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**Deciphering the morphological features underlying breast cancer
behaviour using computational pathology and Artificial
intelligence
(with focus on the role of the tumour microenvironment)**

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DEDICATION

To the wings of my journey, my mum “Tayseer” and my daughter “, Malika”.

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DECLARATION

I declare that this thesis is the result of my own work and has not in this, or any other form, been presented to this, or any other University in support of an application for any degree other than for which I am now a candidate.

Suzan Ghannam

November 2024

APPROVAL

This study was approved by the Yorkshire & the Humber - Leeds East Research Ethics Committee (REC Reference: 19/YH/0293) under the IRAS Project ID: 266925. Data collected were fully anonymised.

This work has been approved by Nottingham Research Ethics Committee 2, Reference number: REC: C20313 Under the title: Development of a molecular genetic classification of breast cancer.

This study obtained ethical approval to use the human tissue samples from the Northwest – Greater Manchester Central Research Ethics Committee under the title; Nottingham Health Science Biobank (NHSB), reference number 15/NW/0685. Informed consent was obtained from all individuals and all samples were anonymised.

ABBREVIATIONS

ACC	Adenoid cystic carcinoma
ADH	Atypical ductal hyperplasia
AFM	Atomic force microscopy
AI	Artificial intelligence
ASCO	The American Society of Clinical Oncology
AUC	The area under the curve
BC	Breast cancer
BCSS	Breast cancer specific survival
BM	Basement membrane
BMZ	Basement membrane zone
BRCA1	BC type-1 susceptibility protein
BRCA2	BC type-2 susceptibility protein
CAP	College of American Pathologists
CI	Confidence intervals
CK5	Cytoskeletal 5
CNN	Convolutional neural network
CNV	Copy number variations
CT	Connective tissues
CTLs	CD8+ T-cell lymphocytes
DAB	3,3'-Diaminobenzidine
DCIS	Ductal carcinoma <i>in situ</i>
DGE	Differential gene expression
DM	Distant metastasis
DMFS	Distant metastasis free survival
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence

ED MT	Endothelial-to-mesenchymal transition
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
EMT	Epithelial-mesenchymal transition
EP	EndoPredict assay
EPC	Encapsulated papillary carcinoma
ER	Oestrogen hormone
ERBB2	The tyrosine-protein kinase receptor erbB-2
FACITs	Fibril-associated collagens with interrupted triple helices
FDA	The US Food and Drug Administration
FDR	False discovery rate
FFEP	Formalin-fixed paraffin-embedded
FN	False negative
FP	False positive
GGI	Genomic grade index
H&E	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor-2
HLA	Human leukocyte antigens
HPF	High-power field
HR	Hormone receptor
HR	Hazard ratios
HS/HT	High STR/High TILs
HS/LT	High STR/Low TILs
H-score	Histo-score
HSPG	Heparan sulphate proteoglycan
ICC	Intercorrelation coefficient
IHC	Immunohistochemistry

ILC	Invasive lobular carcinoma
IMPC	Invasive micropapillary carcinoma
IMS	Industrial methylated spirit
INV	Invasive group
IPC	Invasive papillary carcinoma
IRAC	The International Agency for Research on Cancer
KEGG	Kyoto Encyclopedia of Genes and Genomes
KM	Kaplan Meier
LH2	Lysyl hydroxylase2
LN	Lymph node
LOH	Loss of heterozygosity
LOX	Lysyl oxidase
LRFS	Local recurrence free survival
LS/HT	Low STR/High TILs
LS/LT	Low STR/Low TILs
LVI	Lymph vascular invasion
MACITs	Membrane-anchored collagens
MDSCs	Myeloid-derived suppressor cells
MEC	Myoepithelial cells
MGA	Microglandular adenosis
MMP	Matrix metalloproteinases
MSCs	Mesenchymal stem cells
NHS	National Health System
NK	Natural killer
NPI	Nottingham Prognostic Index
NST	No special type
OM	OncoMasTR

PAS	Periodic acid Schiff
PAS-D	Periodic acid Schiff diastase
pCR	Pathological complete response
PDL1	Programmed cell death 1
PDL1	Programmed cell death ligand-1
PGs	Proteoglycans
PL	Polarised light
PLC	Pregnancy-lactation cycle
PR	Progesterone hormone
RANKL	Nuclear factor kappa-B ligand
RBCs	Red blood cells
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RRFS	Regional recurrence free survival
S.inv	Special invasive group
SD	Standard deviation
SEER	National Cancer Institute's Surveillance Epidemiology and End Results
SEM	Standard error of mean
SEM	Scanning electron microscopy
SHG	Second Harmonic Generation Microscopy
SPC	Solid papillary carcinoma
SPSS	Statistical Package for Social Sciences
STR	Stroma-tumour ratio
TAMs	Tumour associated macrophages
TBS	Tris buffered saline
TCCRP	Tall cell carcinoma with reverse polarity
TDLU	Terminal duct lobular unit

TEM	Transmission electron microscopy
THBS1	Thrombospondin 1
TILs	Tumour infiltrating lymphocytes
TME	Tumour microenvironment
TN	True negative
TNBC	Triple-negative BC
TNF	Tumour necrosis factor
TP	True positive
Tregs	Regulatory T cells
TSR	Tumour stroma ratio
UDH	Usual ductal hyperplasia
VEGF	Vascular endothelial growth factor
VNPI	Van Nuys prognostic index
WebGestalt	WEB-based Gene SeT Analysis Toolkit.
WHO	World Health Organization
WSI	Whole slide image
α -SMA	Smooth muscle actin- α

Abstract

Background:

Breast cancer (BC) is a heterogeneous disease with variable presentations, morphologies, and behaviours. The tumour microenvironment (TME) plays a key role in tumour progression. Molecular and phenotypic alterations in the neoplastic cells and their behaviour usually attracts more attention. However, there is evidence that the stroma of BC plays an important role in tumour behaviour. The basement membrane (BM), which comprises a physical barrier that separates the proliferating epithelial cells from the surrounding stroma is used to distinguish malignant *in situ* and invasive lesions. However, exceptions exist. The stroma of BC also varies widely with a variable degree of fibrosis, collagenisation, and elastosis in addition to other microenvironment components such as immune cells. The stromal cells are relatively genetically stable compared to the tumour cells, therefore they may be targets for therapeutic agents for cancer. The revolution of using digitalised whole slide images (WSI) in pathology developed a way of investigating BC cases. In addition, using artificial intelligence (AI) enables the integration of knowledge beyond human limits. However, this is a recently introduced technology, so further assessment of its uses and applications in the clinical setting is of paramount importance. Therefore, this study integrates digital pathology (DP) and AI to decipher the morphological features of TME in BC and identify novel biomarkers for improved BC prognosis.

Patient and Methods:

This study included multiple large cohorts of primary BCs and ductal carcinoma in situ (DCIS) in either tissue microarray (TMA) or full-face sections. The Nottingham cohort and the online cohort the Cancer Genome Atlas (TCGA) BC dataset. The detailed clinicopathological and outcome data were collected for the Nottingham cohort. For the DP part of the study, the haematoxylin and eosin (H&E)-stained slides were

histologically reviewed and scanned into WSIs, and the same sections were picrosirius (PSR) stained to be examined under polarised microscopy or kept unstained for differential interference contrast microscopy (DIC). Image analysis assessment of the geometric characterisation of collagen was performed using AI applications.

For the immunohistochemical (IHC) part of the study, TMAs were constructed, and full-face sections were also utilised. Mining for stromal-related genes and collagens-related genes as novel biomarkers that may have potential prognostic and predictive values in BC was performed. This included hypoxia-inducible factors alpha 1 (HIF-1 α), Lysyl oxidase (LOX), collagen XV and XIX.

Differential gene expression (DGE) analysis was performed to identify a set of genes associated with stroma in the main molecular BC subtypes and their prognostic roles in each molecular subtype. Stromal features and types were scored in 822 cases and the association to outcome was evaluated.

Results:

This study aimed to decipher the morphological features of TME in BC. Therefore, we started with BM as a part of TME and hypothesised that differentiation of a native BM from a reactive BM surrounding an invasive lesion is clinically important for early diagnosis. The study included 150 cases divided into six groups, each containing 25 patients per group. The six groups included normal breast tissue as a control group, DCIS, encapsulated papillary carcinoma (EPC), invasive carcinoma (INV), special types of invasive carcinoma (S.INV) and metastatic lymph node (LN). Full-face sections were stained with PSR stain and examined using polarized microscopy. Image analysis software was used to assess collagen fibre parameters. DCIS' BMs resembled normal tissue BMs except the collagen fibres were higher in density, straighter, disorganised and less aligned. However, EPC, invasive and special invasive groups had a higher density of collagen with thicker fibres, and more collagen I

content which were wider, shorter, straighter, less aligned and disorganised. The EPC group resembled invasive groups regarding alignment but had longer fibres than special BC but less wide fibres, and less collagen I (thick fibre) content, than invasive BC.

Therefore, the EPC group was further analysed to assess the differences between the inner and outer parts of the thickened capsule and to compare the capsule parts with the other groups with an additional encapsulated papillary thyroid carcinoma (EPTC) control group. The collagen fibre parameters of EPC capsules differed from the BMs of normal breast tissue and DCIS, and EPC was surrounded with BM-like material resembling those surrounding some invasive tumour types. This provides further evidence that EPC is a reactive process rather than a thickened native BM. In addition, most papillary tumours in different organs, such as EPTC which are surrounded with BM-like material showed indolent behaviour with better prognosis.

To study collagen fibre parameters of the stroma, a TMA was generated from 200 cases, comprising ductal carcinoma in situ (DCIS; n=100) and invasive tumours (n=100), with an extra 50 (25 invasive BC and 25 DCIS) cases for validation was unstained for examination using DIC microscopy. The collagen fibres had higher density, and were thinner, straighter more disorganized and less aligned in the invasive BC compared to DCIS. A model considering these features was developed that could distinguish between DCIS and invasive tumours with 94% accuracy. There were strong correlations between fibre characteristics and clinicopathological parameters in both groups. A statistically significant association between fibre characteristics and patients' outcomes was observed in the invasive group but not in DCIS.

Stromal-related genes and collagen-related genes were selected for immunohistochemistry assessment to explore novel biomarkers. The assessment of the selected biomarker panel revealed a significant association between their protein expression and patient outcome. For the assessment of collagen XV and XIX, 100 cases of full-face sections

were used which included both normal and neoplastic breast tissue. The cases were divided into four groups, each containing 25 patients. The four groups included normal breast tissue, DCIS, EPC, and invasive carcinoma. Collagen XV and XIX were significantly involved in the structural and biological changes associated with BC and may act as potential biomarkers. Collagen XIX could be used as a diagnostic tool to differentiate between invasive and non-invasive lesions in challenging cases. For the assessment of HIF-1 α and LOX protein expression, TMA sections of a large well-characterised cohort of BC (n=876) were used. HIF-1 α and LOX could be new biomarkers for BC prognosis and therapy response. HIF-1 α levels in BC may be used to prospectively stratify patients who received neoadjuvant therapy. LOX could be a good therapeutic target specifically for metastasis prevention.

The mechanisms of stroma formation and composition in different molecular subtypes, which could explain the different prognostic values, were evaluated. Two large well-characterized BC cohorts were used, an in-house BC cohort (n=822) and the public domain dataset TCGA, (n=978) as a validation cohort and for differential gene expression (DGE) analysis. DGE was performed to identify a set of genes associated with high stroma tumour ratio (STR) in the three main molecular subtypes. In each subtype, stromal assessment was carried out and tumours were assigned to two groups: high and low STR, and further correlations with tumour characteristics and patient outcomes were investigated. The contribution of tumour-infiltrating lymphocytes (TILs) to the stroma was also studied. High STR was associated with favourable patient outcomes in the whole cohort and the luminal subtype, whereas high STR showed an association with poor outcomes in triple-negative (TNBC). No association with outcome was found in the HER2-enriched BC. DGE analysis identified various pathways in luminal and TNBC subtypes, with immune upregulation and hypoxia pathways enriched in TNBC, and pathways related to fibrosis and stromal remodelling enriched in the luminal group instead. Low STR accompanied by high TILs was shown to carry the most favourable prognosis in TNBC. In line with the DGE

results, TILs played a major prognostic role in the stroma of TNBC, but not in the luminal or HER2-enriched subtypes. The stroma type could be a predictor factor for predicting outcomes. Desmoplastic sclerotic stroma is the most common type and showed a worse prognosis compared to other stromal types in all histological types except lobular carcinoma.

Conclusion

This comprehensive study emphasises the importance of considering all morphological features of TME in tumour behaviour and prognosis. Collagen parameters of BM and stroma could be used as a diagnostic and prognostic tool to differentiate between invasive and non-invasive lesions. Most papillary tumours in different organs which are surrounded with BM-like material showed indolent behaviour with better prognosis, therefore mechanisms which explain these phenomena should be further investigated. Stromal and collagen-related genes could be used as prognostic biomarkers for BC. The underlying molecular mechanisms and composition of the stroma in BC are variable in the molecular subtypes which explain the differences in its prognostic significance in each molecular subtype. Evaluation of tumour stroma types and features may be easily performed in routine clinic practice as a guide in stratifying patient risk.

STUDY RELATED PUBLICATIONS

- 1- **Ghannam, S. F.**, Makhlof, S., Alsaleem, M., Rutland, C. S., Allegrucci, C., Mongan, N. P., & Rakha, E. A. (2024). The Conflicting Prognostic Role of the Stroma-Tumor Ratio in Breast Cancer Molecular Subtypes. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, 37(12), 100607. Advance online publication. <https://doi.org/10.1016/j.modpat.2024.100607>.
- 2- **Ghannam, S. F.**, Rutland, C. S., Allegrucci, C., Mather, M. L., Alsaleem, M., Bateman-Price, T. D., Patke, R., Ball, G., Mongan, N. P., & Rakha, E. (2024). Geometric characteristics of stromal collagen fibres in breast cancer using differential interference contrast microscopy. *Journal of microscopy*, 10.1111/jmi.13361. Advance online publication. <https://doi.org/10.1111/jmi.13361>.
- 3- **Ghannam, S. F.**, Rutland, C. S., Allegrucci, C., Mongan, N. P., & Rakha, E. (2023). Encapsulated papillary carcinoma of the breast: does it have a native basement membrane? *Histopathology*, 83(3), 376–393. <https://doi.org/10.1111/his.14939>.
- 4- **Ghannam, S. F.**, Rutland, C. S., Allegrucci, C., Mongan, N. P., & Rakha, E. (2023). Defining invasion in breast cancer: the role of basement membrane. *Journal of clinical pathology*, 76(1), 11–18. <https://doi.org/10.1136/jcp-2022-208584>. → This review was accepted without any corrections from the reviewer.
- 5- Makhlof, S., Wahab, N., Toss, M., Ibrahim, A., Lashen, A. G., Atallah, N. M., **Ghannam, S.**, Jahanifar, M., Lu, W., Graham, S., Mongan, N. P., Bilal, M., Bhalerao, A., Snead, D., Minhas, F., Raza, S. E. A., Rajpoot, N., & Rakha, E. (2023). Evaluation of tumour infiltrating lymphocytes in luminal breast cancer using artificial intelligence. *British journal of cancer*, 129(11), 1747–1758. <https://doi.org/10.1038/s41416-023-02451-3>.
- 6- Atallah, N. M., Wahab, N., Toss, M. S., Makhlof, S., Ibrahim, A. Y., Lashen, A. G., **Ghannam, S.**, Mongan, N. P., Jahanifar, M., Graham, S., Bilal, M., Bhalerao, A., Ahmed Raza, S. E., Snead, D., Minhas, F., Rajpoot, N., & Rakha, E. (2023). Deciphering the Morphology of Tumor-Stromal Features in Invasive Breast Cancer Using Artificial Intelligence. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, 36(10), 100254. <https://doi.org/10.1016/j.modpat.2023.100254>.

- 7- Lashen, A. G., Toss, M. S., **Ghannam, S. F.**, Makhoul, S., Green, A., Mongan, N. P., & Rakha, E. (2023). Expression, assessment and significance of Ki67 expression in breast cancer: an update. *Journal of clinical pathology*, 76(6), 357–364. <https://doi.org/10.1136/jcp-2022-208731>.
- 8- Lashen, A.G.; Wahab, N.; Toss, M.; Miligy, I.; **Ghannam, S.**; Makhoul, S.; Atallah, N.; Ibrahim, A.; Jahanifar, M.; Lu, W.; Graham, S.; et al. Characterization of Breast Cancer Intra-Tumour Heterogeneity Using Artificial Intelligence. *Cancers* **2024**, 16

Reviewer for:

Journal of Modern Pathology for manuscript Computational Pathology for Accurate Prediction of Breast Cancer Recurrence: Development and Validation of a Deep Learning-based Tool.
Ziyu Su; Yongxin Guo; Robert Wesolowski; Gary Tozbikian; Nathaniel S. O'Connell; M. Khalid Khan Niazi; Metin N. Gurcan.

Computational and Structural Biotechnology Reports for manuscript MSCAnet: A Multi-Scale Cross-Attention Framework for Enhanced Breast Cancer CIN Status Prediction
Liangliang Liu; Ying Wang; Fengjie Zhao.

Accomplishments

- 1- The review article was the choice of editor-in-chief of the Journal of Clinical Pathology for the year 2023.
- 2- Figure was chosen as the cover image for the Journal of Clinical Pathology for the year 2023.



- 3- Course representative student for stem cells and cancer course.
- 4- My testimonials were chosen to represent the course of cancer and stem cells at the University of Nottingham.

Conference Presentations

- 1- The role of capsule in Encapsulated papillary carcinoma of the breast: An image analysis study, (The Winter Meeting of the Pathological Society, 2024, London, United Kingdom)

Poster presentation

- 2- The prognostic significance of stroma-tumour ratio combined with the immunity status in different molecular subtypes of breast cancer, (The Winter Meeting of the Pathological Society, 2024, London, United Kingdom)

Poster presentation

- 3- The prognostic role of LOX in breast cancer, Cancer Research Nottingham Symposium 2024, Nottingham, United Kingdom.

Poster presentation

- 4- Encapsulated papillary carcinoma of the breast: is it in situ or invasive carcinoma? (The 14th Joint Meeting of the BDIAP and The Pathological Society, 2023, Liverpool, United Kingdom).

Oral presentation.

- 5- Characterisation of stromal features in breast cancer using differential interference contrast microscopy, (The 14th Joint Meeting of the BDIAP and The Pathological Society, 2023, Liverpool, United Kingdom).

Oral presentation.

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1 Chapter 1: General introduction

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1.1 Breast cancer

1.1.1 Definition and Background

Breast cancer (BC) is defined as a heterogeneous disease with a diverse of variable presentations, histological entities, molecular profiles, clinical behaviour, outcome, and therapy responses (1-3). BC is considered a group of diseases rather than a single disease although, cancer subtypes arise from the same anatomical organ, the breast. Therefore, this heterogeneity has significant impacts on both the patient's outcome and management (4, 5). BC encompasses many biological entities which are characterised by pathological heterogeneity, various gene expressions, diverse genomic alterations and heterogenous tumour microenvironment (TME)(6, 7). BC is a multi-factorial disease, which is attributed to complex mechanisms between diverse genetics, hormonal, heritable and environmental factors (8-10).

1.1.2 History of Breast Cancer

Humankind has struggled to recognise BC and its treatment since its first documentation of BC more than 3500 years ago. Historically, ancient Egyptians were the first people to term and describe BC however, the word cancer was not used. Edwin Smith and Ebers papyrus demonstrated that a swelling bulging in the breast was a grave illness that they tried to treat with knife cauterisation, salts, and arsenic paste (11, 12).

Herodotus (484–426 B.C.) who is known as ‘the father of history’, reported that Demokedes a famous doctor treated the queen Atosa from a breast carcinoma (phyma). Currently, it is believed that the queen had mastitis which proved that Herodotus knew the difference when he used the word ‘phyma’ which meant either abscess or neoplasm (13).

After that (460-375 B.C.) in the Greek civilisation, Hippocrates documented his theories about the cause of cancers to a “black bile excess” with therapy routes including castor and opium oils. The growth

of the cells reminded Hippocrates of a moving crab, which led to the term” carcinoma”. Galen (129–216), ‘second Hippocrates’, reported the Galenic humoral theory which defined phlegm and black bile due to excess body liquids and proposed treatment of the tumours with lancet and cauterisation (14, 15).

Finally, from these ancient civilisations until the 18th and 19th centuries, it was agreed that BC was present throughout the world and could not be cured, hence more diagnosis and treatment options were required (14).

1.1.3 Epidemiology

BC surpassed lung cancer to become the most common cancer in women worldwide and now ranks as the fifth reason for cancer-related mortality (16, 17). Globally, it has been predicted that an estimated 2,964,197 new BC cases in women will be diagnosed per annum in 2040, an increase of 31% from the corresponding number of cases observed in 2020 (18).

In 2020, nearly 2.3 million new cases and approximately 685,000 deaths were estimated with a ratio of 1 in 4 new cancer cases while 1 in 6 deaths (18-20). The incidence of BC ratios varied by approximately 4-folds across World Health Organization (WHO) areas. The greatest rates of incidence per 100,000 were reported in Australia (95.5), western Europe (90.7) and Central America (89.4). However, the lowest rates were found in Asia (26.2), Middle and Eastern Africa (33) and South America (39.5) (18, 21). In contrast, BC's highest mortality rates were recorded in Melanesia (37.5), Western Africa (22.3) and the Caribbean (18.9) whereas the incidence lowest mortality rates were in Eastern Asia (9.8), Central America (10.4) and Australia (12.1) that showed a difference estimated with a 2.8-fold (18, 21).

In the UK, about 55,200 new BC cases are diagnosed annually. Approximately 1 in 7 women will have BC throughout their lifetime in the UK (22). BC has a financial burden which has recently increased with an

ageing population and increasing management costs. This costs the National Health System (NHS) over £700 million per year (23).

Historically, BC was considered to be a disease of developed countries, in contrast, now over half of the diagnoses and about two-thirds of BC mortality occur in developing countries (24). In developed countries, overall 5-year survival rate of BC is over 80% compared to developed countries where the 5-year survival rate is less than 50%, this difference may be attributed to early diagnosis strategies combined with better access to effective management in the developed countries (24, 25).

The morbidity of BC represents a wide array of symptoms ranging from mild symptoms to devastated health status resulting from the tumour spreading and metastasis both regionally and systemically. BC complications could also occur as pathologic bone fractures, neurologic manifestations, and organ failure. These complications result from tumour metastasis to bones, the brain, and visceral organs such as the liver and lungs (3). Moreover, BC's various therapy modalities have been reported to trigger numerous morbidities including lymphoedema, cutaneous numbness, and impaired pain sensations which could result from the axillary lymphadenectomy carried out for diagnosis, staging and management purposes (26, 27). In addition, the side effects could be caused by systemic chemotherapy (28, 29), hormonal therapy (29, 30), or humanised antibodies against human epidermal receptor protein 2 (HER2) (31, 32).

1.1.4 Anatomy and Histology of normal breast tissue

To understand BC, we need to differentiate the histology of non-malignant mammary tissue. In embryogenesis, a normal female breast develops as a mammary crest forms, followed by nascent mammary buds. Breasts are classified as complex glandular tissues which cover the pectoral muscle at the rib cage. The structural integrity of glands is maintained by the surrounding adipose and connective tissues (CT).

Within each mammary gland, there are between twelve and twenty lobes, which are subdivided into numerous lobules and end with up to 200 acini, which are the milk-secreting units. Acini ducts intersect together forming a terminal duct, which combines with the associated lobule to form the terminal duct lobular unit (TDLU). The TDLU drains into the sub-lobular and lobar ducts draining into the lactiferous duct which opens into the lactiferous sinus and leads to the nipple (33). The functional maturity of breast tissue happens during the pregnancy-lactation cycle (PLC) (34). Puberty, PLC, and post-menopause promote structural and hormonal changes in breast glands [43] **Figure 1-1**.

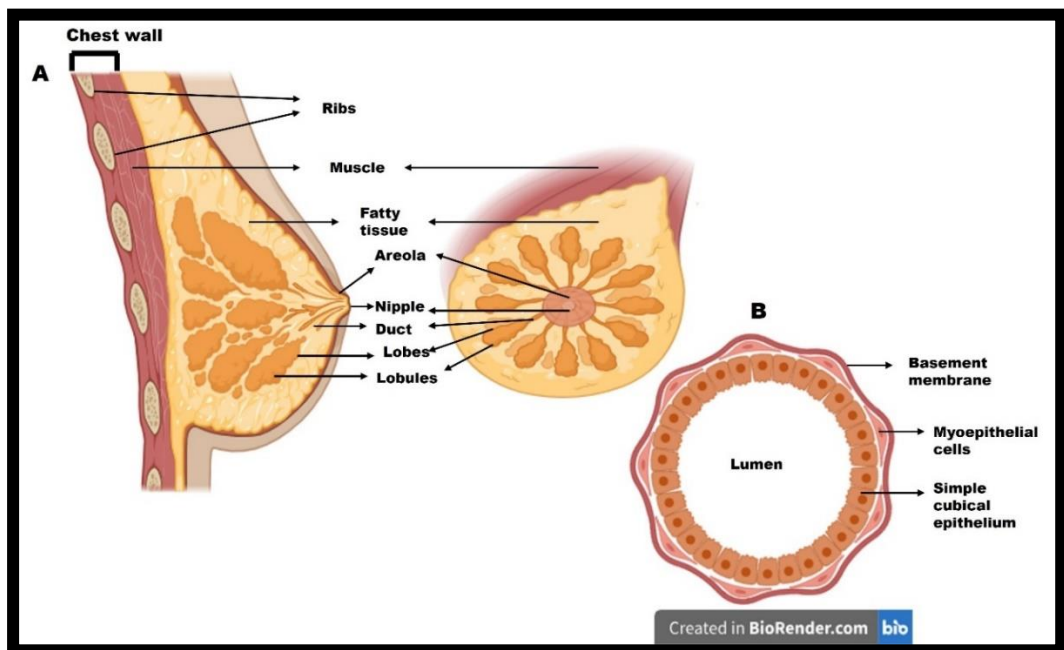


Figure 1-1: Anatomy of the female breast.

A) Longitudinal and transverse sections of the breast show the nipple and areola on the outside of the breast. The lobes, lobules, ducts, and other components of the breast. **B)** The transverse section of tubule duct units shows the lining of luminal epithelial layers surrounded by the basement membrane.

Histologically, the breasts are modified tubulo-alveolar apocrine sweat glands, they consist of epithelial bilayer within adipose CT with support from dense fibrous CT called the Cooper ligaments. The epithelial bilayer is composed of the inner part of luminal cuboidal cells and the outer part of myoepithelial cells (MECs). The epithelial part of the

mammary gland constitutes approximately 10 to 15% of the total volume. Breast size variation is related to the volume of the adipose tissue rather than the epithelial content (35-37). **Figure 1-2.**

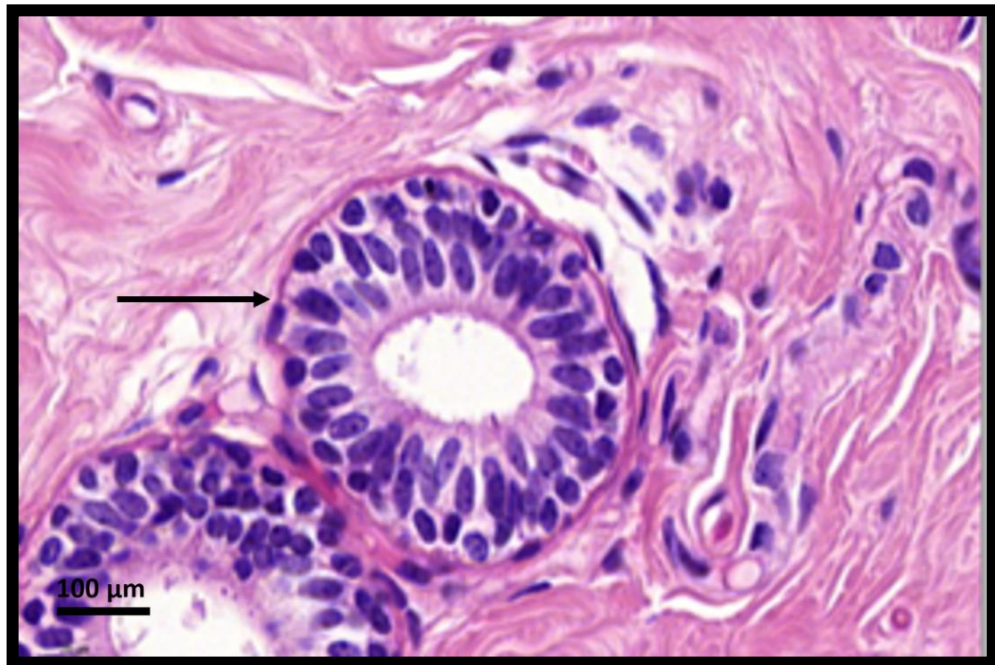


Figure 1-2: Histology of normal breast tissue.

The arrows show the luminal epithelial bilayer surrounded by the basement membrane.

1.1.5 Risk factors

As noted earlier, BC is a multifactorial disease, and the precise mechanisms that cause BC are still not yet completely understood. Therefore, environmental, heritable, and lifestyle factors can predispose the development and progression of BC (38).

1.1.5.1 Hormonal

Initiation and progression of BC are prompted by hormonal factors. The primary female sex hormone is oestrogen, which is associated with a multitude of biological functions. While oestrogen is an endogenous hormone, BC risk increases with external sources that contain oestrogen and can stimulate breast growth as oral contraceptives or hormonal replacement therapy for menopausal women (39, 40). Pregnancy and longer breastfeeding duration result in lobule differentiation, which is

considered a protective mechanism against BC by reducing oestrogen exposure (41). There is a significant difference in BC incidence between nulliparous women and women who have had full-term pregnancies and breastfed and this is significant, with the former group being much more vulnerable to BC (42).

1.1.5.2 Age

The risk of BC increases with age, women over 50 are being more likely to develop BC compared to younger ones. In contrast, BC which develops in young women has a higher tendency to be an aggressive advanced-stage phenotype, such as triple-negative BC (TNBC) or overexpression of HER2, which are strongly associated with poor survival outcomes (43, 44).

1.1.5.3 Heritable and genetic predisposition

A few cases of BC, about 10%, are attributable to the hereditary factors (45). The risk of BC initiation and progression increases for females who have first- or second-degree relatives (i.e., mother and sister) who are diagnosed with BC. In addition, there is a susceptible increased BC risk for women who have many relatives who suffer from ovarian cancer. There is a relationship between hereditary BC and germline mutations in genes for BC type-1 susceptibility protein (BRCA1) and BC type-2 susceptibility protein (BRCA2). A previous study demonstrated that 25% of familial risk is attributed to BRCA1 or BRCA2 mutations (46). BCs that result from BRCA1/BRCA2 mutations are characterised by early-onset and bilateral breast affection (45). Early detection of the mutation carriers in these genes, may play an essential role in BC prevention (47, 48).

1.1.5.4 Environmental factors

Some other factors may increase the risk of BC development including lifestyle. Mammography scans have detected an association between the density level of the scan and regular high alcohol consumption (49). The International Agency for Research on Cancer (IARC) stated that the BC risk increased by about 10% for females who drink 10 grams of

alcohol per day (50). Smoking is also a BC risk factor, active smokers and women who have smoked for a long time are much more vulnerable to BC than non-smokers. Passive smoking is still a risk factor, non-smoking women who are exposed to smoke are more likely to have BC than non-smoking women who are not exposed to smoke (51). Obesity has a higher risk of BC development (52). There are potential mechanisms that could explain the increased risk of developing BC with obesity. For example, the adipose tissue expresses aromatase that converts androgen to oestrogen. In addition, there is an association between obesity and high levels of circulating insulin, which is responsible for the secretion of inflammatory cytokines and insulin-like growth factor I (53).

1.1.6 Pathogenesis and Cytogenesis

Several factors can cause the risk of developing BC. Deoxyribonucleic acid (DNA) damage and genetic mutations have been experimentally linked to the initiation and progression of BC (54). Chromosomal abnormalities and somatic loss of heterozygosity (LOH) have been documented to identify putative tumour-suppressor genes in BC (55). LOH commonly occurs in the chromosomes 17p, 11p and 16q (56). BC shows loss of tumour suppressor genes, such as *TP53* and *p16*, as well as overexpression of proliferation markers such as Ki67 (57-59). In addition, the potential role of other factors such as MECs and surrounding TME in the process of BC progression has been widely reported (60).

1.1.7 Prognostic and predictive factors

The prognostic factor is defined as a factor that could identify a patient group whose prognosis would be good therefore the adjuvant treatment following local surgery would be with no value, additional costs and harmful, however, another poor prognostic group, the adjuvant treatment would be warranted (61). To be clinically applicable, the prognostic factor must show a significant wide separation in identified groups' outcomes with adequate numbers of patients in each group. Nevertheless, no

single prognostic factor could fulfil these criteria (62). The reliability of these prognostic factors on the routinely used techniques and the cost are significant practical criteria for choosing the most appropriate adjuvant therapy. The predictive factors for hormonal therapy are well confirmed, in contrast, those of chemosensitivity are still less well-defined. For example, it is a challenge to identify patients who are more likely to respond to chemotherapy therapy based on prognostic or predictive factors such as tumour size or lymph node (LN) status (63, 64).

1.1.7.1 Prognostic clinicopathological factors

1.1.7.1.1 Histological type

Histological typing, which is defined as the tumour growth pattern, should be routinely performed for each invasive BC case based on published guidelines and strictly revised criteria (65). Concerning patient outcomes, ductal carcinoma of no special type (NST) shows a poor prognosis, whereas tubular carcinoma has an excellent prognosis. In addition, other special types of invasive carcinoma vary between these two extremes (66, 67).

1.1.7.1.1.1 Invasive ductal carcinoma of no special type (NST)

NST is defined as tumours that do not fit in any of the special BC variants, they do not have the eligibility characteristics that enable them to be classified as a specific histologic subtype. NST is known as the most common type of invasive BC, and it represents approximately 70% of BCs. It is defined as a malignant ductal growth and proliferation with stromal invasion. The neoplastic cells are pleomorphic, with various sizes and shapes observed, prominent nucleoli, and several mitoses. They incorporate a big heterogeneous group relating to gross and microscopic features (66, 68), (**Figure 1-3**).

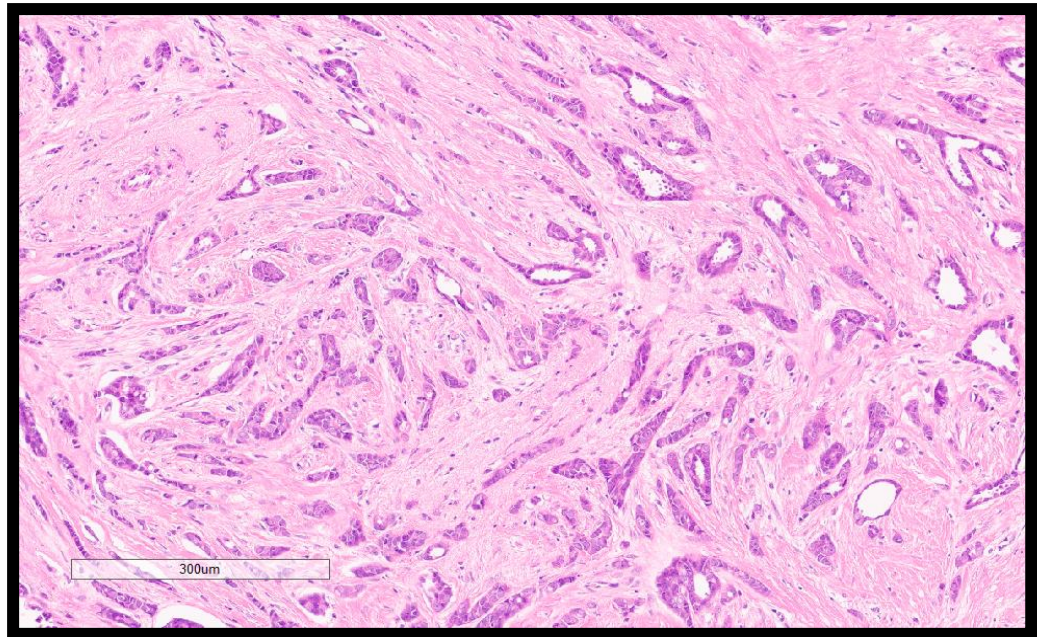


Figure 1-3: Non-special type of breast cancer (NST).

1.1.7.1.1.2 Invasive lobular carcinoma (ILC)

ILC is a special histological type, considered to be the second major biologically invasive BC. It constitutes about 5%–15% of invasive BC. Morphologically, it is a nest-forming tumour where the neoplastic cells infiltrate in the lobular pattern. ILC cells are small, rounded, relatively uniform, and non-cohesive with a specific growth pattern that shows a single-file stromal infiltration. (66, 68). Several variants of ILC have been identified. These include the tubule-lobular type which shows microtubular formation as part of the classic pattern, the alveolar variant which shows small aggregates of about twenty cells or more, the solid variant which shows cell sheets with scanty stroma, the pleomorphic variant exhibits a classic type of growth pattern but has more pleomorphic cytological appearances, and the lobular mixed type which are composed of mixtures of different ILC subtypes (69) **(Figure 1-4)**.

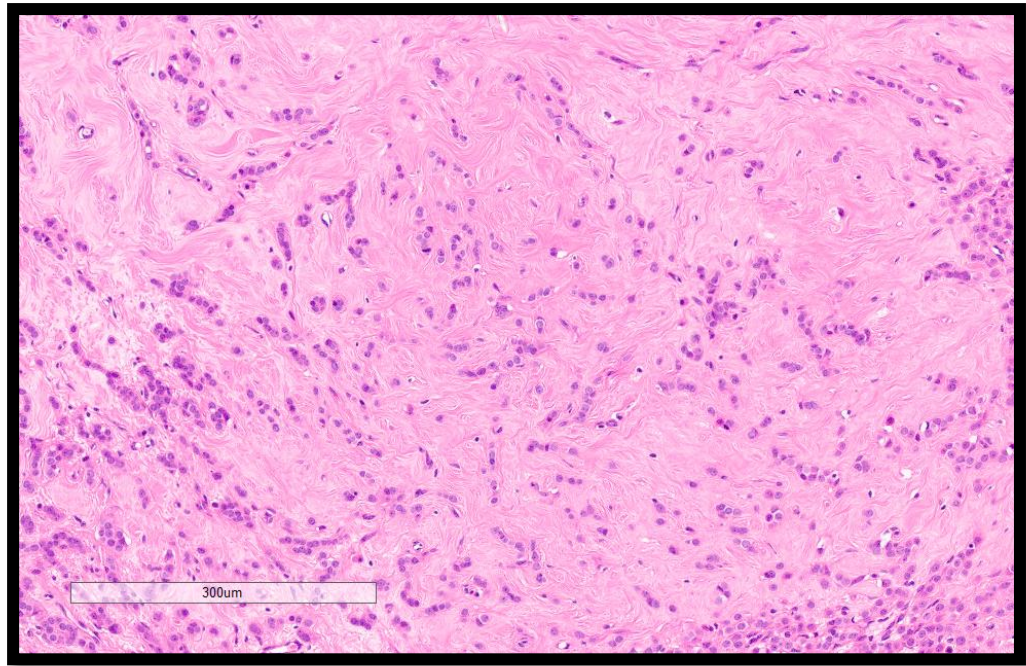


Figure 1-4: Invasive lobular carcinoma of breast cancer, classical type.

1.1.7.1.1.3 Metaplastic carcinoma

Metaplastic carcinoma is a rare and aggressive tumour which accounts for about 0.2–5% of BC and is correlated with poor prognosis and outcomes. Metaplastic means that the cells have transformed their morphology from glandular to non-glandular. A metaplastic carcinoma is a heterogeneous cluster of BCs characterised by differentiation to squamous or mesenchymal cells such as spindle, chondroid, and osseous cells (70, 71), (**Figure 1-5**).

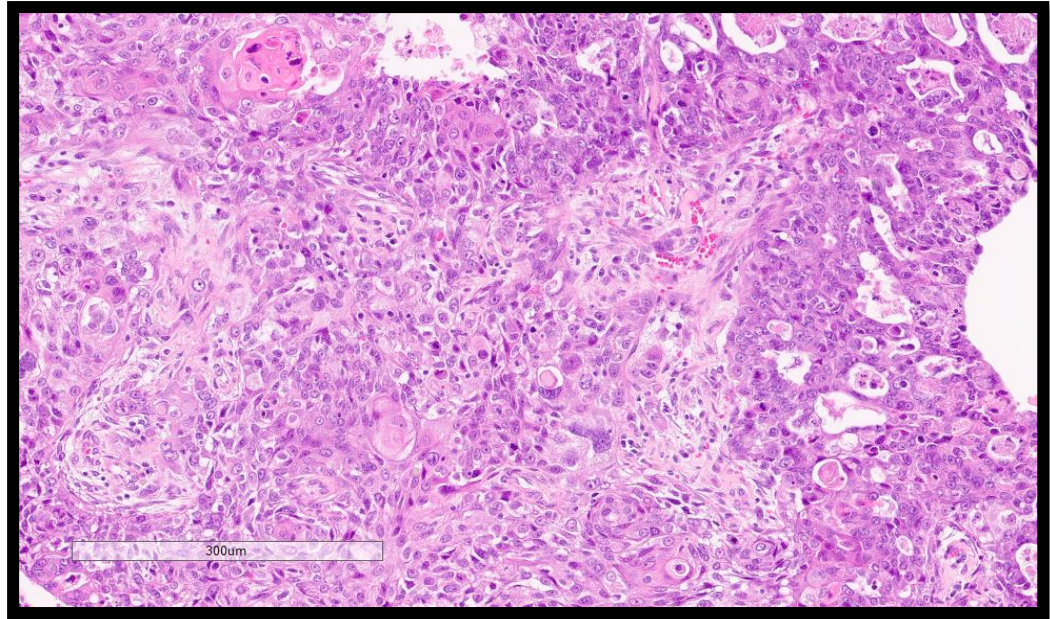


Figure 1-5: Metaplastic type of breast cancer.

1.1.7.1.1.4 Special types of BC

There are several special types of BC which are also described below.

- **Invasive cribriform carcinoma**

Invasive cribriform carcinoma is a rare type of BC which is correlated with low metastatic potential and favourable prognosis and outcomes. Morphologically, it consists of uniform islands of neoplastic cells that show low-grade atypia with a cribriform appearance (68). It is divided into three subcategories: pure, classical, and mixed forms. The pure type is characterised by a cribriform pattern of more than 90% of the tumour, whereas the classical type is accompanied by less than 50% of the lesion with a tubular carcinoma. The mixed type showed about 10-90% of the lesions of any other BC types except tubular (66, 72), **Figure 1-6**.

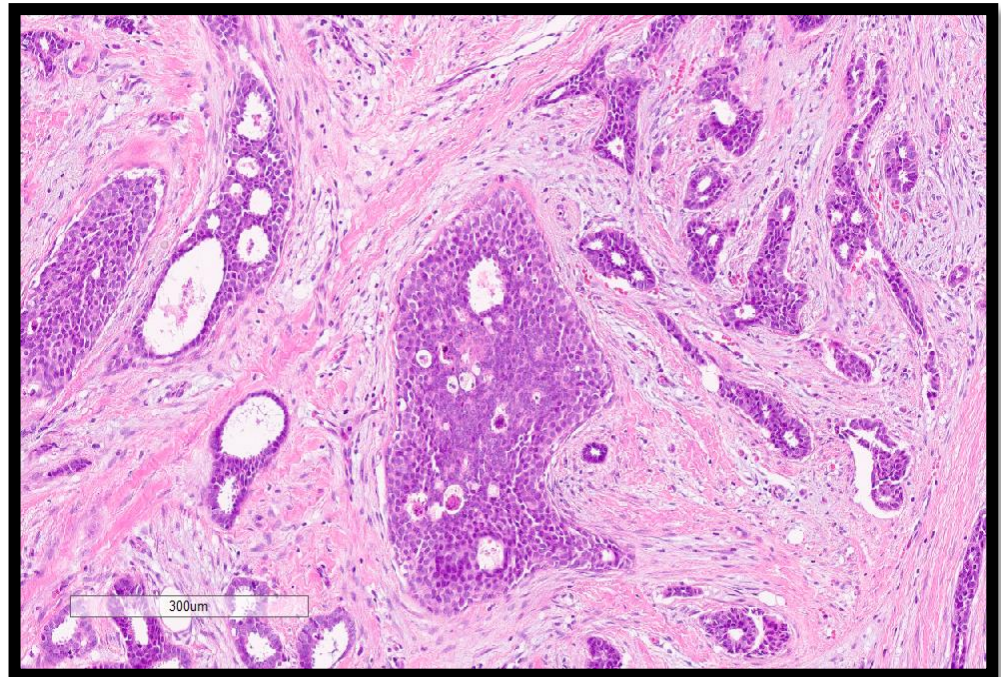


Figure 1-6: Invasive cribriform type of breast cancer.

- **Tubular breast carcinoma**

Tubular carcinoma is a rare special type of BC which accounts for about 1-2% of BC and is characterised by well-differentiation. It is a low-grade tumour that showed an excellent prognosis. Microscopically, it is characterised by the elongated open tubules predominance consisting of a single layer of epithelial cells enclosing a clear lumen with a haphazard arrangement. The neoplastic cells size is small to moderate, with few nuclear pleomorphisms, and scanty mitotic figures. The MEC are absent, but some tubules may have an incomplete BM. A crucial feature of this type is the cellular desmoplastic stroma (68, 73) (**Figure 1-7**).

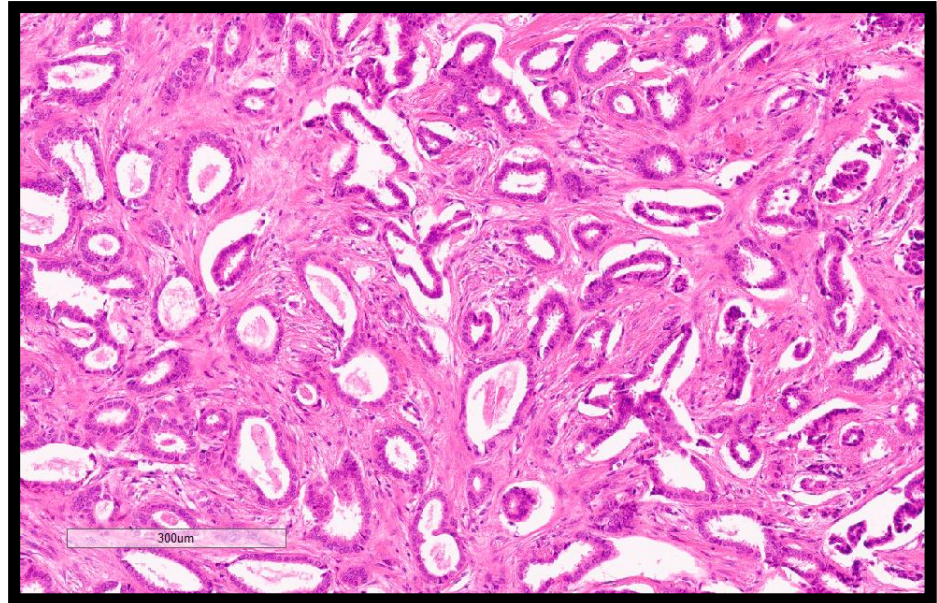


Figure 1-7: Tubular type of breast cancer.

- **Mucinous carcinoma**

Mucinous carcinoma is considered a relatively rare subtype of BC which is associated with a good prognosis. Mucinous carcinoma accounts for about 2% of total BC. Based on the tumour cellularity, it is divided into two subtypes: pure and mixed. The pure type is composed of neoplastic tissue with extracellular mucin over 90% of the lesion whereas the mixed type includes infiltrating epithelial components without mucin (68). Microscopically, it is composed of nests of neoplastic cells floating in mucin lakes partitioned by delicate fibrous septae. The clusters with tubular arrangements with variable shapes and sizes. Neoplastic cells are characterised by low nuclear atypia, but in rare cases, nuclear atypia and mitoses may exist (74), (**Figure 1-8**).

