Negative	IIIA	
			Negative	Positive		
				Negative	IIIB	
		G3	Positive	Positive	Positive	IIB
					Negative	IIIA
				Negative	Positive	
					Negative	
	Negative		Positive	Positive	IIIB	
				Negative		
			Negative	Positive	IIIC	
				Negative		

TNM	Grade	HER2	ER	PR	Stage
T4 N0 M0 T4 N1 M0 T4 N2 M0 Any T N3 M0	G1	Positive	Positive	Positive	IIIA
				Negative	IIIB
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
			Negative	Positive	
				Negative	IIIC
	G2	Positive	Positive	Positive	IIIA
				Negative	IIIB
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
			Negative	Positive	
				Negative	IIIC
	G3	Positive	Positive	Positive	IIIB
				Negative	
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IIIC
				Negative	
			Negative	Positive	
				Negative	
Any T Any N Any M	Any	Any	Any	Any	IV

Pathological prognostic stage

TNM	Grade	HER2	ER	PR	Stage
Tis N0 M0	Any	Any	Any	Any	0
T1 N0 M0 T0 N1mi M0 T1 N1mi M0	G1	Positive	Positive	Positive	IA
				Negative	
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
			Negative	Positive	
				Negative	
	G2	Positive	Positive	Positive	
				Negative	
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	
				Negative	
			Negative	Positive	
				Negative	
	G3	Positive	Positive	Positive	IA
				Negative	
			Negative	Positive	
				Negative	
Negative		Positive	Positive		
			Negative		
		Negative	Positive		
			Negative		

TNM	Grade	HER2	ER	PR	Stage
T0 N1 M0 T1 N1 M0 T2 N0 M0	G1	Positive	Positive	Positive	IA
				Negative	IB
			Negative	Positive	
				Negative	IIA
		Negative	Positive	Positive	IA
				Negative	IB
			Negative	Positive	
				Negative	IIA
	G2	Positive	Positive	Positive	IA
				Negative	IB
			Negative	Positive	
				Negative	IIA
		Negative	Positive	Positive	IA
				Negative	IIA
			Negative	Positive	
				Negative	
	G3	Positive	Positive	Positive	IA
				Negative	IIA
			Negative	Positive	
				Negative	
		Negative	Positive	Positive	IB
				Negative	IIA
			Negative	Positive	
				Negative	

TNM	Grade	HER2	ER	PR	Stage
T2 N1 M0 T3 N0 M0	G1	Positive	Positive	Positive	IA
				Negative	IIB
		Negative	Positive	Positive	
				Negative	IA
		Negative	Positive	Positive	IIB
				Negative	
	G2	Positive	Positive	Positive	IB
				Negative	IIB
		Negative	Positive	Positive	
				Negative	IB
		Negative	Positive	Positive	IIB
				Negative	
	G3	Positive	Positive	Positive	IB
				Negative	IIB
		Negative	Positive	Positive	
				Negative	IIA
		Negative	Positive	Positive	IIB
				Negative	IIIA

TNM	Grade	HER2	ER	PR	Stage
T0 N2 M0 T1 N2 M0 T2 N2 M0 T3 N1 M0 T3 N2 M0	G1	Positive	Positive	Positive	IB
				Negative	IIIA
		Negative	Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IB
				Negative	IIIA
			Positive	Positive	
			Negative	Negative	
	G2	Positive	Positive	Positive	IB
				Negative	IIIA
			Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IB
				Negative	IIIA
			Positive	Positive	
			Negative	Negative	
	G3	Positive	Positive	Positive	IIA
				Negative	IIIA
			Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IIB
				Negative	IIIA
			Positive	Positive	
			Negative	Negative	

TNM	Grade	HER2	ER	PR	Stage
T4 N0 M0 T4 N1 M0 T4 N2 M0 Any T N3 M0	G1	Positive	Positive	Positive	IIIA
				Negative	IIIB
		Negative	Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IIIA
				Negative	IIIB
	G2	Positive	Positive	Positive	IIIA
				Negative	IIIB
		Negative	Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IIIA
				Negative	IIIB
		Negative	Positive	Positive	
			Negative	Negative	IIIC
	G3	Positive	Positive	Positive	IIIB
				Negative	
		Negative	Positive	Positive	
			Negative	Negative	
		Negative	Positive	Positive	IIIC
			Negative	Negative	
Any T Any N Any M	Any	Any	Any	Any	IV

Genomic profile for pathological prognostic staging: when Oncotype Dx score is less than 11

TNM	Grade	HER2	ER	PR	Stage
T1 N0 M0 T2 N0 M0	Any	Negative	Positive	Any	IA

Data from National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology, 2021 (49).