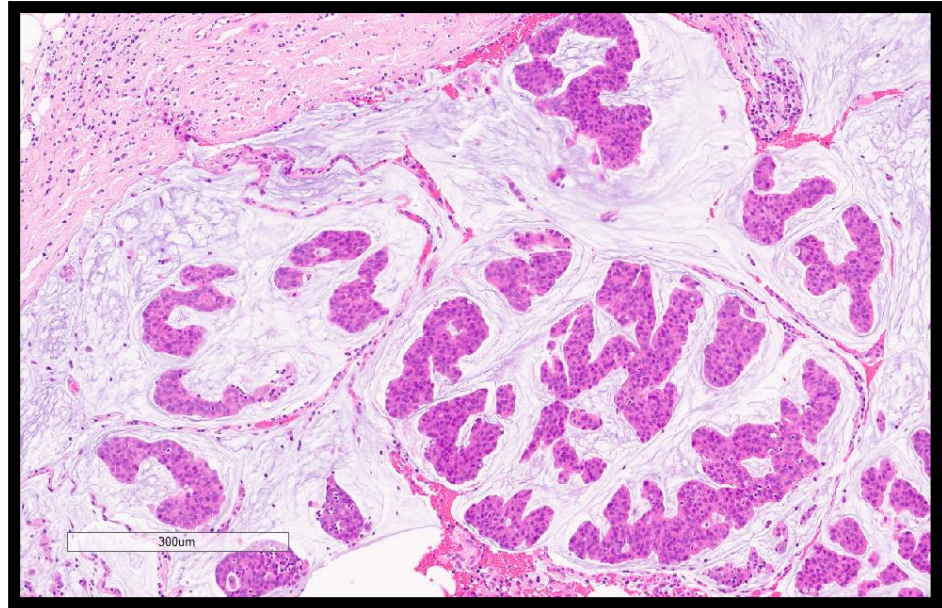


Figure 1-8: Mucinous type of breast cancer.

- **papillary neoplasms and micropapillary carcinoma**

Papillary neoplasms are a group of neoplasms that range from benign lesions to invasive papillary carcinoma (IPC) characterised by specific architectural structures composed of fibrovascular cores which are covered by neoplastic cells showed nuclear atypia according to the latest WHO Classification of BC (5th edition). Papillary neoplasms include encapsulated papillary carcinoma (EPC), solid papillary carcinoma (SPC), and IPC, tall cell carcinoma with reversed polarity (TCCRP) and adenomyoepithelioma. EPC is characterised by an expansile growth pattern with a well-circumscribed margin surrounded by a thick capsule, **Figure 1-9**. SPC is characterised by a solid growth pattern with interspersed delicate fibrovascular cores **Figure 1-10**.

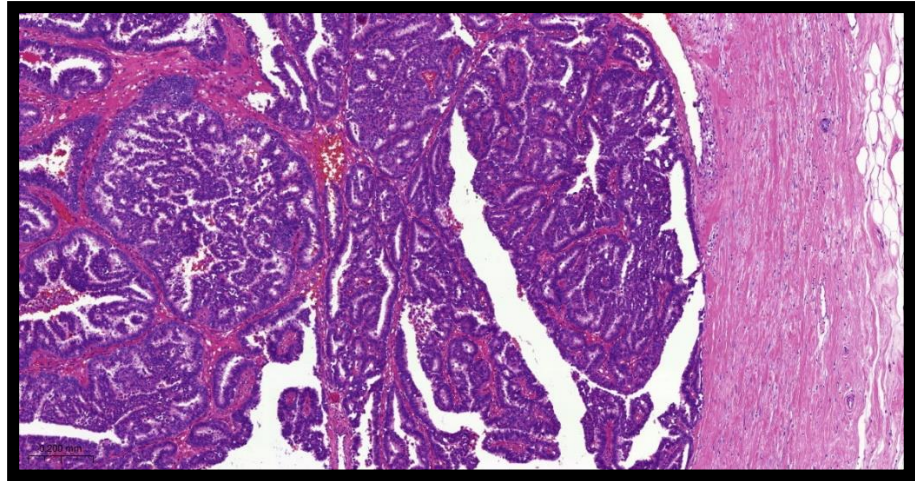


Figure 1-9: Encapsulated papillary carcinoma of the breast.

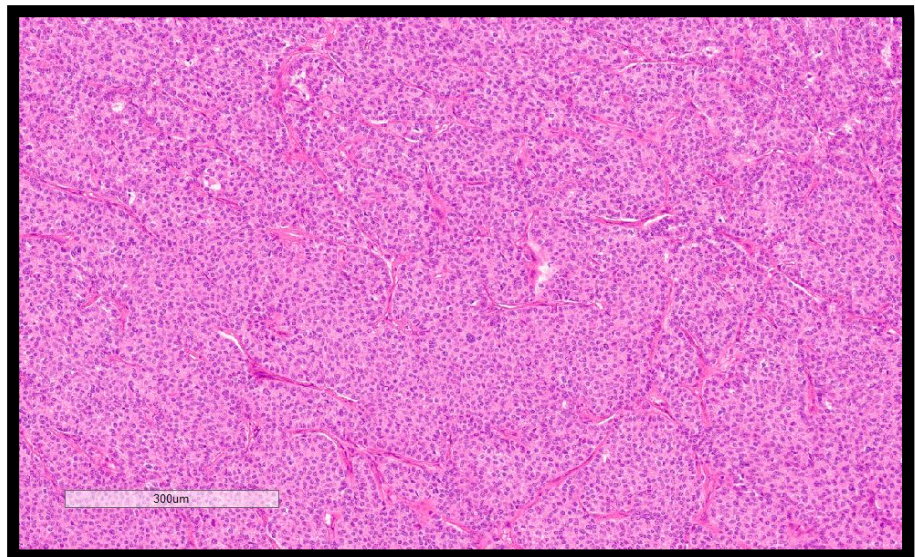


Figure 1-10: Solid papillary type of breast cancer.

IPC is a rare type which accounts for approximately 0.5% of total BCs, **Figure 1-11**. TCCRCP is considered SPC with reverse polarity. Adenomyoepithelioma is a proliferation of both inner epithelial and outer MECs with a variety of architectural patterns (75, 76). Invasive micropapillary carcinoma (IMPC) is excluded from the IPC classification, as it is characterised by tumour cell nests with reverse polarisation in cleft-like spaces. IMPC is associated with frequent LVI, poor prognosis and outcomes (77, 78), **Figure 1-12**.

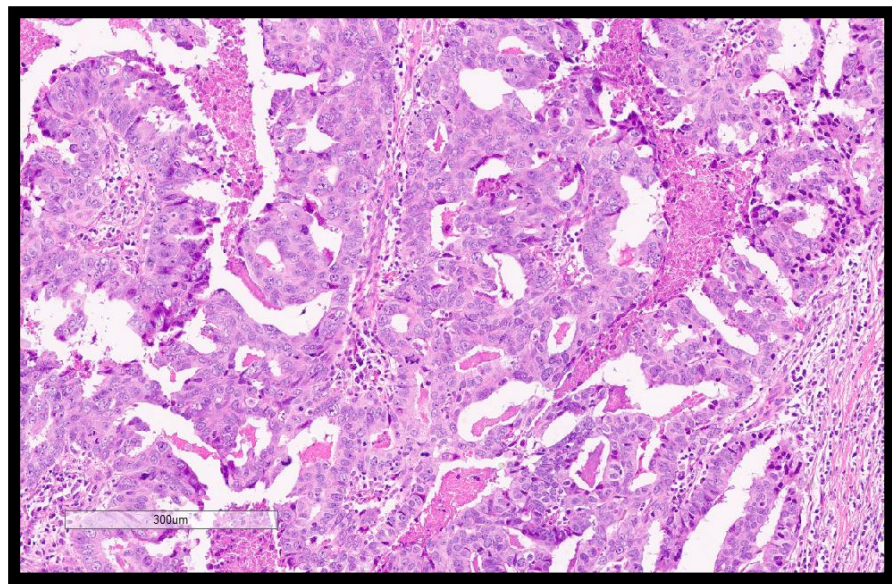


Figure 1-11: Invasive papillary type of breast cancer.

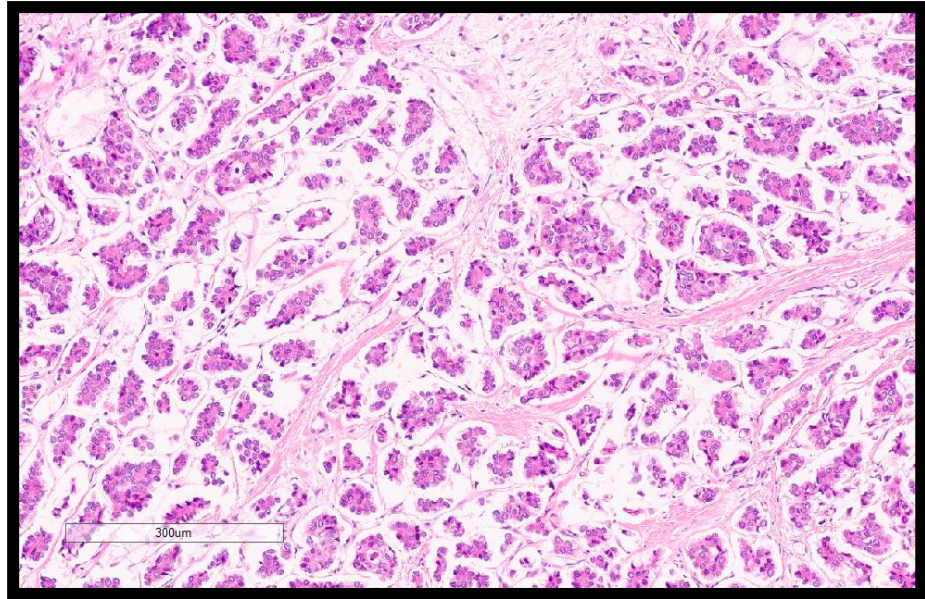


Figure 1-12: Invasive micropapillary type of breast cancer.

- **Adenoid cystic carcinoma (ACC)**

ACC is a rare special BC type that accounts for about 0.1% of BCs with an excellent prognosis. It is characterised by a biphasic morphology showing epithelial cells forming ducts and the modified MEC forming pseudo-lumina filled with stromal mucin and BM-like material. ACC is characterised by variant growth patterns cribriform, tubular, solid and basaloid variants of solid (79, 80), **(Figure 1-13)**.

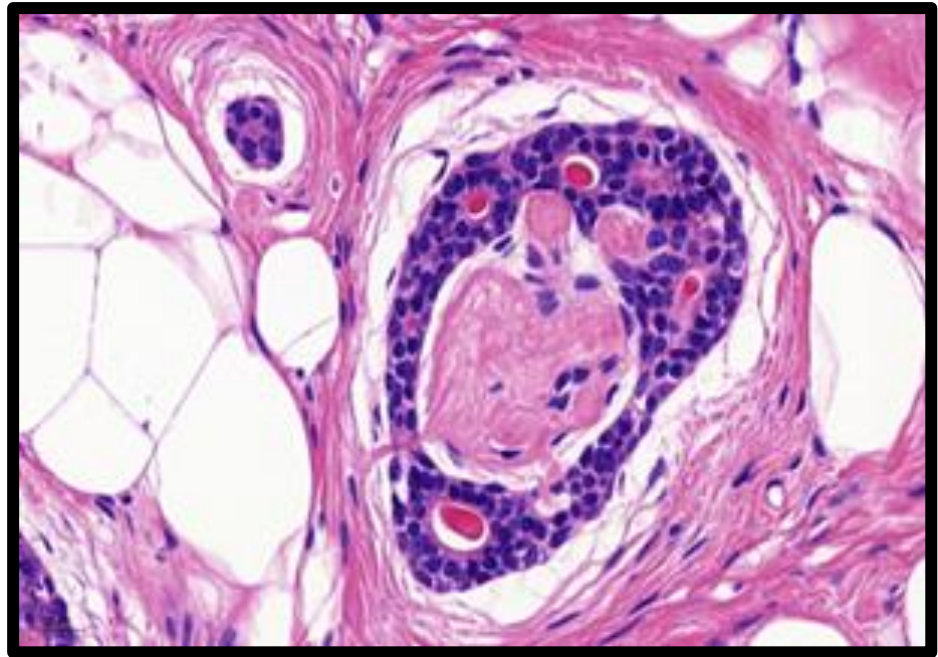


Figure 1-13: Adenoid cystic carcinoma of the breast.

- **Apocrine carcinoma**

Apocrine carcinoma is a rare tumour that accounts for approximately 1% of all BC and is characterised by cytologically apocrine cells in above 90% of the lesion. Microscopically, cells with distinct borders, abundant eosinophilic vacuolated or granular cytoplasm, large size round to oval nuclei and prominent nucleoli. According to the WHO 5th Edition of BC classification, many histological types may show apocrine morphology and immunophenotype which is a characteristic steroid receptor profile, should be listed within carcinoma with apocrine differentiation (76, 81), **Figure 1-14.**

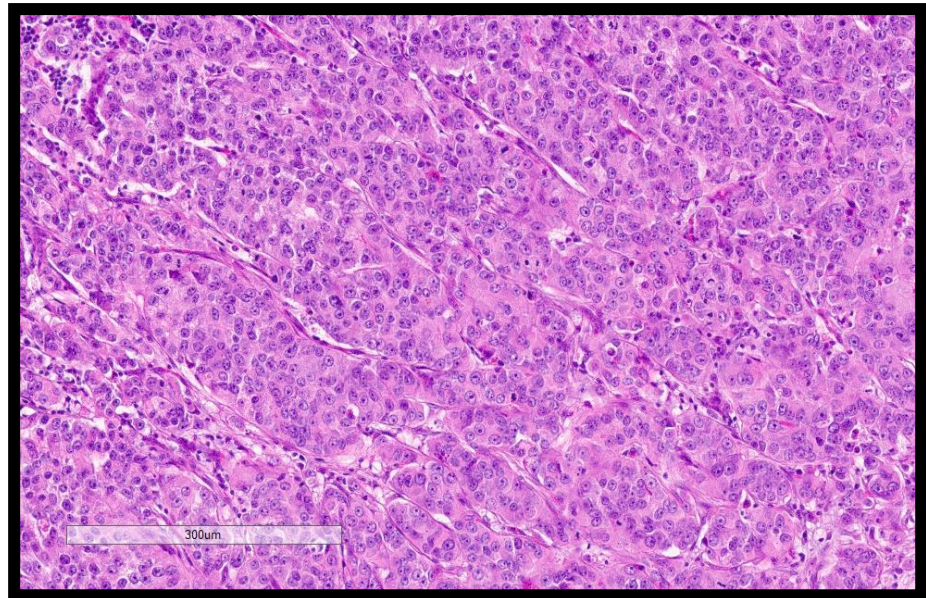


Figure 1-14: Apocrine type of breast cancer.

- **Mixed types**

Mixed types are a relatively common pattern of invasive BC. This category is defined as tumours with specialized patterns within more than 50% of the tumour and the remaining has a nonspecialized pattern (68).

1.1.7.1.2 Tumour size

Tumour size is one of the most powerful prognostic and predictor factors of tumour behaviour in BC. The larger the size of the tumour, the worse the prognosis, and outcomes. Interestingly, it is a robust predictor of nodal metastasis (82). Tumours less than 20 mm have been classified as small tumours, and anything exceeding that was deemed to be a large tumour (62).

1.1.7.1.3 Histological grade

Histologic grading is a simple and inexpensive method to evaluate tumour behaviour and prognosis, thereby identifying patients at risk for adverse outcomes (83). The Nottingham modification of Elston & Ellis of the Scarff-Bloom-Richardson histological grading of BC is one of the most powerful prognostic factors. Grading plays a key role in patient

stratification for adjuvant therapy, being a part of the Nottingham Prognostic Index (NPI) (84-86). It is a three-tier system based on three parameters reflecting the degree of tumour differentiation: degree of tubule formation, mitotic scores and nuclear pleomorphism. The semi-quantitative method to calculate the BC grade includes scoring the three items. The percentage of tubules as score 1 indicates most of the tumour (> 75%) score 2 between (10-75%) and score 3 less than 10%. The scoring of pleomorphism as score 1 indicates small regular uniform cells, score 2 moderate variable size and shape cells with enlarged vesicular nuclei and prominent nucleoli, and score 3 indicates marked variation in size and shape of cells showing enlarged nuclei and prominent nucleoli. Mitotic figure scoring in the high-power field (HPF) on power x40 light microscopy, HPF size of 0.274 mm², mitotic count score 1, indicates less than 10, score 2 indicates the range between 10-19, and score 3 indicates more than 20. The tumour grade of a case is determined by summing the three factors' scores. Therefore, total scores of 3–5 are classified as grade I, and the prognosis is good. A total score of 6 or 7 is classed as grade II, and the prognosis is moderate. Total scores of 8 or 9 are categorised as grade III tumours, and the prognosis is poor (83, 87, 88).

1.1.7.1.4 Lymph node (LN) status

Axillary LN status has been constantly shown to be the most powerful predictor of BC overall survival. Most patients (70%) with positive LN involvement develop distant recurrence within ten years from the initial diagnosis compared to only 20-30% of LN-negative patients. Approximately 83% of BC patients with negative LNs survive at least five years (89). The number of axillary LN involved showed a prognostic significance. Axillary LN of BC specimens are routinely examined, and the number of LN involved is reported. LN staging as tumours with negative LNs was classified as stage 1, tumours with 1-3 positive LNs were classed as stage 2, and tumours with 4 or more positive LNs were stage 3 (90).

1.1.7.1.5 Nottingham Prognostic Index (NPI)

NPI is a survival-based setting and prognostic stratification tool for the assessment of BC patients. There are three prognostic parameters, tumour size, grade and LN stage conferring the tool with robust prognostic power (91). Each patient's NPI score is based on their clinicopathological tumour and LN scores. The formula used to calculate NPI is as follows: $NPI = 0.2 \times \text{tumour size (cm)} + \text{LN stage (1 to 3)} + \text{tumour grade (1 to 3)}$. Regards the calculation results, NPI is categorised as being good, moderate, or poor. The good prognostic NPI group is defined as patients with an NPI score of < 3.4 , while the moderate prognostic NPI classification covers NPI scores of $3.4\text{--}5.4$, and all scores exceeding 5.4 are classed as the poor prognostic NPI group (62). The NPI has been updated, and each of the original three prognostic groups was split into two groups to give a total of six prognostic groups. In the excellent prognostic group, the NPI score ranged from $2.02\text{--}2.4$ while in the good prognostic group, it ranged from $2.41\text{--}3.4$. In the moderate prognostic group 1, the NPI score is $3.41\text{--}4.4$, and in the moderate prognostic group 2, the NPI is $4.41\text{--}5.4$. Poor prognostic scores range from $5.41\text{--}6.4$ and those exceeding 6.4 scores are in the very poor prognostic group (62). Not only does NPI stratify BC patients, but also it plays a role in patient selection for systemic therapies (92). This study utilised the original NPI prognostic system for all cases.

1.1.7.1.6 Lymph vascular invasion (LVI)

LVI reflects tumour cells' presence in blood vessels or lymphatic spaces around the tumour. In BC, LVI correlates strongly with patient survival outcomes (93). Within microscopic examination, LVI is identified by the morphological features of the primary tumour that may or may not contain endothelial-specific markers. LVI is an indicator of metastatic potential if identified in the primary tumour (94-96).

1.1.7.2 Predictive factors

Evaluation of predictive factors or biomarkers is essential for accurately forecasting the prognosis and therapy responsiveness. In conjunction with the prognostic clinicopathological parameters described above, predictive biomarkers are strategic to stratify BC patients and management decisions (97).

1.1.7.2.1 Hormone Receptors (HR)

HR expression is detected by immunohistochemistry (IHC) in tumour nuclei including receptors for oestrogen hormone (ER) and progesterone hormone (PR) in BC. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have issued guidelines recommending that for BC patients, the thresholds for positive detection for ER and PR are set at 1% of tumour nuclei (98). Patients with HR-positive BC showed a better prognosis than those with HR-negative BC (99). Most tumours (80%) with ER and PR positive expression and 50% of ER positive and PR negative patients respond to hormonal therapy (100). Therefore, ER is a powerful predictive indication for adjuvant endocrine management. Clinical practice routinely tests for the co-expression of PR in ER-positive patients. Patients' responses to adjuvant endocrine therapy can be predicted depending on the co-expression of both ER and PR (101).

1.1.7.2.2 Human Epidermal Growth Factor Receptor 2 (HER2)

Encoded by the ERBB2 gene, the tyrosine-protein kinase receptor erbB-2 which is known as HER2 is a cell membrane tyrosine kinase receptor, which is normally involved in the signal transduction pathways for cell growth and differentiation (102). HER2 is overexpressed in 15% of BC (103). A strong correlation between HER2 overexpression and aggressive BC phenotypes and poor survival outcomes has been reported (104). HER2-positive BCs can be treated with trastuzumab (eg Herceptin), which is a humanised monoclonal antibody that targets HER2-positive tumours. Therefore, trastuzumab is a good example of the

promising discipline of precision medicine, where the tumour's molecular features can be targeted by therapeutics (105). However, the efficacy of trastuzumab decreases as the tumour progresses in addition, it is limited to early-stage BC (106).

1.1.7.2.3 Breast cancer molecular classification

Distinct gene expression patterns which are related to the different BC subtypes, were first reported in 2001 (107). HR-positive and HR-negative BCs were identified by DNA microarray experiments using hierarchical clustering analysis with the Prediction Analysis of Microarrays (PAM50) gene panel (108). Clustering analysis demonstrated that HR-positive BC could be sub-classified on the basis of tumour gene expression profiles. Using clustering analysis, five categories of tumours were distinguished: luminal A, luminal B, HER2-amplified, basal-like, and normal breast-like. These subcategories phenotype that represent the expression profiles of ER, PR, and HER2 as luminal A (ER+, PR+/-, HER2-), luminal B (ER+, PR+/-, HER2+), HER2+ (ER-, PR-, HER2+), normal like (ER+ and/or PR+, HER2-), and basal-like (ER-, PR-, HER2-) (109, 110). According to Reis-Filho and Tutt, normal-like BC was not exhibited to be a true subtype (111). The expression of ER, PR, or HER2 is about 15%–54% of basal-like BC. This TNBC and basal-like BC share a similar histological morphology but are not necessarily molecularly similar (112). Therefore, IHC expression of epidermal growth factor receptor (EGFR+) and/or type II cytoskeletal 5 and 6 (CK5/6+) has been used to differentiate between TNBC and basal-like BC (112). In 2007, claudin-low type BC was added to the classification system. It is characterised by low expression of genes that are required in tight junctions and cell-to-cell adhesions (113) **(Figure 1-15).**

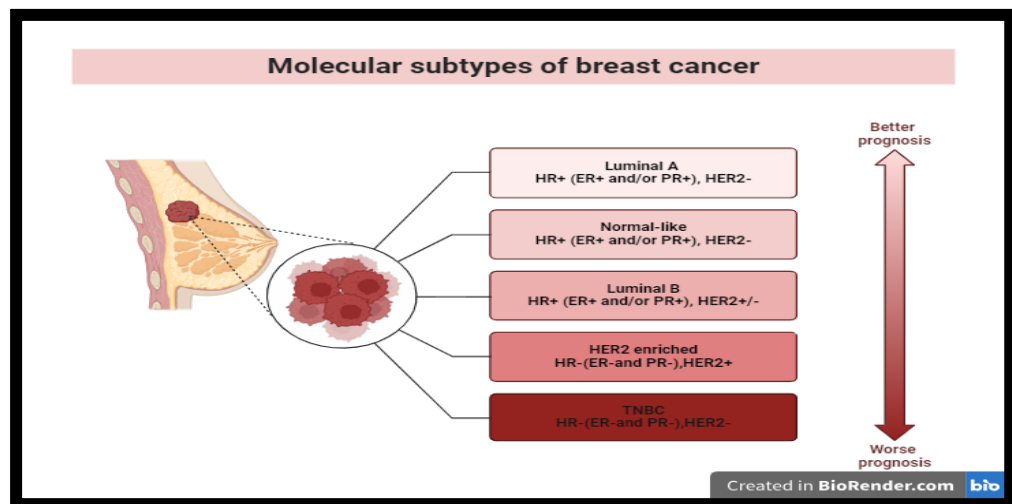


Figure 1-15: Molecular subtypes of breast cancer.

1.1.7.2.4 Multi-gene signatures

Stratification of patients with early-stage BC who should receive adjuvant chemotherapy is a vital clinical issue. Therefore, there are many multi-gene prognostic signatures for BC survival outcomes however, their ability to predict therapy benefits is still less evident (114). Multi-gene signatures provide a prediction for survival, recurrence risk, and treatment benefits from chemotherapy or endocrine therapy (115). They have been identified by expression profiling technology with various statistical approaches, including the MammaPrint (70 gene signature), Oncotype DX recurrence score, EndoPredict assay (EP), the 76 gene signature the genomic grade index (GGI), OncoMasTR (OM), and Mamma Typer assay (116, 117). The use of a gene panel is the shared character of all these multi-gene signatures to predict the outcomes reflecting the overall genetic derangements underlying tumour biology (118, 119). Their prognostic power is time-dependent from five to ten years of follow-up (120).

1.1.8 Breast Cancer Diagnosis Routine Practice

For pathological diagnosis, formalin-fixed paraffin-embedded (FFPE) samples are a cost-effective approach (121). FFPE is routinely collected

and stored with the patients' clinicopathological and outcomes data, for further clinical and research purposes (122). The tissue samples and their clinical data represent a fundamental requirement for research studies, both retrospective and prospective which is essential for and validation and detection of new biomarkers (123). Regarding the UK Royal College of Pathologists, the minimum dataset report for BC practice consists of tumour size and grade, LN status, histological type, presence of *in-situ* component and excision margin status. The strict approach of the guidelines for specimen handling, fixation and processing is necessary for reliable reporting on these parameters (124, 125). IHC of FFPE sections to detect ER, PR, HER2 and Ki67 is usually used in BC prediction for prognosis and management (126, 127). Notably, frozen sections are currently used to obtain a quick decision for surgical margin adequacy and/or axillary nodal status through surgical procedures (128).

1.2 Ductal carcinoma in-situ

1.2.1 Definition and background

Ductal carcinoma *in situ* (DCIS) of the breast is a heterogeneous group of diseases which refers to the progression and proliferation of neoplastic epithelial ductal cells within the confinement of the ductal-lobular system surrounded by an intact MEC layer and Basement membrane (BM) without invasion into the surrounding stroma (129). DCIS was first described in the early twentieth century by a group of surgeons as a transitional stage between hyperplasia and invasive disease (130-132). The early DCIS descriptions outlined the major morphological patterns that are known nowadays, including micropapillary and cribriform patterns, which were described as laciform and cartwheel shapes, respectively. However, the comedo type was first classified as comedo carcinoma (133).

1.2.2 Epidemiology

Several decades ago, DCIS diagnosis was occasional and infrequent, it represented approximately 1-2% of newly diagnosed BCs (134). In 1975, the National Cancer Institute's Surveillance Epidemiology and End Results (SEER) programme data reported that less than 3% of BC cases contained pure DCIS (135). In 1990, mammography was introduced in BC screening programmes, the DCIS incidence has increased severely since then (136). It has been reported that about one in 33 women will likely be diagnosed with DCIS in their lifetimes (137). DCIS is uncommon compared to invasive BC, with DCIS comprising about 20% of all breast malignancies (138).

Regarding DCIS data deriving from the UK, the average number of newly diagnosed cases for each year was reported in 50 to 54 age groups. (139). DCIS is extremely uncommon at a young age, and it is unlikely to be diagnosed in women less than 30 years old. The incidence is approximately doubled compared with the age group 40-50 years (1%) and age group 70- 80 years (<1.5%) as well as the women above 80 years old (6%)(140, 141). The incidence increase of DCIS was observed in all ethnic groups (142, 143).

1.2.3 Risk factors

The risk factors for DCIS are similar to BC risk factors in general as previously discussed, including a family history of BC, early menarche, late menopause, nulliparity, long-term endocrine replacement therapy and obesity (144, 145). The strongest risk factor for DCIS development is a high density of breast tissue which is detected by mammography (146, 147). In addition, mutations of BRCA1 and BRCA2 are risk factors for DCIS as mutations were detected in about 13% of DCIS patients who were diagnosed before the age of 50 years. The *BRCA* mutation was detected in 10% of DCIS patients diagnosed before the age of 50 (148, 149).

1.2.4 Pathogenesis and Cytogenesis

The pathogenesis of DCIS is a complex process with multiple features (150, 151). Chromosomal abnormalities with LOH are observed in approximately 70% of high-grade DCIS (152). The published aneuploidy frequency in DCIS ranged from 21- 71% (153, 154). The overexpression of oncogenes such as *HER2* was detected in DCIS, *HER2* gene amplification was detected in 40-48% of DCIS (155, 156). Similar to BC in general, DCIS showed loss of tumour suppressor genes, such as *TP53* and *p16*, and overexpression of proliferation markers such as Ki67 and LOH in chromosomes 17p, 11p and 16q (157-160). Importantly, the molecular profile of DCIS is usually similar to the adjacent invasive BC which highlights the role of MECs and TME in the process of DCIS progression into an invasive disease (161, 162).

1.2.5 Clinical presentation

Most DCIS cases are diagnosed at an early asymptomatic stage via mammography in the screening programme. In contrast, a few cases may present with a breast lump, nipple discharge or Paget's disease of the nipple (163, 164). However, approximately 10-15% of DCIS cases were discovered incidentally in biopsies for other indications (165). Mammography has a high sensitivity of about 80-90% in DCIS detection (164, 166). As previously discussed, age and breast tissue density play a key role in the sensitivity of mammography (167).

The final diagnosis of DCIS is confirmed through histopathological examination of lesion biopsies either through needle core biopsy, vacuum-assisted core biopsy, or incisional or excisional biopsy. However, there are no definite cytological features that can differentiate DCIS from invasive BC in either fine needle aspiration cytology specimens or nipple discharge smears (168).

1.2.6 Bilaterality, multifocality and multicentricity

The data that describes the frequency of DCIS bilaterality is limited. Approximately 10-20% of DCIS cases present bilaterally, either as a concurrent contralateral disease or as a subsequent episode of DCIS, as reported by Cancer Registration Statistics, England (169).

Multicentricity does not have a uniformly accepted definition in DCIS. DCIS was considered to be multicentric when they were found to be separated from the detected lesion. Lagios and coauthors defined DCIS as multicentric when it is presented as independent foci of neoplastic and separated from the clinically or mammographically evident tumour (170, 171). Others defined multicentricity as when DCIS is detected in more than one quadrant or the distance is five cm from the index lesion (172, 173). Multicentricity refers to the presence of DCIS in multiple quadrants therefore it cannot be examined in lumpectomy. In clinical practice terms, multicentricity refers to DCIS foci in two or more quadrants of the breast (174-176).

Multicentricity should be distinguished from intra-epithelial extensions within a single focus, limited to one quadrant which is referred to as multifocality. The criteria for the presence of multifocality depends on the number of histologic sections that contain tumours. However, unifocal DCIS refers to when it is seen in only one section of two or more sections obtained from different blocks (177, 178).

1.2.7 Pathology of DCIS

1.2.7.1 Gross pathology

Grossly, DCIS may show unremarkable changes within the excised specimens. Classically, on macroscopic examination, DCIS does not make a recognisable lesion. Nevertheless, exceptions include high-grade DCIS with comedo necrosis due to soft centres and a few cases of solid papillary DCIS (179, 180).

1.2.7.2 Microscopic morphology

Diagnostic criteria for DCIS are based on the cytological atypia degree. Generally, it includes architectural features, cytonuclear features, cell population clonality and lesion extent. This criterion could differentiate between DCIS and mimicker lesions such as usual ductal hyperplasia (UDH) and atypical ductal hyperplasia (ADH) (180), **(Figure 1-16)**.

Histologically, DCIS is classified according to the nuclear grade or the morphological pattern. There are classification systems that combine both features in their scheme. Regardless of DCIS prevalence, there is no consistently accepted histological classification system. Nonetheless, a rising consensus admits the importance of nuclear grade over morphological patterns (181).

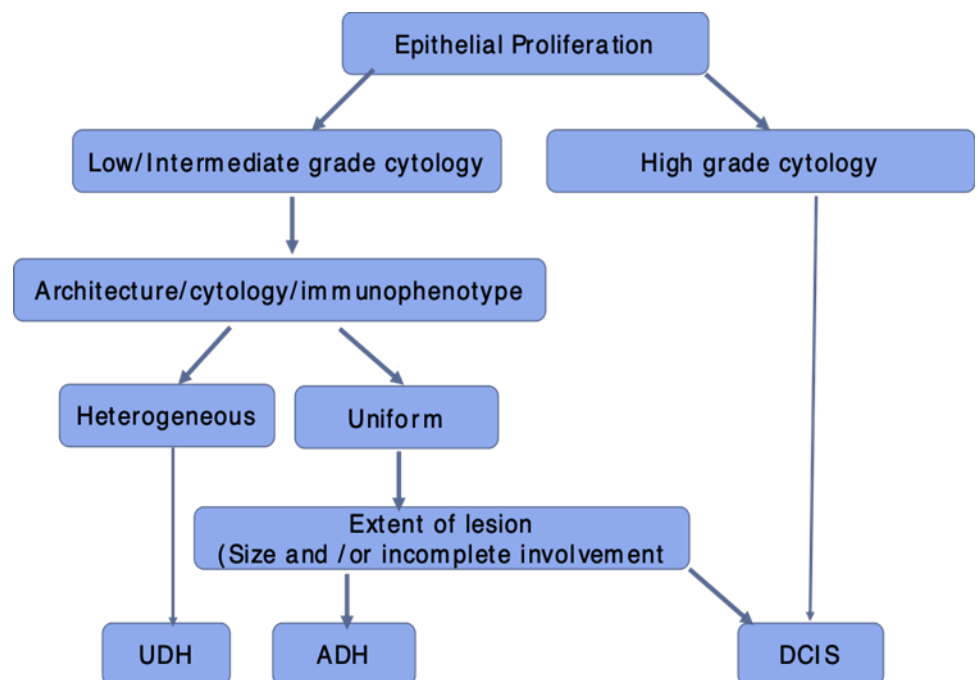


Figure 1-16: Diagnostic criteria used for breast intraductal proliferations.

1.2.7.3 Histological patterns

DCIS exhibits the proliferation of malignant cells within the breast in various morphological patterns (179). The majority of lesions showed a

mixture of patterns (150). Allred classified DCIS into two categories: the more aggressive comedo large cell group and the less aggressive non-comedo small cell group (182). In addition, Poller et al. categorised DCIS into DCIS without necrosis, pure non-comedo DCIS and pure comedo DCIS which correlated with HER2 expression known as the Nottingham classification system 1994 (183). WHO classification of BC in the 5th Edition, described DCIS's different morphological patterns including comedo, cribriform, papillary, micropapillary and clinging in addition to unusual patterns such as signet ring cell, apocrine, spindle, neuroendocrine and clear cell types (184).

1.2.7.3.1 Comedo DCIS

Comedo carcinoma *in-situ* is a distinctive type of high-grade DCIS. Comedo is a term that refers to creamy yellowish material that is secreted from the ducts of the breast and resembles comedones. Histologically, it is characterised by enlarged ducts which are filled with acellular central necrosis with nuclear debris surrounded by proliferative epithelium with high atypia and multiple mitotic figures. Necrosis constitutes beyond 90% of the duct in the cross-section (185), **(Figure 1-17)**.

1.2.7.3.2 Cribriform DCIS

Cribriform DCIS is characterised by malignant cells developing punched-out spaces with rounded rigid extracellular lumens. It should be distinguished from ACC; however, ACC has an infiltrative growth pattern with BM-like material while cribriform DCIS shows a monomorphic appearance with neoplastic cells surrounding these spaces (151), **(Figure 1-17)**.

1.2.7.3.3 Papillary DCIS

Papillary DCIS is defined as papillary proliferation which is characterised by branching fibrovascular cores covered by neoplastic cells with mild to moderate nuclear atypia. On contrary to benign papilloma, papillary DCIS has no MECs (151), **(Figure 1-17)**.

1.2.7.3.4 Micropapillary DCIS

The micropapillary DCIS is manifested by tufts of neoplastic cell proliferations which project into the lumen of TDLUs which lack fibrovascular cores and show a club-shaped end. UDH may show a micropapillary growth pattern but with a broad base and a narrow-pinch tip micro papillae with cells that show larger nuclei at the base and smaller ones at the tip. In contrast, micropapillary DCIS shows a narrow base and a broad bulbous tip (151), (**Figure 1-17**).

1.2.7.3.5 Solid DCIS

The solid DCIS is characterised by monomorphic proliferation sheets of cohesive cells that fill TDLUs lumen. It may form pseudo rosettes or micro acini (151), (**Figure 1-17**).

1.2.7.3.6 Clinging DCIS

Clinging DCIS is manifested by a duct lined by layers of highly atypical tumour cells at the periphery which are accompanied by comedo necrosis (151), (**Figure 1-17**).

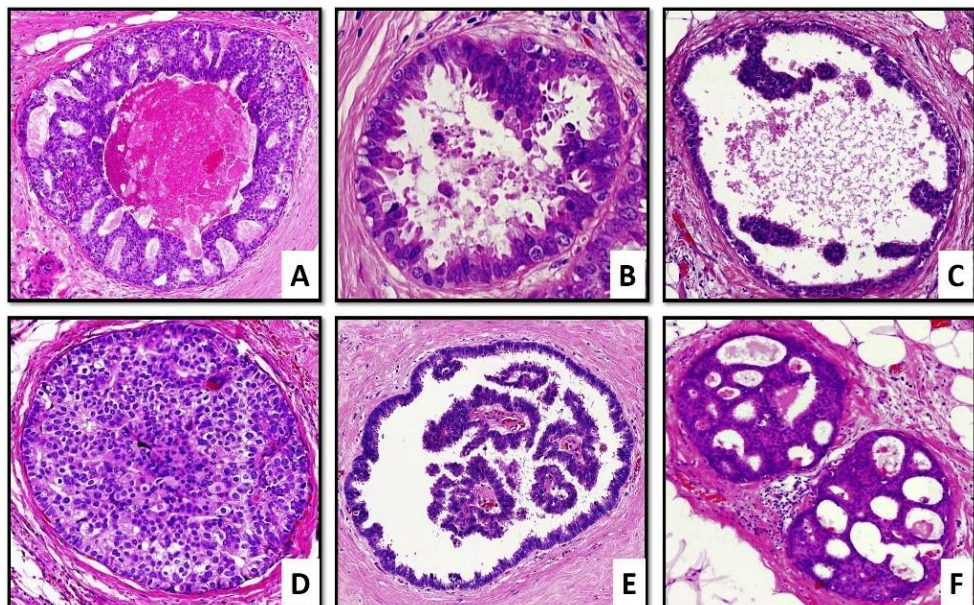


Figure 1-17: Morphological patterns of DCIS.

(A) Comedo, (B) Clinging, (C) Micropapillary, (D) Solid, (E) Papillary, and (F) cribriform.

1.2.7.3.7 DCIS with microinvasion

DCIS with microinvasion is defined as the malignant epithelial cells that breach the BM invading the adjacent stroma to a depth of 1mm or less. This happens in the form of a single cell or cell cluster (186). Microinvasion particularly is associated with high-grade DCIS therefore, it will necessitate a sentinel LN biopsy as up to 20% of cases with LN metastases (187, 188).

1.2.8 Grading Systems of DCIS

Various grading systems for DCIS are available and all of them are subjective. In 1994, Holland *et al.* categorised DCIS into well, intermediate, and poorly differentiated DCIS based on cytonuclear features, growth patterns and calcifications (189), (**Table 1-1**).

Table 1-1: Holland's classification system for DCIS

	Well-differentiated	Moderately differentiated	Poorly differentiated
Nuclei	Monomorphic	Slightly pleomorphic	Markedly pleomorphic
Nucleoli	Inconspicuous	Conspicuous	Prominent
Mitosis	Rare	Occasional	Often
Chromatin	Fine and uniform	Fine to coarse	Coarse and clumped
Cell polarisation	Marked	present	Absent
Central necrosis	Absent or minimal	variable	Present and marked
Cell necrosis	Absent	Focal	Marked
Calcifications	Laminated, psammoma-like and amorphous	Laminated and amorphous	Amorphous
Growth pattern	Clinging, cribriform, micropapillary and solid	Any patterns	Solid and clinging

The Van Nuys (VN) classification system is a scheme for risk stratification of DCIS patients for different treatment modalities. This system includes

tumour size, grade, and resection margin distance. Later, it was modified by adding the patient's age (190), (**Figure 1-18**).

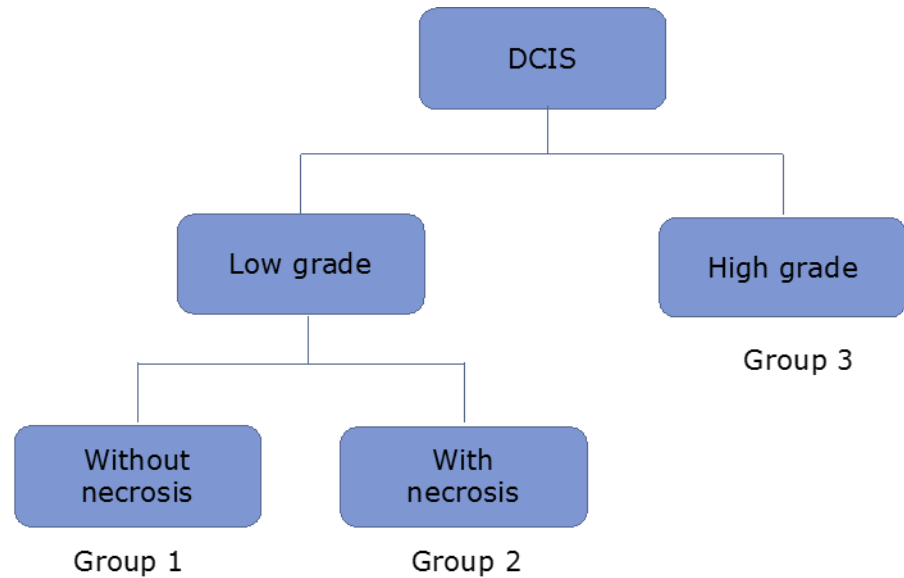
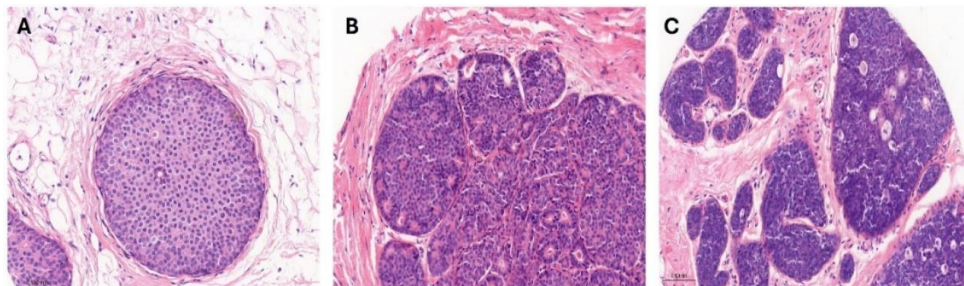


Figure 1-18: Van Nuys grading system of DCIS.

The cytonuclear grading for DCIS was developed in 1997 by Consensus Committee Recommendations (191) and confirmed in 2005 by the National Pathology Co-ordinating Group (192). Despite these advances, the technique remains subjective but it is still the standard and most widely used for DCIS grading (193). It is recommended in the WHO classification of the BC (194). A summary of the features of this grading system is addressed in **Table 1-2, Figure 1-19**.

Table 1-2: Cytonuclear grading of DCIS.

	Low	Intermediate	high
Pleomorphism	Mild	Moderated	Marked
Size	1.5-2 of red blood cells (RBCs) size or normal size of duct epithelial cell nucleus	X2.5 size of RBCs or normal size of duct epithelial cell nucleus	More than 2.5 size of RBCs or normal size of duct epithelial cell nucleus
Chromatin pattern	Fine dispersed diffuse	intermediate	Dense clumped irregularly dispersed
Nucleoli	Rarely conspicuous	Occasionally	Prominent and/or multiple
Mitosis	few	intermediate	numerous
orientation	Towards lumen	Degree of polarisation	No polarisation

**Figure 1-19:** Examples of DCIS nuclear grades.

(A) Low **(B)** Intermediate **(C)** High.

Another grading system for DCIS depends on the differentiation degree combined with the nuclear features, comparable to that used for grading BC, its score consists of four parameters and each item has a score from 1 to 3. The total score ranges from 4 to 12, and 4-7 indicates low-grade DCIS, 8-9 indicates intermediate-grade DCIS and 10-12 indicates high-grade DCIS (84, 182), **Table 1-3**.

Table 1-3: Modified grading system for DCIS

Features	Score 1	Score 2	Score 3
Gland/papillae	>75%	10-75%	<10%
Mitosis	None	1-2	More than 2
Nuclear grade	Low	intermediate	high
Central necrosis	<10%	10-50%	>50%

In 2010, Pinder et al. published a new DCIS classification scheme containing a correlation with the incidence of ipsilateral tumour recurrence which identified a high-grade DCIS subgroup with a worse prognosis compared to the classic classification system based on cytonuclear features only (195). There are many other grading systems for DCIS, however, the reproducibility and reliability of them differ between pathologists and depend on their expertise in breast pathology, hence working on finding a universal objective scheme for DCIS grading may provide a more convenient method for the prediction of DCIS behaviour and understanding its natural history (196).

1.2.9 Molecular classification of DCIS

Routine histological classification of DCIS has remained the gold standard as a prognostic and predictive factor for their behaviour. Recently, new classification methods have been developed based on the molecular signatures of the tumours. Although the molecular classification of DCIS lesions is not widely used in routine practice as BC classification, they share frequent molecular, genotypic, phenotypic, and transcriptomic similarities (197, 198), **Table 1-4**.

Table 1-4: Molecular subtypes of DCIS

Molecular type	ER, PR, HER2 and Ki67 expression
Luminal A	ER+/PR+, HER2- and low Ki67 (less than 14%)
Luminal B	ER+/PR+, HER2-/+ and high Ki67 (more than or equal 14%)
HER2 enriched	ER-/PR-, HER2+
Triple-negative	ER-/PR-/ HER2-

1.2.10 Prognostic and Predictive Factors

The prognostic and predictive factors for DCIS can be referred to in two separate ways: predictive factors for DCIS recurrence and predictive factors for progression to BC.

1.2.10.1 Predictive factors of DCIS recurrence

Although DCIS is a non-fatal disease with a nil mortality rate, about 50% of DCIS recurrences are invasive diseases which are potentially fatal (199), therefore stratification of patients with high risk for recurrence is vital to avoid cancer progression.

Clinicopathological features are associated with an increasing risk of DCIS recurrence at the age of 40 years and above (200), symptomatic patients compared with screen-detected ones (201), and higher mammographic density (202).

.Van Nuys prognostic index (VNPI) for DCIS is one of the most widely used schemes for risk stratification of DCIS patients according to recurrence risk and for adequate therapy decisions (**Table 1-5**), however, there are limitations and a lack of validation in some studies (203).

Table 1-5: Modified Van Nuys Prognostic Index

Scoring parameters	1	2	3
Tumour size (mm)	≤15	16-40	≥41
Margin width (mm)	≥10	1-9	<1
DCIS grade	Non-high nuclear grade, no Comedo necrosis	Non-high nuclear grade, Comedo necrosis present	High nuclear grade, with or without comedo necrosis
Age (years)	>60	40-60	<40
VNPI Score and suggested treatments			
Score		Treatment options	
Low (3-5)		Excision alone	
Intermediate (6-8)		Excision with RT	
High (9-12)		Mastectomy	

Molecular subtypes play a key role in the rate of recurrence. For example, DCIS which are ER-negative, HER2 positive and have a high proliferation index are more susceptible to recurrence (204).

1.2.10.2 Predictive factors for DCIS progression to invasive BC

Although the natural history of DCIS is still not clear, approximately all patients are treated surgically after diagnosis, some reports stated that approximately 15-50% of DCIS cases may progress to invasive BC if left untreated within 10 years (205, 206). High-grade lesions, comedo necrosis, high mitotic rate, high proliferative index, and HER2 positive molecular subtype are more likely to progress to invasive tumours than others (207).

1.3 Basement membrane (BM)

BM is a thin and amorphous sheet-like structure. BM is composed of collagenous and non-collagenous components such as proteoglycans and glycoproteins (208). The molecular structure and arrangement of the BM vary in the tissues along with their functions (209). In malignancy, the normal assembly of the BM is disrupted.

By electron microscopy (EM), BM is characterised by two main layers, the reticular lamina and the basal lamina which are divided into (i) a linear, homogeneous, electron-dense region (lamina densa) and (ii) a clear zone (lamina lucida) directly below MECs. The lamina densa is known as the BM proper and is composed of densely packed fibrils inserted in a dense matrix, while, the lamina lucida is an electron-lucent zone that contacts the cells (210). The lamina densa is an electron-dense zone which is 20–300 nanometres (nm) in thickness and consists of a network of collagen IV fibrils with proteoglycan perlecan. The lamina lucida is about 40 nm thick composed of laminin, nidogens (entactin) and integrins. Anchoring filaments of collagen VII bridges the lamina densa and lucida. The hemidesmosomes, on the basal aspect of MECs, are fixed to the basal lamina by fine filaments. The lamina fibro-reticulars or reticular lamina is the outermost layer formed mainly of collagen III (211-213).

Fixation methods affect the thickness and appearance of the BM structure (214). Cryofixation has demonstrated that the BM is a homogenous layer, not laminated, and the lamina lucida is an artefact produced by fixation (214). BMs thickness is a variable approximately 100nm by EM however this may be an underestimation result from dehydration in the preparation procedure, which was confirmed by atomic force microscopy (AFM) that measured two-fold thickness (215).

1.3.1 Molecular structure of BMs

The molecular components of BMs are categorised into major and minor structures. The major structures are collagen IV, laminin, nidogens and perlecan. The minor components are fibronectin and collagens XV and XVIII (213, 216). Collagen IV is the main component which forms about 50% of the BM (213, 217). It is a non-fibrillar collagen that exhibits a network with different six alpha chains known as $\alpha 1$ (IV) through to $\alpha 6$ (IV). The chains $\alpha 1$ (IV) and $\alpha 2$ (IV) which are known as classical chains represented all BMs tissues, while the other chains show different distribution throughout tissues. The chains of $\alpha 3$ (IV), $\alpha 4$ (IV), and $\alpha 5$ (IV) are in the renal glomerular basement, lung, eye, and testis BMs, however $\alpha 5$ (IV) and $\alpha 6$ (IV) are present in the epidermis, smooth muscle, and mammary gland BMs (218-220).

Laminins are a family of large glycoproteins composed of α , β , and γ chains. There are 15 various heterotrimeric isoforms of laminin resulting from 12 genetic types of these chains. laminin is responsible for cell attachment and differentiation (216, 221). Nidogen is a glycoprotein with two forms N1, and N2 linked with collagen IV and laminin for BM stability (213, 222, 223). Perlecan is a heparan sulphate proteoglycan (HSPG) and binds to collagen IV and laminin influences adhesion and migration(213, 224, 225).

In addition to the major components of BMs, there are minor ones which are variable according to the tissue. The function(s) of the minor components of BMs are still unclear and their significance is less known,

however, they may confer BM properties. They include fibronectin and other collagen types such as collagen XV and collagen XVII. Fibronectin is a glycoprotein in the lamina reticularis joining it with the basal lamina for structural integrity. Collagen XV is responsible for BM maintenance whereas collagen XVII is nonfibrillar collagen that supports cell proliferation and migration. Other minor components such as SPARC (secreted protein acidic and rich in cysteine) and fibulins (213, 225, 226).

Figure 1-20.

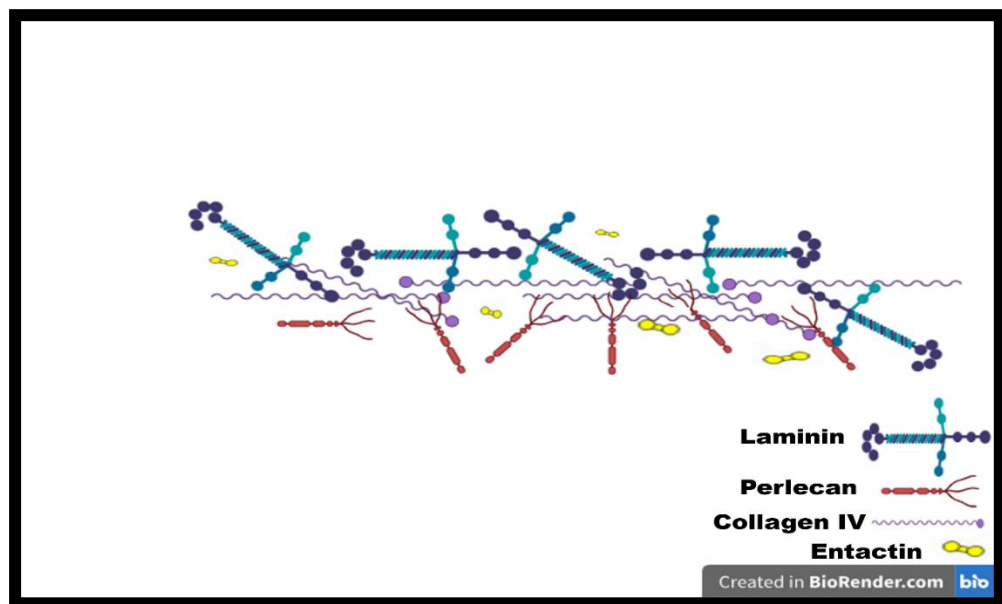


Figure 1-20: Diagram of the molecular structure of a BM.

In the normal breast tissue, there are MECs which are found between the BM and the luminal epithelial cells which produce BM components. They secrete laminin, collagen IV, laminin 5, and fibronectin (227).

1.3.2 Diagnostic use of the BM in breast tissue

When visualised using a haematoxylin and eosin (H&E) stain, which is the gold standard stain in histopathology, BMs appear as a fine line beneath the epithelium at normal TDLU and ducts, which is difficult to demonstrate using light microscopy. Special stains such as periodic acid Schiff (PAS) and silver preparations are often used for BM detection. BMs are PAS+, producing a magenta colour; however, when diastase (PAS-

D) is added to break down glycogen, it appears as a pink line. BMs also contain reticulin fibres, therefore they can be stained using silver stains and appear black under these conditions. IHC, alongside antibodies against collagen IV and laminin, is often used for BM identification (228).

The basic definition of invasive BC is based on BM penetrating with stromal infiltration, with the migration of neoplastic cells in the adjacent stroma with potential local and distant spread (229). While the diagnostic use of BM is still valid, and pathologists use BMs to differentiate *in-situ* from invasive lesions (229), routine assessment of BM in practice is challenging (230).

DCIS with micro invasion shows focal fragmentation and disruption of the BM at the micro invasion site (231). Evaluation of BM using multiphoton microscopy reported that the BM of ADH is complete but enlarged compared to normal BM. In addition, low-grade DCIS BM is similar to ADH with straighter collagen fibres (232). Although the structure, composition, and arrangement of the BM in DCIS are different compared to native BM, it is believed that the DCIS BM is a modification of the native BM rather than a new one.

High-grade DCIS showed thickened BMs resulting in the perception that the well-developed layer of fibrous tissue (pseudo capsule) around EPC may represent native BMs resembling *in situ* lesions (233-235). However, there is much evidence which indicates that this pseudo capsule represents an abnormal proliferation of the BM-like material produced either by the proliferating neoplastic cells or the stromal cells (235). The presence of a similar pseudo capsule peripherally surrounding EPC at metastatic or recurrent areas outside the breast suggests that this structure is a reactive process resulting from tumour (233, 234).

The BM is used regularly when diagnosing microglandular adenosis (MGA) to distinguish it from tubular carcinoma. MGA shows infiltrating round glands surrounded by a BM (236). MGA lacks MECs and is characterised by an infiltrative appearance that mimics BC. MEC

markers do not help distinguish MGA from tubular or low-grade NST with prominent tubule formation. Moreover, there is a lack of desmoplastic stromal reaction, BM markers are used in routine practice. Possibly this is currently the main diagnostic use of BM markers in the BC in routine practice.

Lactational changes and apocrine metaplasia are two benign changes in the breast, that show a focal absence of MEC layers. Apocrine metaplasia can show cytological atypia in addition, lactational changes present with enlarged irregular nuclear raising suspicion of invasive lesions. BM markers can confirm the benign nature of these lesions. Additionally, BM plays a key role in distinguishing sclerosing adenosis from tubular carcinoma that continuous BMs in sclerosing adenosis despite perineural invasion while completely absent in tubular carcinoma (237). Collagenous spherulosis is a benign lesion characterised by several cysts consisting of collagenous material surrounded by MECs and epithelial cells which can be misdiagnosed as ACC. Collagenous spherulosis shows IHC-positive staining for collagen IV and laminin, however, it is composed of only basal lamina which is duplicated (238).

ACC is surrounded by BM-like material (239, 240). The IHC staining reported that pseudo cystic spaces filled with disturbed BM-like material mainly fibronectin and laminin that are lined by tumour cells (241). Ultrastructural EM studies showed ACC was surrounded by uninterrupted thickened BM (242). The BM-like material within the pseudocyst is produced by the surrounding neoplastic cells. The presence of vascular capillaries within the pseudocysts suggests invagination of the surrounding stroma. Nevertheless, the ultrastructural features and material composition in the pseudocysts are consistent with that of the BM and not that of the interstitial ECM. The indolent behaviour of ACC reflects the degree of differentiation that tries to recapitulate the normal breast and secrete BM-like material.

Acinic cell carcinoma (AC) is a type of salivary gland-like BC with well-differentiated acinar-like structures that resemble atypical MGA.

Although both lesions lack an MEC layer, AC lacks a BM (243), (**Table 1-6**).

Table 1-6: Basement membrane (BM) and myoepithelial cells (MEC) in various breast lesions (244)

Breast lesion	Classification	BM	MEC
Microglandular adenosis	Benign but infiltrative	Present	Absent
Infiltrating epitheliosis	Benign /focally infiltrative	Mainly present	Focally absent
Collagenous spherulosis	Benign	Present	Present
Metaplastic apocrine changes	Benign	Present	Can be attenuated or absent in occasional cases
Lactational changes	Benign	Present	Can be attenuated or absent in occasional cases
Ductal Carcinoma <i>in-situ</i> (DCIS)	Malignant <i>in-situ</i>	BM is preserved at the periphery but can be different from the normal BM around the TDLU and breast ducts	Present but can be focally attenuated
Encapsulated papillary carcinoma (EPC)	Low-grade invasive	Typically, thick capsule-like structure at the periphery	Absent in 70-80% of cases at the periphery
Solid papillary carcinoma	Low-grade invasive	Typically, a thin capsule-like structure (BM) at the periphery	Absent in 50-70% of cases at the periphery
Adenoid cystic carcinoma	Malignant invasive	BM-like material at the periphery of the cribriform structures and within the false lumen (pseudocyst)	Absent

1.3.3 Basement membrane and its mimickers

BMs share some ultrastructural features with ECM (245) and with some reactive processes such as scar tissue and pseudo capsules (**Table 1-7**).

Besides, BM-like material is produced by some invasive BC. The different components of the BM can help to distinguish the native BM from its histological mimickers

Table 1-7: Characteristics of the breast basement membrane (BM) and its mimickers (244)

Component	Basement membrane	Normal interstitial tissue stroma	Tumour-associated stroma	Scar tissue	BM-like material in invasive carcinoma
Chemical composition	Laminin 1, 5 Collagen IV Anchoring fibrils XV, XVIII, VII, XIX Fibronectin PGs: Perlecan & agrin	Fibroblasts Laminin 1 Collagen I, III, V Anchoring fibrils VII, VI, XXVI, and XXVIII Fibronectin PGs: hyalectans and small leucine-rich Matrix metalloproteinases Elastic fibres	Myofibroblasts Laminin 1 Collagen I, III, V Anchoring fibrils VII, VI, XXVI, and XXVIII Fibronectin PGs: hyalectans and small Growth factors Matrix metalloproteinases Inflammatory cells	Collagen I Elastic fibres Laminin 5	Collagen iv Laminin 5 Fibronectin Hyaline material Heparan sulphate proteoglycan Entactin
Staining pattern					
Collagen IV	+	—	—	—	+
Laminin	+	-	+	+	+
CD+34	—	+	+	—	—
Alpha SMA	—	+	+	—	—
Special stains					
PAS	+	-	-		+
Silver stain	+	+	+	-	
Van Gieson	-	+	+	+	
Masson's trichrome	+	+	+	+	
acriflavine	+	+	+		

Electron microscopic	Electron dense zone, which is a fibril network and electron lucent zone of lamina lucida, both called basal lamina, with underlying lamina reticularis (246)	Bundles of collagen fibres and elastin with fibroblasts (247)	Collagen fibres with more prominent elastic fibres arranged in bands with some amorphous matrix. four cell types (fibroblasts, myofibroblasts, MECs, and mesenchymal cells (248)	Meshwork of thick collagen bundles with few elastic fibres which are curled or tortuous numerous large fibroblasts with long processes within and between the bundles (249, 250)	Narrow basal lamina area or wider hyaline (loose BM material) area, lamina densa with variable thickness fine fibrillar split into many layers, lamina lucida, bands of fine fibres(242, 251)
Thickness and	100nm, sheet-like, eosinophilic appearance	Wavy basket-shaped collagen bundles	Dense collagen bundles	Thick bundles of disorganised collagen fibres	Eosinophilic BM-like material, variable thickness
Fibre arrangements	Collagen fibres are packed and arranged regularly in lamellas (252)	Parallel collagen fibre Loose packing of collagen (253)	Disorganised, poorly aligned collagen fibres (254).	Disorganised collagen bundles with no specific orientation appear as whorl-like pattern (253)	

1.4 Tumour microenvironment (TME)

Carcinogenesis is a multiplex process with different interactions between the neoplastic cells and the surrounding TME (255). This theory refers to the soil (stroma) and seed (tumour cells) theory which indicates that a proper environment is important to tumour growth through the crosstalk between both components (256). TME composed of cellular and non-cellular components. The cellular component includes cancer and non-cancerous (stromal) cells, immune cells, blood, and lymphatic vessels. The non-cellular component includes chemokines, cytokines, mediators, and growth factors (257). The stromal cells are relatively genetically stable compared to the tumour cells, therefore they may be targets for therapeutic agents for cancer such as cancer immunotherapy (255).

The immune system attacks the tumour cells to get rid of it. Tumour antigens play a role during this process and are recognised by cytotoxic immune cells. Fibroblasts and macrophages within the TME also contribute to this defence mechanism. However, these cells may have a role in tumorigenesis.

1.4.1 Stroma

Stroma in the breast, like many other tissues, comprises stromal cells that secrete ECM, and immune cells, in addition to other structures including blood vessels and nerves. The ECM includes fibres (collagen fibres, elastic fibres, which contain elastin and fibrillin, and reticular fibres) and ground substance (258). The ratio of tumour to stroma showed to be a prognostic factor in BC patients, especially TNBC patients who lack definite prognostic biomarkers (259).

ECM is composed of two main structures which vary in structure and function, interstitial matrix, and BM. Interstitial matrix composition varies between different tissue types in contrast, BM is a more stable structure that lines the cells (260).

1.4.2 Interstitial extracellular matrix (ECM)

While the non-fibrillar collagen IV is the main component in the BM, fibrillar collagens I, III and V are the abundant components of the ECM (261). The ECM includes collagens that act as anchoring fibrils (VII, VI, XXVI, and XXVIII), which interconnect matrix components. Chondroitin sulphate and glycoprotein fibronectin, which is a minor component of the BM, are major components of the ECM and play a key role in matrix and cell interactions (262). ECM contains glycoproteins which interact with matrix components, growth factors, and cell surface receptors and have a regulatory function in matrix to cell communications (263, 264).

There are two main families of proteoglycans (PGs) in interstitial ECM, hyalactans and small leucine-rich PGs (SLRPs). They are responsible for cell signalling, inflammation processes and collagen fibrillogenesis. Unlike BMs, elastin is a main component in ECM and appears as microfibrils anchoring the BM to interstitial ECM (265, 266).

The interstitial ECM contains several proteins which can be detected by IHC and are different from those in the BM. These include collagen I, laminin 1, thrombospondin 1 (THBS1), and smooth muscle actin- α (α -SMA) (267, 268), in addition to the stromal cell markers such as CD73, CD90 and CD105 (269). However, collagen IV and laminin are markers for the BM, laminin is expressed in stromal cells (270, 271). However, α -SMA is one of the markers for MECs (229). Some collagens are presented in the BM zone and have been used recently to detect BMs, such as collagen XV, XIX (272) and VII (273).

Evaluation of the ECM by picrosirius red staining (PSR) revealed that the ECM deposited in the presence of cancer-associated fibroblasts (CAFs) was not only more abundant which indicates ECM stiffness, but also more aligned. These data suggest that the aligned ECM deposited by CAFs could be used by cancer cells as tracks for invasion (274, 275). In addition, TME may contribute to therapy resistance via different mechanisms such as dense ECM or limited blood flow that could limit

drug delivery to tumour cells, and activation of survival pathways through ECM-epithelial cell interactions (276). A study reported that tumour-associated collagen signature-3 (TACS-3) is correlated with poor outcomes in BC. TACS-3 is characterised by bundles of aligned and straight collagen fibres that were oriented perpendicular to the tumour boundary (277, 278).

1.4.2.1 Cancer-associated fibroblasts (CAFs)

Fibroblasts are the cornerstone in the body tissue homeostasis through ECM production, BM deposition, epithelial cell differentiation and immune response modulation(279). CAFs a major component of the TME in BC CAFs differ from normal fibroblasts, they are mainly characterised by overexpression of fibroblastic markers such as fibroblast-specific protein 1 and fibroblast-activating protein, and matrix metalloproteinase 3 (MMP3) alongside growth factors and cytokines (280). The exact origin of CAFs is still unclear, they may be derived through endothelial-to-mesenchymal transition (End MT) or from epithelial-mesenchymal transition (EMT) (281, 282). Malignant epithelial cells acquire a mesenchymal phenotype when cultured with CAFs, supported by an increase in mesenchymal cell markers and epithelial cell markers decline via TGF- β actions (275). Another mechanism that is supported through the study of murine BC models, is the modulation of the immune TME which enhances tumour growth and angiogenesis (283).

1.4.2.2 Myoepithelial cells (MECs)

As mentioned previously in the histology of the breast, the duct-lobular system of the breast is lined by two cell layers, the inner epithelial and the outer MECs. MECs are spindle-shaped cells oriented parallel to the long axis of the ducts forming a continuous layer around the inner luminal layer. Ultra-structurally, they contain smooth muscle filaments with contractile functions to help milk ejection. In addition, they express intermediate filaments like epithelial keratins forming desmosomes, and

gap junctions with cadherin-mediated interactions between each other besides the formation of hemidesmosomes the BM (227, 284-286).

Some studies have shown that MECs produce protease inhibitors that inhibit tumour invasion besides their role in growth and angiogenesis suppression (287). However, MECs in the DCIS appear to be distinct genetically from those in normal ducts and secrete many chemokines which were implicated in cell growth induction and metastasis (288, 289). The exact mechanism of how DCIS cells invade the MECs layer and the BM to the surrounding stroma is not fully understood but two main theories have been proposed. The first is based on proteolytic enzyme action with degradation of these layers to invade. The other theory depends on genetic defects in the MECs due to genetic abnormalities (mutations) leading to partial disruption of the MECs layer and stroma invasion (290, 291).

1.4.2.3 Endothelial cells

Endothelial cells (ECs) are cells that line the inner wall of blood vessels and play a role in controlling nutrient diffusion from blood into tissues, maintaining blood flow and regulating leukocytes migration (292). Dissimilar to the normal ECs, tumour ECs are irregular in size and shape and comparatively longer, participating in intussusceptive angiogenesis which is the process where trans-vascular extension of the vessel wall in the lumen splits the vessel into two halves. This is a rapid process rather than sprouting to increase the TME vessel density (292). ECs abnormalities may result in tumour hypoxia that induces several vascular growth factor-mediated angiogenesis which supports tumour growth and metastasis. ECs are also the main source of mesenchymal stem cells (MSCs) (293, 294).

1.4.2.4 Other cells

In addition to the cells already described, there are also other cell types in the TME such as MSCs, adipocytes and neuroendocrine cells, which

have gradually been considered important regulators of tumour progress and development (295).

1.4.3 Immune cells

1.4.3.1 Tumour infiltrating lymphocytes (TILs)

TILs are indicators of the adaptive immune response against tumours. They play a key role in cancer immunotherapy (296, 297). TILs are defined as the most common mononuclear immune cell infiltrates. TILs which are mainly T cells reported in the majority of BC patients (298). There are two main categories of TILs: stromal and intratumoral TILs. Stromal TILs are defined as TILs within the tumour boundaries within the stroma between neoplastic cells without directly contacting or infiltrating tumour nests (299). In comparison, intratumoral TILs refer to TILs inside the tumour bulk which have direct contact with the tumour cells without stromal intervention (300). Although the count of intratumoral TILs strongly correlates with stromal TILs count (301), The International TILs Working Group recommends assessing stromal TILs in the pathological evaluation due to their reproducibility with less interobserver variability (302). In previous BC studies, dense TILs were shown to augment the effect of chemotherapy providing a better prognosis mainly in TNBC (303, 304). Following the demonstration of TILs prognostic significance in BC and the potential clinical application, guidelines recommendations for TILs evaluation have been published (302, 305) and an update for TILs assessment has been published in 2018 (306, 307).

The immunity of TME plays a key role during carcinogenesis (308). Triggering of an immune response in the form of lymphocyte infiltration with other inflammatory mediators is a phenomenon known as “cancer immuno-editing” (309). It is known that subtype, density, and location of TILs play a role in cancer prognosis. Infiltration of T lymphocytes develops an anti-tumour response via the cytotoxic CD8⁺ T-cell lymphocytes (CTLs) however, an immunosuppressive response arises through regulatory T cells (Tregs) (310, 311).

An influential component of the immuno-editing phenomenon is the development of immunosuppressive TME mediated through the activation of the immune checkpoint pathway. The programmed cell death 1 (PD1) protein which is a member of the B7/CD28 family of co-stimulatory receptors present mainly in T cells. It sends inhibitory signals to T cells to suppress the anti-tumour response (309). PD1 functions mainly through its ligand programmed cell death ligand-1 (PDL1), which is expressed in various immune cells such as antigen-presenting cells and both activated T and B lymphocytes and tumour cells (312, 313). Revolutionary results have been achieved from clinical trials involving monoclonal antibodies that target PD1 and block the PD1/PDL1 pathway (314).

1.4.3.2 Other immune cells

During carcinogenesis, myeloid-derived suppressor cells (MDSCs), including myeloid precursors, macrophages and dendritic cells play a role in tumour progression and angiogenesis, through suppressing T cells and natural killer (NK) cells by producing cytokines such as IL-6, IL-10. Tumour associated macrophages (TAMs) play a key role in tumorigenesis. Although macrophages may have a role in tumour immunity, they have an evident role in tumour progression, especially as a driver for invasive phenotypes (315, 316). The dual reverse functions of the TMA that switch from tumour suppressors to tumour promoters are still not fully understood. It may be explained by their plasticity from classically activated M1 (proinflammatory) cells which trigger anti-tumour response to activated M2 (anti-inflammatory) which release immunosuppressive cytokines (295). In addition, hypoxia may also play a role in this transition (317).

1.5 Role of collagen in breast cancer

1.5.1 Collagen properties

Collagen is a helix glycoprotein composed of three-helix α chains that may be homologous or nonhomologous. The amino acid sequences of

these α chains are characterised by the presence of repeats of glycine–proline –hydroxyproline with or without interruptions. The hydroxyproline contributes to collagen thermal stability (318). The collagen stability assembly is affected by both intermolecular and intramolecular linkages, specifically covalent linkages, including lysyl oxidase (LOX) crosslinks, glycosylation crosslinks and transglutaminase crosslinks (319-321).

Generally, collagen types are about 28 types which are categorised into four subfamilies based on supramolecular assembly, including the fibril-forming collagens (I, II, III, V, XI, XXVI, XXVII); fibril-associated collagens with interrupted triple helices (FACITs) which link to collagen fibrils surface rather than forming fibrils and include (IX, XII, XIV, XVI, XIX, XX, XXI, XXII, XXIV); network-forming collagens (IV, VIII, X), and membrane-anchored collagens (MACITs) include (XIII, XVII, XXIII, XXV) (322). Among non-fibrillar collagens, BM collagens (IV, XV and XVIII), beaded filaments-forming collagens (VI) hexagonal network-forming collagens (type X), and anchoring fibrils-forming collagens (type VII) (323).

Collagen IV is the main constituent in BC. IHC antibodies against collagen IV and laminin are often used for BM identification (228). However, previous studies showed positive collagen IV surrounding BC and LN staining. Expression of collagen IV was shown around the tumour nests, either continuous or fragmented, in 42.9% of BC cases, and 39% of cases showed positive expression of Laminin (324, 325). Anthers' study showed that 80% of invasive BC cases, which were mostly of special types, showed positive immunoreactivity for collagen IV either linearly or fragmentarily around the tumour nests. In addition, 54% of cases showed positive expression of Laminin, either strong linear expression or fragmented and dotted expression (326). Additionally, Laminin 8 was detected to be increasingly expressed in invasive BC (88%) and its metastases (100%) (327). Another study demonstrated that laminin 5 was expressed in the cytoplasm of tumour cells of BC (328). Moreover, Albrechtsen et al. reported strong positive expression of laminin in BC cells and demonstrated that BM produced by these tumour

cells could be detected by laminin staining, but they were thinner and discontinuous compared to normal BM (329). In addition, laminin expression in BM was observed in invasive in various patterns and was significantly correlated with histological grade (330). Therefore, laminin and collagen IV are not effective method to differentiate between invasive BC and non-invasive lesions in many conditions.

EM, including transmission electron microscopy (TEM) and scanning electron microscopy (SEM), could be a powerful tool for studying collagen fibres. However, several limitations have been identified. The preparation process of the samples for EM, such as fixation, dehydration, embedding, and sectioning, can cause artefacts that may distort the native structure of collagen (331). For example, in BM, the lamina lucida was discovered as an artefact caused by fixation; in addition, BM thickness was proved to be underestimated with EM due to dehydration, as proved by AFM, which showed twofold higher measurements (244). The challenge is to have sufficient contrast and resolution to measure crowded by other fibrils, and to measure the lengths of fibrils (332). The limiting random errors in the measurements which are caused by the noise in the scattered electron signal from the fibrillar mass and the underlying support film, therefore, less accuracy can be achieved for collagen fibril measurements (333). A main limitation of TEM is the necessity for a thin section, and resolution is limited to twice the section thickness (334). While the major drawback of SEM is the small field of view and limited view depth (335).

1.5.2 Effect of cancer on collagen

During carcinogenesis, the ECM undergoes some structural changes. The distribution and content of collagen are modified to further coordinate the biological properties of cancer cells including gene mutations.

Mutation of oncogenes which are divided into two main categories: tumour suppressor genes and proto-oncogenes, alters the collagen properties in the TME. Mutated tumour suppressor genes play a role in

the alteration of the architecture and content of the collagen as the P53 pathway which is responsible for TACS3 referred to as collagen bundle orientation ranged between 60-90 ° indicated tumour aggressiveness and potential invasion (336). During cancer progression, collagen displays different signatures TACS-1 which refers to the presence of dense collagen near the tumour, TACS-2 indicates collagen fibres parallel to the tumour boundary, and TACS-3 represents radially aligned collagen fibres (278). The higher the density and depth of collagen fibre deposition, the higher the proliferation and invasion of tumour cells (337, 338). Studies showed that there is significant alterations in collagen constituents were observed in BC as the increment of fibrillar collagens types I, III, and V while collagen type IV was decreased (339-341). In line with these findings, the fibrotic reaction, which is associated with tumours, termed desmoplasia, correlated with poor prognosis in BC (342).

Previous studies on BC indicated that over-deposition of collagen bundles of type I and III were localized at the tumour invasion front (343, 344). There was an increment of collagen content of collagen V content to about 10% of total collagen contents however, it accounts for approximately 1% of normal breast tissue (345). The perturbation of collagen I in the presence of high concentrations of collagen V is associated with a physiological organisation and derangement of collagen I fibres which have a role in BC invasion (346).

Collagen acts as a scaffold which facilitates the migration of invading tumours or stromal cells (347). Collagen organisation and stiffness are affected by some enzymatic modifications such as crosslinking, thus, the high tissue density observed in BC provides evidence that the high tissue tension influences tumour initiation and progression (348, 349).

Furthermore, the high tissue density in normal breasts detected with mammography has been linked to an increased risk of BC occurrence (350). ECM density may promote invadopodia, which is an actin-rich protrusion valuable for cell invasion and metastasis (351).

LOX crosslinking of collagen fibres leads to increased ECM stiffness that promotes, PI3K activity through the focal adhesions pathway which was associated with tumour progression in BC. Therefore, LOX inhibition results in reduced tumour incidence and size, and decreased tissue desmoplasia which delays tumour progression (349). The collagen fibres which are adjacent to the lesions showed different architecture and mechanics resulting in stiff fibrotic stroma (352, 353).

1.5.3 Effect of collagen on cancer behaviour

In hypoxic conditions, ECM is collagen-rich and intensifies tumour progression based on the interaction between tumour cells and collagen. Hypoxia-inducible factor (HIF)-1, MMPs, and LOX participate in this process. Collagen I fibres are reduced in the hypoxic microenvironment by high LOX expression and decreased MMP-3 expression in BC (354). Hypoxia increases the procollagen-lysine expression as a collagen crosslinker to further enhance collagen fibre size which promotes metastasis (355). Collagen I fibres exhibited covalent crosslinking with both prolyl 4-hydroxylase alpha 1 and prolyl 4-hydroxylase alpha 2 in TNBC differentiated cell types resulting in increased chemoresistance (356).

Among the six collagen parameters which are, density, width, length, alignment, orientation and straightness, the increased collagen fibre width is the most powerful parameter in cancer prognosis prediction (357). This was proved with the study as width was an independent factor to predict BC prognosis and main characteristic to differentiate between invasive and non-invasive lesions.

The desmoplastic reactions in various cancers were divided into three types mature, intermediate, and immature depending on the presence of keloid-like collagen and myxoid (358). Fibrosis grade was correlated with metastasis seeming a more reliable predictive factor for bone metastasis rather than LN status (359).

Collagen plays a vital role in cancer therapy resistance. For example, increased collagen density was associated with chemotherapy resistance in oesophageal cancer through the MAPK and PI3K/AKT pathways (360). In addition, high collagen density contributes to doxorubicin resistance in pancreatic cancer (361). Generally, the collagen crosslinking results in high stromal stiffness which promotes treatment resistance (362). Besides, collagen I result in therapy resistance in other cancer types (363-365).

1.5.4 Role of collagen in cancer therapy

Collagen can be used as a targeted therapy strategy by combining collagen inhibitors with traditional therapeutics, including chemotherapy and radiotherapy, which is seen as a promising strategy. Studies which used collagenase combined with trastuzumab through thermosensitive hydrogels exerted anticancer results on experimental animals (366). Other trials such as losartan combined with photodynamic nanoplateforms caused tumour volume suppression in a BC mouse model (367).

Preclinical studies on collagen-related therapy have exhibited inspiring outcomes, unfortunately, few clinical trials have been conducted to date. There are some collagen depletion or collagen alignment changing drugs, but their effects are still not clear and further experimentation is needed. There are also many conflicting issues such as collagen functioning as a barrier in certain stages of cancer yet enhancing other stages of cancer. Moreover, degraded products of collagen can continue to function and promote cancer angiogenesis and metastasis (368).

1.6 Effects of breast cancer (BC) on the tumour microenvironment (TME)

Previous sections briefly mentioned the roles of TME components in BC tumorigenesis and progression. However, the tumour cells interact with the TME in different ways to invade the surrounding BM and stroma and modulate the host immune response. The following sections will briefly discuss some of these interactions that help cancer cell invasion and play

a main role in the progression from localised invasive disease to metastatic behaviour.

1.6.1 Degradation of basement membranes and invasiveness to surrounding tissues

The hallmark of any malignant tumour is its ability to penetrate the resting BM and the surrounding stroma (369). Epithelial malignant cells express many proteolytic enzymes such as cathepsins and MMPs which facilitate BM degradation and stromal invasion (370, 371). Numerous studies on CNV showed that there is a significant difference in these genes between normal, DCIS and BC epithelial cells (372-374).

1.6.2 Epithelial-mesenchymal transition (EMT) and its role in tumour progression

Recently, there has been a breakthrough in understanding the EMT mechanisms which may help clarify the tumour's metastatic capability and explain the unusual phenotype of some tumours (375). EMT has a key role in tumour progression from DCIS to invasive disease (376). EMT gene profile is highly expressed in DCIS with invasive BC lesions rather than pure DCIS cases which implicate their role in tumour progression (376).

1.6.3 Stromal fibrosis

Stromal fibrosis is considered as a histopathological benign diagnosis of breast tissue which is characterised by fibrous tissue proliferation and mammary duct obliteration. Stromal fibrosis has been defined by various names including fibrous tumour of the breast, fibrosis of the breast, focal fibrous disease of the breast and fibrous mastopathy (377). Stromal fibrosis is a clinicopathological feature that was originally termed as a palpable mass, which can be detected via imaging including breast ultrasound and screening mammography followed by diagnostic core biopsy (378).

Large BCs are associated with hypoxia resulting from an imbalance between supply and demand leading to central necrosis which is

replaced by scar-like area or what is called fibrotic foci. Fibrotic foci appear as hyalinized collagenous bundles associated with fibroblasts and neoplastic cells that extend radially may contain remnants of necrotic tissue and are surrounded by high vascular density (379). Stromal collagenisation is demonstrated in some benign BC as myofibroblastoma and appears as thick bundles of collagen with less cellular stroma (380). Hasebe *et al.* demonstrated that the central scar is an indicator of tumour aggressiveness in BC, it is associated with tumour size and higher grades and it is correlated with reduced overall survival (381).

The reason behind the development of stromal fibrosis is still unknown however some theories suggested several causes including oestrogen-dependant fibroblastic proliferation, an end stage of inflammatory processes, and a variant type of mammary involution (377).

The transitions of normal stroma into a dense fibrotic tumour stroma result from progressive accumulation and alignment associated with post-translational cross-linking of ECM fibrillar, especially collagen types I and III. The cross-linking of collagen is a multi-step process which is initiated by lysyl hydroxylase2 (LH2) catalyse hydroxylation (Hyl) of lysine (Lys). LOX-L family members catalyse the Hyl and Lys oxidative deamination to produce reactive aldehydes which result in the creation of links with the opposing Hyl/ Lys residues. LH2 is upregulated in various tumours including breast and lung cancers. This leads to increased LH2 levels which are associated with ECM stiffness and tumour aggression (382).

Studies have described the development of stromal fibrosis in cancers as being similar to the processes in wound healing (383). It has been stated that tumours are “wounds with no healing” and it is known that chronic fibrosis is a risk factor for cancer (382). Chang highlighted that patients with wound response signatures (stromal fibrosis) had a poor prognosis (384).

1.6.4 Stromal elastosis

Stromal elastosis is characterised by an accumulation of large quantities of elastic fibres mainly in the stroma. While less common in younger women, elastosis may be found in around half of all women over age 50 years with no evidence of breast disease (385). Periductal and stromal elastosis can be seen in benign and malignant lesions while marked perivascular elastosis is uncommon and is frequently associated with malignancy (385).

The amount of these elastic fibres increases in benign breast proliferative diseases more than in malignant lesions (386). The most common lesions in the breast that show a significant degree of elastosis are radial scar/complex sclerosing lesions however, a variable degree of elastosis is seen in BC particularly low-grade tumours. Elastosis in the breast was described decades ago but since then has received less attention. Unlike in the normal and the benign lesions, elastic fibre production in BCs is ascribed to the tumour cells rather than to stromal cells (387). BC elastosis includes both deposition and degradation of elastin. The elastin fragments originating from a degraded matrix provide cells with different signals compared to elastin, as stromal cells adhere to elastin but only can migrate and/or proliferate when incubated with elastin fragments (388).

Elastosis in the BC remains an unexplained phenomenon as its morphogenesis has not been determined. There is a hypothesis that explains this phenomenon as the presence of protease inhibitors in neoplastic cells might influence elastic fibre metabolism, which facilitates their proliferation via elastinolytic enzyme inhibition (389). Khatun *et al.*, (389) have reported that the lack of elastase in the benign proliferating epithelial cells may play a role in the occurrence of elastosis whereas in invasive carcinoma, the degree of expression of alpha 1-antichymotrypsin in cancer cells is correlated well with stromal elastosis, although there was no correlation with elastase. Elastosis has also

another diagnostic role as deposition of elastin material in stroma is associated with early detection in mammography.

Elastosis is common in BC, and it is a helpful feature to differentiate primary from metastatic BC, as the distinction of extramammary metastasis to the breast from primary BC in diagnosis is very important in management strategies and could help the prevention of unnecessary surgery. Hajdu *et al.*, proved that elastosis is considered an indicator of primary cancer (390).

Shivas and Douglas determined that elastosis is a prognostic factor in BC patients, and as a result, they developed an Elastica index on their observational histopathology of elastosis. They showed that moderate or massive elastosis correlated with good overall survival rates (391). Breast elastosis also correlates with good prognostic markers such as ER positivity, lower Ki-67, and HER2 negativity, and elastosis was increased in low-grade tumours compared to high-grade ones (392-394).

1.7 Digital pathology (DP) and artificial intelligence (AI) in BC

DP is defined as the usage of a computer to visualize histopathological slides in whole slide image (WSI) format which is obtained by the high-resolution scanning of tissue slides (395). The automated scanner is a robotic microscope which digitises an entire glass slide using specific software to merge the individually captured images into one digital image (396). It can store and analyse the images and their associated meta-data for diagnostic purposes using general techniques of “pattern recognition”, particularly through “deep learning” techniques (395). With availability of faster networks, and cheaper storage solutions has made it easier for pathologists to manage digital slide images and share them for clinical use (397). Many studies on histopathological diagnoses using WSIs have shown non-inferior to microscopic diagnosis (398-400). It has been shown that a percentage of concordance of between 89 and 99% was detected between using WSIs and the traditional methods, which

use slides and optical or EM (401). Moreover, there are many advantages of WSI over glass slides and a conventional microscope; including easy image accessibility, annotating and manageability to automated image analysis which has been shown to improve objectivity and productivity within pathology practice (402). In addition, WSI can be read by multiple examiners in multiple locations, allowing remote consultations and reducing the time and financial costs of transferring glass slides (403). Furthermore, image digitalisation provides a great opportunity to develop algorithms for automatic quantifications, which leads to improved reproducibility with the comparison to the naked eye examination by pathologists (404). Several important guidelines on the validation and implementation of WSIs for primary diagnosis have been developed by professional bodies such as the CAP, the Canadian Association of Pathologists, the Royal College of Pathologists in the United Kingdom, the Royal College of Pathologists of Australasia, and the American Telemedicine Association (396). In Europe, several WSI scanners are approved (given the CE mark), and in the US, the US Food and Drug Administration (FDA) has approved the Philips IntelliSite Pathology Solution to be marketed (405). Notably, WSI serves as an enabling platform for the application of AI in DP. AI became a significant part of the pipeline in research and clinical laboratories as high-resolution digital images can be prepared from histology slides and with the aid of computational tools, is that of “computational pathology”, which regards the computerised analysis of large imagistic data volumes and meta-data, acquired through multiple sources, to extract relevant biological and clinical features and to identify specific patterns (406). Researchers have developed deep learning (DL) algorithms to detect and classify BCs. A DL model was proposed to automate the multiclassification of BC histopathologic types(407). Cruz-Roa and his colleagues built a convolutional neural network (CNN) model to estimate the extent of invasive foci and the degree of infiltration on entire WSIs (408). Similarly, several fully automated systems to count mitosis for accurate histologic grading were developed (409, 410). Additionally, computational assessment of ER/PR IHC using AI algorithms provides opportunities to

improve precision performance (411-413). Furthermore, AI provides new information in predicting prognosis and therapy response in BC (414).

There are many web prognostics tools, molecular profiling, and gene expression testing techniques, which facilitate the prediction of the behaviour of BC disease, therefore the era of precision medicine in breast cancer has been developed using AI (415). Quantification of stromal morphological changes is difficult to assess using light microscopy. Studies have indicated that using WSI analysis and automated recognition systems could help identify tumour stromal changes including objective evaluation and quantification. However, the development of an algorithm to distinguish stromal components in histological slides is still a challenge as there have been no valid criteria for TME. However, with the use of computer vision, image analysis software, AI and machine learning, approaches could set algorithms without pre-defined criteria from a large set of classified WSI (416).

Digital image analysis and machine learning have been used to develop algorithms that could identify stromal alterations in benign and malignant BCs in addition to grading carcinomas *in-situ* based on stromal characteristics to predict invasion and malignancy (417). Moreover, these techniques have been used to detect the value of tumour stroma ratio (TSR) when predicting outcomes of both luminal and TNBC (418). Tumour cellularity is time-consuming when estimated microscopically, but cellularity is an important factor in neoadjuvant response evaluations and assessment of residual cancer burden indexes. AI could improve the performance and efficiency of cellularity assessment (419). Stromal remodelling is the first step of the invasion process, and the main factor here is collagen fibre restructure and alignment. As a result, the quantification of collagen fibres and the development of signatures of TACS have a predictive prognostic role (278).

Computational algorithm assessment for collagen distribution is a challenge for the clinical utility of image-based collagen fibre biomarkers as collagen fibre matrices such as alignment, density, orientation,

individual fibres measurements and their relation to tumour gland have diagnostic and prognostic value (420). However, Keikhosravi *et al.*, developed a model that extracted these characteristics from H&E sections based on a trained neural network of Second Harmonic Generation microscopy (SHG) data, but their clinical value requires more validation (421)

In conclusion, the diagnostic and prognostic roles of TME are still not well-defined and are presently underestimated, especially given that deciphering TME features is still complex and a challenge. Technological revolutions, such as the use of DP and AI, could open up the route to quantitative and qualitative assessment of the TME. Further research may lead to advanced clinical approaches, new strategies, and novel modalities that could enhance patient quality of life and advance the treatment of cancers.

1.8 Hypothesis and aims of the thesis

The pathology field of BC is challenging; therefore, the use of digitalised WSI integrated with AI in clinical pathology may extend the frontiers of the pathologist's view beyond a microscopic slide. Therefore, this study aimed to decipher the morphological features of TME of BC using DP and AI and investigate their diagnostic and prognostic roles in BC, highlighting the role of collagen fibres in BC.

The hypothesis of this thesis is:

Investigating the morphological features of TME and novel stromal biomarkers in BC will act as useful diagnostic, prognostic or predictive tools to be used in routine clinical practice.

In Chapter 3, 3.1.1, : It was hypothesised that differentiation of a native BM from a reactive BM surrounding an invasive lesion has significant clinical importance.

In Chapter 4, 4.1.1: It was hypothesised that elucidation of the structural composition of the EPC capsule could provide insights into the underlying behaviour.

In Chapter 5, 5.1.1: characterization of stromal ultrastructural features, particularly collagen fibre architecture, could serve as a novel methodology for differentiating between non-invasive and invasive breast lesions and act as a prognostic tool to predict outcomes.

In Chapter 6, 6.1.1: Collagen XV and collagen XIX could be potential diagnostic biomarkers to distinguish invasive and non-invasive BC lesions, In addition, HIF-1 α and LOX could provide a predictive tool for BC prognosis.

In Chapter 7, 7.1.1: The mechanisms driving stroma with the immune composition are different in BC subtypes which could explain the conflicting roles of the prognostic value of stroma ratio in different molecular subtypes.

In Chapter 8, 8.1.1: the interplay between cancer cells and TME is exemplified by the morphological changes observed in the stroma, could have a prognostic role in BC

The specific aims of this thesis are:

- 1- Compare the BM between normal, in situ and invasive breast tissue, to understand its role with cancerous cells and to determine the process of invasion that makes *in-situ* act as an intermediate stage between normal tissue and invasive carcinoma.
- 2- Refine the definition of invasiveness by characterisation of the geometric (physical) features of collagen in BC and precursor lesions to develop a signature that potentially could differentiate between native BM and BM-like material.
- 3- Identify criteria to distinguish *in-situ* from invasive disease based on BM characteristics.
- 4- Elucidate the nature of the EPC based on BM collagen fibre geometric characteristics.

- 5- Identify distinguishing characteristic features like a signature of stromal collagen fibres in DCIS and invasive carcinoma and assess the clinical and prognostic value of stromal collagen fibres in BC
- 6- Explore novel potential biomarkers closely relevant to BC biology and progression.
- 7- Clarification of the conflicting prognostic roles of stroma and its immune composition in different molecular subtypes.
- 8- Characterise the stroma types in BC and their prognostic role.

2 Chapter 2: Patients and Methods

2.1 Patient cohort and data collection

A large cohort (n=3030) of primary operable BCs presenting to the Nottingham Breast Institute, Nottingham City Hospital, Nottingham, UK between 1999 and 2018 according to the ethical approval to use the cases in these years and the availability of TMA blocks to cases in these years, was included in this study. This is a well-characterised cohort with a long-term follow-up with a median of 138 months ranging from (0-216 months). Full detailed clinicopathological data are available in each chapter, including patient age at diagnosis, menopausal status, tumour size, histological tumour grade, histological tumour type, molecular subtypes and NPI risk groups (422). Moreover, the outcome data were available in terms of breast cancer specific survival (BCSS), which is defined as the time from the date of the primary surgery to the time of death from BC in months, and distant metastasis free survival (DMFS), defined as the time from the primary surgery until the first event of distant metastasis in months. For the analysis, the cohort was classified into subcategories, therefore the study cohort for each project is described in detail in each chapter **Table 3-1**, **Table 5-1**, **Table 5-2**, **Table 6-1**, **Table 7-1**, **Table 8-1**.

2.2 Histological review

The author and two experienced pathologists (Dr Mohammed Aleskandarany and Dr Wakkas Fadhil) performed histological reviews. Histologic review was initiated by retrieving FFPE blocks from storage archives. These blocks were marked as containing tumour tissue and 4-6 were retrieved for each case. One full-face 4-µm thick section was cut from each block and stained with H&E. A quality check was done to exclude slides with air bubbles, where there was no invasive tumour tissue, and for those with extensive tissue folding or slides with poor staining. Re-sectioning was performed in case of air bubbles and poor staining. The presence of DCIS was scored into 3 grades: 0=no DCIS, 1=DCIS within the invasive tumour, or 2=DCIS was 1mm beyond the invasive tumour. TILs were evaluated by estimation of the percentage of

TILs within the invasive tumours and were scored as 1 (<10%), 2 (10-50%) or 3 (>50%).

Additionally, the suitability of IHC staining was evaluated in each slide based on the presence of sufficient tumour tissue. Tumour histological types and the grade were evaluated and recorded if there was a difference compared to those originally assigned in the patient record. Specific findings were recorded including, biopsy side changes, previous TMA empty cores, areas of haemorrhage, calcification, and fibrocystic changes.

2.3 Slide digitalisation and image acquisition

After the histologic review, H&E-stained slides were scanned into digital images. High-resolution digital slide scanning was performed with the Panoramic digital slide scanner (250 Flash III, 3DHISTECH®, Budapest, Hungary), at 20x magnification. This was followed by checking the quality through image viewing using Panoramic Viewer software v 1.15.4 (3D HISTECH®, Budapest, Hungary). As discussed in each chapter, 3.2.3, 4.2.3 4.2.4, 5.2.1, 5.2.2, 7.2.2, 8.2.2.

2.4 Tissue Microarray Construction (TMA)

The TMA construction process was the collection of the cohort to be arrayed, preparation of the recipient array blocks, designing into array grids, construction of individual array blocks, and preparation of the array block for sectioning.

2.4.1 Preparation of the donor blocks

For proper tissue array construction, sampling the representative site from the donor block was a critical step. To identify suitable cases for TMA construction, previously reviewed slides were selected and scanned.

FFPE tumour blocks (donor) that were characteristic of the specimens from each patient were retrieved from the tissue block archives. The

blocks included were ≥ 4 mm in depth, bearing viable tumour tissue, with a suitable tumour burden. Priority was given to blocks showing adequate tumour tissue which had not been frequently sectioned for previous studies.

2.4.2 Matching of donor blocks with their corresponding slides

To identify the tissue areas most appropriate for the TMA sampling, the presence of the tumour was confirmed by reviewing the digital slides and comparing them with the retrieved blocks. Visual assessment of blocks against the slides was performed to find matched blocks with slides. To avoid the potential risk of utilising a patient's diagnostic material, tumours with only one block or small tumour tissue samples were excluded to ensure the diagnostic patient's materials would not be depleted.

2.4.3 Digital image marking

The TMA cores were marked by Panoramic Viewer Software v. 1.15.4 (3D HISTECH®, Budapest, Hungary). TMA cores were selected based on the higher density of invasive tumours. Annotation of 0.6 mm tissue core diameter in the centre of the tumour was undertaken and 4-12 cores were marked per image to use one after that.

2.4.4 Recipient block construction

TMA construction was accomplished using the ordinary paraffin wax-based blocks which had been prepared by pouring the paraffin into blocks about 5–10 mm in depth and then extruding the tissue array cores into the block.

To prepare the recipient blocks, plain molten paraffin wax (56–58°C) was poured into a mould and then topped with a histopathological tissue cassette. The blocks were cooled on ice until the wax hardened and the wax was checked for the presence of air bubbles, striations or any fault lines (423). The block was checked to ensure that it was covered

completely without any excess wax at the edges or back. Finally, the block was trimmed smooth using a microtome to ensure a flat surface.