Solutions and Reagents

Development of stable cell lines

1× Phosphate buffered saline (PBS)

Dissolve 1 phosphate buffered saline (PBS) tablet (BR0014G; Oxoid, Cheshire, UK) in 100 ml of deionized water. Sterilize solutions by autoclaving. Store the buffer at RT.

50× Tris-Acetate-Ethylene-di-amine-tetra-acid (TAE) buffer (pH 8.3)

Dissolve 242 gm of Tris-base in 700 ml of deionized water and mix well. Carefully add 57.1 ml of 100% acetic acid and 100 ml of 0.5 M ethylene-di-amine-tetra-acid (EDTA) (pH 8.0). Adjust the volume of the solution to 1 L with deionized water. The pH of this buffer is not adjusted and should be about 8.3. Store the buffer at RT. This stock solution is diluted 49:1 with deionized water to make 1× working solution. This 1× solution will contain 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA.

0.5 M Ethylene-di-amine-tetra-acid (EDTA)

Dissolve 18.61 gm of EDTA in 60 ml of deionized water and mix well. Adjust pH to 8. Adjust the volume of the solution to 100 ml with deionized water. Sterilize solutions by autoclaving. Store the buffer at RT.

RNA isolation

Diethyl pyro-carbonate (DEPC)-treated high-performance liquid chromatography (HPLC) grade water

Add 1 ml of 0.1% diethylpyrocarbonate (DEPC) to 1 L high-performance liquid chromatography (HPLC) grade water and mix well until the globules of DEPC disappear. Incubate at 37 °C overnight and then autoclave. Store at RT for up to 12 months.

3M sodium acetate (pH 5.2)

Dissolve 40.83 gm of sodium acetate in 40 ml of HPLC grade water and mix well. Allow the solution to be at RT. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume of the solution to 100 ml with HPLC grade water. Add 100 µl of 0.1% DEPC to the solution and mix well. Incubate at 37 °C overnight and then autoclave. Store the solution at RT.

Protein isolation

Radioimmunoprecipitation assay (RIPA) buffer

Mix together 2 ml of 1.5 M sodium chloride, 500 µl of 1 M Tris-hydrochloride (pH 7.6), 2 ml of 10% Triton X-100, 2 ml of 10% sodium deoxycholate, 200 µl of 10% sodium dodecyl sulfate (SDS) and 13.3 ml deionized water. Aliquot and store at -20 °C. Before using RIPA buffer, add 40 µl of 25× Complete Protease Inhibitor Cocktail stock solution (11697498001; Roche, Penzberg, Germany) in 1 ml of RIPA buffer.

1.5 M sodium chloride (NaCl)

Dissolve 8.766 gm of NaCl in 80 ml of deionized water and mixed well. Adjust the volume of the solution to 100 ml with deionized water. Sterilize solutions by autoclaving. Store the buffer at RT.

1 M Tris-hydrochloride (pH 7.6)

Dissolve 12.11 gm of Tris-base in 80 ml of deionized water and mix well. Adjust pH to 7.6. Allow the solution to be at RT. Final adjust the pH to 7.6. Adjust the volume of the solution to 100 ml with deionized water. Sterilize solutions by autoclaving. Store the buffer at RT.

10% Triton X-100

Mix together Triton X-100 10 ml and 90 ml deionized water. Store the solution at 4 °C.

10% sodium deoxycholate (Na deoxycholate)

Dissolve 1 gm of Na deoxycholate in 5 ml of deionized water and mixed well. Adjust the volume of the solution to 10 ml with deionized water. Protect the solution from light and store at RT.