2.4.5 TMA layout design

TMA Grand Master 2.4-UG-EN software (3D HISTECH®, Budapest, Hungary) was used to design the block layouts. The creation of a new layout followed by a new layout name was inserted. The standard block size was 24x35 mm. Four replications were prepared for each block, each was given a unique version number, that was added to the block.

Each array contained 120 samples arranged in three subarrays, with a 1mm space between each core. The first subarray was composed of the 53 cores (6 rows, 10 columns) in the upper half of the block. The second subarray of the block was made up of 40 cores (4 rows, 10 columns), while the third subarray contained 23 cores (3 rows, 10 columns). In each subarray, cores were arranged at a centred distance, and the subarrays were separated by a single gap for orientation and macroscopic identification of the TMA block. Positive internal controls were introduced by adding orientation cores from the normal kidney, normal liver and tonsil tissues outside of the geometric margin of the array on the top and bottom left-hand sides.

Documentation of both the donor block and the TMA position of each core was done. An array map grid was performed for each array block. Each grid had a unique identifier and was organised in the same sequence corresponding to its position in the array block.

2.4.6 TMA block annealing and sectioning

After TMA block construction, blocks were placed face-up in an oven for three hours at 37°C to anneal them, helping the tissue cores to adhere to the walls of the holes in the array block and making the wax flexible for handling.

A clean glass microscope slide was placed on the face surface of the block, then gentle pressure was applied to push all the cores on the array

to the same level. The blocks were left to cool at room temperature for three hours. The annealing cycle was replicated, and the blocks were stored overnight at 4°C before sectioning.

Using a standard microtome sectioning technique, 4µm sections were cut from TMA blocks and transferred into permanent positively charged glass slides (Surgipath, X-tra™ Adhesive, Peterborough, UK). To ensure the quality of the TMA construction, the first section of each block was stained with H&E and was scanned to be reviewed histologically.

2.5 Optimisation of antibodies used in the study.

2.5.1 Validation of Antibody Specificity by Western Blot

Western blotting (WB) was done to determine the antibodies' specificity before IHC staining. Cell lysates from 6 cell lines: MCF-7, MCF10A- MD-MBA-231, BT-549, SKBR3, and ZR-751 which were obtained from the American Type Culture Collection; Rockville, MD, USA were used to reflect the selected protein expression. MCF-7 is a slow growing BC cell line. MCF-10 is a non-tumorigenic epithelial cell line resemble DCIS and fibrocystic changes. MD-MBA-231 is a model of last stage BC which represents TNBC. BT-549 is a papillary BC. SKRB3 is a cell line expresses HER2. ZR-751 represent luminal BC.

2.5.2 Preparation of samples, electrophoresis and transfer

Cell line lysates were prepared in a maximum volume of 24µl (15.6µl of lysate, 6µl of LDS sample buffer (4x), and 2.4µl of reducing agent, (10x)). All reagents were obtained from Thermo Fisher Scientific.

This mixture was heated at 70°C for 5 minutes and then left to cool on ice. The prepared sample was loaded onto 10% SDS–NuPAGE (4-12%) (Thermo Fisher Scientific) in 20x MES SDS running buffer (Thermo Fisher Scientific). The pre-stained Thermo Scientific Protein Ladder LC5602 (Magic ladder) was used as a molecular weight standard and

gels were run at 200 volts for 20 minutes. Proteins were transferred to a nitrocellulose membrane by wet transfer and electro-blotting at 10 volts for 60 minutes in a transfer buffer. To confirm that the transfer was successfully done, the membrane was placed in a 1 ml Ponceau S solution (P7170-1L, SLS; Sigma).

For blocking, the membranes were incubated for 60 minutes in 5% non-fat dry milk (Marvel dried skimmed milk, UK). Blocking solution was prepared by adding 2.5mg of milk to 50ml washing solution (Phosphate Buffered Saline (PBS; Sigma) with 0.1% Tween-20 (Sigma, P 2287, Polyethylene glycol sorbitan monolaurate, Polyoxyethylene sorbitan monolaurate, Polysorbate 20). Then membranes were washed three times with the washing solution and then incubated with the primary antibody overnight at 4°C using the optimal concentration as instructed by the manufacturer (**Table 2-1**). The next day, membranes were washed with a washing solution for 10 minutes three times. The membranes were incubated with the secondary antibody in a dark room. A secondary antibody was used (anti-mouse or anti-rabbit, Licor Biosciences). Then membranes were washed three times with the washing solution. The membrane was visualised using Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA) using Enhanced Chemiluminescence (ECL) solution, 1705061, (Bio-Rad). Mouse β -Actin (A5441, Sigma-Aldrich; Clone AC-15; Sigma, UK) or Rabbit β -Actin (SAB5600204, Sigma-Aldrich; Clone RM112; Sigma, UK) were used as a house-keeping protein at a concentration of 1:5000 and 1:15000, respectively.

Table 2-1: The selected antibodies for the project

Antibody	Molecular Weight	Concentration	Supplier
LOX	47	1:1000	Thermo Fisher Scientific
HIF-1 α	95	1:500	Thermo Fisher Scientific
Collagen XV	140.7	1:500	Affinity Biosciences
Collagen XIX	115	1:500	Affinity Biosciences
Mouse β -Actin	42	1: 5000	Sigma-Aldrich
Rabbit β -Actin	42	1: 15000	Sigma-Aldrich

2.5.3 Optimisation of antibodies by IHC

For each antibody, staining conditions optimisation was performed by altering some variables in the staining protocol until the optimal results were obtained, which was considered as the specific subcellular antibody's localisation with no background staining.

These variables included; 1- the dilution of the antibody, starting with the recommended dilution by the manufacturer and dilutions above and below, 2- antigen retrieval solution: microwave pre-treatment in citrate buffer at pH 6.0, or water bath pre-treatment in EDTA buffer (AR9640, Bond™ Epitope Retrieval Solution 2), pH 9.0, 3- the incubation period started with the duration recommended by the manufacturer, then change the period regarding the obtained staining results ranged from 30 minutes to overnight incubation. For the optimisation of antibodies, the slides were stained using Sequenza trays (Thermo Scientific). As discussed in 6.2.3 and 6.2.4.

For the specificity testing of an antibody, cells should express immunoreactivity within the specific cellular location (nuclear, membranous, or cytoplasmic) with no or minimal background staining, which allows the most accurate assessment of protein expression status. All staining procedures including optimisation were done by the author except ER, PR and KI67 staining in core biopsies, which were conducted by the Histopathology Department, Queen's Medical Centre,

Nottingham. Ki67 staining was performed by the department, for Pathlake project, and scored with my colleague (Ayat Lashen).

2.6 Immunohistochemistry (IHC) technique

IHC was conducted on 4µm thick, full-face tissue or TMA sections mounted on permanent positive charged glass slides (Surgipath, X-tra™ Adhesive, Peterborough, UK). For the antibodies studied, sections were finally stained using Novolink™ Polymer Detection System (RE7150-K). To affirm tissue section adhesion with the slide surface, sections were placed on a hot plate (60°C) for 20 minutes and then left to cool. Slides were dewaxed in xylene (Genta Medica, York, UK) twice for 5 minutes, and rehydrated twice in industrial methylated spirit (IMS) or methanol (2 minutes/each) followed by 5 minutes in running tap water.

Heat-induced retrieval of antigen epitopes was performed, using Whirlpool JT359 microwave (1000 W) treatment of the slides in 1 litre of sodium citrate buffer (pH 6.0), using full power for 20 minutes. Then, slides were cooled for 10 minutes under tap water and were rinsed with tris buffered saline (TBS) (0.05 mol/L Tris HCL, 0.15 mol/L NaCl; pH 7.6) twice, each for 5 minutes. Endogenous peroxidase activity was blocked by peroxidase block (RE7101, 3-4% hydrogen peroxide) for 5 minutes. Slides were incubated in protein block (RE7102, 0.4% casein in phosphate-buffered saline, with stabilisers, surfactant, and 0.2% Bronidox L as a preservative) for 5 minutes and primary antibodies, optimally diluted using Leica antibody diluent (AR9352). The optically diluted primary antibody was added to each slide. Each of these steps was followed by 3×5 minutes of washing with 0.1% TBS-Tween 20. Post-primary block (RE7111, rabbit anti-mouse IgG (<10µg/mL) in 10% animal serum in TBS/0.09% ProClin™ 950) was incubated for 30 minutes followed by 5 minutes washing three times with TBS-Tween 20 0.1% and then Novolink Polymer (RE7112, anti-rabbit Poly-HRP-IgG (<25µg/mL) containing 10% animal bovine serum in TBS/0.09% ProClin™ 950) was added for 30 minutes. After washing sections with TBS, the peroxidase reaction was visualised using a freshly prepared filtered solution of 3,3'-

Diaminobenzidine (DAB) working solution (DAB chromogen, RE7105, 1.74% w/v 3,3'-diaminobenzidine, in a stabiliser solution) and DAB substrate buffer (RE7143, buffered solution containing $\leq 0.1\%$ hydrogen peroxide and preservative) of a ratio 1:20 was added for 5 minutes and then followed by 5 minutes of washing three times. Counterstaining with Meyer's haematoxylin (RE7107, $<0.1\%$ haematoxylin) for 6 minutes was done. The slides were dehydrated with alcohol and cleared with xylene (Genta Medica, York, UK). Finally, slides were mounted with distyrene, a plasticiser, and xylene. Tissue sections used as negative and positive controls were included in the staining of each antibody, as discussed in 6.2.3 and 6.2.4, **Table 2-2**.

Table 2-2: The optimal conditions of the study's antibodies.

Antibody	Supplier	Species	Incubation time	Dilution	Positive control	Negative control
Collagen XV	Affinity Biosciences	Rabbit polyclonal	90 minutes	1:100	Placenta	Tonsil
Collagen XIX	Affinity Biosciences	Rabbit polyclonal	90 minutes	1:100	Testicular tissue	Skin
LOX	Thermo Fisher Scientific, UK	Rabbit polyclonal	30 minutes	1:200	Skin	Thyroid
HIF-1 α	Thermo Fisher Scientific, UK	Mouse Monoclonal	60 minutes	1:50	Tonsil tissue	Lung

2.6.1 Assessment of immunoreactivity

Prior to the application of each primary antibody to the TMA using IHC, staining of randomly chosen 14 slides of full-face tumour sections was done from FFPE tissues with each primary antibody to assess immunoreactivity homogeneity in terms of staining distribution and intensity. The staining distribution across the tissue was ensured followed by IHC staining to TMA sections or the full-face sections according to the study.

High-resolution digital images ($0.45\mu\text{m}/\text{pixel}$) of stained slides were obtained using a Panoramic digital slide scanner (250 Flash III, 3DHISTECH®, Budapest, Hungary), at 20x magnification and accessed

using Xplore viewing software viewer (Philips) or by direct visualisation of the digital slides using Pannoramic Viewer software. Immunoreactivity was manually assessed at 20x magnification.

Each core in the TMA was assessed to determine whether the invasive tumour occupied >20% of the total core area for scoring eligibility. Assessment of staining was performed by assessing the positivity with a cutoff>1% then the percentage of protein expression was estimated. In addition, the semiquantitative approach Histo-score (H-score) was completed taking into consideration the intensity (0=negative, 1=weak, 2=moderate and 3=strong staining) and the percentage of stained tumour cells (0-100%) was also calculated to obtain the final H-score with a range from 0-300 (424). All cases were scored blindly without previous knowledge of patients' clinicopathological or outcomes data, 6.2.5 and 6.2.6.

2.6.2 Determination of the cutoff

For the dichotomisation of the studied biomarkers into low and high expression, various cutoffs were used. Cutoffs were individually measured by numerous methods using: 1) cutoffs from previous studies that assessed large data, 2) the mean or median according to data distribution whether normally or not and, 3) a non-commercial research tool; X-tile software (version 3.6.1, 2003-2005, Yale University, USA <http://x-tile.software.informer.com>). This software randomly divided the cohort into two equal distinct sets; training and validation by producing separate lists of 'censored' and 'uncensored' observations, ranked by patients' follow-up time (425). The patients who developed distant metastasis or died were considered as an event (uncensored), while patients who did not have metastasis or lost follow-up during this study were considered censored during the analysis at the time of that event. The optimal cutoff was determined by the brightest pixel located on the X-tile plot diagram of the training set (426). Statistical significance was tested by applying the obtained cutoff to the validation set, 5.2.3, 6.2.7, and 7.2.5.

2.7 Statistical approach

To test the significance of the results, many statistical approaches were used as required by the data set, and as specified for each set of analysis. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 27 (SPSS Inc., Chicago, IL, USA) and Graph Pad Prism. For all statistical tests, the level of significance was set as $p < 0.05$ (two-tailed).

3 Chapter 3: Characterisation of basement membrane associated collagen morphology in breast cancer

3.1 Introduction

BC is a heterogeneous disease with variable presentations, molecular profiles, morphologies, and clinical behaviours. Molecular and phenotypic alterations in the neoplastic cells and their behaviour usually attract more research attention. However, there is evidence that the stroma in BC plays an important role in the tumour (1). Stroma in the breast, like many other tissues, comprises stromal cells that ECM, and immune cells (258). The ECM is composed of two main structures, the interstitial matrix and the BM, that can vary in both structure and function (260). The BM is characterised by a complex structure comprising collagenous and non-collagenous proteoglycans and glycoproteins. The main constituents of the BM are collagen type IV, laminin, and heparan sulphate proteoglycan (209).

The BM is an amorphous, sheet-like structure which can be demonstrated with EM separating the epithelium from stroma (208). Microscopically, BMs are composed of two main layers, the reticular lamina and the basal lamina which is subdivided into the lamina densa and the lamina lucida. The lamina densa is known as the BM proper and is formed from densely packed fibrils embedded in a dense matrix, however lamina lucida is an electron-lucent zone that contacts the cells (210). The lamina densa consists of a network of collagen IV fibrils with proteoglycan perlecan while the lamina lucida consists of laminin, nidogens (entactin) and integrins. A bridge of anchoring filaments of collagen VII connects the lamina densa and lucida. The lamina fibroreticularis or reticular lamina is the outermost layer formed mainly of collagen type III (211-213).

BM is a physical barrier that separates the proliferating epithelial cells from the surrounding stroma and is used to distinguish malignant *in-situ* and invasive lesions in BC (427). It has been proven that the fundamental definition of invasive carcinoma is based on BM penetration with stromal growth, whereas benign and *in-situ* lesions show an intact layer of BM (229). Some *in-situ* carcinoma associated with micro invasion

show focal BM fragmentation, thinning, and at the site of the micro invasion but not elsewhere which may support the use of BMs to differentiate *in situ* from invasive tumours in the breast (231). However, exceptions exist for these basic generalities. Despite the complete absence of BM found in invasive BC (237), there is evidence that some invasive BCs can show variable retention of a BM structure at the epithelial stroma interface (428, 429) and some studies have described BM staining around clusters and groups of the metastatic invasive BC in the lymph nodes (430).

EPC, which is considered a variant of *in situ* shows low metastatic potential and is surrounded by a capsule which consists of BM-like material that mimics the BM, together with the indolent behaviour of EPC results in staging this tumour as an *in situ* lesion (233-235). Some special types of invasive BC, such as salivary gland-like tumours, show BM-like material despite their invasive nature. For example, ACC shows thickened BMs surrounding their neoplastic cells in comparison to normal, benign or *in situ* lesion BMs (239, 240).

Despite the knowledge gained to date, the data on the nature of BM materials associated with invasive tumour foci remains to be elucidated. Tumour-associated BM materials and structures are likely to be different from the native BMs present around breast tissue and benign and *in situ* lesions (431). It has also been reported that decreased deposition of BM in invasive BC is associated with decreased tumour differentiation and poor prognosis (209). However, tubular, lobular, and mucinous carcinomas, have a relatively good prognosis despite the complete absence of the BM (209).

Although the diagnostic use of BM is now being replaced by MEC IHC markers in routine pathology practice, some pathologists consider BM detection to be a specific finding, useful for distinguishing *in situ* from invasive lesions, especially in challenging cases (229). For example, the detection of BM in MGA is used to indicate its benign nature despite the lack of MEC, cell proliferation and infiltrative patterns (432). Therefore,

MEC markers do not help differentiate MGA from tubular or low-grade NST carcinomas with prominent tubule formation (244). Another challenging case is sclerosing adenosis, the BM has a key role in differentiating sclerosing adenosis from tubular carcinoma (237). Notably, lactational changes and apocrine metaplasia are two benign metaplastic breast changes that show a focal absence of MEC layers and have cytological atypia raising suspicion of being invasive carcinoma. Therefore, BM markers help to prove the benign nature of these lesions (244).

In practice, IHC is used to distinguish the BM components to differentiate between *in-situ* and invasive tumours in doubtful cases. However, distinguishing between the native BM and BM-like structures in invasive lesions still remains challenging (229).

PSR is a stain used for collagen detection, it is the most widely practical histochemical procedure for selectively visualizing and highlighting collagen fibres (433). When PSR is combined with polarized light microscopy it is sensitive to fibrillar birefringence. Collagen fibres, when hit by light, refract into two rays with different speeds and directions of vibration that orientate at 90° to each other. PSR itself is composed of elongated birefringent molecules that can bind to the collagen fibres in parallel positions to the fibres, thus improving the natural birefringence (433). PSR has a higher specificity for collagen fibre detection compared to other commonly used stains (such as Masson's trichrome, Movat's Penta chrome, and Van Gieson) and provides interference colour differences and birefringent intensity which can help distinguish collagen subtypes (434). Antibody labelling has also been used for collagen characterisation; however, this method presents complications due to the cross-reactivity of collagen types (435).

Collagen stained by PSR, when examined under polarized microscopy, detects thinner fibres (type III collagen) which are visualised as green to green-yellow colours, while thicker fibres (type I collagen) present as yellowish orange to orange-red colours (436).

3.1.1 Aims and hypothesis

In this chapter, it was hypothesised that differentiation of a native BM from a reactive BM surrounding an invasive lesion is clinically important for early diagnosis and proper management. Therefore, this study aimed to:

1. compare the BM between normal, *in-situ* and invasive breast tissue, to understand its role with cancerous cells and to determine the process of invasion that makes *in situ* act as an intermediate stage between normal tissue and invasive carcinoma.
2. Refine the definition of invasiveness by characterisation of the geometric (physical) features of collagen in BC and precursor lesions to develop a signature that potentially could differentiate between native BM and BM-like material.
3. Identify BM characteristics that could be used to distinguish *in-situ* from invasive disease .

3.2 Patient cohort and methods

3.2.1 Study cohort

This study was conducted on primary operable BCs presenting to Nottingham City Hospital, Nottingham, UK between 1999 and 2006. The study included 150 cases which were divided into six groups containing 25 patients per group. The six groups included normal breast tissue as a control group, DCIS, EPC, invasive carcinoma (INV), special types of invasive carcinoma (S.INV) and metastatic lymph node (LN). The special types of invasive carcinoma included ACC, invasive cribriform and salivary gland-like carcinoma as these meet the criteria of being invasive tumours and showed BM-like material surrounding the neoplastic cells. Regarding the invasive group that had no BM, the collagen fibres that surrounded the neoplastic cells were measured.

Cases of normal breast tissue were either from reduction mammoplasty or normal tissue adjacent to the tumour. Reduction mammoplasty tissue is regarded as the best representation of the normal breast tissue as the patient does not have cancer and the tissue removal is not targeted to a specific abnormality; however, some ducts showed histological abnormalities and proliferative changes with or without atypical hyperplasia were presented in tissue adjacent to tumours (437). This was excluded from the study.

DCIS was associated with invasive tumours (72%; 16 of the cases) while the remaining cases were pure DCIS.

DCIS cases presented as high grades in 54% of cases (n=12/25) and 46% were low to intermediate grades. DCISs were solid in 72% of the cases, and comedo necrosis was observed in 68% (n=17/25) of the cases. The adjacent invasive tumour was NST in 20 cases (80%; n=20/25), while the others were tubular.

The original tumours in the LN metastatic cases were NST and their sizes ranged from 2 cm to 4.5 cm with a median size of 2.5 cm. The tumours were grade III in 92% (n=22/25) of the cases and the remaining cases were grade II.

The cohort of papillary lesions consisted of 25 cases, 18 cases were pure EPC with 40% (10 cases) low grade and 32% (8 cases) were intermediate grade. Half of the cases were associated with DCIS (n=12/25). Of the remaining 7 cases (28%) of EPC associated with invasive tumours, all of them were intermediate grade except one case which was considered low grade.

The invasive cohort (n=50) age range was 33-75 years (median 54), with 33 cases older than 50 years, and the tumour size ranged from 0.2 cm to 4.3 cm (median 2 cm). Full detailed clinicopathological data were available, including patient age at diagnosis, primary tumour histological grade, tumour size, histological tumour type, NPI, and molecular subtypes (**Table 3-1**).

Table 3-1: Clinicopathological characteristics of the invasive cohort.

Parameter	Cohort N (%)
Patient Age	
≤ 50 years	17 (34%)
> 50 years	33 (66%)
Tumor grade	
Grade 1	30 (60%)
Grade 2	11 (20%)
Grade 3	9 (18%)
Lymph node stage	
Stage I	38 (76%)
Stage II	10 (20%)
Stage III	2 (4%)
Lymph vascular invasion (LVI)	
Definite	5 (10%)
Negative	45 (90%)
ER receptor status	
Positive	32 (64%)
Negative	18 (36%)
PR receptor status	
Positive	43 (86%)
Negative	7 (14%)
HER2 receptor status	
Positive	11 (22%)
Negative	39 (78%)
Histological type	
NST	18 (36%)
Lobular	3 (6%)
Mixed NST with other types	4 (8%)
Special type	25 (50%)
Nottingham Prognostic Index (NPI)	
Good prognosis	38 (76%)
Moderate prognosis	9 (18%)
Poor prognosis	3 (6%)

3.2.2 Tissue processing and staining

Freshly cut, 4µm thick, full-face sections for the cohort were stained with H&E and PSR, and one section per case from each tumour block was stained with each stain. Collagen fibres were assessed using tissue sections stained with PSR. A preparation of PSR solution was undertaken by mixing 200mg of Sirius Red (Direct red 80, lot#MKCJ4451, 365548, Sigma-Aldrich, Darmstadt, Germany) into

200ml of saturated aqueous solution of picric acid 2.1% (lot:// 7883, RRSP143-E, Atom-Scientific, Hyde, England).

Sections were deparaffinised then rehydrated and immersed in the picrosirius red solution for 60 min. After staining, the excess stain was removed by shaking the slide and then blotting it against filter paper. Slides were rinsed in acidified water twice and then dehydrated using three changes of 100% ethanol, the sections were then cleared using xylene and mounted with coverslips and DPX. A liver section was used as a control positive, when examined using light microscopy epithelial cells appeared in yellowish and the collagen fibres within the stroma were stained on a red colour spectrum.

3.2.3 Microscopy and imaging

H&E-stained slides were examined, via standard light microscopy with a 10× objective, for histopathological verification of the section before being stained with PSR, the glands were identified which served as a reference once the tissue sections had been stained with PSR.

The PSR-stained tissue was examined under the polarized microscope and visualised using accompanying software (Leica, IL 5000B, and LAS VERSION 3.8.0, Leica Microsystems, Switzerland) and a camera. Under polarized light, PSR-stained collagen type I presents within the red-orange-yellow range and type III as green, under light microscopy total collagen is visualised within the red spectrum and the two types cannot be differentiated. Tissue sections were split into four quadrants. From each quadrant a single gland was chosen, and systematic, non-overlapping, random areas were examined in a Z-shaped pattern, 4-6 photomicrographs were captured (24 photomicrographs per slide) for each gland to represent each gland during analysis. This method was identical between imaging sessions to ensure comparable quantification of images, as previously mentioned in other experiments (438). Each gland was further split into 4 quadrants, 2 regions of interest (ROIs; 75x75 pixels) were randomly selected per quadrant and 2 measurements were taken from each quadrant one measurement per ROI, (8

measurements per gland, 32 measurements per slide) **Figure 3-1** & **Figure 3-2**. Then the mean value of these measurements was calculated for each case. All photomicrographs were taken at 100x magnification.

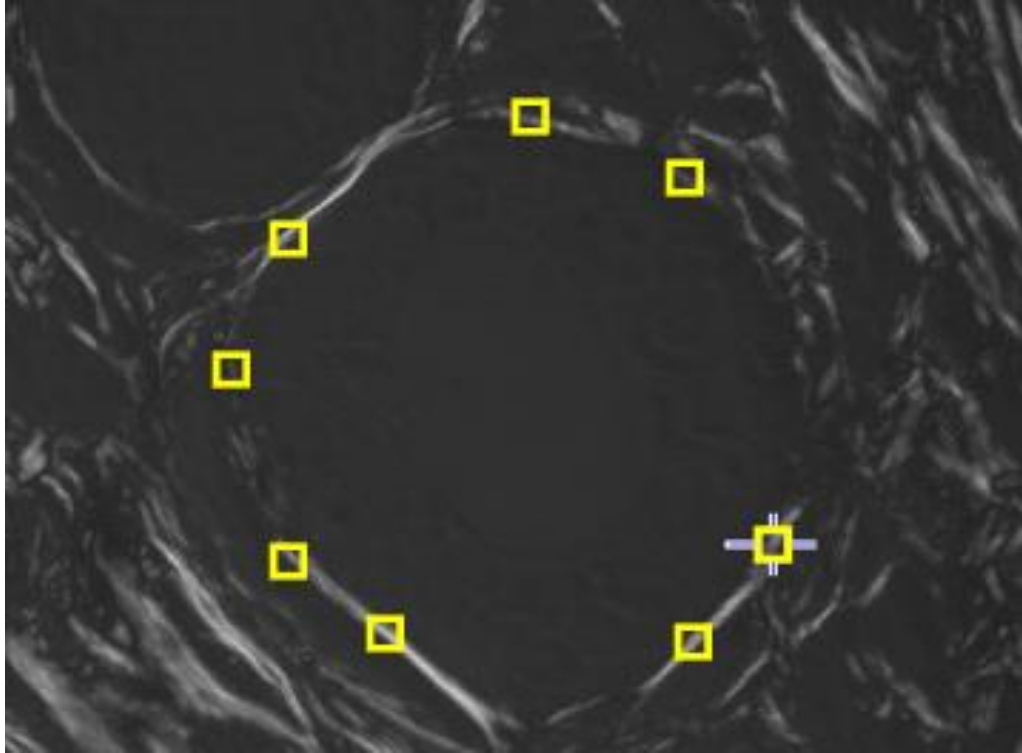


Figure 3-1: Normal gland BM on the grey scale in CT-Fire shows ROIS selection.

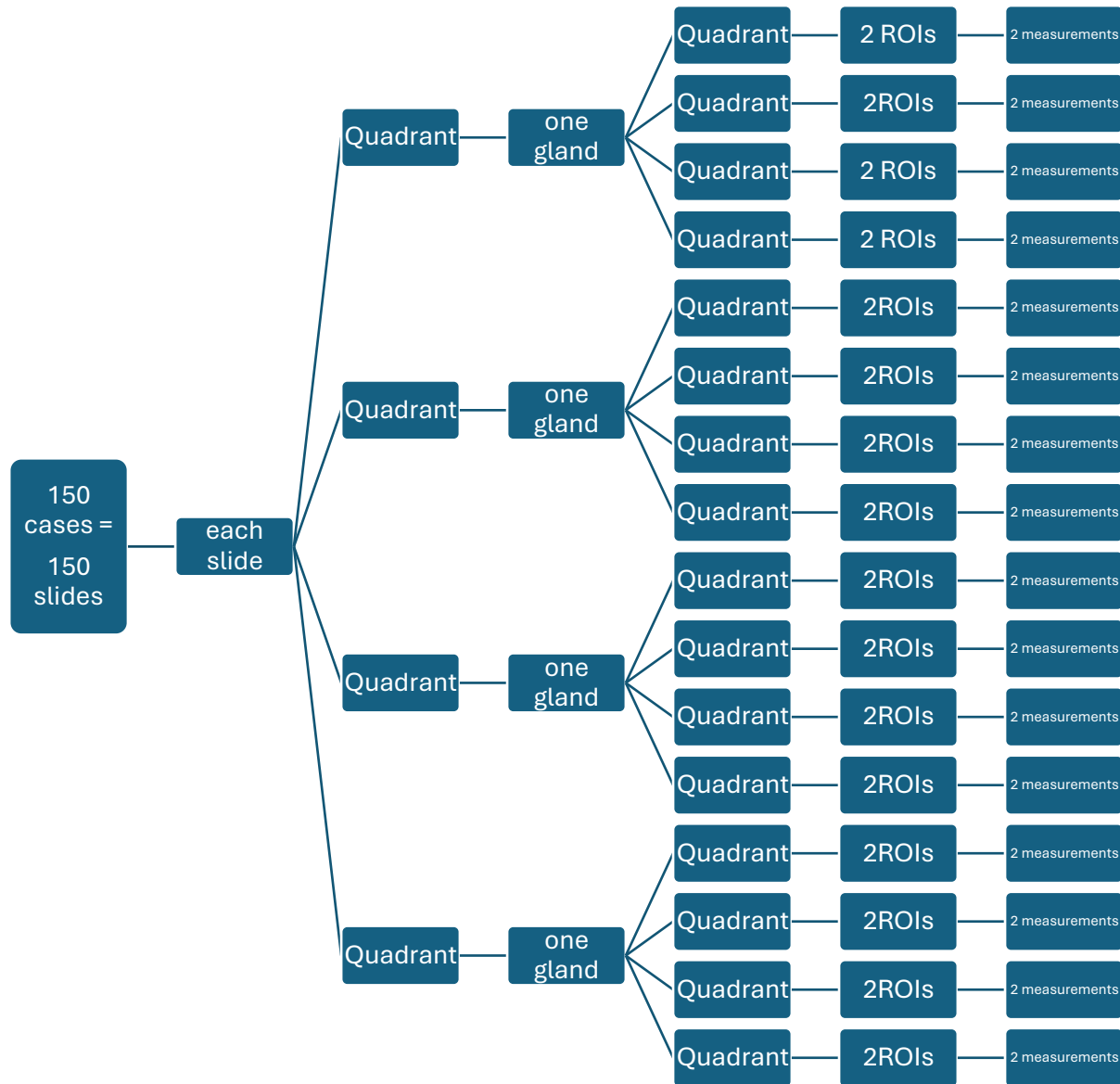


Figure 3-2: Systematic random sampling method.

3.2.4 Choosing the number of ROIS

It was essential to choose the number of ROIs that could effectively represent the whole BM measurements as this type of systematic random sampling has not been conducted for these variables on this tissue type before. It was also noted that variance between regions of the BMs existed in these tissues. Therefore, a preliminary study was undertaken to calculate the number of regions required.

BM measurements were taken for a whole control cohort in one gland for orientation angle, alignment, fibre width, fibre length, fibre density and

fibre straightness. Then differing numbers of regions were analysed: 4 measurements (one from each quadrant), 6 measurements (3 from each half) and 8 measurements (2 from each quadrant) in every measurement throughout the BM for the entire gland, in the normal breast tissue cohort. Comparing the average of the whole BM measurements with the average of each of the systematic random sampling methods (4, 6 and 8 measurements) was undertaken using an independent t-test.

There was a significant difference between the whole BM and the average of four and six measurements while there was no significant difference observed with the eight measurements.

3.2.5 Comparison between whole BM and 4 measurements

There was a significant difference in fibre alignment (t-test; $t = 9.7$; $p < 0.0001$), fibre width (t-test; $t = 4.9$; $p < 0.0001$) and fibre density (t-test; $t = 6.4$; $p < 0.0001$) between whole BM and four measurements. There was no significant difference in each orientation angle (t-test; $t = 0.84$; $p < 0.4$), fibre length (t-test; $t = 0.69$; $p < 0.34$) and straightness (t-test; $t = 1.5$; $p < 0.13$) between whole BM scores and the four measurements.

3.2.6 Comparison between whole BM and 6 measurements

There was a significant difference in fibre alignment (t-test; $t = 0.97$; $p < 0.03$) and density (t-test; $t = 6.5$; $p < 0.001$) between the whole BM and six measurements. There was no significant difference in each orientation angle (t-test; $t = 0.30$; $p < 0.76$), fibre width (t-test; $t = 0.49$; $p < 0.62$), fibre length (t-test; $t = -0.59$; $p < 0.55$) fibre straightness (t-test; $t = -0.9$; $p < 0.37$) between whole BM and six measurements.

3.2.7 Comparison between whole BM and 8 measurements

There was no significant difference in each orientation angle (t-test; $t = 0.18$; $p < 0.85$), alignment (t-test; $t = -0.14$; $p < 0.88$), fibres width (t-test;

$t = -0.19$; $p < 0.84$), fibres length (t-test; $t = -0.84$; $p < 0.4$), straightness (t-test; $t = -0.98$; $p < 0.22$), and density (t-test; $t = -0.76$; $p < 0.45$) between whole BM and eight measurements. This indicated that eight measurements were an appropriate systematic random sampling method to continue with.

3.2.8 Image analysis and quantification of collagen

Assessment of collagen distribution was undertaken using Image j, (NIH, Bethesda, Maryland), which is available for free download at (<https://imagej.nih.gov/ij/download.html>).

3.2.8.1 Proportion of collagen I and collagen III fibres

The collagen fibres were distinguished as either thin or thick fibres using the polarized light, thin fibres were green-yellow and thick fibres appeared orange-red (439). An Image J Macro plugin Recorder was used to automatize channel separation for red, green and background colours with quantification of the pixel area for each feature (ImageJ, NIH, Bethesda, Maryland). Using the raw data output, the proportions and mean values of thin and thick collagen fibres were calculated for each case.

3.2.8.2 Collagen density

Collagen fibre density (the concentration of collagen fibres in a given area). Assessment of the collagen density via Image J (NIH, Bethesda, Maryland) was split into RGB channels (Red, Blue, Green), the blue and red images were then excluded. The green image was adjusted into a binary image with foreground pixels which were red designated as intensity 0 and the background expressed as white pixels (intensity 255). All images were adjusted to a threshold of 150 to decrease noise. Integrated density was measured using the automated tools in Image J. The mean values for each case were calculated (**Figure 3-3**)

3.2.8.3 Collagen length, straightness, and width

Assessment of collagen fibre features using CT-FIRE (ctFIRE_V2.0Beta_WIN64_MCR2014b.exe) to extract individual fibres and to quantify the following:

- Mean length of fibres: the total end-to-end length of the fibre.
- Mean straightness of fibres - fibre curvature degree.
- Mean width of the fibres.

Finally, all the values for each of the fibres, from each of the images, for each tumour, were averaged to determine one value for each case (**Figure 3-3**).

3.2.8.4 Collagen fibre alignment and orientation

Assessment of collagen fibre features using Curve Align (CurveAlign_V4.0Beta_WIN64_MCR2014b.exe) was used to extract individual fibres and then quantify the following measures:

- The alignment coefficients range from 0-1 for each fibre, with 0 used to determine random alignment and 1 assigned as perfect alignment (alignment of fibres to each other).
- Mean orientation angle (expressed as a degree unit) was calculated and defined as the alignment of each fibre to a horizontal line resembling the duct border, with angles ranging from 0 to 180° (**Figure 3-3**).

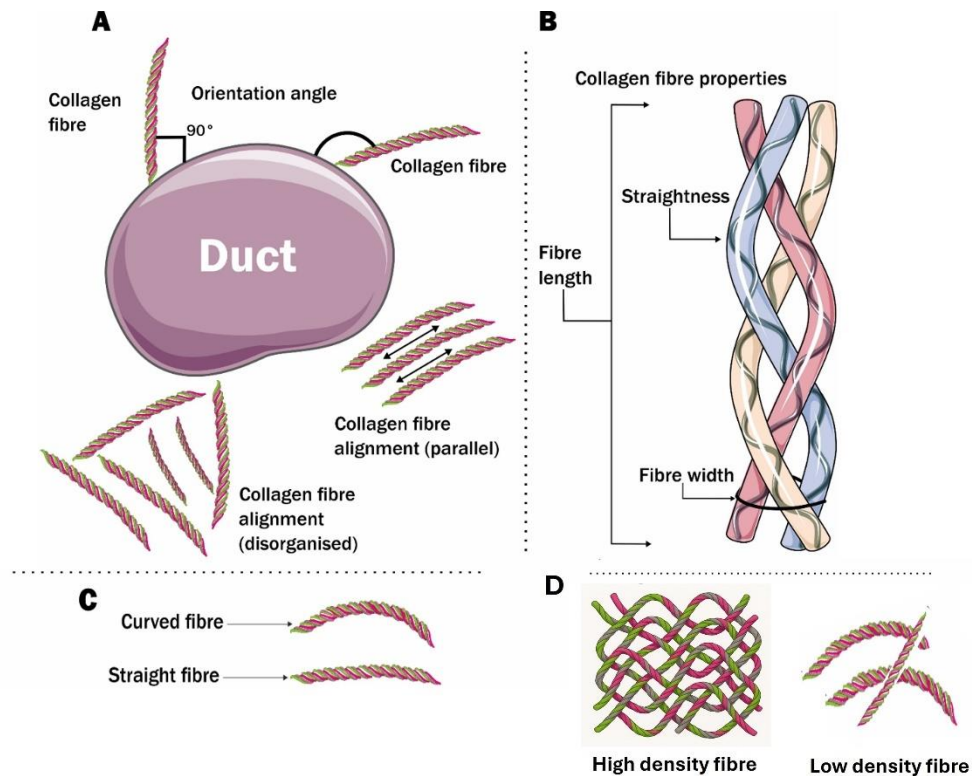


Figure 3-3: Schematic depicting collagen parameter definitions and measurements.

A) Describes the orientation angle which is the angle between collagen fibres and duct border which is perpendicular in right angle (90 degrees) or parallel, in addition, it defines the coefficient alignment as the direction of the fibres to each other either good alignment as parallel to each other or disorganised. **B)** Graphic describes the characteristics of individual fibres which are formed of three chains including the fibre length which is the end-to-end measurement, fibre width which is the fibre diameter and straightness which is the curvature degree of the fibre. **C)** Graphic describes the fibre straightness and curved fibre. **D)** Graphic shows high and low density fibres.

3.2.9 Quantification, graphical and statistical analysis

Excel was used to collate all the data. For all statistical analyses, $p < 0.05$ was considered statistically significant and undertaken either in SPSS v27 or GraphPad Prism. One-way ANOVA with post hoc tests and chi-square tests were used according to each data set. Graphs and tables were constructed as applicable using GraphPad Prism. Results are expressed as the mean $\pm$ standard deviation (SD) of the mean throughout unless otherwise stated.

3.3 Results

3.3.1 Collagen fibre density

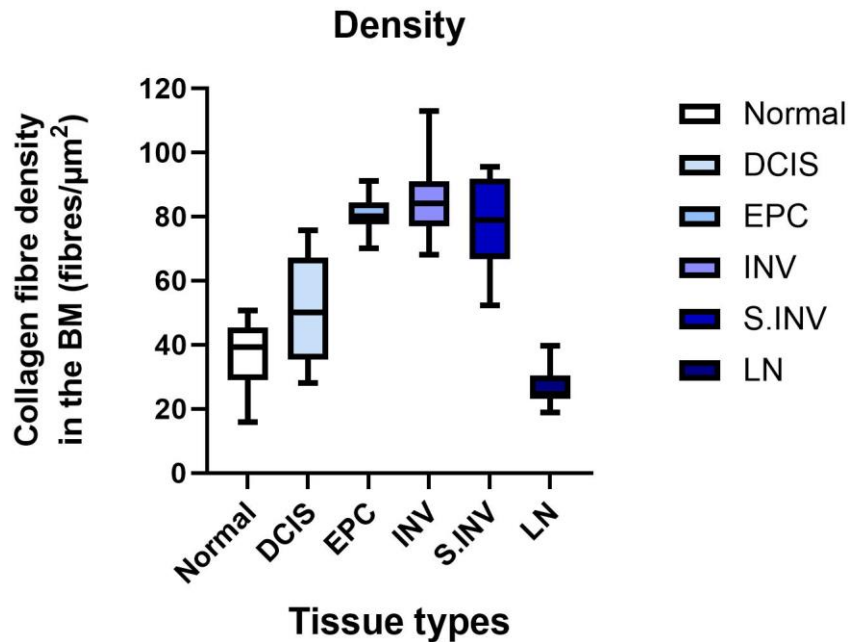


Figure 3-4: Mean density of collagen fibres in the basement membrane in breast and metastatic lymph node tissue.

In the comparisons between the normal breast tissue control group (36.86 ± 10.12 fibres/ μm^2 ; **Figure 3-4**) and the other groups, there was a statistically significant higher collagen fibre density within the BM of the DCIS (51.88 ± 16.04 fibres/ μm^2 ; $p < 0.001$), EPC (80.85 ± 5.24 fibres/ μm^2 ; $p < 0.001$), invasive group (84.69 ± 9.64 fibres/ μm^2 ; $p < 0.001$) and special types invasive carcinoma (77.8 ± 12.92 fibres/ μm^2 ; $p < 0.001$). On the other hand, there was lower collagen fibre density in the BM-like material of the metastatic lymph node group (26.61 ± 4.8 fibres/ μm^2 ; $p < 0.001$).

There was a significant decrease in density between of DCIS (51.88 ± 16.04 fibres/ μm^2) and EPC (80.85 ± 5.24 fibres/ μm^2 ; $p < 0.001$), invasive group (84.69 ± 9.64 fibres/ μm^2 ; $p < 0.001$) and special type invasive carcinoma (77.8 ± 12.92 fibres/ μm^2 ; $p < 0.001$). However, there was a significant increase in the fibre density of DCIS compared to the

BM-like material of the metastatic lymph node group (26.61 ± 4.8 fibres/ μm^2 ; $p < 0.001$).

EPC capsule (80.85 ± 5.24 fibres/ μm^2) showed a significant increase in the fibre density compared to both special types (77.8 ± 12.92 fibres/ μm^2 ; $p < 0.001$), collagen fibres surrounding the invasive tumour (84.69 ± 9.64 fibres/ μm^2 ; $p < 0.001$) and metastatic lymph node group (26.61 ± 4.8 fibres/ μm^2 ; $p < 0.001$).

Notably, there was an increase in the fibre density of collagen fibres surrounding the invasive group (84.69 ± 9.64 fibres/ μm^2) and both the BM-like material of special types of carcinoma (77.8 ± 12.92 fibres/ μm^2 ; $p < 0.001$) compared to the metastatic lymph node group (26.61 ± 4.8 fibres/ μm^2 ; $p < 0.001$). However, there was no significant difference between both invasive groups. One-way ANOVA test $F = 140.13$, $p < 0.001$; (**Table 3-2 and Figure 3-4**)

Table 3-2: Comparisons of mean fibre density in the basement membrane in breast and metastatic lymph node tissue

Group	Mean Density	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = $p < 0.05$)
Normal	36.86	10.12	2.02	DCIS	0.001*
				EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001
DCIS	51.88	16.04	3.20	EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001*
EPC	80.85	5.24	1.05	Invasive	0.001
				Special invasive	0.001
				Metastatic LN	0.001*
Invasive	84.69	9.64	1.92	Special invasive	0.35
				Metastatic LN	0.001*
Special invasive	77.8	12.92	2.58	Metastatic LN	0.001*
Metastatic lymph node	26.61	4.8	0.97	-	-

3.3.2 Collagen fibre distribution

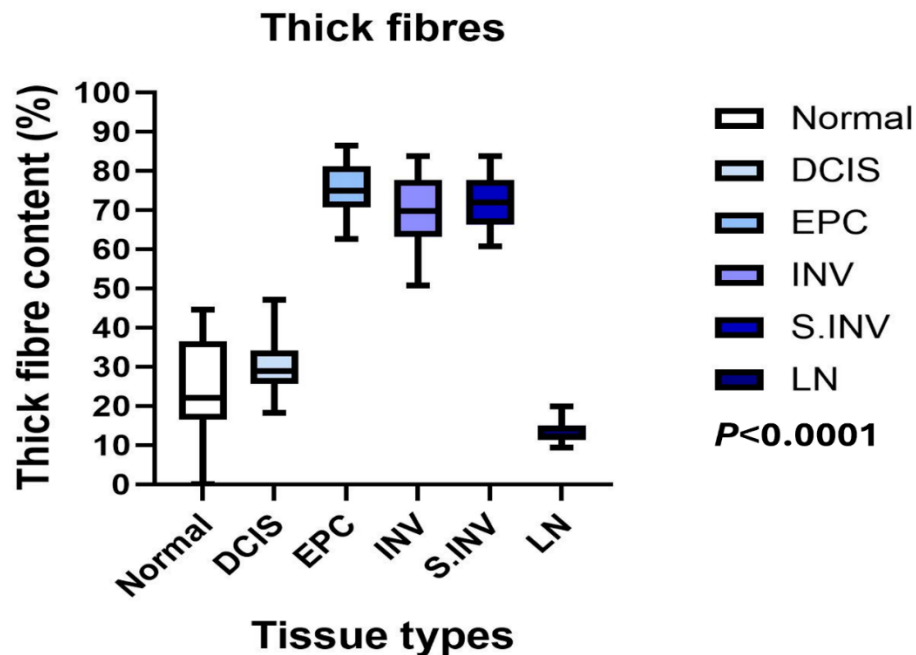


Figure 3-5: Percentage of thick fibre content in the basement membrane in breast and metastatic lymph node tissue.

According to the measurements taken for thick fibre content, in the comparisons between the normal breast tissue control group (25.25 ± 12.27 %; **Figure 3-5**) and the other groups. There was a statistically significant increase in the percentage of thick fibres within the EPC capsule (75.46 ± 7.15 %; $p < 0.0001$), the collagen fibres surrounding the invasive group (69.52 ± 9.88 %; $p < 0.0001$), and the BM-like material of special types (71.92 ± 7.05 %; $p < 0.0001$). However, there was a significant decrease in the thick fibre content in the metastatic lymph node group (13.21 ± 2.33 %; $p < 0.001$) compared to normal breast BMs. On the other hand, there was no significant difference in thick fibre content within BMs in the DCIS group (29.86 ± 6.81 %; $p < 0.51$) compared to the normal group.

There was a significant decrease in the thick fibre content in the DCIS group (29.86 ± 6.81 %) compared to the other groups except for the

metastatic lymph node group where DCIS BMs showed a significant increase in thick fibre content ($p < 0.0001$).

Interestingly, there were no significant differences in the thick fibre content between EPC, and the special invasive group ($p < 0.388$). Similarly, there was no significant difference between the two invasive groups ($p < 0.9$). However, there was an increase in thick fibre content in the EPC ($p < 0.01$) compared to the invasive group.

However, all these groups showed a significantly higher thick fibre content compared to the metastatic lymph node group ($p < 0.0001$). One-way ANOVA, $F = 409.18$, $p < 0.0001$, **Table 3-3**, **Figure 3-6** & **Figure 3-7**.

Table 3-3: The comparison of mean thick fibre content in the basement membrane in breast and metastatic lymph node tissue

Group	Mean thick fibres	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P value</i> (*= <i>significance</i> = $p < 0.05$)
Normal	25.25	12.27	2.45	DCIS	0.514
				EPC	0.0001*
				Invasive	0.0001*
				Special invasive	0.0001*
				Metastatic LN	0.0001*
DCIS	29.86	6.81	1.26	EPC	0.0001*
				Invasive	0.0001*
				Special invasive	0.0001*
				Metastatic LN	0.0001*
EPC	75.46	7.15	1.43	Invasive	0.01*
				Special invasive	0.388
				Metastatic LN	0.0001*
Invasive	69.52	9.88	1.97	Special invasive	0.9
				Metastatic LN	0.0001*
Special invasive	71.92	7.05	1.41	Metastatic LN	0.0001*
Metastatic lymph node	13.21	2.33	0.4	-	-

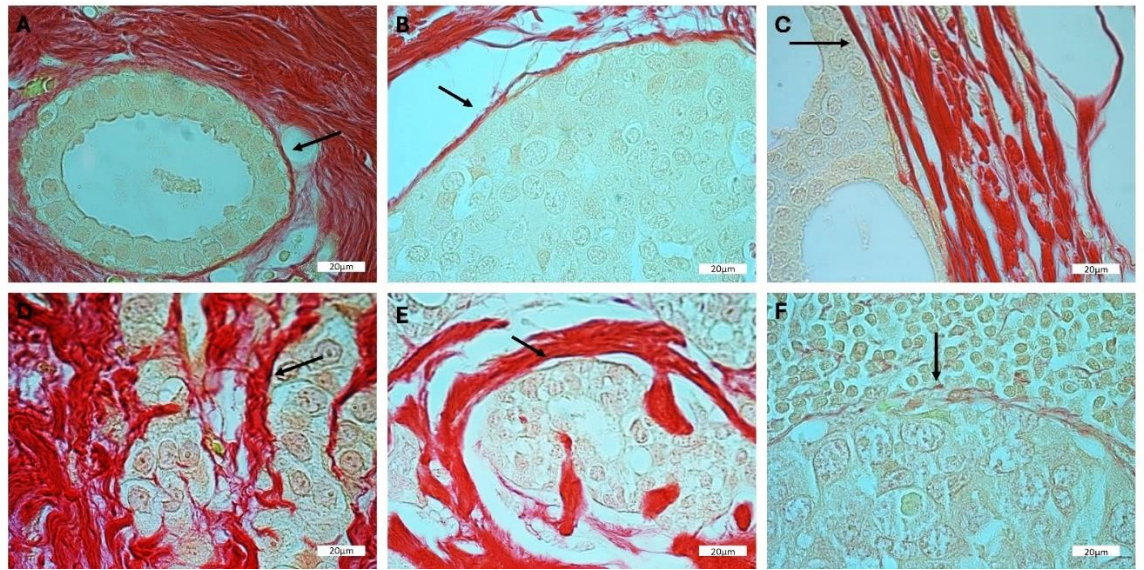


Figure 3-6: Photomicrographs of Picrosirius red stained tissue sections.

A) Normal breast tissue depicting a normal gland lined with simple cubical epithelium surrounded by a well-defined basement membrane (BM). **B)** DCIS depicting a part of a breast gland with cell proliferation surrounded by a definite BM. **C)** EPC tissue with part of the capsule that surrounds the distended gland. **D)** INV, invasive carcinoma tissue with clusters of tumour cells separated with stroma. **E)** S.INV, a special invasive type (adenoid cystic carcinoma) with tumour cells expressing a pseudo lumen containing eosinophilic BM material surrounded by BM-like material. **F)** LN, metastatic lymph node with large circular tumour cells separated by red stained BM-like material from the lymphocytes (yellow in colour). Magnification x100, scale bar 20µm.

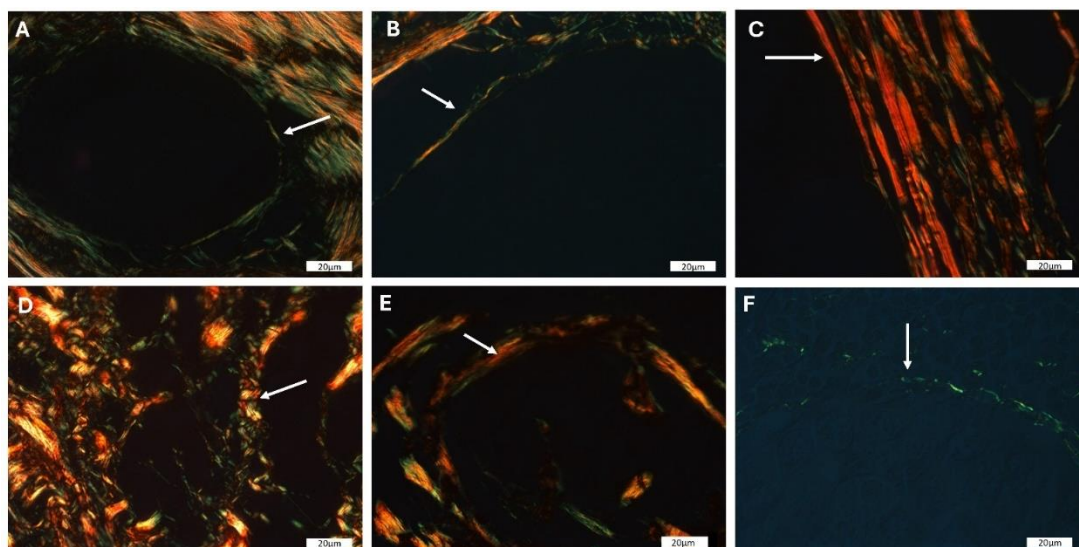


Figure 3-7: PSR-stained sections examined under a polarised microscope x100, scale bar 20µm.

A) The section of normal breast tissue shows green collagen fibres in the BM surrounding the gland that appear as black space. **B)** Section of DCIS shows part of BM with yellow to orange collagen fibres surrounding the gland which appear as black space. **C)** Section of EPC shows part of the capsule which appears as orange to red wavy collagen fibres. **D)** Section of INV invasive carcinoma shows the stroma separated the tumour cells as ranged from greenish to yellowish orange collagen fibres. **E)** Section of S.INV special invasive tumour (adenoid cystic carcinoma) shows the BM-like material which appears as a variation from green to orangish red fibres surrounding the cells which appear as black space interrupted with red to orange columns that represent the pseudocysts of BM-like material. **F)** Section of LN metastatic lymph nodes shows a green line of BM-like material that separates the tumour and lymphocyte cells which appear as black space, scale bar 20µm.

Conversely for thin fibre content, in the comparisons between the normal breast tissue control group (80.30 ± 12.27 %; **Figure 3-8**) and the other groups, there was a statistically significant decrease in the percentage of thin fibres within the EPC capsule (24.48 ± 7.15 %; $p < 0.0001$), the collagen fibre surrounding the invasive group (30.49 ± 9.88 %; $p < 0.0001$), BM-like material of special types (28.08 ± 7.05 %; $p < 0.0001$). However, there was a significant increase in thin fibre content in the metastatic lymph node group (87.56 ± 2.02 %; $p < 0.0001$). On the other hand, there was no significant difference in thin fibre content within BMs in the DCIS group (78.86 ± 7.88 %; $p < 0.12$) compared to the normal group.

There was a significant increase in the thin fibre content in the DCIS group (78.86 ± 7.88 %) compared to the other groups except for the metastatic lymph node group where there was a significant decrease in thin fibres in DCIS BMs ($p < 0.0001$).

Interestingly, there was a significant increase in the thin fibre content between EPC, and invasive ($p<0.01$). However, there was no significant difference in thin fibre content between both EPC and the special invasive group ($p<0.367$), similarly, there was no significant difference between both invasive groups ($p<0.09$). However, all these groups showed a significantly higher thick fibre content compared to the metastatic lymph node group ($p<0.0001$). One-way ANOVA, $F= 487.34$, $p<0.0001$, Table 3-4, Figure 3-6 & Figure 3-7).

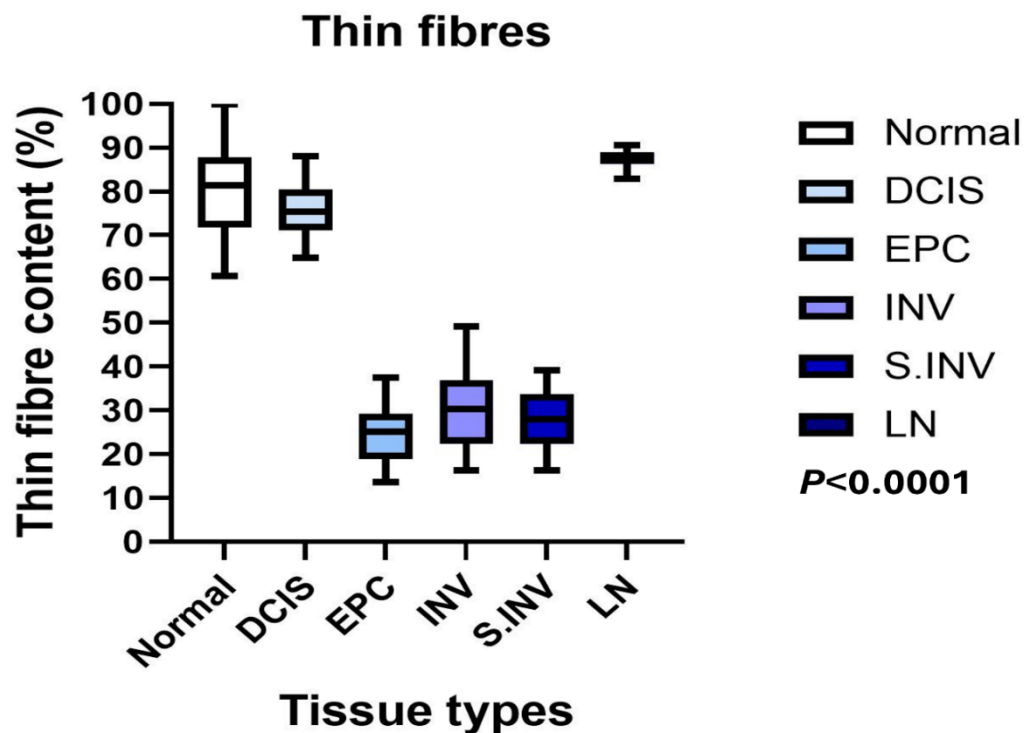
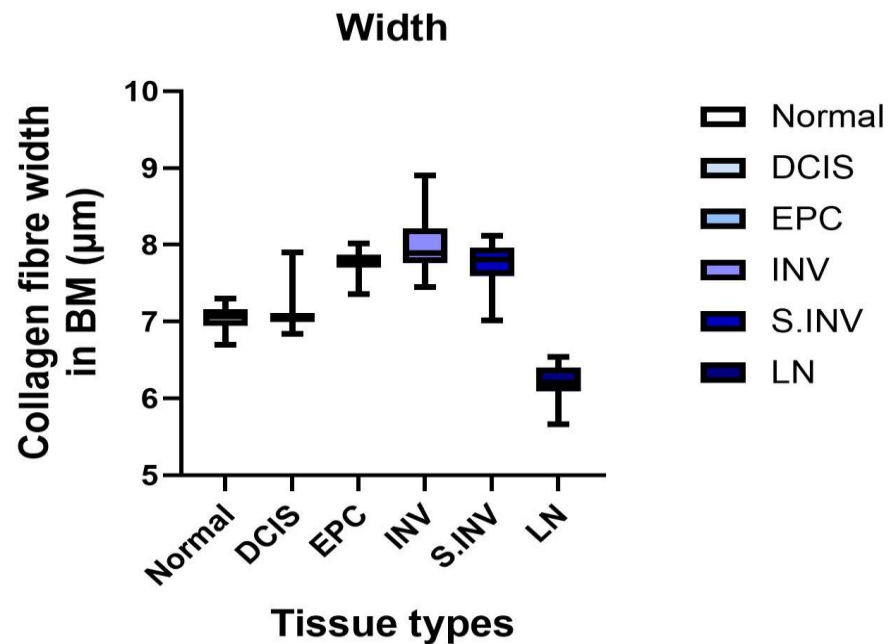


Figure 3-8: Percentage of thin fibre content in the basement membrane in breast and metastatic lymph node tissue.

Table 3-4: Comparisons of mean thin fibre content in the basement membranes in breast and metastatic lymph node tissue

Group	Mean thin fibres	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	80.30	11.08	2.421	DCIS	0.128
				EPC	0.0001*
				Invasive	0.0001*
				Special invasive	0.0001*
				Metastatic LN	0.0001*
DCIS	78.86	7.88	1.55	EPC	0.0001*
				Invasive	0.0001*
				Special invasive	0.0001*
				Metastatic LN	0.0001*
EPC	24.48	7.15	1.43	Invasive	0.01*
				Special invasive	0.367
				Metastatic LN	0.0001*
Invasive	30.48	9.88	1.97	Special invasive	0.09
				Metastatic LN	0.0001*
Special invasive	28.08	7.05	1.41	Metastatic LN	0.0001*
Metastatic lymph node	87.56	2.02	0.40	-	-

3.3.3 Collagen fibre width within the BM

**Figure 3-9:** Mean width of collagen fibre in the basement membrane in breast and metastatic lymph node tissue.

In comparison to the normal breast tissue control group, ($7.07 \pm 0.15 \mu\text{m}$; **Figure 3-9**) with other groups, there was a statistically significant increase in collagen fibre width in the BMs of EPC cases ($7.77 \pm 0.15 \mu\text{m}$; $p < 0.001$), collagen fibres surrounding invasive carcinoma ($7.98 \pm 0.36 \mu\text{m}$; $p < 0.001$), and BM-like material of special types of BC ($7.75 \pm 0.27 \mu\text{m}$; $p < 0.001$). On the other hand, compared to the normal group there was decreased fibre width in the metastatic lymph node group ($6.23 \pm 0.11 \mu\text{m}$; $p < 0.02$). In contrast, there was no difference between both DCIS ($7.07 \pm 0.20 \mu\text{m}$) and normal breast tissue BMs ($p < 1$).

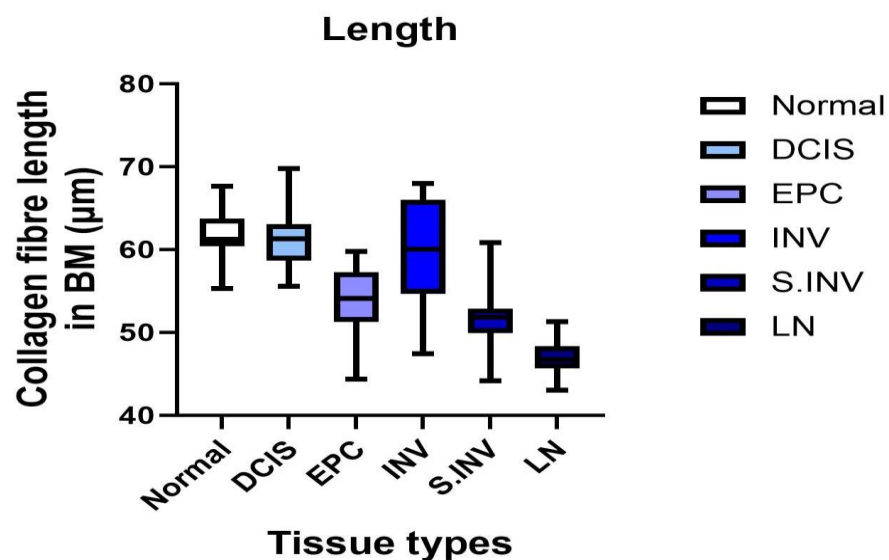
There was a significant decrease in the fibre width in DCIS compared to all of the other groups except the metastatic lymph node group ($6.23 \pm 0.11 \mu\text{m}$; $p < 0.001$), which showed a decrease in the fibre width compared to DCIS's BM.

In addition, collagen fibres surrounding invasive carcinoma ($7.98 \pm 0.36 \mu\text{m}$) showed a significant increase in fibre width compared to both the EPC ($7.77 \pm 0.15 \mu\text{m}$; $p < 0.001$) and special types of groups ($7.75 \pm 0.27 \mu\text{m}$; $p < 0.001$). There was no significant difference between the EPC and special type group ($p < 1$), however, both of them showed significantly wider fibres than the metastatic lymph node group ($p < 0.001$). One-way ANOVA test, $F = 189.37$, $p < 0.001$ (**Table 3-5**).

Table 3-5: Comparisons of mean fibre widths in the basement membrane in breast and metastatic lymph node tissue

Group	Mean Density	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	7.074	0.15	0.30	DCIS	1
				EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.02*
DCIS	7.075	0.20	0.039	EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001*
EPC	7.77	0.15	0.03	Invasive	0.001*
				Special invasive	1
				Metastatic LN	0.001*
Invasive	7.98	0.36	0.07	Special invasive	0.001*
				Metastatic LN	0.001*
Special invasive	7.75	0.27	0.05	Metastatic LN	0.001*
Metastatic lymph node	6.23	0.21	0.04	-	-

3.3.4 Collagen fibre length within the BM

**Figure 3-10:** Mean length of collagen fibres in the basement membranes in breast and metastatic lymph node tissue

When the normal breast tissue control group ($61.66 \pm 2.78 \mu\text{m}$; **Figure 3-10**) was compared to the other groups, there was a statistically significant decline in collagen fibre length within the EPC ($56.63 \pm 0.54 \mu\text{m}$; $p < 0.001$), invasive group ($51.22 \pm 0.6 \mu\text{m}$ $p < 0.001$), special type group ($51.22 \pm 3.38 \mu\text{m}$ $p < 0.001$) and metastatic lymph node group ($47 \pm 1.91 \mu\text{m}$; $p < 0.001$). In contrast, there was no significant difference compared to the BMs in the DCIS group ($61.61 \pm 2.78 \mu\text{m}$; $p < 1$).

On the other hand, the DCIS group showed a significant increase in fibre length compared to all other groups ($p < 0.001$). Conversely, EPC showed significantly shorter fibres than the invasive group ($p < 0.03$) however, there was a significant increase in length compared to the other groups (INV, $P < 0.03$; S.INV $P < 0.01$ and metastatic LN $p < 0.001$). There was also a significant difference in fibre length between invasive carcinoma and special type BC ($p < 0.001$), while both the groups showed significantly longer fibres than the metastatic lymph node group. Comparisons with one-way ANOVA, $F = 59.24$, $p < 0.001$ (**Table 3-6**).

Table 3-6: The comparison of mean fibre length in the basement membrane in breast and metastatic lymph node tissue

Group	Mean fibre length	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	P value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	61.66	2.78	0.557	DCIS	1
				EPC	0.001*
				Invasive	0.01*
				Special invasive	0.001*
				Metastatic LN	0.001*
DCIS	61.61	2.78	0.779	EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001*
EPC	56.53	0.54	1.30	Invasive	0.03*
				Special invasive	0.01*
				Metastatic LN	0.001*
Invasive	51.22	0.66	0.62	Special invasive	0.001*
				Metastatic LN	0.01*
Special invasive	51.22	3.38	0.67	Metastatic LN	0.003*
Metastatic lymph node	47	1.91	0.19	-	-

3.3.5 Mean collagen fibre straightness within the BM

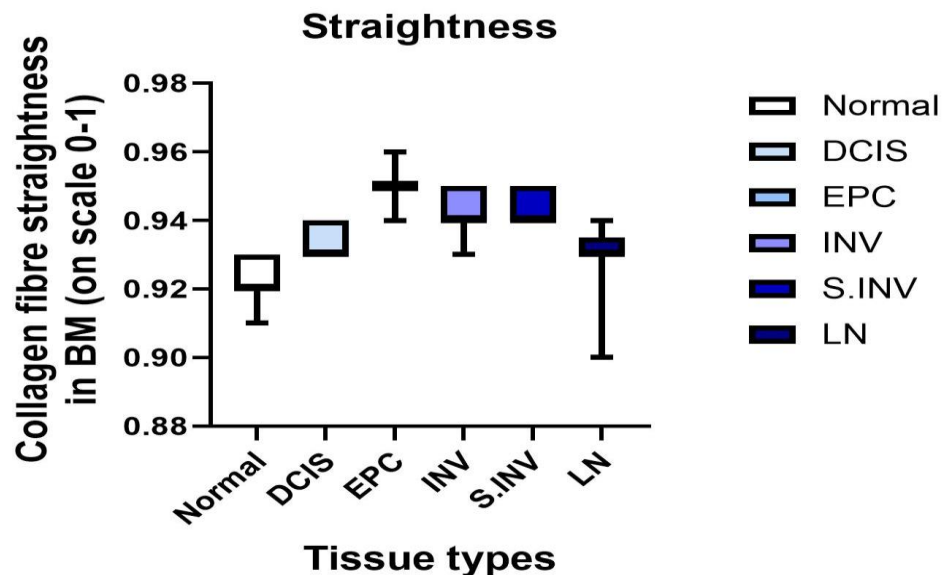


Figure 3-11: Mean fibre straightness in the basement membrane in breast and metastatic lymph node tissue

In comparison to the normal breast tissue control group, (0.92 ± 0.007 on a scale 0-1) with other groups, there was a statistically significant increase in collagen fibre straightness of each DCIS (0.93 ± 0.005 on a scale 0-1; $p < 0.001$), EPC (0.95 ± 0.0074 on scale 0-1; $p < 0.001$), invasive carcinoma (0.94 ± 0.007 on scale 0-1; $p < 0.001$) and special types BC group (0.94 ± 0.004 on scale 0-1; $p < 0.001$). However, the metastatic lymph node group showed less straight fibres (0.92 ± 0.01 on a scale 0-1; $p < 0.025$) compared to normal tissue. Similarly, DCIS BMs showed more curved fibres (0.93 ± 0.005 on a scale 0-1) than all other groups except the metastatic lymph node group (0.92 ± 0.01 on a scale 0-1), which had a more curved fibre than the DCIS group ($p < 0.025$).

There was a significant increase in collagen fibre straightness in EPC BMs compared to BMs in all of the other groups. Additionally, all the groups showed straighter fibres compared to the metastatic lymph node group. In contrast, there was no significant difference in fibre straightness between invasive carcinoma and special type BC ($p < 1$). One-way ANOVA test, $F = 56.41$, $p < 0.0001$, **Figure 3-11 & Figure 3-12, Table 3-7.**

Table 3-7: The comparison of mean fibre straightness in the basement membrane in breast and metastatic lymph node tissue

Group	Mean fibre straightness	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	0.922	0.007	0.001	DCIS	0.001*
				EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.025*
DCIS	0.934	0.005	0.001	EPC	0.001*
				Invasive	0.025*
				Special invasive	0.001*
				Metastatic LN	0.025*
EPC	0.95	0.004	0.0008	Invasive	0.001*
				Special invasive	0.006*
				Metastatic LN	0.001*
Invasive	0.94	0.007	0.001	Special invasive	1
				Metastatic LN	0.001*
Special invasive	0.943	0.004	0.0009	Metastatic LN	0.001*
Metastatic lymph node	0.92	0.01	0.002	-	-

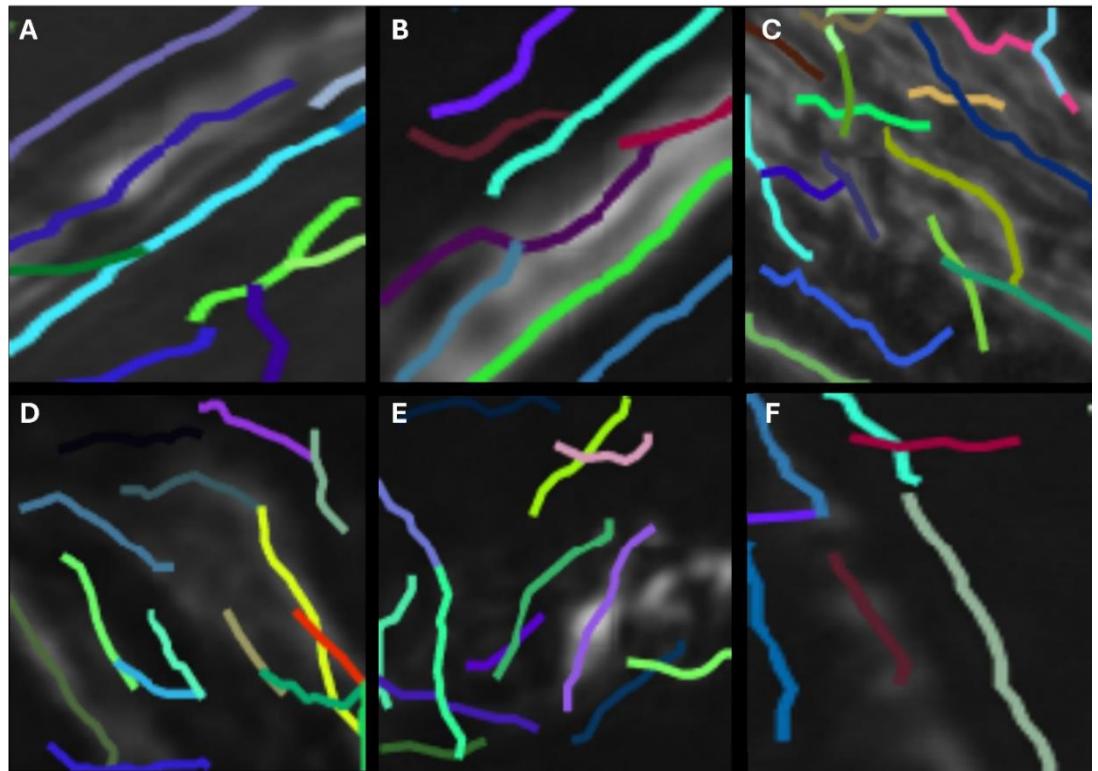


Figure 3-12: Image analysis using CT-fire software highlighting extracted fibres in different colours.

A) Normal breast tissue BM with parallel long fibres. **B)** DCIS BM with more thickened, straighter and longer fibres than normal tissue. **C)** EPC BM with a straighter, shorter fibres than normal tissue. **D)** BM-like material between clusters of neoplastic cells in invasive carcinoma with scattered, non-parallel, straighter and thinner fibres. **E)** Special invasive carcinoma (adenoid cystic carcinoma) BM-like material with shorter, thinner, random, intersected fibres compared to normal tissue. **F)** Metastatic lymph node BM-like material with thin, scattered fibres.

3.3.6 Orientation angle of collagen fibres within the BM

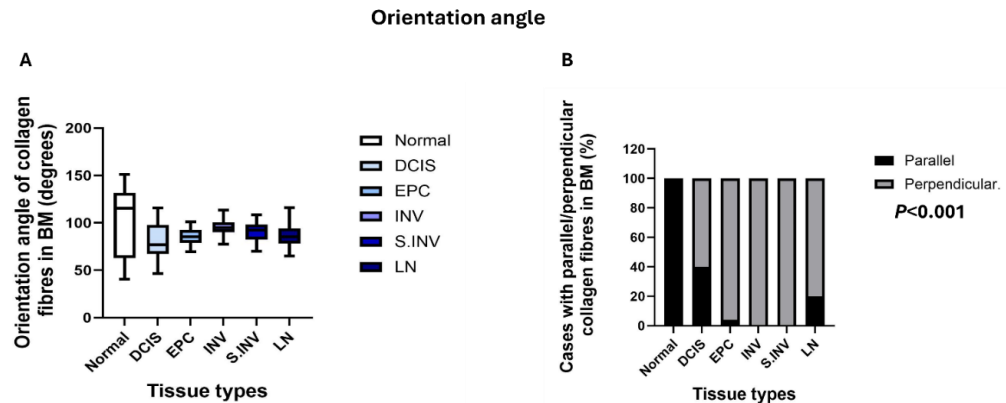


Figure 3-13: Mean orientation angle of collagen fibres of basement membrane in breast and metastatic lymph node tissue.

A) The orientation angle of collagen fibres in each case. **B)** The percentages of cases with parallel and perpendicular fibres according to $70 < \text{angle} < 110$ were considered as perpendicular and < 70 or > 110 were considered parallel fibres.

In comparison to the normal breast tissue control group (**Figure 3-13 & Figure 3-14**), all the cases within the DCIS, EPC, and metastatic lymph node groups had fewer cases where 'parallel' fibres were observed (40%, 4%, and 20% respectively, compared to 100% in the control) while the invasive group and special types group had no cases where parallel fibres were observed.

Conversely, all the groups showed a higher number of cases with 'perpendicular' fibres and all the cases within the invasive group and special invasive, while 96% of the cases in the EPC capsule and 60% of DCIS cases contained 'perpendicular' fibres (chi-square test $\chi^2 = 87.01$, $p < 0.001$, **Table 3-8**).

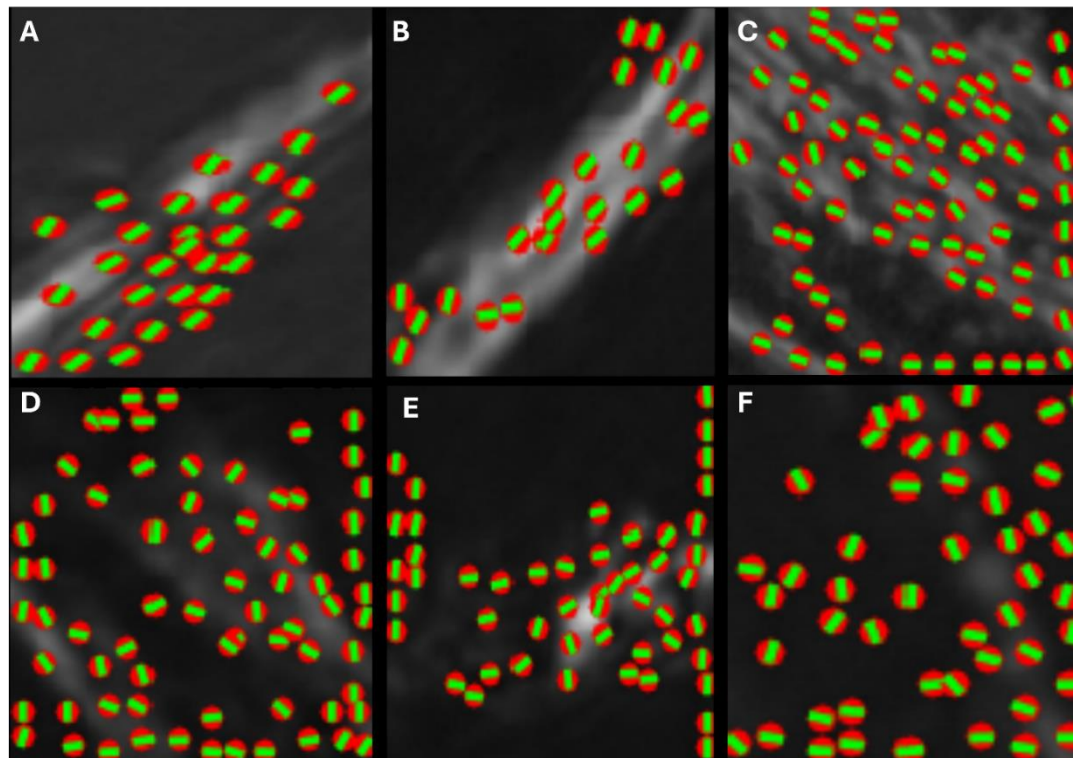


Figure 3-14: Image analysis using curve align.

The red dots indicate the centre of each fibre, and the green lines indicate fibre alignment. **A)** Normal breast tissue BM with green lines lying parallel to the BM fibres. **B)** DCIS BM with green lines lying parallel to the BM fibres, but to a lesser extent than normal tissue. **C)** EPC BM with green lines lying parallel with the BM fibres. **D)** BM-like material between clusters of neoplastic cells in invasive carcinoma, with green lines that are horizontal lines which are perpendicular or with varying angles within the BM-like material fibres. **E)** Special invasive carcinoma (adenoid cystic carcinoma) with green lines at various angles within the BM-like fibres. **F)** Metastatic lymph node with green lines at various angles in the BM-like material fibres, but generally more horizontal.

Table 3-8: Comparisons of the mean orientation angle of collagen fibres in the basement membrane in breast tissue and metastatic lymph node tissue

Group	Mean orientation angle	Standard deviation (SD)	Parallel fibres	Perpendicular fibres
Normal	102.8	32.20	100%	0%
DCIS	80.58	19.48	40%	60%
EPC	86.28	12.09	4%	96%
Invasive	95.07	8.17	0%	100%
Special invasive	90.79	9.42	0%	100%
Metastatic lymph node	86.91	11.94	20%	80%

3.3.7 Alignment of collagen fibres within the BM

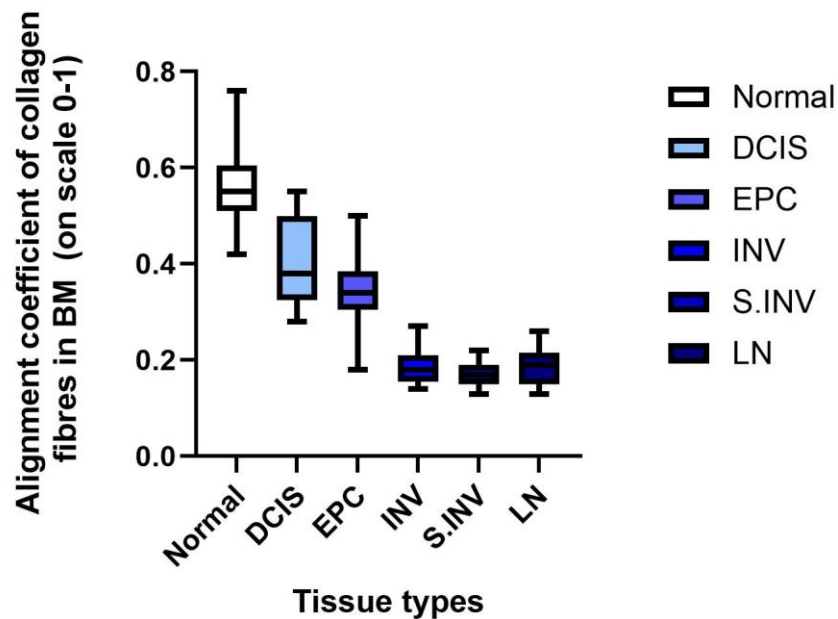


Figure 3-15: Mean orientation angle of collagen fibres of basement membrane in breast and metastatic lymph node tissue

There was a significant decrease in fibre alignment in all the groups, DCIS (0.40 ± 0.09 on a scale of 0-1; $p < 0.001$), EPC (0.3 ± 0.01 ; on a scale of 0-1; $p < 0.001$), the invasive group (0.23 ± 0.06 ; on a scale of 0-1; $p < 0.0001$), the special types group (0.18 ± 0.03 ; on a scale of 0-1) and metastatic lymph node group (0.23 ± 0.03 ; on a scale of 0-1) compared to normal breast tissue group (0.57 ± 0.08 on a scale of 0-1). Similarly, there was a significant decline in fibre alignment in all the groups compared to the DCIS group. There was not any significant difference in the fibre alignment between cancerous groups including EPC, invasive carcinoma, special types BC and metastatic lymph nodes. One-way ANOVA test, $F = 133.21$, $p < 0.001$, **Figure 3-15**, **Figure 3-16** and **Table 3-9**.

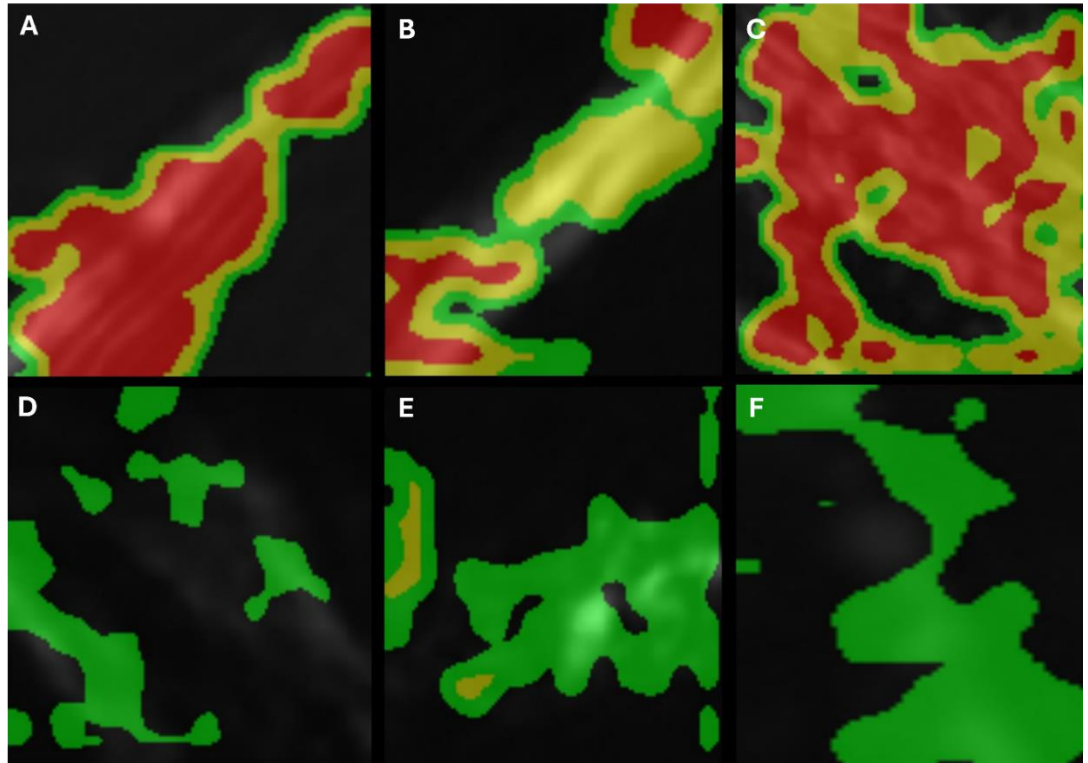


Figure 3-16: A heat map indicating fibre alignment well-aligned fibre areas are depicted in red, moderately aligned fibres are shown in yellow, and green areas designate areas with poorly aligned fibres

Curve align software determines the centre of the fibre extracted by CT-FIRE as mentioned before, then measures the distance between the fibres to each other and within the boundary and the algorithm generates a heat map with the regions according to the alignment of the fibres (440).

A) Normal breast tissue with red areas indicating a well-aligned region, with areas of moderate and poor alignment. **B)** DCIS tissue heat map resembles normal breast tissue, with a smaller good alignment area. **C)** EPC tissue heat map indicating large areas of well-aligned fibres, in addition to moderate and poorly aligned areas. **D)** Invasive carcinoma with green areas indicating poor fibre alignment. **E)** Special invasive carcinoma (adenoid cystic carcinoma) with mostly poor alignment in green and some smaller yellow areas indicating moderate alignment. **F)** Metastatic lymph node with green areas indicating poor alignment areas.

Table 3-9: The *comparison* of mean alignment coefficients in the basement membrane in breast and metastatic lymph node tissue

Group	Mean alignment coefficients	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	P value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	0.57	0.08	0.01	DCIS	0.001*
				EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001*
DCIS	0.4	0.05	0.01	EPC	0.001*
				Invasive	0.001*
				Special invasive	0.001*
				Metastatic LN	0.001*
EPC	0.3	0.01	0.01	Invasive	1
				Special invasive	1
				Metastatic LN	1
Invasive	0.23	0.06	0.01	Special invasive	0.06
				Metastatic LN	1
Special invasive	0.18	0.03	0.0006	Metastatic LN	0.05
Metastatic lymph node	0.23	0.03	0.007	-	-

3.4 Discussion

The BM plays a key role in diagnosing and differentiating *in situ* and invasive lesions. Finding differences between the native BM and a reactive BM surrounding an invasive lesion is clinically important for early diagnosis and proper management of each patient, especially when considering the implications of management for *in situ*, benign tumour versus an invasive diagnosis. Observations relating to birefringence fibres in PSR-stained tissue sections could assist us in differentiating BM collagen fibres via their differing polarized light colours (441). Collagen maturation changes the proteoglycan content within fibres, which results in fibre dehydration, as a result, the diameter of the collagen fibres alters thus resulting in differential colour expression when examined using polarized microscopy (442).

The observations from the present study found that the BM in the normal breast tissue had more thin fibres (type III collagen) than thick fibres (type I collagen). The DCIS BM was similar to normal BM in the collagen

distribution but higher in density. In contrast, the EPC cases and special invasive cases capsules were surrounded with a high content of thick fibres. One possibility is these thick fibres may act as a defensive mechanism with the body to prevent neoplastic cell invasion, or it may be a mechanism employed by neoplastic cells to prevent immune cell responses (443). For example, in the special types of BC, the cases had the lowest thick fibre proportion compared to all groups. This could be explained by the presence of BM remnants following invasion and BM destruction alternatively, the neoplastic cells may mimic the normal tissue trying to construct a BM for protection.

The invasive carcinomas were not surrounded by any BM-like material; however, they did exhibit small, interrupted fibres between the clusters which were most probably stromal fibres and showed a high content of thick collagen fibres which was higher than that observed in the other groups. The metastatic lymph node (LN) cases were surrounded by thin fibres and their overall content was less than the thin/thick ratio of the BM fibre content observed in the normal breast tissue, this is consistent with the theory that the neoplastic cells secrete BM constituents. One explanation for this may be that the collagen proportion could be influenced by ECM remodelling enzyme activity which contributes towards cancer progression (444) with protease expression as MMPs (445) especially in breast cancer invasion as with MMP-2, MMP-3, MMP-9, and MMP-14 (446).

The observations in the present study are consistent with other studies relating to canine mammary cancer which examined the stroma and demonstrated that there was a higher proportion of collagen I (thick fibres) and a lower percentage of collagen III (thin fibres) (447). On the other hand, a published study observed that there was a high percentage of thin fibres in canine prostate cancer compared with normal tissue, and a higher level of thick fibres in prostate cancer, but these were not statistically significant differences (448). Another study demonstrated that human prostate sections stained with PSR when examined under polarized microscopy, showed higher proportions of the thicker stromal

collagen type I fibres in prostate glandular hyperplasia compared to normal prostate tissue (449). This finding was similar to the present study where the number of thick collagen type I fibres increased in the EPC and invasive BC cases compared to normal breast tissue.

The thin/thick fibre observations in the present study were in agreement with Kallage et al (450). Their work showed there was a correlation between tumour differentiation and the thickness of the collagen fibres in human oral squamous cell carcinoma, and that differentiated tumours were associated with thicker collagen I fibres whereas poorly differentiated ones showed thinner collagen III fibres. In addition, this previously published study also showed a significant correlation between high inflammatory cell number and the thick (coloured red to orange) collagen type I fibres. Many studies demonstrated a gradual change in collagen fibre thickness, with polarizing colours changing from green to red/orange, with tumour differentiation progression, again poorly differentiated tumours were associated with the thinner collagen III fibres (441, 451-460). This potentially explains the higher ratio of thick fibre (collagen I) in the EPC group compared to both invasive groups and the presence of higher content of collagen III (thin fibres) in special types of BC.

The results from the present study showed that collagen density increased in the DCIS, EPC, and special invasive and invasive groups, this was in agreement with previously published work evaluating ECM stiffness in normal, benign, and invasive BC. The previous work revealed that BC aggressiveness was associated with an incremental increase in collagen deposition and density (461). Many studies have shown that increased collagen density was associated with tumour metastasis and poor outcome in human BC (462-465).

On the other hand, the results from the present study were not consistent with another study in human breast cancer demonstrating an inverse correlation between stromal collagen density, fibre width and lesion severity as density and fibre thickness decreased with conversion from normal, benign to carcinoma (466). However, the results were consistent

with some aspects of this study, for example concerning orientation, alignment, length, and straightness, decreases in these factors correlated with increased lesion severity (466). This could be explained as the study investigated the density of stromal collagen which decreased with conversion to carcinoma due to collagen destruction and ECM remodelling for invasion.

Individual characteristics of the collagen fibres may also modulate tumour cell-microenvironment interactions. For example, it was revealed that compared to non-neoplastic pancreatic tissue, there was an increased length of collagen fibres in pancreatic ductal adenocarcinoma (467), which was consistent with the increased fibre length observed in the DCIS cohort compared to invasive groups. In gastric tumours, collagen characteristics such as width, and straightness as well as density and alignment were revealed to be increased compared to non-neoplastic tissues (357, 468). The present study observations concurred with these findings, density, fibre thickness and straightness were increased in the invasive group.

The present study results showed that collagen fibres became straighter in the DCIS, EPC and invasive groups compared to the normal breast tissue group, where the fibres were more curved. These results concurred with a study by Arwert and colleagues which demonstrated that tumour cells migrate with macrophages along straighter, unidirectional, collagen fibres towards blood vessels during invasion (469). In addition, collagen fibre straightness seems to be an important parameter as the straightest collagen fibres were found in the intertumoral region while the most curved fibres were present in the extra tumoral region in human BC (468). The straightening, and increased width, of the collagen fibres could result in ECM porosity changes which limit the ability of cells to randomly move within the tumour, which enhances the cell's requirements to leave the tumour via invading adjacent tissues. Furthermore, these changes have a powerful impact on the biomechanical characteristics of the TME (470).

Mammary collagen fibres activate signalling pathways which result in stimulating the proliferation and progression of tumour cells (277). Collagen fibre organization and density also influence tumorigenesis and tumour invasiveness (471, 472). In addition, the realignment of collagen fibres and the straightening of these fibres has been correlated with local invasion, metastasis, and poor outcomes in BC (277). This was consistent with study results as all the groups showed poor alignment compared to normal BM.

The observations in the present study showed that there were increases in the percentages of perpendicular fibres in all of the cancer groups compared to the normal group, with a concomitant decline in the number of parallel fibres. This could be explained, as evidenced in a previous study, that deposition of collagen fibres with a perpendicular orientation angle to the tumour gland boundary (TACS-3) resulted in building highways for tumour cell migration (278), which correlated with increased metastasis in experimental animals (465). It was hypothesised that this radial orientation of the fibres resulted from contractile and morphogenic processes from tumour boundaries, to reorganise the ECM for local invasion and metastasis. This hypothesis was supported by research relating to collagen structure analysis within regions of invasion (278).

The alignment results from the present study showed poorly aligned fibres within the tumour groups (invasive, special invasive and metastatic lymph node groups). These results are consistent with other studies, which showed that well-aligned collagen fibres are a protective prognostic factor in BC (473). In vitro studies on tumour cell mechanisms with various collagen fibre architectures showed that regions of small tumours showed random 'curly' fibres but within larger tumours, the fibres become straighter and more aligned under expansion tension and more perpendicular (474, 475). This previous observation helps explain the presence of more perpendicular fibres in the EPC group, it may result from the tension and stress of capsule fibres expansion.

3.5 Conclusion

The study demonstrated that DCIS's BM resembled normal tissue BM except with notable exceptions of increased density, straightness, disorganisation, and reduced alignment. In contrast,

All invasive groups had higher density, thicker fibre, more collagen I content, wider, shorter, straighter, less aligned and disorganised.

The EPC group demonstrated alignment patterns similar to invasive groups with higher density and straighter fibres, while possessing longer fibres than special BC, thinner fibres, and reduced collagen I (thick fibre) content compared to invasive BC.

This chapter identifies collagen features that could differentiate between invasive and non invasive lesions in BC Future work studying the mechanisms behind these collagen features could further assist the patient outcome prediction and help develop novel therapies.

4 Chapter 4: Characterisation of encapsulated papillary carcinoma capsule in breast cancer

This study has been published in the Journal of Histopathology with the corresponding <https://doi.org/10.1111/his.14939>.

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4.1 Introduction

Papillary lesions of the breast are a group of heterogeneous tumours which are characterised morphologically by fibrovascular cores that are covered by epithelial cells ranging from benign to malignant (476). PCs include papillary DCIS, SPC, EPC, a neuroendocrine feature of papillary neoplasm, IPC and a new entity called tall cell carcinoma with reverse polarity (TCCRP) characterised by apical nuclei (75, 476). EPCs present as a well-circumscribed and encapsulated mass characterised by their lack of a MEC layer at their periphery in most of the cases (80%) (234). EPC is a term that has been recently introduced to define the previously named cystic variants (intra-cystic or encysted) of PC in order to reflect its encapsulation (234). EPC displays a peripheral thick capsule-like structure and was interpreted as a thickened BM as it contains some structures of the BM namely collagen type IV and laminin. Subsequently, this feature is known to be one of the indications of its *in-situ* nature (229).

BMs are thin pliable sheet-like materials which is located between either normal or proliferative intraductal epithelial and MEC layers and the surrounding stromal CT (477). In malignant tumours, the preservation of BM refers to *in situ* disease whereas BM absence is considered a feature of invasive tumours (477). Although there are some DCIS which show BM expansion, this is different from the reactive fibrous tissue capsules which can be observed around some benign and malignant tumours in several tissues and organs such as hepatocellular carcinomas, renal cell carcinomas, and thyroid follicular carcinomas with their benign counterparts (478).

Tumour encapsulation denotes the presence of a fibrous capsule surrounding the neoplasm regardless of whether it is a true or pseudo capsule, a true capsule is defined as having functional and morphological integrity and is often associated with indolent clinical behaviour (478). The concept of capsule pathogenesis suggests that tumour encapsulation is not a passive condensation of fibrous tissue caused by expansive cell growth or a mechanism for a body defence mechanism

against tumours that resembles a foreign body response, however, it is a biological process that acts like the response to wound healing (478, 479). The importance of understanding the tumour encapsulation nature is that it increases the perspective of the knowledge about the tumour invasion process (478). Many tumours exhibit an encapsulation process, including renal cell carcinoma, hepatocellular carcinoma, thyroid carcinoma, and their benign counterparts (478).

WHO recommends that EPC is staged as carcinoma in situ in cases of the absence of frank stromal invasion or infiltrative growth despite the lack of a MEC layer (184). This was supported by several previous studies that reported the expression of certain BM structures, such as collagen type IV and laminin in EPC cases, inferring that this structure surrounds EPC may represent BM expansion and hence the tumour's intraductal nature (235, 476).

The BM outer layer which faces the ECM is named the reticular lamina, or lamina fibro-reticularis, and is mainly formed of collagen III (244). Previous studies have revealed that collagen III is co-expressed with collagen I, resulting in the regulation of fibril dimensions that influence their functional properties and mechanical stiffness (223, 480). Collagen III plays a key role in normal tension maintenance and scar formation modulation (480). Additionally, stiffening of BM has been demonstrated to trigger cell invasiveness in some tumours such as prostate cancer (481) and squamous cell carcinoma (482).

PC is still a diagnostic challenge due to overlapping histological features and unique biological features. The diagnostic challenges of PC not only categorisation as *in situ* or invasive disease but also under-estimation as benign lesions (483).