10% sodium dodecyl sulfate (SDS)

Dissolve 10 gm of SDS in 70 ml of deionized water and mixed well. Leave the solution at RT to allow bubbles to disappear. Adjust the volume of the solution to 100 ml with deionized water. Store the solution at RT.

Western blotting

2× Laemmli buffer

Mix together 1 ml of 1 M Tris-hydrochloride (pH 6.8), 4 ml of 10% SDS, 0.31 gm of dithiothreitol (DTT), 2 ml of glycerol, 20 µl of 0.5M EDTA and 2 mg of bromophenol blue. Adjust the volume of the buffer to 10 ml with deionized water. Aliquot and store at -20 °C.

5× Laemmli buffer

Mix together 3.75 ml of 1 M Tris-hydrochloride (pH 6.8), 1.5 gm of SDS, 1.16 gm of DTT, 7.5 ml of glycerol and 7.5 mg of bromophenol blue. Adjust the volume of the buffer to 15 ml with deionized water. Aliquot and store at -20 °C.

6× Laemmli buffer

Mix together 4.5 ml of 1 M Tris-hydrochloride (pH 6.8), 1.8 gm of SDS, 1.39 gm of DTT, 9 ml of glycerol and 9 mg of bromophenol blue. Adjust the volume of the buffer to 15 ml with deionized water. Aliquot and store at -20 °C.

1 M Tris-hydrochloride (pH 6.8)

Dissolve 12.1 gm of Tris-base in 80 ml of deionized water and mix well. Adjust pH to 6.8. Adjust the volume of the solution to 100 ml with deionized water. Store the buffer at RT.

10× Running buffer

Dissolve 75.7 gm of Tris-base, 360.5 gm of glycine, and 25 gm of SDS in 1.5 L of deionized water and mix well. Adjust the volume of the solution to 2.5 L with deionized water. Store the stock buffer at RT and dilute to 1× before use.

10× Transfer buffer

Dissolve 75.7 gm of Tris-base and 360.5 gm of glycine in 1.5 L of deionized water and mix well. Adjust the volume of the solution to 2.5 L with deionized water. Store the stock buffer at RT. To make 1× transfer buffer, mix 100 ml of 10× transfer buffer, 200 ml of methanol and 700 ml of deionized water per litre.

10× Tris-buffered saline (TBS)

Dissolve 75 gm of Tris-base, 200 gm of sodium chloride and 5 gm of potassium chloride in 1.5 L of deionized water and mix well. Adjust pH to 7.6. Adjust the volume of the solution to 2.5 L with deionized water. Store the stock buffer at RT.

Tris-buffered saline and 0.1% Tween 20 (TBST)

Mix 100 ml of 10× TBS with 900 ml of deionized water. Add 1 ml of Tween 20 and mix well.

RNA *in situ* hybridization (ISH) and immunofluorescence (IF) staining

0.02% ammonia water (bluing reagent)

Add 1.43 ml of 1N ammonium hydroxide to 250 ml deionized water and mixed well.

1N ammonium hydroxide

Dilute 1 ml of 28-30% ammonium hydroxide (423305000; Acros Organics, Geel, Belgium) in deionized water 16 ml and mixed well.

Sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0)

Dissolve 2.94 gm of Tri-sodium citrate (dihydrate) in 800 ml of deionized water and mix well. Adjust pH to 6.0 by adding 1 M HCl. Adjust the volume of the solution to 1 L with deionized water. Add 0.5 ml of Tween 20 and mix well. Store the buffer at RT for 3 months or at 4 °C for longer storage.

0.2 M Phosphate buffer (PB)

Dissolve 184 gm of sodium phosphate dibasic and 42 gm of sodium phosphate monobasic in 1 L of deionized water and mixed well. Adjust pH to 7.2-7.4. Adjust the volume of the solution to 8 L with deionized water. Store the buffer at RT.

Phosphate buffered saline with Triton X-100 (PBST)

Add 18 gm of sodium chloride and 3 ml of Triton X-100 to 1 L of 0.2 M PB and 1 L of deionized water and mixed well. Store the buffer at RT.

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