PSR stain is a special histochemical stain for collagen as mentioned previously. It has a higher specificity for the detection of collagen fibre compared to other stains that are commonly used due to the ability to stain finer collagen fibrils, which in turn avoids collagen density

underestimation (434). PSR comprises of birefringent elongated dye molecules which can bind in parallel positions to the collagen fibre, consequently enhancing the natural birefringence under a polarising microscope. Therefore, PSR can detect thinner fibres (collagen III) which present as green to green-yellow colours, whereas thicker fibres (collagen I) are visualised as yellowish orange to orange-red colours (433, 436).

4.1.1 Aims and Hypothesis

In this chapter, it was hypothesised that the deciphering of the structural composition of EPC capsule could provide insights into the underlying behaviour. To test this hypothesis, this chapter aims to determine whether the EPC capsule represents a reactive process similar to that shown in some breast invasive tumours, or native BM expansion which mimics that seen in some DCIS cases, and therefore elucidate the nature of the EPC based on BM collagen fibre geometric characteristics.

4.2 Patient and Methods

4.2.1 Study cohort

This study included 100 cases of normal and neoplastic breast tissue from patients who presented to Nottingham City Hospital, Nottingham, UK between 1999 and 2006. The cases were divided into four groups, each containing 25 patients. The four groups included EPC, in addition to DCIS, with normal breast tissue, and invasive carcinoma (INV) as control groups. The INV group was composed of invasive cribriform, ACC, and salivary gland-like carcinoma, as these types fulfil the criteria of being invasive tumours and showed BM-like material surrounding the neoplastic cells. Encapsulated papillary thyroid carcinoma (EPTC) cases were also stained and analysed to be used as a control group for comparison against the EPC group, random selection of cases of EPTC were included (n=10) with exclusion of cases which associated with any other type of thyroid cancer

4.2.2 Tissue processing and staining

Sections were freshly cut, full-face 4µm thick for the cohort and were stained with H&E and PSR special stain. For each tumour block, one section per case was prepared for each stain. Collagen fibres were assessed using tissue sections stained with PSR as mentioned in Chapter 2.

4.2.3 Microscopy and Imaging

H&E-stained slides were examined for section histological verification, via standard light microscopy with a 10× objective. The glands were identified which worked as a reference once the tissue sections had been stained with PSR.

The PSR-stained sections were examined under a polarized microscope and visualised using accompanying software (Leica, IL 5000B, and LAS VERSION 3.8.0, Leica Microsystems, Switzerland) and a camera. ROIs: 75x75 pixels were chosen for image analysis, as mentioned in Chapter 2.

The EPC group was analysed to assess the difference between the inner and outer parts of the thickened capsule. As the BM of normal and DCIS samples were thin, this splitting into inner and outer parts was not possible and the whole BM was analysed as one part, and the difference was considered between different ROIs. The inner capsule part was defined as the most adjacent part to tumour cells whereas the outer part of the capsule was defined as the fibrous part adjacent to the stroma.

This method was validated by repeating the mean calculation using different randomly selected measurements (always n=32) with no significant differences in the mean values obtained. The mean values of the measurements were calculated for each case. All photomicrographs were taken at 100x magnification as mentioned previously.

4.2.4 Image analysis and quantification of collagen

The BM thickness and collagen density and distribution assessment was performed using Image J, (NIH, Bethesda, Maryland, available for free download at <https://imagej.nih.gov/ij/download.html>). Collagen parameters such as width, length, straightness, alignment, and orientation angle, were measured using Ct-Fire and Curve align as mentioned before in Chapter 2.

4.2.4.1 Basement membrane thickness

BM thickness and the EPC capsule were measured by calculating the average of 20 measurements for each case at 20X magnification.

4.2.5 Statistical analysis

For all statistical analyses, $p < 0.05$ was considered statistically significant and undertaken either in SPSSv26 or GraphPad Prism. Paired t-test, independent t-test and Wilcoxon signed ranks tests Kruskal-Wallis, one-way ANOVA, and chi-square test were used as specified in each result. Graphs and tables were constructed as applicable using GraphPad Prism10. Results are expressed as the mean $\pm$ SD of the mean throughout unless otherwise stated.

4.3 Results

4.3.1 Heterogeneity of EPC capsule

The comparisons between the mean collagen fibre density inside and outside parts of the EPC capsule showed that the outer part of the capsule had significantly higher collagen density (85 ± 3.56 fibres/ μm^2 ; paired t-test, $t=5.88$, $p < 0.001$) compared to the inner part of the capsule (80.94 ± 2.62 fibres/ μm^2 ; **Figure 4-1 & Figure 4-2**).

The content of collagen type I was higher in the outer part of the capsule ($75.46 \pm 7.16\%$; Wilcoxon signed ranks $Z = -4.238$, $p < 0.001$; **Figure 4-1**).

compared to the inner part ($59.31 \pm 13.79\%$). Conversely, the inner capsule had more collagen type III ($40.69 \pm 13.79\%$; Wilcoxon signed ranks, $Z = -4.238$, $P < 0.0001$) compared to the outer part ($24.54 \pm 7.16\%$; **Figure 4-1 & Figure 4-2.**

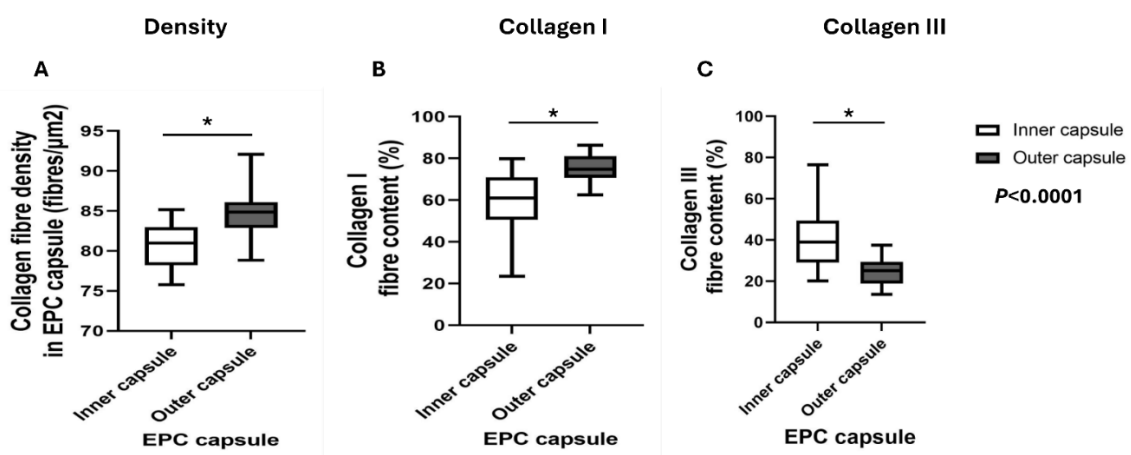


Figure 4-1: Collagen fibre density and distribution in the capsule of encapsulated papillary carcinoma (EPC).

A) Collagen fibre density, paired t-test, $t = -5.88$ $p < 0.0001$. **B)** Collagen I fibre content, Wilcoxon signed ranks test, $Z = -4.238$, $P < 0.0001$. **C)** Collagen III fibre content, Wilcoxon signed ranks test $Z = -4.238$, $P < 0.0001$.

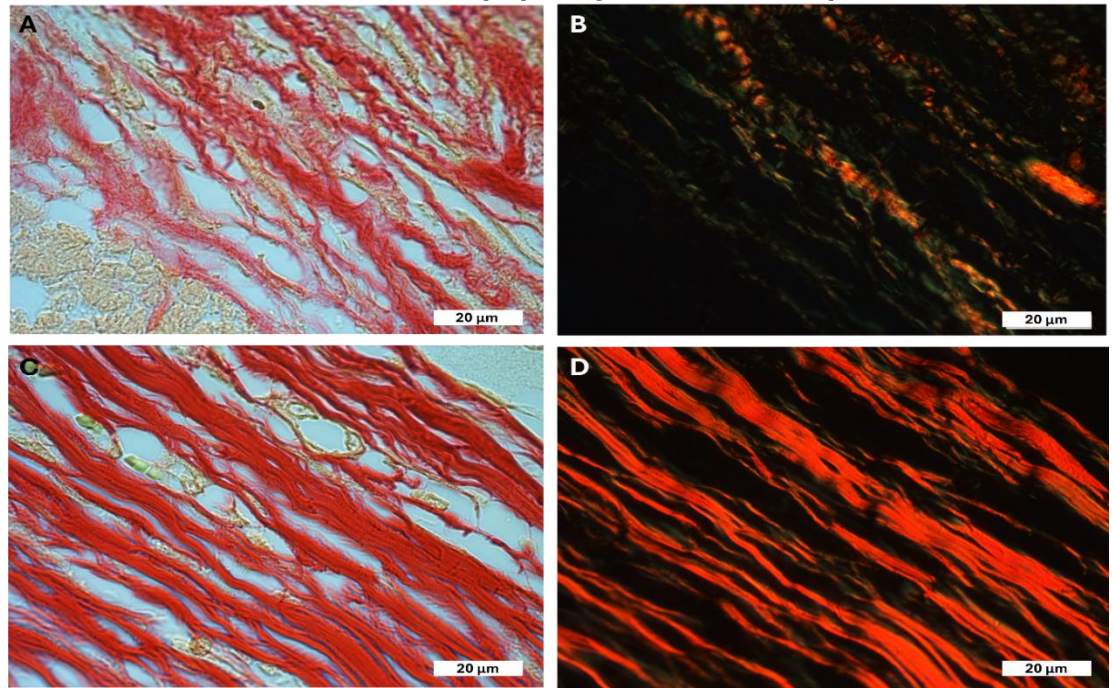


Figure 4-2: Encapsulated papillary carcinoma (EPC) capsular heterogeneity.

A) Inner part of EPC stained with picrosirius red (PSR) stain showing collagen fibres. **B)** The inner part of EPC stained with PSR and examined under polarized light (PL) showing part of the capsule with straight collagen fibres ranging in colour from green to orange. **C)** The outer part of the EPC capsule stained with PSR demonstrates wavy collagen fibres. **D)** The outer part of the EPC capsule was stained with PSR and examined under PL showing collagen expressed as orange to red wavy fibres. Magnification x100, Scale bars depict 20µm.

There was a statistically significant increase in mean fibre width in the outer part of the capsule ($7.78 \pm 0.015 \mu\text{m}$) compared to the inner part of the capsule ($7.36 \pm 0.094 \mu\text{m}$; paired t-test, $t = -11.90$, $p < 0.0001$; **Figure 4-3**.

There were no significant differences in mean fibre lengths within both the inner and outer capsules (57.59 ± 1.62 compared to $57.05 \pm 1.99 \mu\text{m}$, respectively; $t = -1.35$, $p < 0.19$; **Figure 4-3**.

The outer capsule fibres showed more straightness (0.95 ± 0.005 on a scale of 0-1) compared to the inner part (0.94 ± 0.007 ; Wilcoxon signed ranks test, $Z = -4.32$, $P < 0.0001$; **Figure 4-3**.

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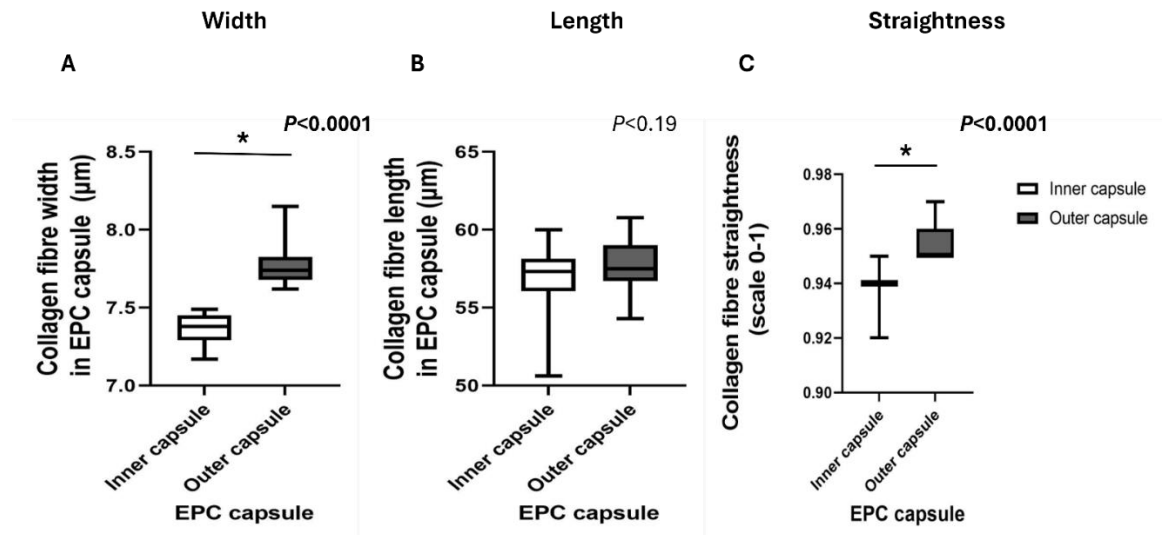


Figure 4-3: Collagen fibre characteristics in the capsule of encapsulated papillary carcinoma (EPC)

A) Collagen fibre width, paired t-test, $t = -11.90$, $p < 0.0001$. **B)** Collagen fibre length, paired t-test, $t = -1.35$, $p < 0.19$. **C)** Collagen fibre straightness, Wilcoxon signed ranks test $Z = -4.32$, $P < 0.0001$.

Capsular collagen fibres of the outer part were also less aligned than the inner part (paired t-test, $t = -6.07$, $p < 0.0001$; **Figure 4-4**).

The orientation angles of the collagen fibres were classified as either 'parallel' or 'perpendicular' for each case observed based on the dominant angle measurement. Angles of $70-110^\circ$ were considered 'perpendicular' fibres and measurements outside of this were considered 'parallel'. Comparisons were made between the numbers of cases in each group classified as having either 'parallel' or 'perpendicular' fibres in the BM. There was no significant difference in the orientation angle between the inner and outer parts ($Z = 3.42$, $p < 0.1$), **Figure 4-4**.

Additionally, the outer part had more cases where 'perpendicular' fibres were observed (96% perpendicular vs 4% parallel), whereas the inner part had 'perpendicular' fibres in 92% of cases (8% parallel), these results were not statistically different (chi-square; $\chi^2 = 0.355$; $p < 0.55$; **Figure 4-4**).

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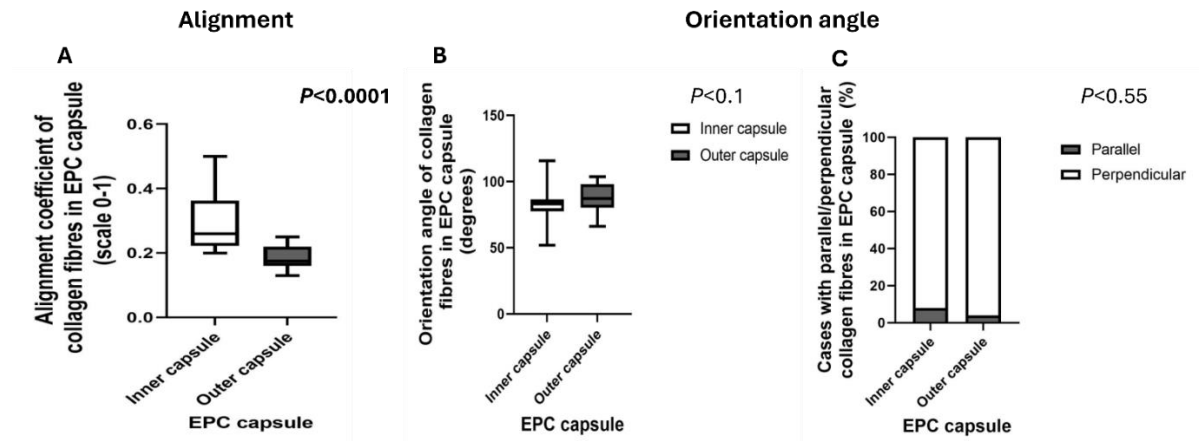


Figure 4-4: Collagen directionality in the capsule of encapsulated papillary carcinoma (EPC).

A) The coefficient alignment of collagen fibres, **paired t-test**, $t = -6.07$, $p<0.0001$. **B)** The orientation angle of collagen fibres for each case, Wilcoxon signed ranks test, $Z=3.42$, $p<0.1$. **C)** The percentages of cases with parallel ($<70^\circ$ or $>110^\circ$) or perpendicular fibres ($\geq 70^\circ$ - $\leq 110^\circ$), Chi-square, $\chi^2=0.355$, $p<0.55$.

4.3.2 Comparison between EPC capsular heterogeneity and other groups

4.3.2.1 BM thickness

A significant increase in the thickness of the EPC capsule ($59.65 \pm 18.50 \mu\text{m}$) was found in comparison to the BMs in the normal breast tissue group ($0.032 \pm 0.009 \mu\text{m}$; $p<0.0001$) and BMs from DCIS cases ($0.058 \pm 0.017 \mu\text{m}$; $p<0.0001$; **Figure 4-5**). In contrast, there was no difference between the BM thicknesses in the DCIS samples ($0.058 \pm 0.017 \mu\text{m}$) and normal breast tissue ($0.032 \pm 0.009 \mu\text{m}$; $p<0.995$; **Figure 4-5** :).

Capsule thickness

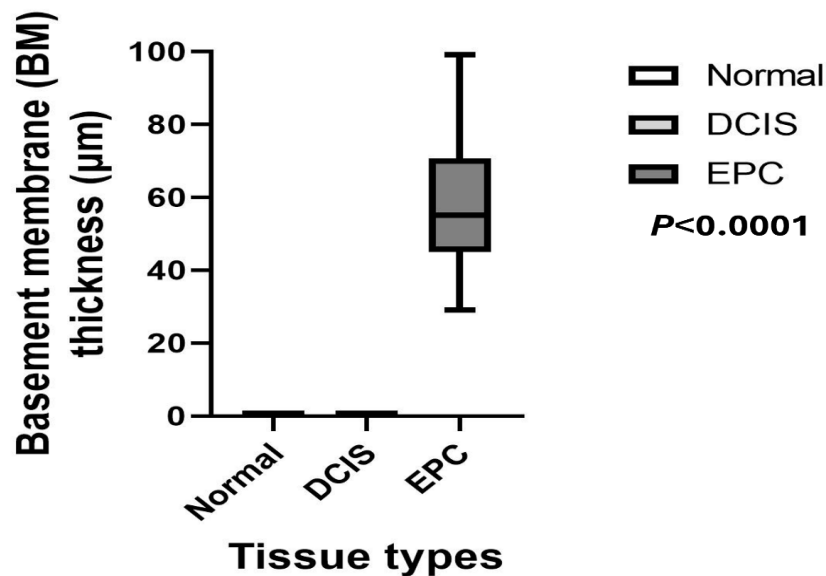


Figure 4-5 : Thickness of the basement membrane in breast tissue.

4.3.2.2 Collagen fibre density within the BM

The density of collagen fibres in the EPC capsule and the BM of normal breast tissue comparisons (**Figure 4-6**), showed there was a significant increase in collagen fibre density in both the inner (80.94 ± 2.62 fibres/ μm^2 , $p < 0.0001$) and outer parts of the EPC capsule (85 ± 3.56 fibres/ μm^2 ; $p < 0.0001$) compared to normal BM (36.86 ± 10.12 fibres/ μm^2). In addition, there was a significant increase in collagen fibre density in both the inner and outer parts of the capsule (80.94 ± 2.62 fibres/ μm^2), and (85 ± 3.56 fibres/ μm^2), respectively; $p < 0.0001$) compared to those in the BM from DCIS samples (51.88 ± 16.04 fibres/ μm^2 ; $p < 0.0001$), and also in the BM-like material in the invasive carcinoma cells (77.8 ± 12.92 fibres/ μm^2 ; $p < 0.0001$). There was a significant increase in collagen fibre density in the invasive group (77.8 ± 12.92 fibres/ μm^2) compared to both normal tissue BMs (36.86 ± 10.12 fibres/ μm^2 ; $p < 0.0001$) and DCIS BMs (51.88 ± 16.04 fibres/ μm^2 ; $p < 0.0001$). Moreover, there was a significant increase in collagen fibre density within BMs in DCIS

(51.88 ± 16.04 fibres/ μm^2) compared to BMs within normal breast tissue (36.86 ± 10.12 fibres/ μm^2 ; $p < 0.0001$). One-way ANOVA, $F = 61.05$, $p < 0.0001$; (Table 4-1) & (Figure 4-6).

Table 4-1: Collagen fibre density within breast tissues basement membranes.

Group	Mean Density	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = $p < 0.05$)
Normal	36.86	10.12	2.02	DCIS	0.0001*
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	51.88	16.04	3.20	In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	80.94	2.62	0.52	Out EPC	0.044
				Invasive	0.827
Out EPC	85	3.56	0.71	Invasive	0.011
Invasive	77.8	12.92	2.58	-	-

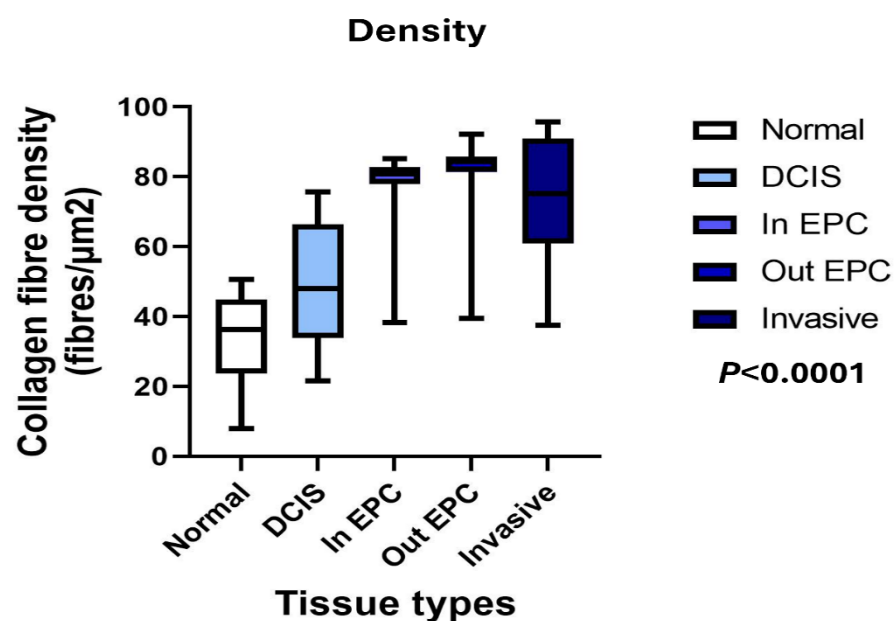


Figure 4-6: Collagen fibre density within breast tissue basement membranes.

4.3.2.3 Collagen fibre distribution within the BM

According to the measurements taken for type I collagen, in the comparisons between the normal breast tissue control group (25.25 ± 12.27 %; **Figure 4-7 & Figure 4-8**).

and the other groups, there was a statistically significant increase in the percentage of collagen I within the BMs in the inner capsule of EPC cases (59.31 ± 13.79 %; $p < 0.0001$), the outer part of EPC capsule cases (75.46 ± 7.16 %; $p < 0.0001$), and in the invasive group (71.92 ± 7.05 %; $p < 0.0001$) compared to the normal tissue group. On the other hand, there was no significant difference in collagen I content within BMs in the DCIS group (29.86 ± 6.81 %; $p < 0.514$) compared to the normal group.

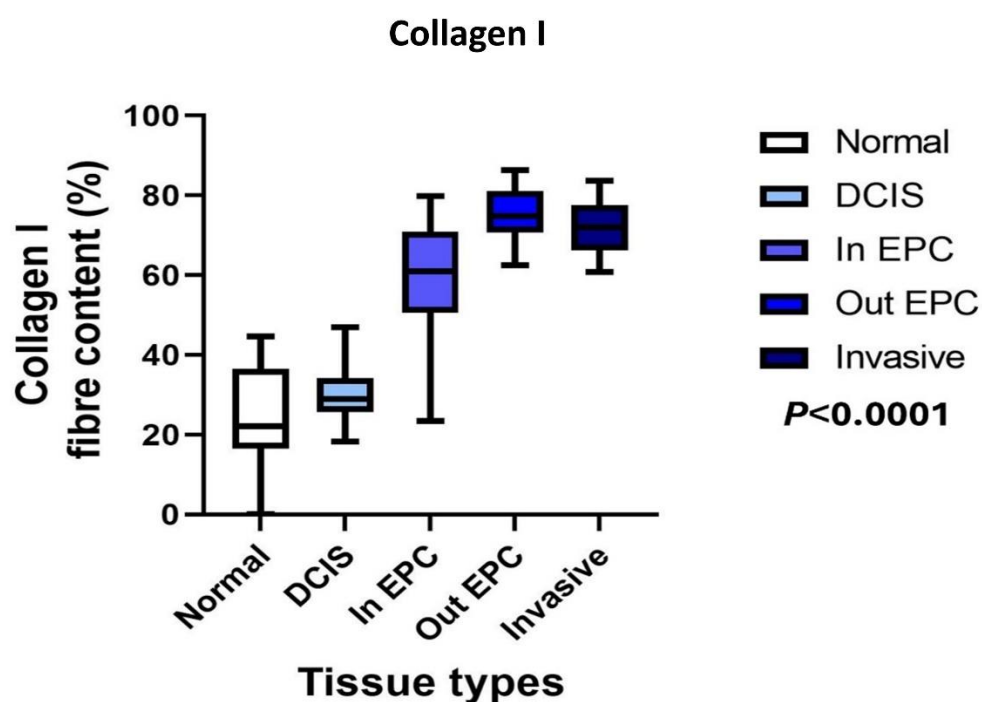
There was a significant increase in the collagen I content in the inner capsule of EPC (59.31 ± 13.79 %; $p < 0.0001$), outer part of EPC capsule (75.46 ± 7.16 %; $p < 0.0001$), and the invasive group (71.92 ± 7.05 %; $p < 0.0001$) groups compared to the DCIS group (29.86 ± 6.81 %).

The invasive group (71.92 ± 7.05 %) showed a significant increase in collagen I content compared to the inner part of the EPC capsule (59.31 ± 13.79 %; $p < 0.03$) however, there was no significant difference between the invasive group and the outer part of the capsule (75.46 ± 7.16 %; $p < 0.381$). Kruskal-Wallis test, $H=94.655$, $p < 0.0001$;

Table 4-2).

Table 4-2: Comparisons between collagen I fibre content in the basement membrane in breast tissue

Group	Collagen I percentage	Standard deviation (SD)	Standard error of mean (SEM)	Comparisons	<i>P</i> value (*= <i>significance</i> = $p < 0.05$)
Normal	25.25	12.27	2.454	DCIS	0.514
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	29.86	6.81	1.362	In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	59.31	13.79	2.759	Out EPC	0.0001*
				Invasive	0.03*
Out EPC	75.46	7.16	1.431	Invasive	0.381
Invasive	71.92	7.05	1.410	-	-

**Figure 4-7:** Percentage of collagen type I fibre content in the breast tissue basement membrane.

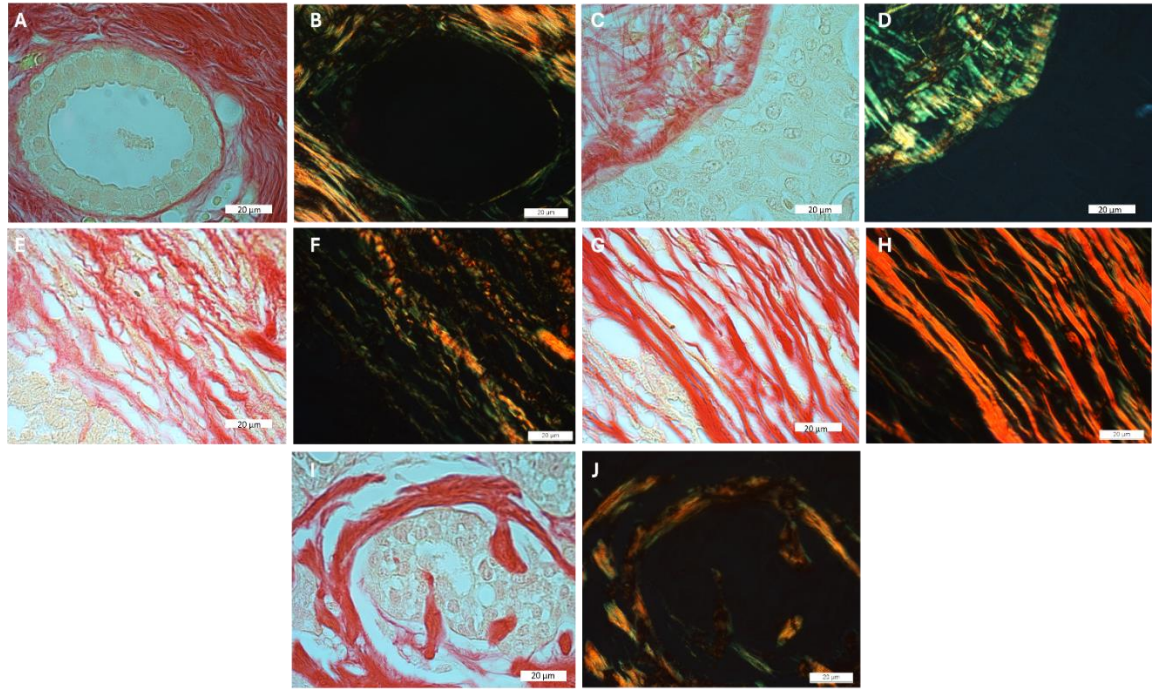


Figure 4-8: Collagen I and collagen III in the breast tissue basement membrane.

A) Normal breast tissue depicting a normal gland stained with Picrosirius red stain (PSR) lined with simple cubical epithelium surrounded by a well-defined basement membrane (BM). **B)** Normal breast tissue examined under polarised light (PL) indicating greenish collagen fibres in the BM surrounding the gland (shown in black). **C)** Ductal carcinoma in situ (DCIS) stained with PSR depicting part of a breast gland undergoing cell proliferation surrounded by a definite BM. **D)** DCIS tissue examined under PL showing BM with green collagen fibres surrounded by the gland (shown in black). **E)** The inner part of encapsulated papillary carcinoma (EPC) capsule stained with PSR showing wavy collagen fibres. **F)** The inner part of EPC examined under PL capsule shows part of capsule with wavy greenish collagen fibres. **G)** The outer part of EPC capsule stained with PSR showing collagen fibres. **H)** The outer part of EPC capsule with collagen examined under PL expressed as orange to red straight fibres. **I)** Invasive carcinoma stained with PSR showing thick BM-like material surrounding neoplastic cells with BM-like material aggregation between the cells. **J)** Invasive carcinoma examined under PL showing collagen fibres ranging in colour from green to orange. Scale bars depict 20μm.

With regards to type III collagen, the comparisons between the normal breast tissue control group (80.30 ± 11.08 %) and the other groups showed there was a statistically significant decrease in the percentage of collagen III within the BM between the inner capsule of EPC (40.69 ± 13.79 %; $p < 0.0001$), outer part of EPC capsule (24.54 ± 7.16 %;

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$p < 0.0001$), and the invasive (28.08 ± 7.05 %; $p < 0.0001$) group compared to the normal group. In contrast, there was no difference in collagen III within BMs in the DCIS group cases (68.86 ± 7.88 %; $p < 0.128$) compared to control. There was a significant decrease in the percentage of collagen III present in the inner capsule of EPC (40.69 ± 13.79 %; $p < 0.001$), outer part of EPC capsule (24.54 ± 7.16 %; $p < 0.0001$), and invasive group (28.08 ± 7.05 %; $p < 0.0001$) compared to the DCIS group (68.86 ± 7.88 %).

The invasive group (28.08 ± 7.05 %) showed a significant increase in collagen III compared to the inner part of the EPC capsule (40.69 ± 13.79 %; $p < 0.032$), however, there was no significant difference between the invasive group and the outer part of the capsule (24.54 ± 7.16 %; $p < 0.381$). Kruskal-Wallis test, $H = 96.876$, $p < 0.0001$, **Figure 4-8 & Figure 4-9, Table 4-3**).

Table 4-3: Comparison between percentages of collagen III fibres in the basement membrane in breast tissue

Group	Collagen III percentage	Standard deviation (SD)	Standard Error of mean (SEM)	Comparisons	<i>P value</i> (*= <i>significance</i> = $p < 0.05$)
Normal	80.30	11.08	2.217	DCIS	0.128
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	68.86	7.88	1.557	In EPC	0.001*
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	40.69	13.79	2.759	Out EPC	0.0001*
				Invasive	0.032*
Out EPC	24.54	7.16	1.431	Invasive	0.381
Invasive	28.08	7.05	1.410	-	-

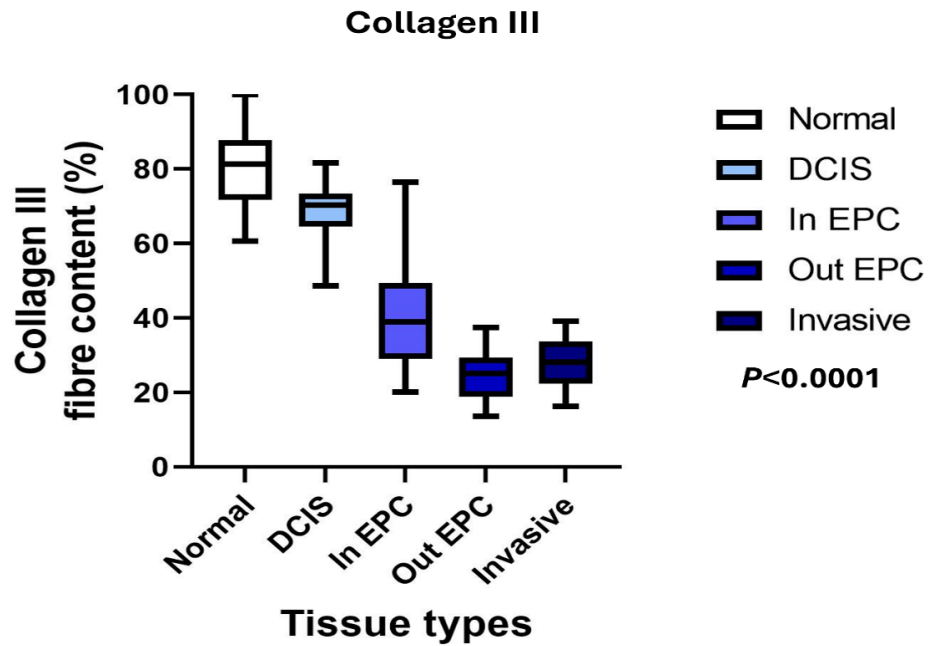


Figure 4-9: Percentage of collagen type III fibre content (compared to collagen type I) in breast tissue basement membranes.

4.3.2.4 Collagen fibre width within the BM

In comparison to the normal breast tissue control group, ($7.047 \pm 0.15 \mu\text{m}$) there was a statistically significant increase in collagen fibre width in the inner part ($7.365 \pm 0.09 \mu\text{m}$; $p < 0.0001$) and outer part of the EPC capsule ($7.785 \pm 0.15 \mu\text{m}$; $p < 0.0001$), and in the invasive group ($7.755 \pm 0.27 \mu\text{m}$; $p < 0.0001$). In contrast, compared to the normal tissue group, there was no difference in fibre width with BMs present in the DCIS samples ($7.075 \pm 0.20 \mu\text{m}$; $p < 0.982$).

There was a significant increase in collagen fibre width in both the inner part ($7.365 \pm 0.09 \mu\text{m}$; $p < 0.0001$), outer part of the EPC capsule ($7.785 \pm 0.15 \mu\text{m}$; $p < 0.0001$), and in the invasive group ($7.755 \pm 0.27 \mu\text{m}$; $p < 0.0001$) compared to BMs of DCIS group ($7.075 \pm 0.20 \mu\text{m}$).

The invasive group ($7.755 \pm 0.27 \mu\text{m}$) showed a significant increase in fibre width compared to the inner part of the capsule ($7.365 \pm 0.09 \mu\text{m}$; $p < 0.0001$) however, there was no significant difference between the

invasive group and the outer part of the capsule ($7.785 \pm 0.15 \mu\text{m}$; $p < 0.978$). One-way ANOVA test, $F = 93.111$, $p < 0.0001$, **Figure 4-10**, **Table 4-4**).

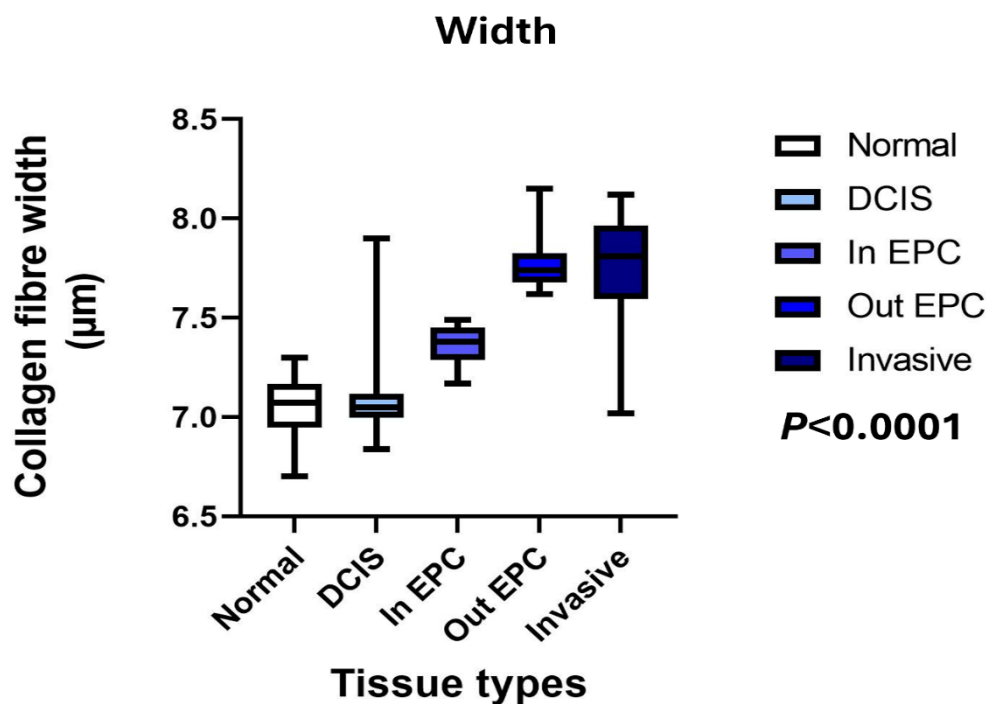


Figure 4-10: Mean width of collagen fibres of the basement membrane in breast tissue.

Table 4-4: Comparisons of the mean width fibre of the basement membrane in breast tissue.

Group	Mean of fibres width	Standard deviation (SD)	Standard Error of mean (SEM)	Comparisons	<i>P</i> value (*= <i>significance</i> = $p < 0.05$)
Normal	7.074	0.15	0.030	DCIS	0.982
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	7.075	0.20	0.039	In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	7.365	0.09	0.019	Out EPC	0.0001*
				Invasive	0.0001*
Out EPC	7.785	0.15	0.029	Invasive	0.978
Invasive	7.755	0.27	0.055	-	-

4.3.2.5 Mean length of collagen fibres within the BM

In comparison to the normal breast tissue control group ($61.66 \pm 2.78 \mu\text{m}$) with other groups, there was a statistically significant decrease in collagen fibre length of both the inner part ($57.05 \pm 1.20 \mu\text{m}$; $p < 0.0001$), and outer part of the EPC capsule ($57.59 \pm 1.62 \mu\text{m}$; $p < 0.0001$) and in the invasive carcinoma group ($51.22 \pm 3.38 \mu\text{m}$; $p < 0.0001$). However, there was no statistically significant difference with BMs in the DCIS group ($61.61 \pm 2.78 \mu\text{m}$; $p < 1$).

There was a significant decrease in collagen fibre length within the groups inner ($57.05 \pm 1.20 \mu\text{m}$; $p < 0.0001$) and outer parts of EPC capsule ($57.59 \pm 1.62 \mu\text{m}$; $p < 0.0001$), and the invasive group ($51.22 \pm 3.38 \mu\text{m}$; $p < 0.0001$) compared to those in DCIS group ($61.61 \pm 2.78 \mu\text{m}$).

The invasive group ($51.22 \pm 3.38 \mu\text{m}$) showed a significant decrease of fibre length compared to both parts of EPC capsule, inner ($57.05 \pm 1.20 \mu\text{m}$; $p < 0.0001$) and outer ($57.59 \pm 1.62 \mu\text{m}$; $p < 0.0001$), however, there was no significant fibre length difference between the different parts of the EPC capsule ($p < 0.963$). One-way ANOVA test, $F = 55.851$, $p < 0.0001$. **Figure 4-11.**

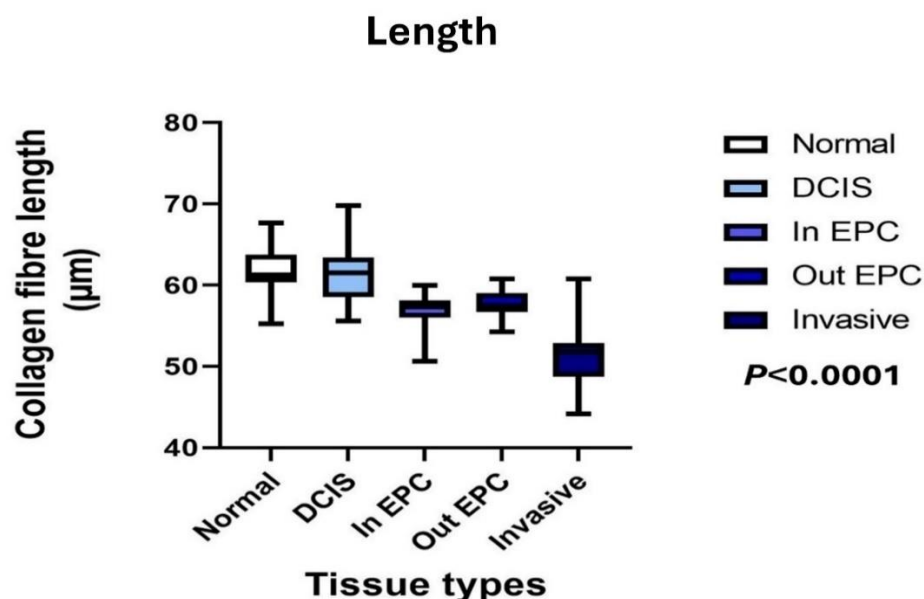


Figure 4-11: Mean collagen fibre length of the basement membrane in breast tissue.

Table 4-5: Comparisons of the mean of length fibre of the basement membrane in breast tissue.

Group	Mean of fibres length	Standard deviation (SD)	Standard Error of mean (SEM)	Comparisons	P value (*=significance = $p<0.05$)
Normal	61.66	2.78	0.557	DCIS	1
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	61.61	2.78	0.779	In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	57.05	1.20	0.399	Out EPC	0.963
				Invasive	0.0001*
Out EPC	57.59	1.62	0.325	Invasive	0.0001*
Invasive	51.22	3.38	0.677	-	-

4.3.2.6 Mean collagen fibre straightness within the BM

In comparison to the normal breast tissue control group, (0.923 ± 0.007 on a scale of 0-1) with other groups, there was a statistically significant increase in collagen fibre straightness of each DCIS (0.935 ± 0.005 on a scale of 0-1; $p < 0.0001$), inner capsule part (0.939 ± 0.007 on a scale of 0-

1; $p<0.0001$), outer capsule part (0.953 ± 0.005 on a scale of 0-1; $p<0.0001$) and the invasive group (0.943 ± 0.001 on a scale of 0-1; $p<0.0001$).

There was a significant increase of collagen fibre straightness within outer part of EPC capsule (0.953 ± 0.005 on a scale of 0-1; $p<0.0001$), and the invasive group (0.943 ± 0.0001 on a scale of 0-1; $p<0.0001$) compared to BMs of DCIS group (0.935 ± 0.005 on a scale of 0-1). In contrast, There was no significant difference in fibre straightness of the inner part of EPC capsule (0.939 ± 0.007 on a scale of 0-1; $p<0.131$) and BMs of DCIS samples.

The outer part of the EPC capsule showed increased fibre straightness compared to the invasive group (0.943 ± 0.001 on a scale of 0-1; $p<0.0001$). In addition, there was no significant difference in fibre straightness between the capsule's inner part and the invasive group (0.943 ± 0.001 on a scale of 0-1; $p<0.076$). One-way ANOVA test, $F=87.342$, $p<0.0001$, **Figure 4-12**.

Straightness

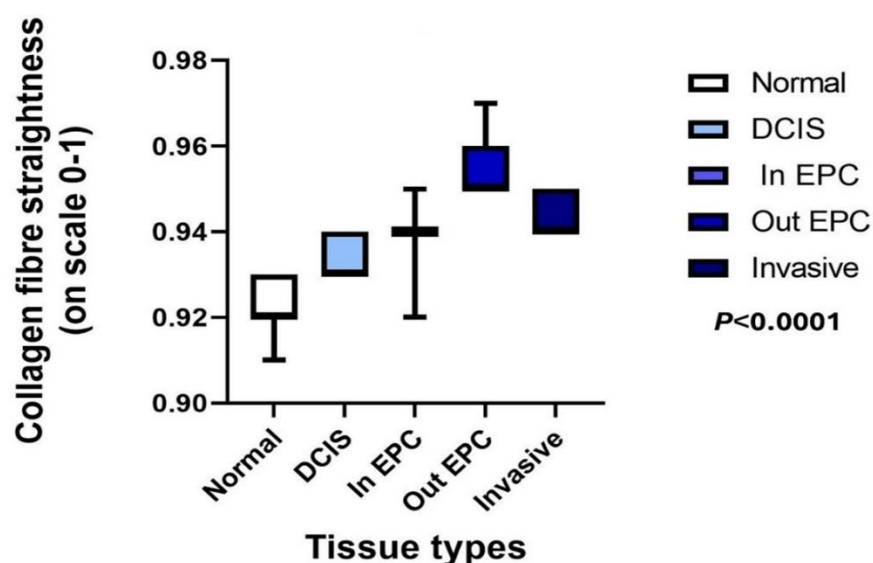


Figure 4-12: Mean fibre straightness of the basement membrane in breast tissue.

Table 4-6: Comparisons of the mean of fibre straightness of the basement membrane in breast tissue

Group	Mean of fibre width	Standard deviation (SD)	Standard Error of mean (SEM)	Comparisons	P value (*=significance = $p<0.05$)
Normal	0.923	0.007	0.001	DCIS	0.0001*
				In EPC	0.0001*
				Out EPC	0.0001*
				Invasive	0.0001*
DCIS	0.935	0.005	0.001	In EPC	0.131
				Out EPC	0.0001*
				Invasive	0.0001*
In EPC	0.939	0.007	0.001	Out EPC	0.0001*
				Invasive	0.943
Out EPC	0.953	0.005	0.001	Invasive	0.0001*
Invasive	0.943	0.001	0.001	-	-

4.3.2.7 Orientation angle of collagen fibres within the BM

In comparison to the normal breast tissue control group (Figure 4-13), all the cases within the DCIS group and inner part of EPC capsule had fewer cases where 'parallel' fibres were observed (32%, and 4% respectively, compared to 96% in the control) while outer part of EPC capsule and the invasive group had no cases where parallel fibres were

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Characterisation of Encapsulated papillary carcinoma capsule in BC

observed. Conversely, all the groups showed a higher number of cases with 'perpendicular' fibres and all the cases within the invasive group and outer part of EPC, while 96% of the cases in the inner part of EPC capsule and 32% of DCIS cases contained 'perpendicular' fibres (chi-square test $\chi^2 = 83.409$, $p < 0.0001$, **Table 4-7**).

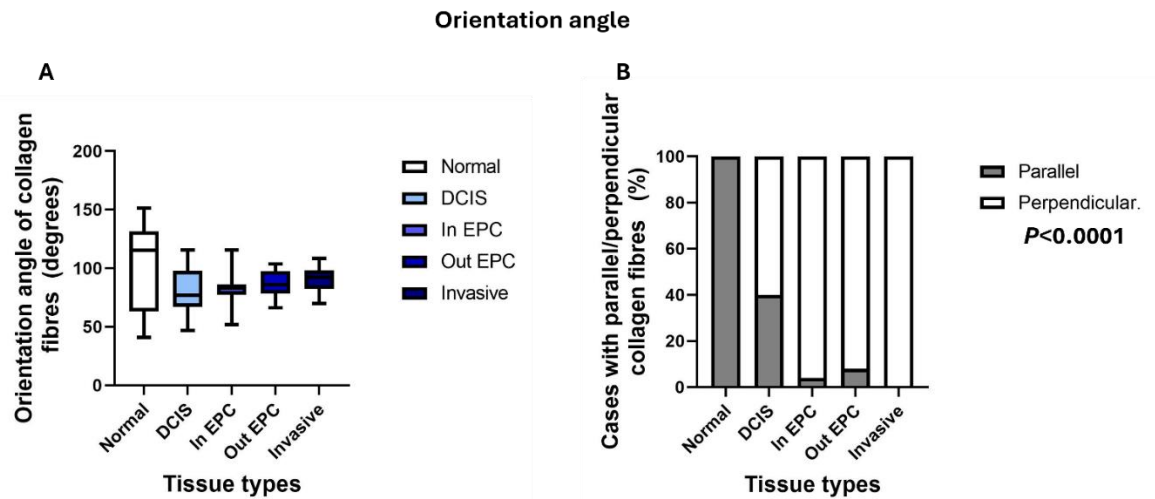


Figure 4-13: Mean orientation angle of collagen fibres of basement membrane in breast tissue.

A) The orientation angle of collagen fibres in each case. **B)** The percentages of cases with parallel and perpendicular fibres according to $70 < \text{angle} < 110$ were considered as perpendicular and < 70 or > 110 were considered parallel fibres. **Chi-square; $\chi^2 = 83.409$, $p < 0.0001$.**

Table 4-7: Comparisons of the mean orientation angle of collagen fibres in the basement membrane in breast tissue.

Group	Mean orientation angle	Standard deviation (SD)	Parallel fibres	Perpendicular fibres
Normal	102.8	32.20	100%	0%
DCIS	80.58	19.48	40%	60%
In EPC	82.73	12.09	4%	96%
Out EPC	86.77	10.70	8%	92%
Invasive	90.79	9.42	0%	100%

4.3.2.8 Alignment of collagen fibres within the BM

There was a significant decrease in fibre alignment in all the groups, DCIS (0.40 ± 0.09 on a scale of 0-1; $p < 0.0001$), inner part (0.19 ± 0.04 ; on a scale of 0-1; $p < 0.0001$) and outer parts of EPC capsule (0.29 ± 0.09 ; $p < 0.0001$) and the invasive group (0.18 ± 0.03 ; on a scale of 0-1; $p < 0.0001$) compared to normal breast tissue group (0.57 ± 0.08 on a scale of 0-1).

There was significant decrease of collagen fibres alignment within the groups outer (0.19 ± 0.04 ; on a scale of 0-1; $p < 0.0001$) and inner parts of EPC capsule (0.29 ± 0.09 ; on a scale of 0-1; $p < 0.0001$) and the invasive group (0.18 ± 0.03 ; on a scale of 0-1; $p < 0.0001$) compared to those in BMs of DCIS group (0.40 ± 0.09 on a scale of 0-1).

Outer part of the EPC capsule showed a decrease in fibre alignment compared to both the inner part (0.29 ± 0.09 ; on a scale of 0-1; $p < 0.0001$). In addition, there was no significant difference in fibre alignment between the inner part of the capsule (0.19 ± 0.04 ; on a scale of 0-1; $p < 0.0001$) and the invasive group (0.18 ± 0.03 ; on a scale of 0-1; $p < 1$). One-way ANOVA test, $F = 136.592$, $p < 0.0001$; **Figure 4-14**).

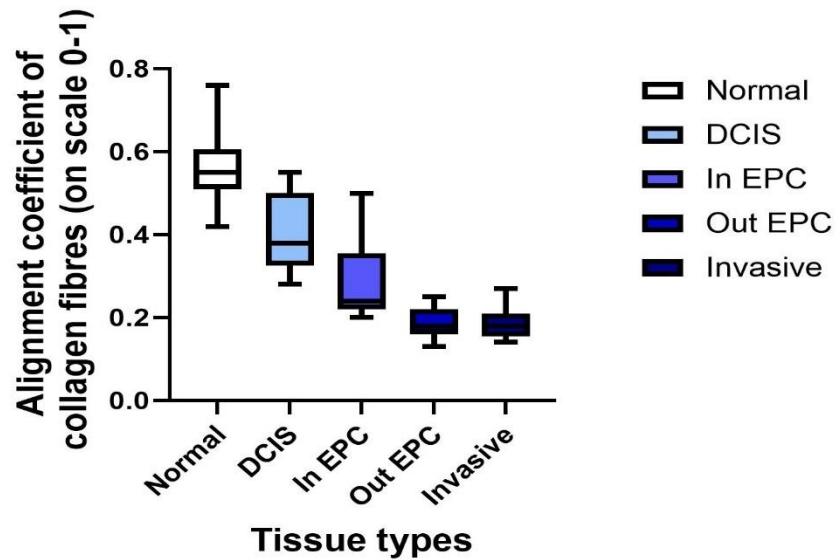


Figure 4-14: The coefficient alignment of collagen fibres in the basement membrane in breast tissue.

4.3.3 Comparison between EPC capsule and encapsulated papillary thyroid carcinoma (EPTC) capsule.

There was no significant difference between the EPC ($59.65 \pm 18.50 \mu\text{m}$) and EPTC ($57.76 \pm 13.86 \mu\text{m}$) in the thickness of their capsules ($p < 0.77$),

Figure 4-15.

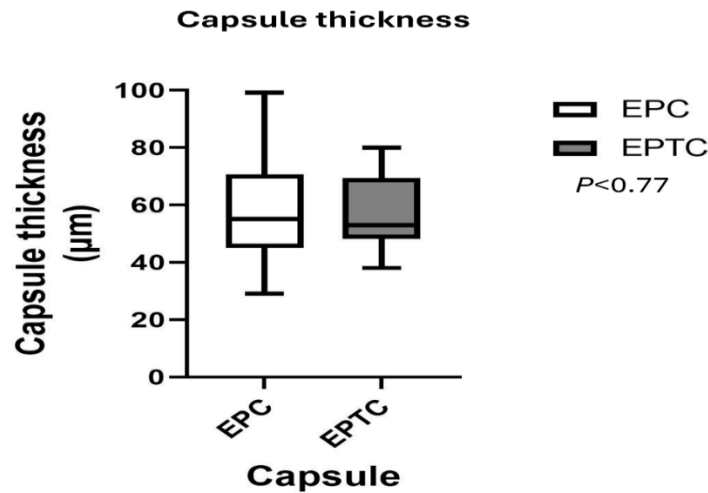


Figure 4-15: Capsule thickness.

Regarding the fibre density, there were no significant differences between EPC and EPTC capsules (106.8 ± 8.5 fibres/ μm^2 and 110.4 ± 7.4 fibres/ μm^2 ; respectively, $p < 0.25$). Regarding the fibre types, most of the collagen within both the EPC and EPTC capsules was collagen I ($75.46 \pm 7.16\%$ vs $76.43 \pm 10.31\%$ respectively; $p < 0.36$) and collagen III ($24.48 \pm 7.14\%$ vs $19.89 \pm 6.08\%$ respectively; $p < 0.06$) without any significance between both capsules, **Figure 4-16** and **Figure 4-17**.

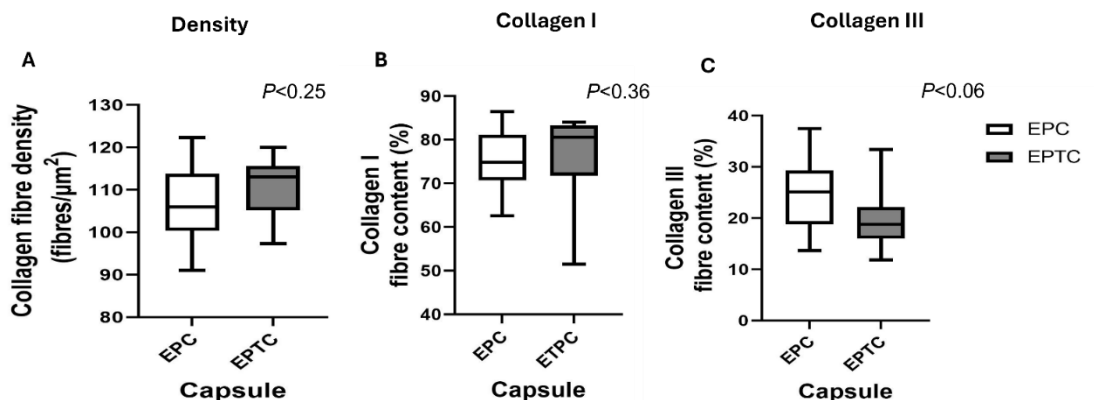


Figure 4-16: Comparison of fibre distribution between encapsulated papillary carcinoma (EPC) and encapsulated papillary thyroid carcinoma (EPTC).

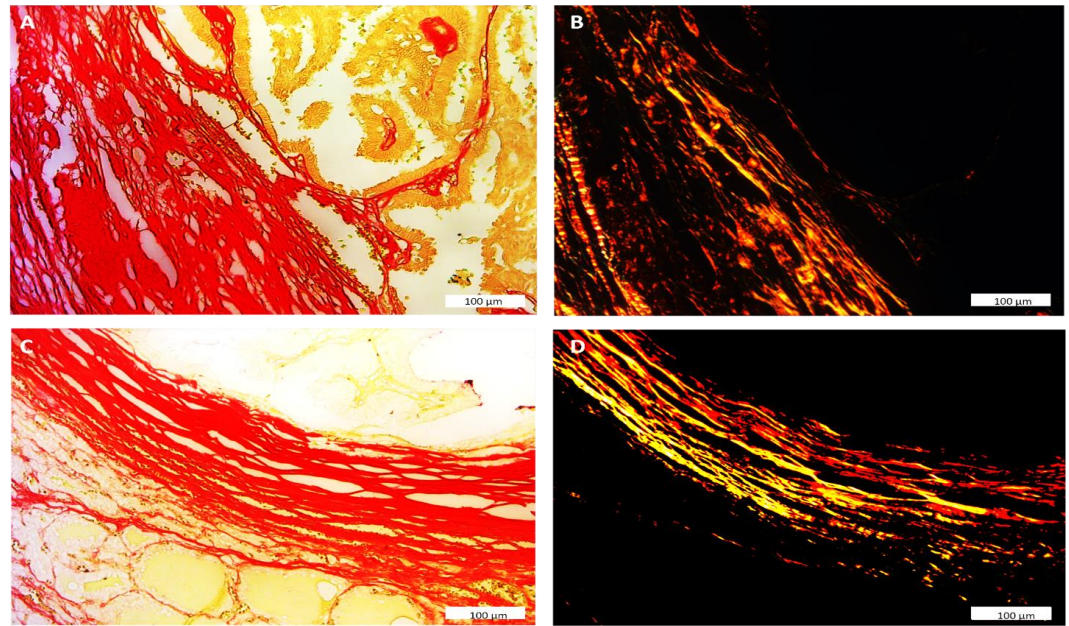


Figure 4-17: Photomicrographs of capsules within different cancer tissues.

A) Encapsulated papillary carcinoma (EPC) stained with PSR depicting part of the capsule. **B)** EPC stained with PSR and examined under polarised light showing collagen fibres expressed as orange to red fibres. **C)** Encapsulated papillary thyroid carcinoma (EPTC) stained with PSR depicting a part of the capsule surrounded by thyroid follicles. **D)** EPTC stained with PSR and examined under polarised light showing collagen fibres whose colour ranged from orange to red.

Concerning fibre characteristics, no significant differences were shown between EPC capsule fibre width ($7.78 \pm 0.15 \mu\text{m}$) and length ($56.53 \pm 2.74 \mu\text{m}$) compared to EPTC capsule fibre width ($7.61 \pm 0.35 \mu\text{m}$; $p < 0.06$, **Figure 4-18** and length ($54.85 \pm 3.59 \mu\text{m}$, $p < 0.14$), **Figure 4-18**.

In contrast, the EPC capsules had straighter fibres (0.95 ± 0.004 on a 0-1 scale) compared to the EPTC capsule (0.94 ± 0.005 on a 0-1 scale; $p < 0.034$; **Figure 4-18**).

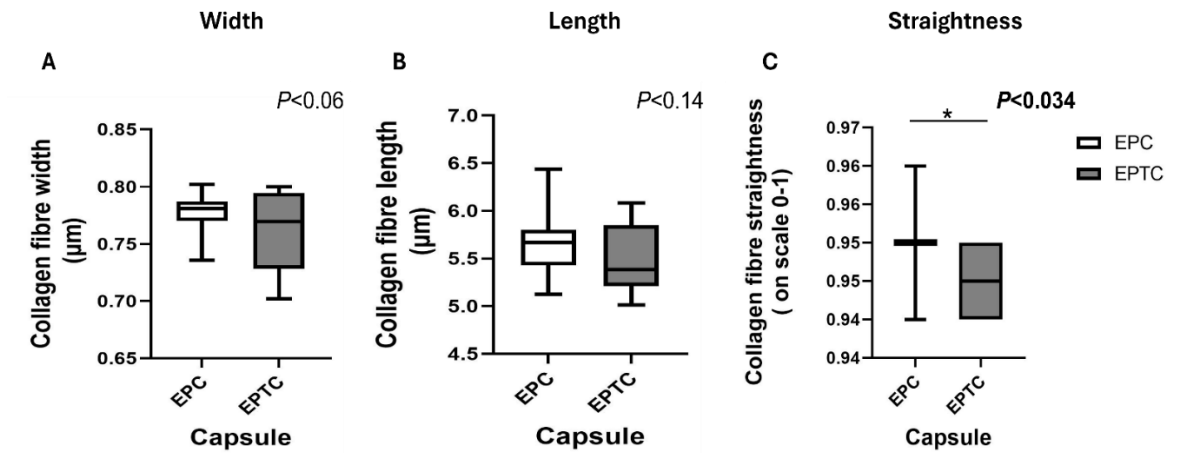


Figure 4-18: Comparison of fibre characteristics between encapsulated papillary carcinoma (EPC) and encapsulated papillary thyroid carcinoma (EPTC).

A) Collagen fibre width Independent t-test, $t = 10.46$, $p < 0.06$. **B)** Collagen fibre length Independent t-test, $t = 1.69$, $p < 0.14$. **C)** Collagen fibre straightness, Mann-Whitney U test, $U = 67.5$, $P < 0.034$.

In terms of the fibres' directionality, the fibres of EPC capsules expressed with less alignment (0.34 ± 0.07 on a scale of 0-1) compared to EPTC capsules fibres (0.38 ± 0.04 on a scale of 0-1), however, this difference was not significant ($p < 0.12$). Regards the orientation angle, about 96% of EPC capsule fibres had 'perpendicular' fibre compared to those in the EPTC capsule (80%) but also this difference was not significant, **Figure 4-19**.

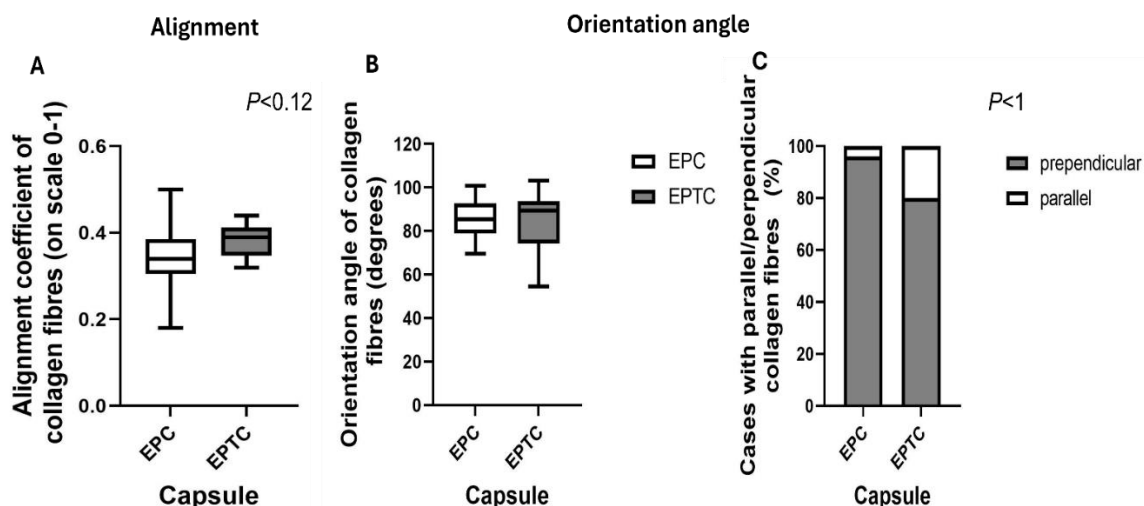


Figure 4-19: Comparison of fibre directionality between encapsulated papillary carcinoma (EPC) and encapsulated papillary thyroid carcinoma (EPTC).

A) The coefficient alignment of collagen fibres Independent t-test, $t = 2.4$, $p < 0.16$. **B)** Collagen fibre orientation angle. **C)** The percentages of cases with parallel ($< 70^\circ$ or $> 110^\circ$) or perpendicular fibres ($\geq 70^\circ - \leq 110^\circ$), Chi-square; $\chi^2 = 0.41$; $p < 1$.

4.4 Discussion

PC remains a controversial entity regarding histological categorisation, morphological features, and clinical management. Although the biological behaviour of papillary DCIS and invasive PC is well established, the categorisation of EPC and SPC into *in situ* or invasive disease is still problematic and challenging, which results in management implications, as classifying as an invasive lesion may trigger the need for adjuvant systematic therapy (484). PC is still a point of debate as research results showed a low level of concordance in pathological reports (484). Research has shown the presence of collagen IV and laminin in all cases of pure EPCs and most cases of SPCs (including invasive SPC) whilst their absence in a significant percentage of SPCs or EPCs which are associated with invasive breast cancer supports that these tumours may characterise as *in situ* lesions (476).

A study of EPC reported the presence of both collagen IV and laminin in all pure EPC cases with the absence of their expression in a significant proportion in the cases of EPCs that were associated with invasive BC. These results were used to indicate that these lesions that had a positive

expression of collagen IV and laminin may be characterised as *in situ* lesions (476). However, collagen IV and laminin can be seen in the wound healing process therefore it is not specific to the native BM characteristic of *in situ* tumours (485, 486). Additionally, a similar capsule-like structure can be observed surrounding some invasive tumour foci in tissues other than the breast which supports the theory that this is a reactive process rather than an expansion of BM (229). Moreover, some cases of typical EPC had well-developed capsules have been reported with infiltration of local muscle (233), lymph node metastasis (175, 233, 487), and distant metastases (191, 488, 489). Similar capsule-like structures were seen surrounding some of the EPC foci at these distant sites (233). These findings claim against the capsule of EPC being a native but thickened BM characteristic of the *in situ* lesions and provide further evidence that these lesions may be invasive carcinomas with an expansile growth pattern and indolent behaviour (234).

Not all sheet-like structures at the epithelial-stromal interface represent a BM. A true capsule is defined as a rim of pure fibrous tissue (composed mainly of collagen) surrounding a tumour, while a pseudo capsule is defined as a relatively mixed fibrous tissue which entraps normal structures around the tumour. A pseudo capsule is predominantly caused by the tumour's expansive growth and a combination of the mechanical effect of the surrounding tissue compression and scarring condensation which appears as a body defence mechanism against tumours. Meanwhile, the true capsule is a biological process resembling wound healing response with morphological integrity. Several tumours, including benign and malignant, even *in situ* or invasive, display an encapsulation process but, the distinction between true and pseudo capsule differs based on context (478).

The present study compared the characteristics of collagen fibres in the inner parts adjacent to the neoplastic cells and the outermost part of the thick capsule of EPC in each case showed that the outer part of the

capsule had significantly higher collagen density with more collagen I compared to the inner part. Outer capsule fibres were thicker, straighter and less aligned than the inner part with no differences in fibre length. In addition, the inner part had more 'perpendicular' fibres (88%) while the outer part of the capsule had more 'parallel' fibres (68%).

The study results showed that the BM that surrounds the normal lobules and ducts in normal breast tissue showed predominantly collagen type III fibres which were characterised by thinner, shorter, more curved fibres parallel to the duct border with good alignment. The BMs in DCIS were similar to normal tissue but revealed higher density, straighter fibres which were less aligned. Conversely, the BM-like material in the invasive group demonstrated a high collagen fibre density with increased collagen I and decreased collagen III which were characterised by shorter, thicker, straighter and less aligned fibres. EPC capsules showed heterogeneity between their inner and outer parts, the inner part exhibited the BM of DCIS only in fibre straightness while the outer part of EPC capsules showed no significant differences relating to fibre density, collagen distribution (I and III) and width compared to the invasive tumour.

A previously published study demonstrated that EPC expresses higher levels of tumour growth factor $\beta 1$ (TGF $\beta 1$) compared with DCIS and invasive tumours, which plays a key role in the development of thick fibrous capsule which supports the hypothesis that the EPC capsule is a reactive process rather than an expanded thickened native BM caused by distention of the proliferation of neoplastic papillary (234). These observations were consistent with our study results. All of the EPC capsule (inner and outer) and the invasive group cases had thicker fibres compared to the control group. In addition, this present study revealed that most of the EPC capsule collagen was type I compared to normal and DCIS BMs, with no difference shown compared to the BM-like material in invasive tumours.

Various previous studies focused on the collagen IV distribution around EPC to investigate whether this tumour was *in situ* or invasive. Wynveen and colleagues detected continuous, highly intense layers (3+ positivity) of collagen IV in only three cases of EPC however, most cases showed fragmented and discontinuous (1+, 2+) collagen IV expression and only one case showed no collagen IV reactivity (490). Golestani and colleagues reported the presence of continuous, strong, and uniform collagen IV immunostaining surrounding all cases of EPC (491). Additionally, Akladois and colleagues demonstrated the presence of BM around EPC cases via the positive staining of collagen IV (492). However, the presence of BM components around EPC cannot be considered evidence of an *in situ* nature, while this finding has been detected in a subset of invasive BC and some nodal metastases (493-496). Therefore, many authors have described that EPCs are indolent invasive cancers with a small percentage of *in situ* (235, 490, 497).

Esposito and his colleagues in their study about EPC characterisation as *in situ* or invasive on a BM basis demonstrated that there was continuous, intense collagen type IV positivity surrounding most of these lesions suggestive that EPCs are *in situ* lesions confined within a well-defined BM, this was supported by patient's outcome as pure EPC without adjacent invasive carcinoma were node-negative except one case showed micrometastasis. In this case, the possibility of an occult invasive carcinoma cannot be completely excluded (235). Esposito *et al.*, also discovered an unexpected finding which was the absence of MEC markers at the periphery papillary DCIS in three cases, however, all were surrounded by continuous, intense layers of collagen type IV. This may indicate an "early" EPC or EPC extension into smaller ducts. An explanation is that papillary lesions exert a paracrine effect on the MECs which results in the alteration of proteins used for immunohistochemical detection. However, this hypothesis is not restricted to papillary DCIS as MEC cells can be variably present at the periphery of other types of DCIS as well (235).

On the contrary, several studies have described metastases with cases of EPC, one of these reports showed lung metastases in one patient from a cohort of 21 patients, whereas in all of the others, there was no evidence of any metastasis (488). In another report, two EPC cases with lymph node micro metastases were described, one case lacking papillary architecture and the other was extensively sampled (487). Correspondingly, another study revealed that (4%) of the cohort (77 patients) with pure EPC without any stromal invasion showed distant metastasis (191). Rakha and his group described a study on pure EPC cases not associated with frank invasion, there was one case of high-grade EPC associated with recurrence, metastasis and death which reflects an underestimation of the aggressive nature of these lesions (498). In addition, Rakha *et al.*, also examined 208 cases of EPC, and there was only a 3% incidence of nodal metastasis and infrequent local recurrence or distant recurrence, thus presenting an invasion nature probability of these lesions despite excellent prognosis (233).

Renal papillary carcinoma is known as an encapsulated tumour in the kidney (499) which is surrounded by a fibromuscular capsule and has a complex composed of collagen fibres and smooth muscle (500). Previous studies have identified the presence of collagen IV in the renal pseudo-capsule (501-503). However, Lohi *et al.*, in their investigations about the expression of collagen type IV $\alpha 1(IV)$ – $\alpha 6(IV)$ in renal carcinoma, reported that renal papillary carcinoma capsules had only collagen IV alpha 5 chain (504). Another study characterised renal papillary carcinoma capsule using multi-photon microscopy and documented that there was a significant difference in fibre thickness and collagen area variation within each tumour, however, the thickest and thinnest parts showed no significant differences within collagen fibre density or fibre directionality (505). Moreover, comparisons of papillary renal carcinoma with other types showed a lower rate of capsule presence and continuous (505). Stamatiou and colleagues revealed that the capsule of renal carcinoma showed expression of collagen I & IV, myofibroblast-like SMA- and vimentin-positive cells using IHC

suggesting that this capsule was produced from mesenchymal cells (506). These conclusions are consistent with this present study's results that the EPC capsule was enriched with collagen I.

Encapsulated papillary thyroid carcinoma (EPTC), is an indolent carcinoma with rare recurrence or lymph nodal metastases (507-511), is associated with an excellent prognosis (512, 513), and is surrounded by fibrous capsules similar to the EPC. Quantitative analysis of collagen fibres via SHG microscopy demonstrated that EPTC capsules showed dispersed collagen fibres, more than observed in benign tumour capsules. In line with our results, Bayadsi *et al.*, (514) stated that EPTC capsules contained higher amounts of collagen I using IHC. In addition, some invasive tumours with indolent behaviour are characterised by the presence of fibrous capsules that contain both collagen IV and collagen I fibres including hepatocellular carcinoma (515-519) and some types of prostate adenocarcinoma (520). This was consistent with the present study results.

4.5 Conclusion

In conclusion, EPC is surrounded by BM-like material resembling that surrounding some invasive tumour types, but it is significantly distinctive from the native BM of normal breast and DCIS, which provides further evidence that EPC is an indolent invasive carcinoma, not an *in situ* lesion.

Most papillary tumours in various organs which are surrounded by BM-like material show indolent behaviour with a good prognosis; therefore, mechanisms which may explain this phenomenon should be further investigated.

5 Chapter 5: Geometric Characteristics of Stromal Collagen Fibres in Breast Cancer

This study has been published in the Journal of Microscopy with the corresponding <https://doi.org/10.1111/jmi.13361>.

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5.1 Introduction

BC, the most commonly diagnosed cancer in women worldwide (17). The diversity of BC at the morphological level is decided by evaluating particular features mainly based on the histological type of the tumour and grade, which are mainly dependent on the tumour neoplastic cell features (521). Nonetheless, the tumour cell-centric view of BC does not fully explain the heterogeneity and progression. It is progressively appreciated that the tumour-associated stroma is an integral part of tumour initiation, progression and growth (522). The response of tumour-associated stromal can act either as a physical barrier to stop cancer progression or as a facilitator of cancer progression and invasion (522).

BC stroma not only affects tumour behaviour and progression and has a prognostic significance but also influences diagnosis utilising clinical, pathological and radiological methods (1). Stromal fibrosis is the key role factor responsible for the tumour hard consistency which makes it clinically detectable via palpation and easily identified on imaging (378). On histopathological examination, the response of tumour-associated stroma, which is termed desmoplastic reaction, is considered one of the features of malignancy (523).

The stroma of most BC types is characterised by dense fibrous tissue deposition composed of newly synthesised constituents of ECM mainly the collagen fibres with or without elastic fibres (377). Moreover, the desmoplastic reaction of the stroma is not only observed in invasive BC but also seen in several cases of DCIS, specifically the high nuclear grade.

Most of the previous studies described the prognostic role of some collagen fibre characteristics in BC (348, 349) however, not all of the collagen fibre characteristics have been investigated. Moreover, the role of collagen fibres to differentiate between non-invasive and invasive lesions has not been covered. The differences between ultrastructure features of stroma in invasive and DCIS especially the collagen fibre

characteristics and their influence on tumour behaviour remains to be defined. Differentiating invasive lesions from DCIS using stromal features is clinically valuable, especially in the preoperative core biopsy specimens (244). To this end, the use of advanced microscopic techniques can be instrumental in dissecting these morphological characteristics and shed light on stroma features in different disease states.

Differential interference contrast (DIC) microscopy is a label-free imaging technique well suited to the visualization of weak optical phase objects such as cells and tissue. It is a contrast-enhancing technique based on beam shearing interference. Contrast is generated by variations in the sample's optical path (combination of refractive index and physical path length) that alter the extent of light interference between orthogonally polarised beams of light, brightening or darkening the resulting image. DIC microscopy characteristically produces pseudo-three-dimensional relief shading, or shadow relief appearance, and has become an extensively used label-free method to visualize fibrillar structures as it is inexpensive and can be superimposed on other techniques when studying fibrils without the need for staining (524).

Therefore, to understand the tumour stroma and the differences between DCIS and invasive BC stroma fibres, we investigated the collagen fibre characteristics, the most predominant constituents in the stroma, using DIC microscopy, which has the advantage of visualising collagen fibres without staining.

5.1.1 Aims and hypothesis of Work

This chapter hypothesised that characterisation of stromal ultrastructural features, particularly collagen fibre architecture, could serve as a novel methodology for differentiating between non-invasive and invasive breast lesions with prognostic role on outcomes.

To test this hypothesis, this chapter aimed to identify distinguishing characteristic features like a signature of stromal collagen fibres in DCIS and invasive carcinoma and assess the clinical and prognostic value of stromal collagen fibres in BC.

5.2 Patient and Methods

5.2.1 Specimens, preparation, and staining

TMA containing 200 cores (0.6 mm) as mentioned in **Chapter 2: Patients and Methods**, 2.4 from primary operable BC (pure DCIS, n=100 and invasive BC, n=100) patients who presented to Nottingham City Hospital between 1999 to 2006 was used in this study. Model validation was performed on an additional 50 cases including DCIS (n=25) and invasive (n=25) BC.

The samples were selected randomly, including all cases of invasive breast cancer or pure DCIS that had available tumour blocks in the archive and their TMA cores were acceptable with an adequate amount of tumour cells and tumour-associated stroma. Exclusion criteria were cores that had any missing parts or parts with normal breast tissue and those with no invasive neoplasm. In addition, DCIS cases with invasive tumours were excluded. Full and detailed clinicopathological data were available (**Table 5-1**) and (**Table 5-2**).

Table 5-1: Clinicopathological characteristics of the two study cohorts DCIS and invasive BC

Parameters	DCIS cohort Cases N (%)	Invasive cohort cases N (%)
Patient Age ≤ 50 years > 50 years	32 (32%) 68 (68%)	28 (28%) 72 (72%)
Tumor Size ≤ 2cm > 2cm	29 (29%) 71 (71%)	65 (65%) 35 (35%)
Estrogen receptor Positive (>1%) Negative (<1%) Missing	75 (75%) 15 (15%) 10 (10%)	82 (82%) 18 (18%) 0 (0%)
Progesterone receptor Positive (>1%) Negative (<1%) Missing	61 (61%) 29 (29%) 10 (10%)	53 (53%) 46 (46%) 1 (1%)
HER2 status Positive (+2, +3) Negative (< +2)	21 (21%) 79 (79%)	21 (21%) 79 (79%)
Ki67 score High (>14%) Low (<14%) Missing	14 (14%) 75 (75%) 11 (11%)	43 (43%) 38 (38%) 19 (19%)
Molecular subtypes Luminal Her2 enriched Triple negative	70 (70%) 5 (5%) 9 (9%)	72 (72%) 7 (7%) 10 (10%)
Tumour Grade Grade I / low grade Grade II / Intermediate grade Grade III / High grade	51 (16%) 33 (33%) 51 (51%)	18 (18%) 37 (37%) 45 (45%)
Histological type Ductal NST Lobular Special types		66 (66%) 6 (6%) 28 (28%)
Lymph vascular invasion (LVI) Definite Negative		35 (35%) 65 (65%)

This is the clinicopathological data of the study cohort excluding the validation cohort. The table describes the percentage of cases according to different clinicopathological parameters

Table 5-2 Clinicopathological characteristics of the validated cohorts DCIS and invasive BC

Parameters	DCIS cohort cases N (%)	Invasive cohort Cases N (%)
Patient Age ≤ 50 years > 50 years	8 (32%) 17 (68%)	10 (40%) 15 (60%)
Tumor Size ≤ 2cm > 2cm	16 (64%) 9 (36%)	5 (20%) 20 (80%)
Estrogen receptor Positive (>1%) Negative (<1%)	20 (80%) 5 (20%)	19 (76%) 6 (24%)
Progesterone receptor Positive (>1%) Negative (<1%) Missing	15 (60%) 10 (40%) 0 (0%)	10 (40%) 13 (52%) 2 (8%)
HER2 status Positive Negative	7 (79%) 18 (72%)	5 (20%) 20(80%)
Ki67 score High (>14%) Low (<14%) Missing	10 (40%) 5 (20%) 10 (40%)	13 (52%) 5 (20%) 7 (28%)
Molecular subtypes Luminal Her2 enriched Triple negative	16 (64%) 4 (16%) 5 (20%)	14 (56%) 5 (20%) 6 (24%)
Tumour Grade Grade I / low grade Grade II / Intermediate grade Grade III / High grade	10 (18%) 3 (37%) 12 (45%)	6 (16%) 8 (33%) 11(51%)
Histological type Ductal NST Lobular Special types		11(44%) 9 (36%) 5 (20%)
Lymph vascular invasion (LVI) Definite Negative		9 (36%) 16 (64%)
DCIS type - Pure DCIS - DCIS with microinvasion - DCIS with invasive tumour	12 (48%) 4 (16%) 9 (36%)	

Two 4 µm sections per case were prepared from each TMA block; one was stained with H&E stains for histological review, and the second

unstained section was examined using DIC microscopy. An Olympus IX83 inverted epifluorescent microscope fitted with a Marzhauser Scan IM 120x80 automated scanning stage was used for imaging, using a 20x/0.75 NA objective (Olympus UPlanSApo) together with a c-mounted 2x amplifying lens (Cairn Research) provided a total magnification of 40x. A sCMOS camera (Photometrics Prime 95b) was used for detection, providing a maximum field-of-view of 200 μm x 200 μm . Image acquisition (microscope stage and illumination) was synchronised using an Olympus Real-Time Controller (RTC) via Olympus CellSens software (Tokyo, Japan). ROIs were selected within each core at random from two non-overlapping areas, one centrally and the other peripherally, each measuring 64x64 pixels, the mean value for the two ROIs of each parameter was calculated (**Figure 5-1**).

Stromal collagen fibres, which are defined as the thick fibre bundles at the tumour-stromal interface, beyond the BM (525).

Outcome data in the form of BCSS, identified as the time from the initial surgical diagnosis to death related to BC, and distant metastasis-free survival DMFS, defined as the time from the initial surgical diagnosis to the event of distant metastasis (DM) were collected. The time from surgery to local recurrence is defined as breast or chest wall recurrence while regional recurrence is defined as regional lymph node recurrences, and these were calculated as local recurrence free survival (LRFS) or regional recurrence free survival, (RRFS) respectively. Mean patient follow-up in the DCIS cohort was 146 months while in invasive cohort was 372 months. Mean patient follow-up in the DCIS cohort was 146 months and the number of cases with recurrence was 15 cases while in the invasive cohort the mean follow-up was 372 months and the number of cases that died was 21.

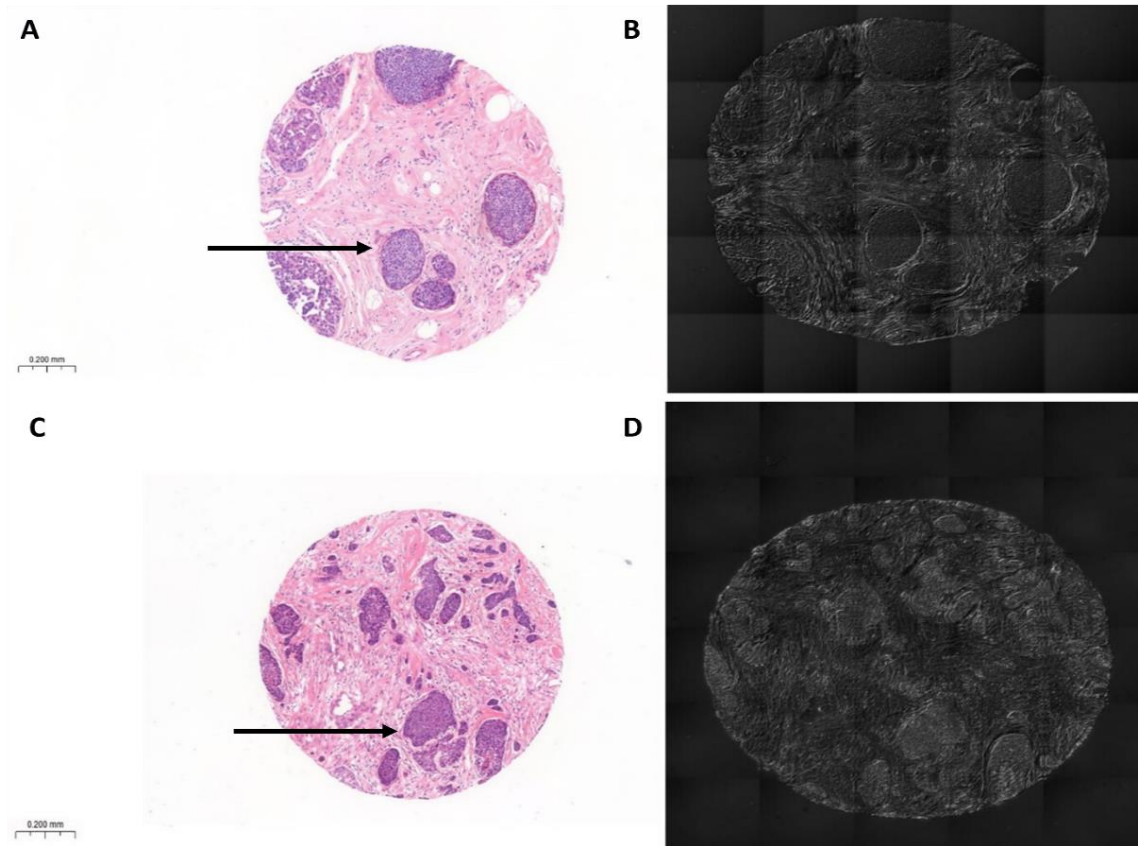


Figure 5-1: Tissue microarray cores of ductal carcinoma in situ (DCIS) and invasive breast cancer tissue.

A) Haematoxylin & eosin (H&E) stained DCIS core. **B)** Unstained DCIS section examined using differential interference contrast (DIC) microscopy showing surrounding stromal fibre features. **C)** H&E-stained invasive breast cancer core containing neoplastic cells (arrows) invading the stroma without myoepithelial cells at the epithelial stroma interface. **D)** Unstained invasive breast carcinoma section examined by DIC microscopy showing the intervening stroma between the invasive cell clusters. Magnification x40.

5.2.2 Assessment of collagen fibre features

Collagen fibre parameters such as fibre density, width, length, straightness, alignment and orientation were measured using CT-Fibre, Curve align, and Image J software as discussed before in Chapter 2 (**Figure 5-2**).

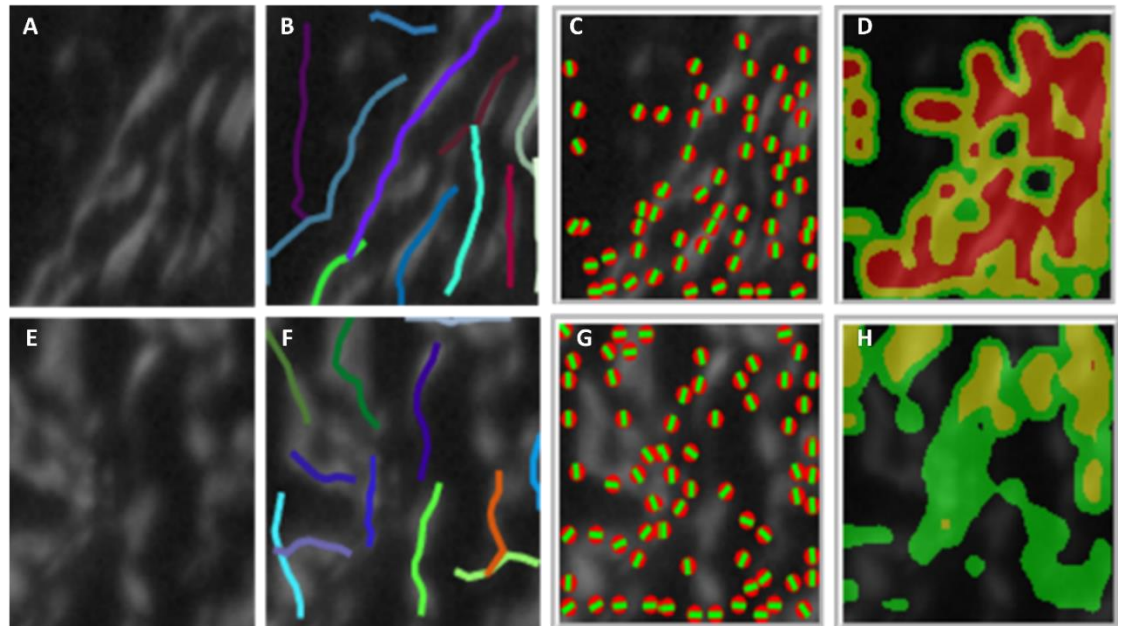


Figure 5-2: The measurement process in the CT-Fire and Curve Align programs.

A) Region of interest (ROI) area in ductal carcinoma in situ (DCIS) stroma. **B)** The same ROI after analysis using CT-fire shows highlighted extracted fibre in several colours to measure width, length, and straightness. **C)** The same ROI analysed with curve align, the image shows red dots which indicate fibres centre and green lines that indicate fibres orientation. **D)** A heat map of DCIS-ROI shows red indicating a well aligned region, whilst yellow indicates moderate aligned areas and green indicates poor aligned area. **E)** ROI in stroma of invasive breast cancer. **F)** The same image after analysis using CT-fire shows highlighted extracted fibre in several colours to measure width, length, and straightness. **G)** The same ROI analysed with curve align, the image shows red dots which indicate fibres centre and green lines that indicate haphazard fibres orientation. **H)** A heat map of invasive group ROI shows areas of green colour with poorly aligned fibres and yellow areas with moderate alignment.

5.2.3 Definitions of the fibre measurements and cut-offs

Cut-off values for collagen characteristics, including dimensions and orientations, were generated in X-tile bioinformatics software (Yale University, version 3.6.1). In the DCIS cohort based on the LRFS while in an invasive cohort based on BCSS (**Table 5-3**).

Table 5-3: The cut-offs of collagen parameters in the cohorts

Collagen parameter		DCIS cohort	Invasive cohort
Orientation angle (degrees)	Narrow	< 85.6	< 92.9
	Wide	> 85.6	> 92.9
Fibre alignment (On scale 0-1)	Poor	< 0.4	< 0.2
	Good	> 0.4	> 0.2
Fibre width (μm)	Thin	< 13.4	< 12
	Thick	>13.4	> 12
Fibre length (μm)	Short	< 132	< 122.4
	Long	> 132	> 122.4
Fibre straightness (On scale 0-1)	Curved	< 0.92	< 0.96
	Straight	> 0.92	> 0.96
Fibre density (fibre/ μm^2)	Low	<135.4	<160.6
	High	>135.4	> 160.6

The mean orientation angle (angle between fibre and horizontal line resembling the duct border), fibre alignment (the fibre direction compared to each other), mean fibre length (defined as the end-to-end length of the fibre), mean straightness (defined as the rate of curvature ranged from 0 more curve to 1 straighter), fibre density (the concentration of collagen fibres in a given area).

5.2.4 Statistical analysis

Statistical analyses were performed on the collagen fibre features in SPSS v27 (Chicago, IL, USA) using the Shapiro-Wilk test to check variable distribution and all parameters were normally distributed. Independent t-tests to compare collagen characteristics in DCIS as compared to invasive tumour lesions. Associations between collagen features and clinicopathological parameters were analysed using chi-square. Univariate survival analysis was completed using a log-rank test and Kaplan–Meier survival estimates. The Cox regression model was used for multivariate analysis. GraphPad Prism was used to create graphs and undertake these statistical test analysis (526). $P < 0.05$ was considered statistically significant.

A parametric regression model was developed in SPSS using binary logistic regression to predict whether the case is invasive or not (DCIS) based on stromal fibre characteristics.

5.3 Results

5.3.1 Collagen geometric characteristics in DCIS and invasive BC

Comparisons of the stromal collagen fibre characteristics between the DCIS and invasive BC groups showed a significantly lower fibre width and higher mean collagen fibre density ($t=10.64$, independent t-test, $t=-10.09$, respectively $p=0.0001$; **Table 5-4, Figure 5-3**) in the invasive group compared to DCIS. The invasive tumour stroma showed straighter collagen fibres than those in the stroma surrounding DCIS ducts (independent t-test, $t=-27.39$; $p=0.0001$). In contrast, there was no significant difference in stromal collagen fibre lengths between the DCIS and invasive groups (independent t-test=0.65; $p=0.45$; **Table 5-4, Figure 5-3**).

Table 5-4: Comparisons of the stromal collagen fibre geometric characteristics in the invasive and DCIS cohorts showing the comparison in the means value of each collagen parameter

Stromal collagen fibre characteristics	DCIS (N=100) (mean ± SD)	Invasive (N=100) (mean ± SD)	P value (* = significance p < 0.05)
Mean fibre width (µm)	13.78±1.08	11.9±1.38	0.0001*
Mean fibre length (µm)	126.8±15.36	125.8±15.18	0.45
Mean fibre density (fibre/µm²)	141.22±17.18	161.68±11.2	0.0001*
Mean fibre straightness (0-1 scale)	0.93±0.009	0.96±0.006	0.0001*
Mean orientation angle (degree)	85.40±9.26	95.07±11.39	0.0001*
Alignment coefficient of fibres (0-1 scale)	0.39±0.11	0.20±0.09	0.0001*

DCIS=ductal carcinoma in situ, Invasive=invasive breast carcinoma, independent t-test used to assess statistical significance. * indicates $p < 0.05$. Fibre straightness ranged between 0.90-0.93 in the DCIS cohort while it ranged from 0.95-0.98 in the invasive group producing a significant difference between the cohorts.

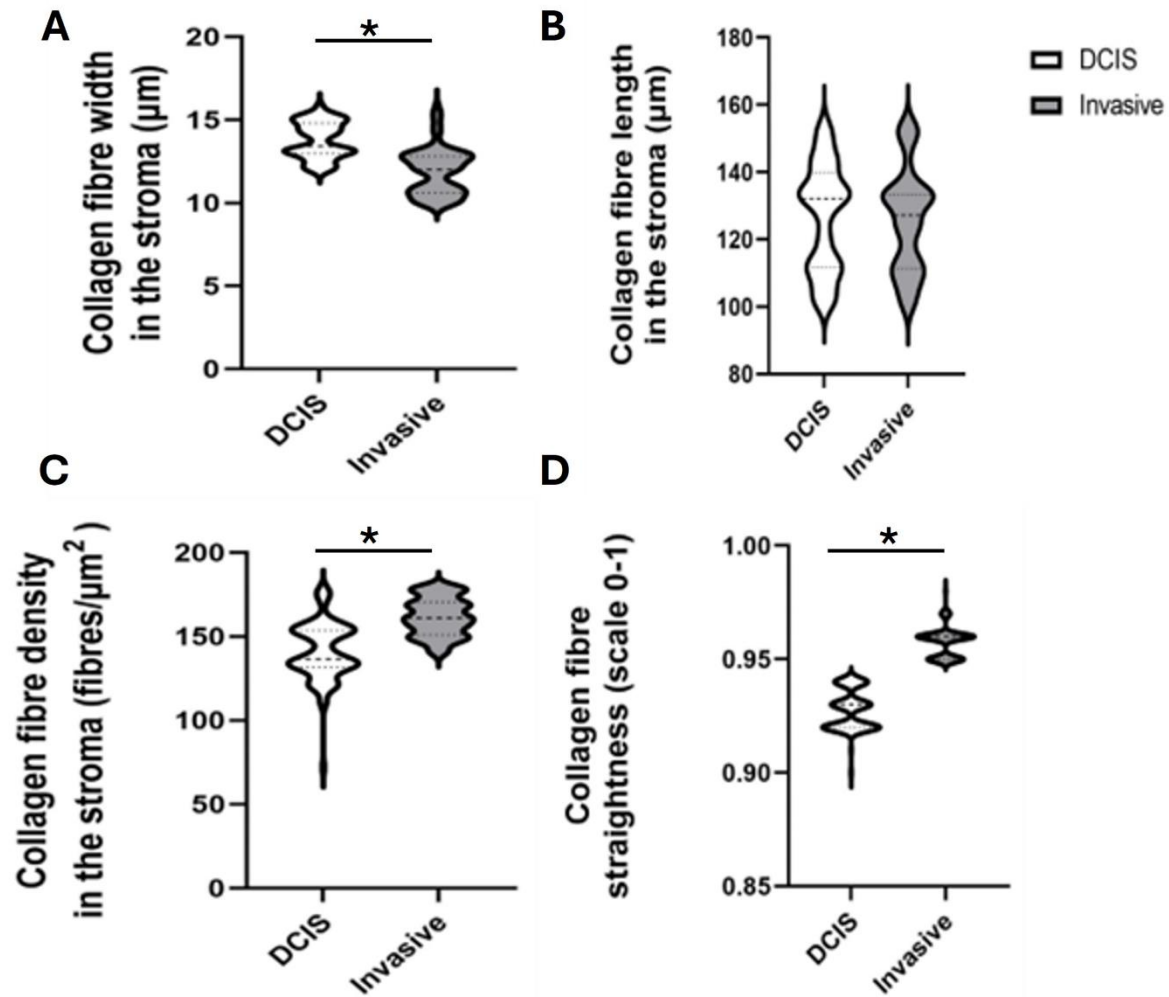


Figure 5-3: Geometric features of stromal collagen fibres in the DCIS and invasive tissues.

A) Fibre width, $t=10.64$; $p<0.0001$. **B)** Fibre length, $t=0.45$; $p<0.65$. **C)** Fibre density, $t=-10.09$; $P<0.0001$. **D)** Fibre straightness, $t=-27.39$; $p<0.0001$. **E)** Fibre orientation angle, $t=-6.59$; $p<0.0001$.

When comparing the fibres within each sample to each other, the DCIS stromal fibres were more parallel and exhibited organised fibre alignment (0.39 ± 0.11 on a 0-1 scale) compared to the fibres observed in the invasive stroma (0.20 ± 0.09 on a 0-1 scale independent t-test; $t=12.82$; $p=0.0001$; **Table 5-4, Figure 5-4**).

Concerning the direction of the stromal collagen fibre orientations, the fibres within the invasive carcinoma stroma were significantly more perpendicular, as measured against the duct border (95.07 ± 11.39

degrees), compared to those in the DCIS stroma (85.40 ± 9.26 degrees; independent t-test, $t = -6.59$; $p = 0.0001$; **Table 5-4, Figure 5-4**). Conversely, the invasive group showed a higher number of cases with 'perpendicular' fibres (95%) while the DCIS group contained more cases with 'parallel' fibres (chi-square test $\chi^2 = 47.36$, $p < 0.0001$; **Table 5-4, Figure 5-4**).

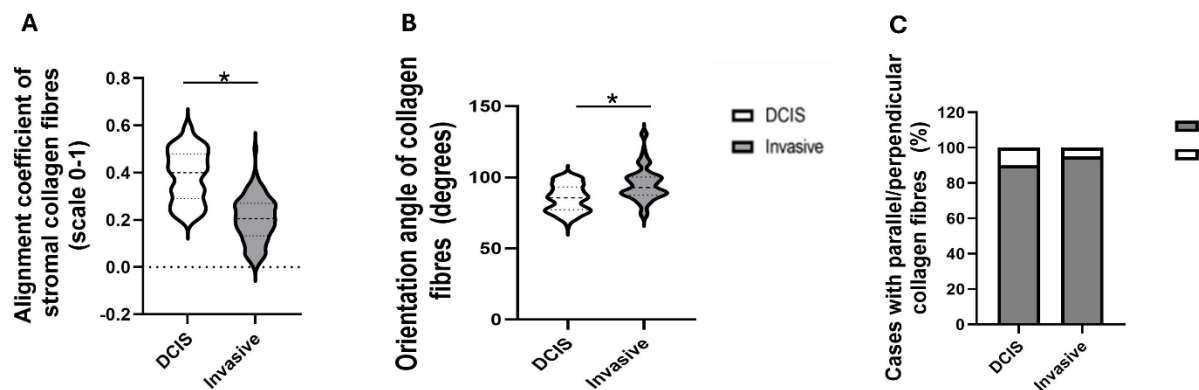


Figure 5-4: The directionality of collagen fibre in the DCIS and invasive tissues

A) Fibre orientation angle, $t = -6.59$; $p < 0.0001$. **B)** Fibre alignment, $t = 12.82$; $p < 0.0001$. **C)** The percentages of cases with parallel and perpendicular fibres according to $70 < \text{angle} < 110$ were considered as perpendicular and < 70 or > 110 were considered parallel fibres, chi-square test $\chi^2 = 47.36$, $p < 0.0001$

5.3.2 Model to differentiate between DCIS and invasive tumours

A parametric regression model was developed in SPSS using binary logistic regression to predict whether the case is invasive or not (DCIS) based on stromal fibre characteristics. The regression coefficients of the model were estimated to optimise the ability of the model to differentiate between DCIS and invasive tumour orientation angle, alignment, fibre width, and fibre density (**Table 5-5**). Nagelkerke R Square was 0.928 and Cox and Snell R square was 0.696 which indicates a good fit of the model. The goodness of fit Hosmer and Lemeshow test showed that the chi-square value was 1.23 with a significance of 0.996 which indicates a good fit model. A large value of chi-squared with a p -value < 0.05

indicates poor fit while a small chi-squared value (with a *p*-value closer to 1) indicates a good logistic regression model fit.

Table 5-5: Variables included in the model equation.

	Unstandardised coefficient (B)	Standard error (S.E.)	Significance (p-value)	Standardised coefficient Exp(B)	95% Confidence interval for EXP(B)	
					Lower	Upper
Orientation angle (degree)	0.216	0.068	0.001	1.241	1.087	1.416
Alignment (Scale 0-1)	-39.505	9.826	0.0001	0.000	.000	.0001
Width (µm)	-2.276	0.613	0.0001	0.103	.031	.341
Density (fibres/µm ²)	0.121	0.034	0.0001	1.129	1.056	1.207
Constant	3.567	7.058	0.613	35.421		

The equation would therefore be calculated as $P_{Invasive} = (3.567) + (0.216 * \text{orientation angle}) + ((-39.505) * \text{alignment}) + ((-2.276) * \text{width}) + (0.121 * \text{density})$ if the $P_{Invasive}$ score was less than 0.5, so the case would be DCIS, whereas in cases more than 0.5 it would be an invasive tumour.

To test the model, performance analysis based on receiver operating characteristic (ROC) was used. The area under the curve (AUC) was 0.984 ($p=0.0001$) with sensitivity 98 and specificity 95 according to the AUC table which indicated good discrimination of the model (**Figure 5-5**). A classification matrix table was formed to measure the model performance, **Table 5-6**.

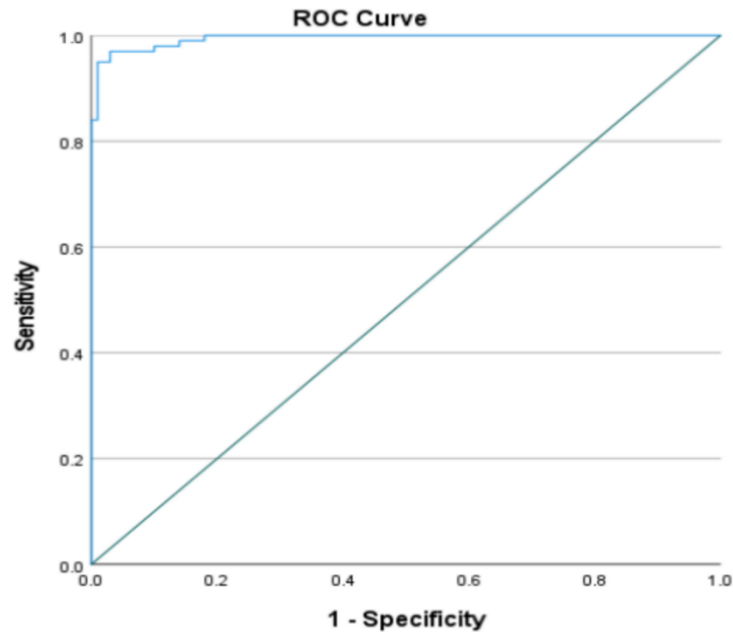


Figure 5-5: The Receiver operating characteristic (ROC) curve showed model performance for internal validation of the training cohort, AUC 0.984, $p < 0.0001$.

Table 5-6: Classification matrix for sensitivity and specificity of the collagen characteristic model for the main cohort.

		Predicted		Percentage of true predicted
		DCIS	Invasive	
Observed	DCIS (N=100)	97 (TN)	3 (FP)	97.0
	Invasive (N=100)	3 (FN)	97 (TP)	97.0
Overall Percentage				97.0
Sensitivity		$(TP)/(TP+FN) = 97/(97+3) = 97\%$		
Specificity		$(TN)/(TN+FP) = 97/(97+3) = 97\%$		
Accuracy		$TP+TN/TP+TN+FP+FN = 97+97/200 = 97\%$		
Precision		$TP/(TP+FP) = 97\%$		

The cut value was 0.500. DCIS=ductal carcinoma in situ. TN=True negative, FP=False positive, FN=False negative, TP=True positive. TP=number of cases that had a probability to be invasive and were invasive. TN=number of cases that had a probability to be DCIS and were DCIS. FP=number of cases that had a probability to be invasive but were DCIS. FN=number of cases that had a probability to be DCIS but were invasive.

An independent (validation) cohort (50 cases) was analysed for collagen fibre features, then the estimated equation was used to predict their type. ROC was performed to measure the performance of the model and AUC

was 0.894 ($p=0.001$) which indicates good discrimination. A classification matrix table was formed to measure the model performance. Sensitivity was 96% while specificity was 92%, accuracy was 94% and precision was 96% (Table 5-7).

Table 5-7: Classification matrix for sensitivity and specificity of the collagen fibre characteristics model for the validation cohort.

		Predicted		Percentage of true predicted
		DCIS	Invasive	
Observed	DCIS (N=25)	23 (TN)	2 (FP)	92.0
	Invasive (N=25)	1 (FN)	24 (TP)	96.0
Overall Percentage				94.0
Sensitivity		$(TP)/(TP+FN) = 24/(24+1) = 96\%$		
Specificity		$(TN)/(TN+FP) = 23/(23+2) = 92\%$		
Accuracy		$TP+TN/TP+TN+FP+FN = 24+23/50 = 94\%$		
Precision		$TP/(TP+FP) = 96\%$		

The cut value was 0.500. DCIS=ductal carcinoma in situ. TN=True negative, FP=False positive, FN=False negative, TP=True positive. TP=number of cases that had a probability to be invasive and were invasive. TN=number of cases that had a probability to be DCIS and were DCIS. FP=number of cases that had a probability to be invasive but were DCIS. FN=number of cases that had a probability to be DCIS but were invasive.

5.3.3 Association of stromal collagen characteristics with clinicopathological data in DCIS

Correlations between collagen features and clinicopathological parameters are presented in (Table 5-8 & Table 5-9). Specific fibre features including wide orientation angle (defined as $>90^\circ$), poorly aligned, thicker, longer straighter fibres with a higher fibre density, as described in the materials and methods, were associated with features of aggressive DCIS behaviour including higher nuclear grade, larger DCIS size, HER2 positivity, hormone receptor negativity, TNBC subtype and high proliferation status (high ki67 score). DCIS heterogeneity in pattern and grade was only associated with a wide orientation angle and longer fibres; but not with the other parameters (Table 5-8 & Table 5-9).

Table 5-8: Correlation of fibre directionality against clinicopathological data in the DCIS cohort.

Parameter	Orientation angle (Degrees)			Alignment (Scale 0-1)			Straightness (Scale 0-1)		
	Narrow	Wide	<i>P</i> value	Poor	Good	<i>P</i> value	Low	High	<i>P</i> value
Patient age <50 years >50 years	18(57%) 38(56%)	14(43%) 30(44%)	<i>P</i> <0.97	18(56%) 33(48%)	14(44%) 35(52%)	<i>P</i> <0.47	13(41%) 33(49%)	19(59%) 35(51%)	<i>P</i> <0.45
Size < 2cm >2cm	20(69%) 36(51%)	9(31%) 35(49%)	<i>P</i> <0.09	8(28%) 43(61%)	21(72%) 28(39%)	<i>P</i> <0.003*	21(72 %) 25(35%)	8(28%) 46(65%)	<i>P</i> <0.001*
Grade Low Intermediate High	11(69%) 30(91%) 15(29%)	5(31%) 3(9%) 36(71%)	<i>P</i> <0.001*	0(0%) 2(6%) 49(96%)	16(100%) 31(94%) 2(4%)	<i>P</i> <0.001*	16(100%) 27(82%) 3(6%)	0(0%) 6(18%) 48(94%)	<i>P</i> <0.001*
Heterogeneity Absence Presence	52(55%) 4(80%)	43(45%) 1(20%)	<i>P</i> <0.026	47(49%) 4(80%)	48(51%) 1(20%)	<i>P</i> <0.18	45(47%) 1(20%)	50(53%) 4(80%)	<i>P</i> <0.23
Comedo Necrosis type Absence Presence	28(70%) 28(47%)	12(30%) 32(53%)	<i>P</i> <0.021*	8(20%) 43(72%)	32(80%) 17(28%)	<i>P</i> <0.001*	31(77%) 15(25%)	9(23%) 45(75%)	<i>P</i> <0.001*
Type Not mixed Mixed	24(55%) 32(57%)	20(45%) 24(43%)	<i>P</i> <0.79	23(52%) 28(50%)	21(48%) 28(50%)	<i>P</i> <0.82	21(48%) 25(45%)	23(52%) 31(55%)	<i>P</i> <0.75
Molecular subtypes Luminal A Luminal B Her2 enriched TNBC	47(75%) 2(29%) 1(20%) 1(11%)	16(25%) 5(71%) 4(80%) 8(89%)	<i>P</i> <0.001*	23(37%) 5(71%) 4(80%) 9(100%)	40(63%) 2(29%) 1(20%) 0(0%)	<i>P</i> <0.001*	40(63%) 2(29%) 1(20%) 0(0%)	23(37%) 5(71%) 4(80%) 9(100%)	<i>P</i> <0.001*
ER receptor status Negative Positive	2(13 %) 51(68%)	13(87%) 24(32%)	<i>P</i> <0.001*	14(93%) 30(40%)	1(7%) 45(60%)	<i>P</i> <0.001*	1(7%) 43(57%)	14(93%) 32(43%)	<i>P</i> <0.001*
PR receptor status Negative Positive	6(21%) 47(77%)	23(79%) 14(23%)	<i>P</i> <0.001*	25(86%) 19(31%)	4(14%) 42(69%)	<i>P</i> <0.001*	2(7%) 41(67%)	27(93%) 20(33%)	<i>P</i> <0.001*
HER receptor status Negative Positive	48(63.2%) 4(28.6%)	28(36.8%) 10(71.4%)	<i>P</i> <0.03*	36(47.4%) 10(71.4%)	40(52.6%) 4(28.6%)	<i>P</i> <0.25	40(53%) 3(21%)	36(47%) 11(79%)	<i>P</i> <0.032*
Ki67 score Low High	49(65%) 2(14%)	26(35%) 12(86%)	<i>P</i> <0.001*	32(43%) 13(93%)	43(57%) 1(7%)	<i>P</i> <0.003*	41(55%) 1(7%)	34(45%) 13(93%)	<i>P</i> <0.001*

* indicates *p*<0.05.

Table 5-9: Correlation of fibre characteristics compared to the clinicopathological data in the DCIS cohort.

Parameter	Fibre width (μm)			Fibre length (μm)			Fibre density (fibres/ μm^2)		
	Thin	Thick	<i>P</i> value	Short	Long	<i>P</i> value	Low	High	<i>P</i> value
Patient age <50 years >50 years	14(44%) 36(53%)	18(56%) 32(47%)	<i>P</i> <0.93	15(47%) 37(54%)	17(53%) 31(46%)	<i>P</i> <0.48	15(47%) 31(46%)	17(53%) 37(54%)	<i>P</i> <0.90
Size < 2cm >2cm	22(76%) 28(39%)	7(24%) 43(61%)	<i>P</i> <0.001*	22(76%) 30(42%)	7(24%) 41(58%)	<i>P</i> <0.002*	19(66%) 27(38%)	10(34%) 44(62%)	<i>P</i> <0.012*
Grade Low Intermediate High	16(100%) 26(79%) 8(16%)	0(0%) 7(21%) 43(84%)	<i>P</i> <0.001*	15(94%) 22(67%) 15(29%)	1(6%) 11(33%) 36(71%)	<i>P</i> <0.001*	16(100%) 27(82%) 3(6%)	0(0%) 6(18%) 48(94%)	<i>P</i> <0.001*
Heterogeneity Absence Presence	49(52%) 1(20%)	46(48%) 4(80%)	<i>P</i> <0.16	47(49%) 5(100%)	48(51%) 0(0%)	<i>P</i> <0.028	45(47%) 1(20%)	50(53%) 4(80%)	<i>P</i> <0.23
Comedo necrosis type Absence Presence	31(78%) 19(32%)	9(22%) 41(68%)	<i>P</i> <0.001*	29(73%) 23(38%)	11(27%) 37(62%)	<i>P</i> <0.001*	31(78%) 15(25%)	9(22%) 45(75%)	<i>P</i> <0.001*
Type Not mixed Mixed	24(55%) 26(46%)	20(45%) 30(54%)	<i>P</i> <0.42	27(61%) 25(45%)	17(39%) 31(55%)	<i>P</i> <0.09	20(46%) 26(46%)	24(54%) 30(54%)	<i>P</i> <0.92
Molecular subtypes Luminal A Luminal B Her2 enriched TNBC	42(67%) 2(29%) 1(20%) 1(11%)	21(33%) 5(71%) 4(80%) 8(89%)	<i>P</i> <0.001*	42(67%) 3(43%) 0(0%) 2(22%)	21(33%) 4(57%) 5(100%) 7(78%)	<i>P</i> <0.002*	38(60%) 2(28.6%) 1(20%) 0(0%)	25(40%) 5(71%) 4(80%) 9(100%)	<i>P</i> <0.001*
ER receptor status Negative Positive	2(13%) 45(60%)	13(87%) 30(40%)	<i>P</i> <0.002*	2(13%) 47(63%)	13(87%) 28(37%)	<i>P</i> <0.001*	1(7%) 42(56%)	14(93%) 32(44%)	<i>P</i> <0.001*
PR receptor status Negative Positive	4(14%) 41(67%)	25(86%) 20(33%)	<i>P</i> <0.001*	5(17%) 43(70%)	24(83%) 18(30%)	<i>P</i> <0.001*	3(10%) 41(67%)	26(90%) 20(33%)	<i>P</i> <0.001*
HER receptor status Negative Positive	43(57%) 3(21%)	33(43%) 11(79%)	<i>P</i> <0.043*	44(58%) 3(21%)	32(42%) 11(79%)	<i>P</i> <0.043*	38(50%) 4(29%)	38(50%) 10(71%)	<i>P</i> <0.30
Ki67 score Low High	43(57%) 0(0%)	32(43%) 14(100%)	<i>P</i> <0.001*	44(59%) 3(21%)	31(41%) 11(79%)	<i>P</i> <0.034*	41(55%) 1(7%)	34(45%) 13(93%)	<i>P</i> <0.004*

* indicates $p < 0.05$.

5.3.4 Association of stromal collagen characteristics with clinicopathological data in invasive group

Associations between specific stromal collagen parameters and the clinicopathologic data in the invasive BC group were presented in **Table 5-10** and **Table 5-11**.

In invasive tumours, the orientation angle was associated with histological grade only ($p < 0.001$). Poor collagen fibre alignment, thicker collagen fibre widths, longer fibres and a higher degree of fibre straightness were associated with the clinicopathological parameters characteristic of aggressive behaviour such as higher histological grade, higher NPI scores, molecular subtypes (TNBC), NST tumour subtype, LVI, high stage, ER and PR negativity, and high ki67. High collagen fibre density was associated with some clinicopathological parameters characteristic of aggressive behaviour such as large tumours, high grade, high stage poor NPI, LVI, NST histological subtype, ER negativity, HER2 positivity, and high ki67.

Table 5-10: Correlation of fibre directionality compared to the clinicopathological data in the invasive cohort.

Parameter	Orientation angle (Degrees)			Alignment (Scale 0-1)			Straightness (Scale 0-1)		
	Narrow	Wide	<i>P value</i>	Good	Poor	<i>P value</i>	Low	High	<i>P value</i>
Patient age <50 years >50 years	10 (36%) 28 (39%)	18(64%) 44(61%)	<i>P</i> <0.76	20(71%) 62(86%)	8(29%) 10(14%)	<i>P</i> <0.08	6(21) 28(39%)	22(79%) 44(61%)	<i>P</i> <0.098
Tumour size < 2cm >2cm	23(36%) 15(43%)	42(64%) 20(57%)	<i>P</i> <0.46	56(86%) 26(74%)	9(14%) 9(26%)	<i>P</i> <0.14	30(46%) 4(11%)	35(54%) 33(89%)	<i>P</i> <0.001*
Tumour grade Grade I Grade II Grade III	17(94%) 11(30%) 11(24%)	1 (6%) 26 (70%) 34(76%)	<i>P</i> <0.001*	18(100%) 36(97%) 28(62%)	0(0%) 1(3%) 17(38%)	<i>P</i> <0.001*	18(100%) 14(38%) 2(4%)	0(0%) 23(62%) 43(96%)	<i>P</i> <0.001*
Stage Stage I Stage II Stage III	16(33%) 18(51%) 4(24%)	32(66.7%) 17(48.6%) 13(76.5%)	<i>P</i> <0.09	40(83%) 29(83%) 13(77%)	8(17%) 6(17%) 4(23%)	<i>P</i> <0.80	18(37%) 15(43%) 1(6%)	30(63%) 20(57%) 16(94%)	<i>P</i> <0.024*
NPI groups Good Moderate Poor	11 (32%) 20 (46%) 7 (32%)	23(68%) 24(54%) 15(68%)	<i>P</i> <0.39	33(97%) 35(79%) 14(64%)	1(3%) 9(20%) 8(36%)	<i>P</i> <0.005*	22(65%) 12(27%) 0(0%)	12(35%) 32(73%) 22(100%)	<i>P</i> <0.001*
LVI Negative Definite	28 (43%) 10 (29%)	37(57%) 25(71%)	<i>P</i> <0.15	58(89%) 24(69%)	7 (11%) 11(31%)	<i>P</i> <0.01*	31(48%) 3(9%)	34(52%) 32(91%)	<i>P</i> <0.001*
Histological type NST Lobular Special	24(36%) 3(50%) 11(39%)	42(64%) 3(50%) 17(61%)	<i>P</i> <0.79	49(74%) 6(100%) 27(96%)	17(26%) 0(0%) 1(4%)	<i>P</i> <0.019*	15(23%) 3(50%) 16(57%)	51(77%) 3(50%) 12(43%)	<i>P</i> <0.004*
Molecular subtypes Luminal A Luminal B Her2+ TNBC	14(41%) 17(45%) 2(29%) 1(10%)	20(59%) 21(55%) 5(71%) 9(90%)	<i>P</i> <0.21	32(94%) 29(76%) 2 (29%) 4(40%)	2(6%) 9(24%) 5(71%) 6(60%)	<i>P</i> <0.055	20(59%) 10(26%) 0(0%) 1(10%)	14(41%) 28(73%) 7(100%) 9(90%)	<i>P</i> <0.001*
ER receptor status Negative Positive	3(17%) 35(43%)	15(83%) 47(57%)	<i>P</i> <0.039*	12(67%) 70(85%)	6(33%) 12(15%)	<i>P</i> <0.06	1(6%) 33(40%)	17(94%) 49(60%)	<i>P</i> <0.005*
PR receptor status Negative Positive	14 (30%) 24 (45%)	32(70%) 29(55%)	<i>P</i> <0.13	34(74%) 47(89%)	12(26%) 6(11%)	<i>P</i> <0.057	10(22%) 24(45%)	36(78%) 29(55%)	<i>P</i> <0.014*
HER receptor status Negative Positive	31 (39%) 7 (33%)	48(61%) 14(67%)	<i>P</i> <0.62	68(86%) 14(67%)	11(14%) 7(33%)	<i>P</i> <0.04*	31(39%) 3(14%)	48(61%) 18(86%)	<i>P</i> <0.032*
Ki67 score Low High	15 (39%) 17 (39%)	23(61%) 26(61%)	<i>P</i> <0.99	35(92%) 33(77%)	3(8%) 10(23%)	<i>P</i> <0.06	21(55%) 9(21%)	17(45%) 34(79%)	<i>P</i> <0.001*

* indicates *p*<0.05.

Table 5-11: Correlation of fibre characteristics compared to clinicopathological data in the invasive cohort.

Parameter	Fibre width (µm)			Fibre length (µm)			Fibre density (fibres/µm ²)		
	Thin	Thick	<i>P</i> value	Short	Long	<i>P</i> value	Low	High	<i>P</i> value
Patient age <50 years >50 years	11(39%) 43(59%)	17(61%) 29(40%)	<i>P</i> <0.06	18(64%) 21(29%)	10(36%) 51(71%)	<i>P</i> <0.001*	11(39%) 29(40%)	17(61%) 43(60%)	<i>P</i> <0.33
Tumour size < 2cm >2cm	42(64%) 12(34%)	23(36%) 23(66%)	<i>P</i> <0.004*	22(34%) 17(49%)	43(66%) 18(51%)	<i>P</i> <0.15	38(58%) 2(6%)	27(42%) 33(94%)	<i>P</i> <0.004*
Tumour grade Grade I Grade II Grade III	18(100%) 27(73%) 9(20%)	0(0%) 10(22%) 36(80%)	<i>P</i> <0.001*	0(0%) 6(16%) 33(73%)	18(100%) 31(84%) 12(27%)	<i>P</i> <0.001*	18(100%) 20(54%) 2(4%)	0(0%) 17(46%) 43 (96%)	<i>P</i> <0.001*
Stage Stage I Stage II Stage III	31(65%) 21(60%) 2(12%)	17(35%) 14(40%) 15(88%)	<i>P</i> <0.001*	15(31%) 12(34%) 12(71%)	33(69%) 23(66%) 5(29%)	<i>P</i> <0.01*	27(56%) 12(34%) 1(6%)	21(44%) 23(66%) 16(94%)	<i>P</i> <0.005*
NPI groups Good Moderate Poor	31(91%) 20(46%) 3(14%)	3 (9%) 24(54%) 19(86%)	<i>P</i> <0.001*	3(9%) 19(43%) 17(77%)	31(91%) 25(87%) 5(23%)	<i>P</i> <0.001*	28(82%) 10(23%) 2(9%)	6(18%) 34(77%) 20(91%)	<i>P</i> <0.001*
LVI Negative Definite	43(66%) 11(31%)	22(34%) 24(69%)	<i>P</i> <0.001*	18(28%) 21(60%)	47(72%) 14(40%)	<i>P</i> <0.002*	35(54%) 5(14%)	30(46%) 30(86%)	<i>P</i> <0.004*
Histological type NST Lobular Special	29(44%) 3(50%) 22(79%)	37(56%) 3(50%) 6(21%)	<i>P</i> <0.009*	32(49%) 3(50%) 4(14%)	34(51%) 3(50%) 24(86%)	<i>P</i> <0.007*	20(30%) 2(33%) 18(64%)	46(70%) 4(67%) 10(36%)	<i>P</i> <0.001*
Molecular subtypes Luminal A Luminal B Her2 TNBC	31(91%) 14(37%) 2(29%) 1(10%)	3(9%) 24(63%) 5(71%) 9(90%)	<i>P</i> <0.001*	2(6%) 20(52%) 5(71%) 8(80%)	32(94%) 18(48%) 2(29%) 2(20%)	<i>P</i> <0.001*	24(71%) 9(24%) 2(29%) 0(0%)	10(30%) 29(76%) 5(71%) 9(100%)	<i>P</i> <0.001*
ER receptor status Negative Positive	3(17%) 51(62%)	15(84%) 31(38%)	<i>P</i> <0.001*	14(78%) 25(31%)	4(22%) 57(69%)	<i>P</i> <0.001*	0(0%) 40(49%)	18(100%) 42(51%)	<i>P</i> <0.016*
PR receptor status Negative Positive	19(41%) 35(66%)	27(86%) 18(34%)	<i>P</i> <0.014*	24(52%) 14(26%)	22(48%) 39(74%)	<i>P</i> <0.009*	11(24%) 29(55%)	35(76%) 24(45%)	<i>P</i> <0.18
HER receptor status Negative Positive	50(63%) 4(19%)	29(37%) 17(81%)	<i>P</i> <0.001*	25(32%) 14(67%)	54(68%) 7(33%)	<i>P</i> <0.003*	36(46%) 4(19%)	43(44%) 17(81%)	<i>P</i> <0.003*
Ki67 score Low High	32(84%) 15(35%)	6(16%) 28(65%)	<i>P</i> <0.001*	5(13%) 26(60%)	33(87%) 17(40%)	<i>P</i> <0.001*	27(71%) 15(35%)	11(29%) 28(65%)	<i>P</i> <0.001*

* indicates *p*<0.05.

5.3.5 Association of stromal collagen characteristics with outcome in DCIS group

No associations between any of the fibre parameters (orientation angle, alignment, width, length, straightness, or density) and patients' outcomes in the DCIS cohort were identified ($p>0.05$).

5.3.6 Association of stromal collagen characteristics with outcome in invasive group

In invasive BC, a wide fibre orientation angle was associated with shorter BCSS (HR=0.17, 95% CI= 0.05 to 0.51, $p=0.042$; **Figure 5-6**) and shorter RRFS (HR=0.07, 95% CI= 0 to 0.009, $p=0.012$; **Figure 5-7**).

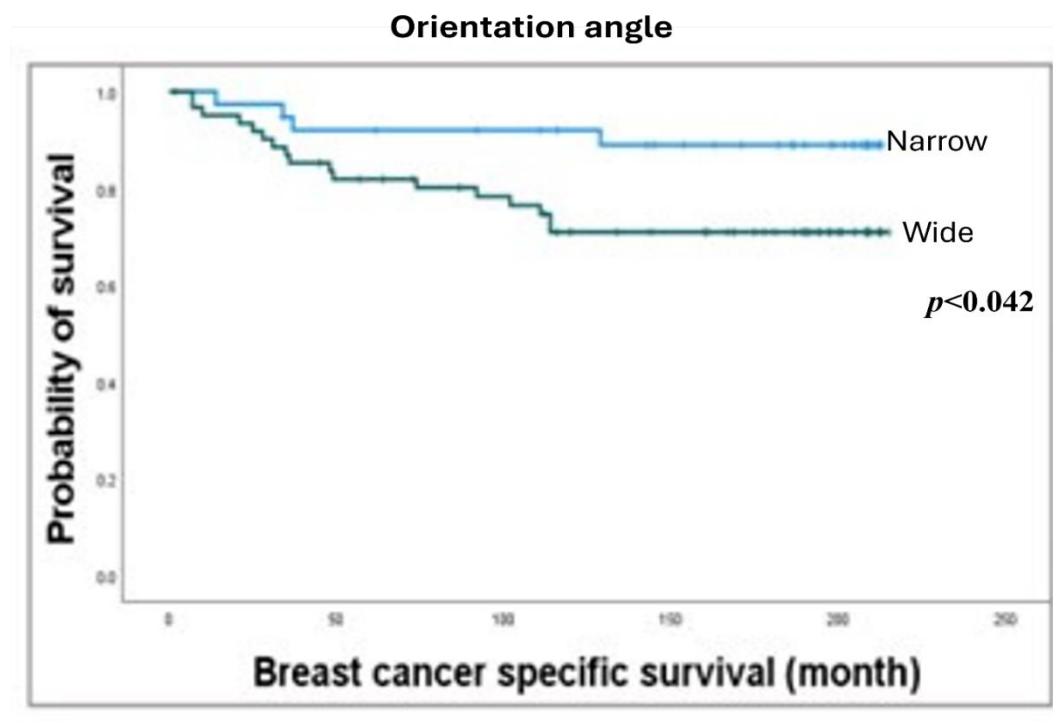


Figure 5-6: Kaplan Meier survival plots showing breast cancer specific survival (BCSS) of cases with orientation angle.

There was no significant association between fibre orientation angle and LRS period ($p<0.43$) or DMFS ($p<0.19$), **Figure 5-7**.

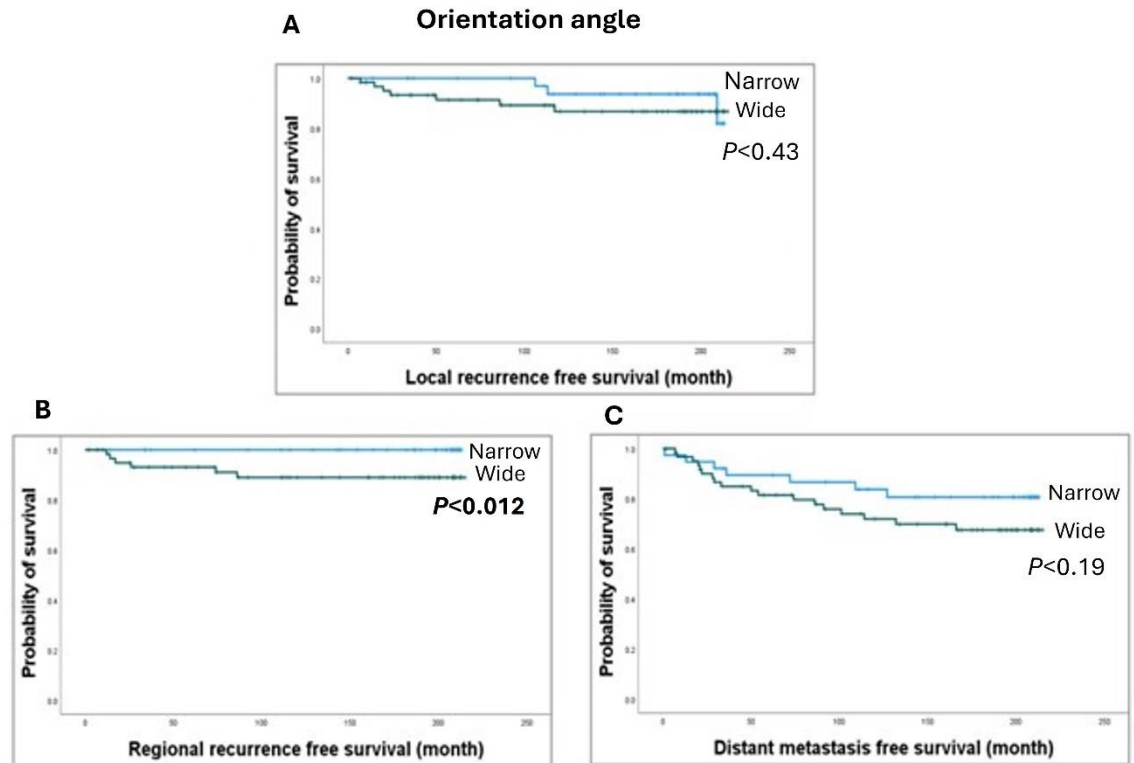


Figure 5-7: Kaplan Meier survival plots showing survival outcomes of cases with narrow and wide orientation angles.

(A) Local recurrence free survival (LRFS; $p < 0.43$). **(B)** Regional recurrence free survival (RRFS; $p < 0.02$). **(C)** Distant metastasis free survival, (DMFS; $p < 0.19$).

Patients whose BC stroma showed poor fibre alignment, or had straighter fibres, had shorter BCSS (HR=3.7, 95% CI= 1.35 to 10.13, $p=0.006$ and HR=0.29, 95% CI=0.08 to 0.98, $p=0.04$ respectively; **Figure 5-8**).

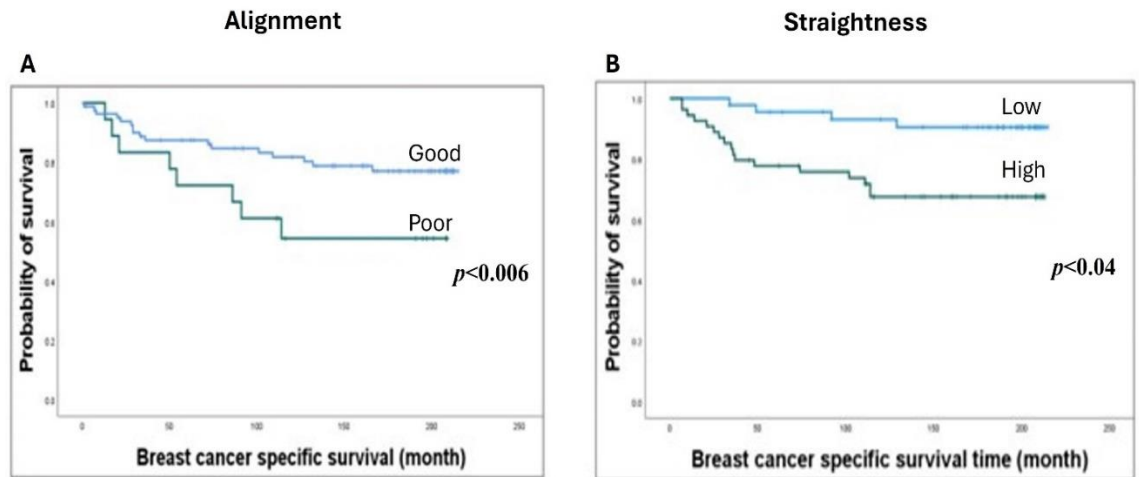


Figure 5-8: Kaplan Meier survival plots showing Breast cancer specific survival (BCSS) of cases with stromal collagen parameters.

(A) Alignment ($p < 0.006$). **(B)** Straightness ($p < 0.04$).

Poor alignment fibres showed shorter DMFS ($p < 0.03$; **Figure 5-9**) while straightness showed no association with other outcomes (**Figure 5-10**).

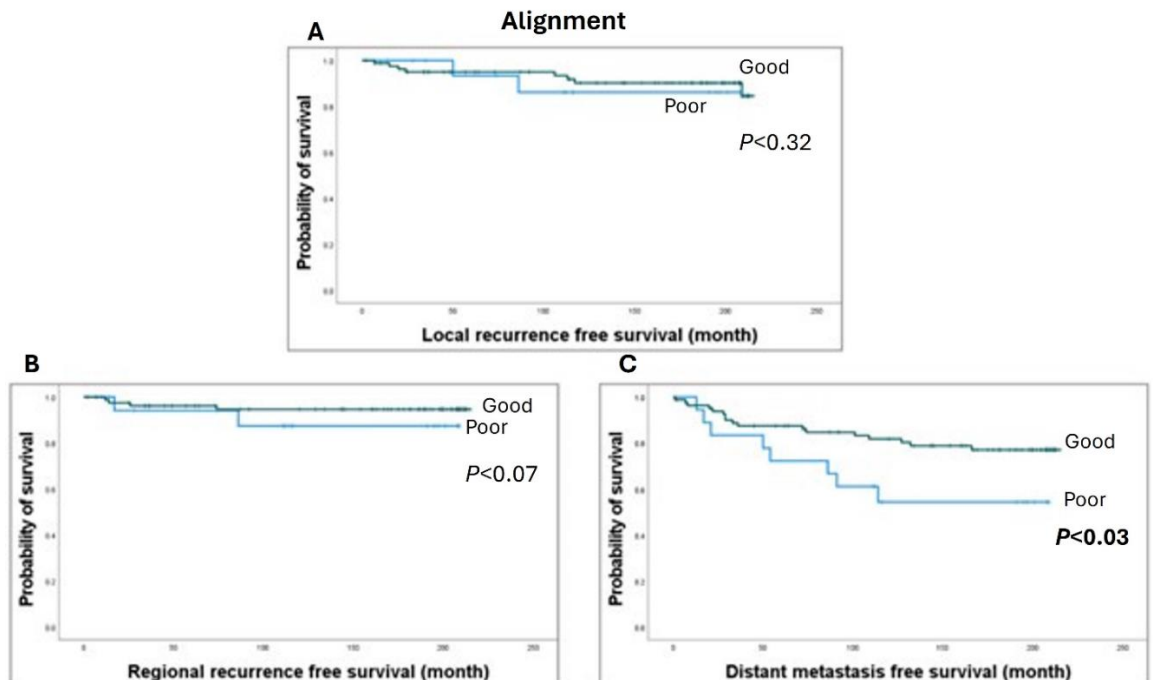


Figure 5-9: Kaplan Meier survival plots showing survival outcomes of cases with poor and good alignments.

(A) Local recurrence free survival (LRFS; $p < 0.32$). **(B)** Regional recurrence free survival (RRFS; $p < 0.07$). **(C)** Distant metastasis free survival (DMFS; $p < 0.03$).

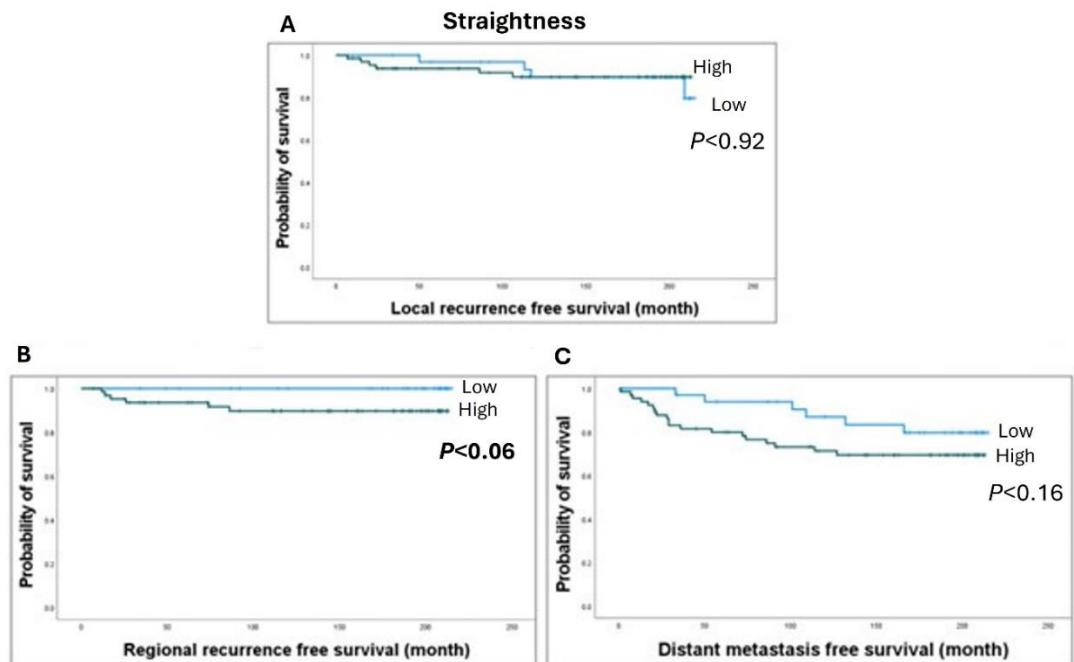


Figure 5-10: Kaplan Meier survival plots showing survival outcomes of cases compared to fibre straightness.

(A) Local recurrence free survival (LRFS; $p < 0.92$). **(B)** Regional recurrence free survival (RRFS; $p < 0.06$). **(C)** Distant metastasis free survival (DMFS; $p < 0.16$).

Stroma with thicker collagen fibres (**Figure 5-11A**) or shorter fibres (**Figure 5-11B**) were associated with shorter BCSS (HR=0.24, 95% CI= 0.09 to 0.62, $p=0.003$, and HR=4.64, 95% CI= 1.79 to 11.97, $p=0.001$ respectively), however, thick fibres showed shorter DMFS (HR=0.17, 95% CI= 0.06 to 0.45, $p < 0.029$; **Figure 5-12**). Fibre length did not show any significant correlation with LRFS ($p < 0.31$), RRFS ($p < 0.11$) or DMFS ($p < 0.06$; **Figure 5-13**). Similarly, fibre width did not show any significance with either LRFS ($p < 0.19$) or RRFS ($p < 0.49$).

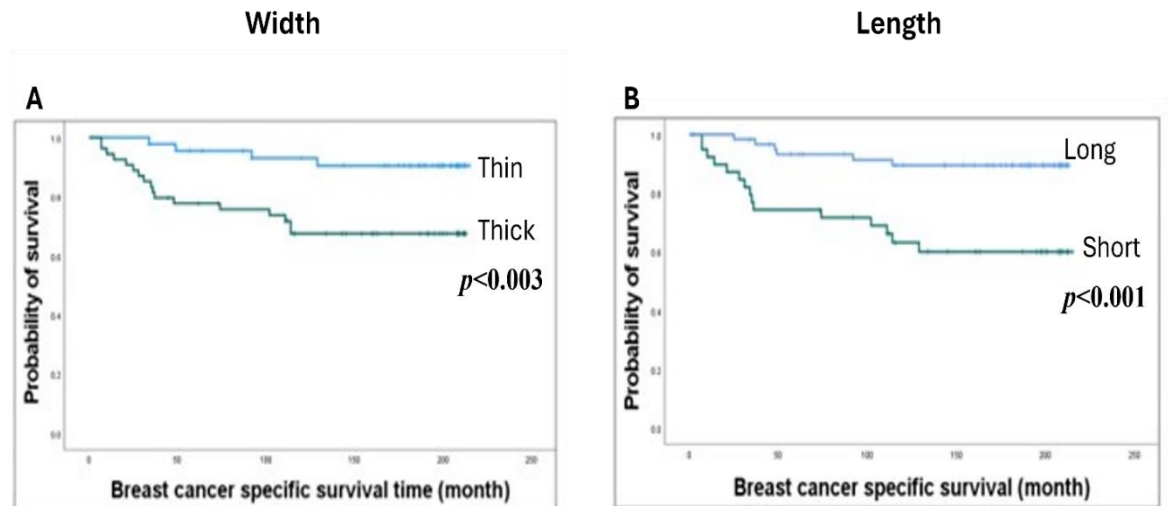


Figure 5-11: Kaplan Meier survival plots showing breast cancer specific survival (BCSS) of cases with stromal collagen parameters.

(A) Fibre width ($p < 0.003$), (B) Fibre length ($p < 0.001$).

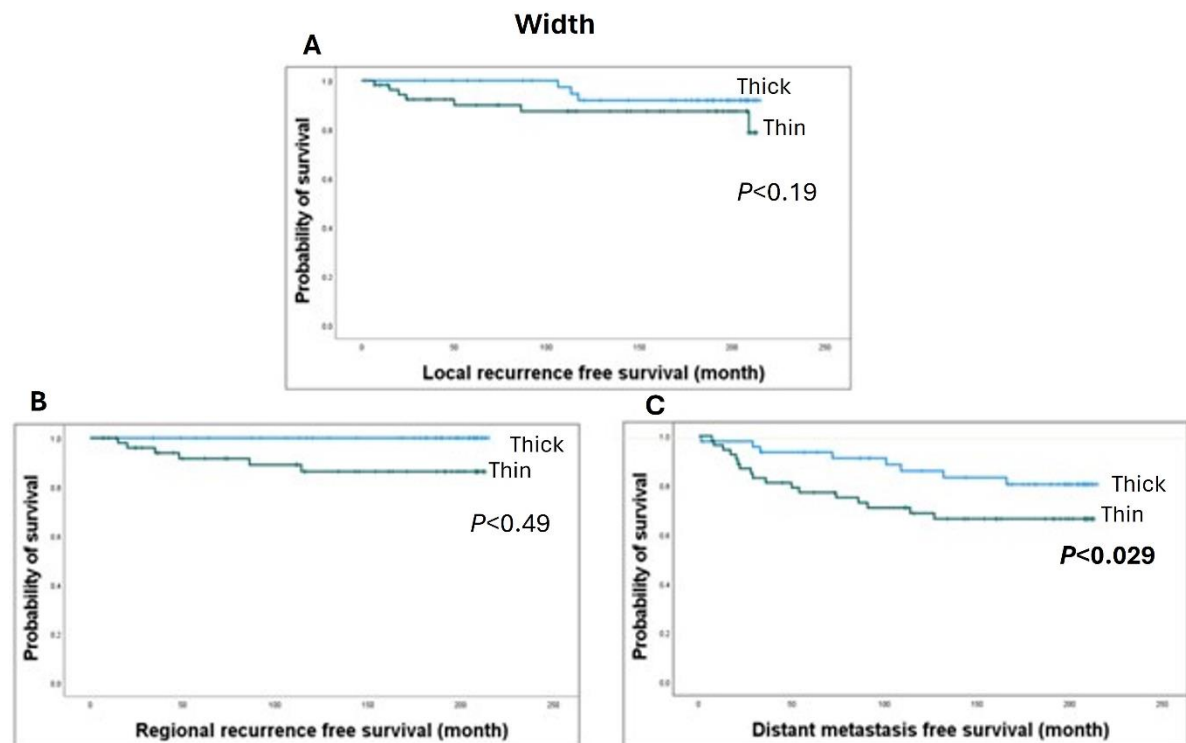


Figure 5-12: Kaplan Meier survival plots showing survival outcomes of cases with fibre width.

(A) Local recurrence free survival (LRFS; $p < 0.19$). (B) Regional recurrence free survival (RRFS; $p < 0.49$). (C) Distant metastasis free survival (DMFS; $p < 0.029$).

Length

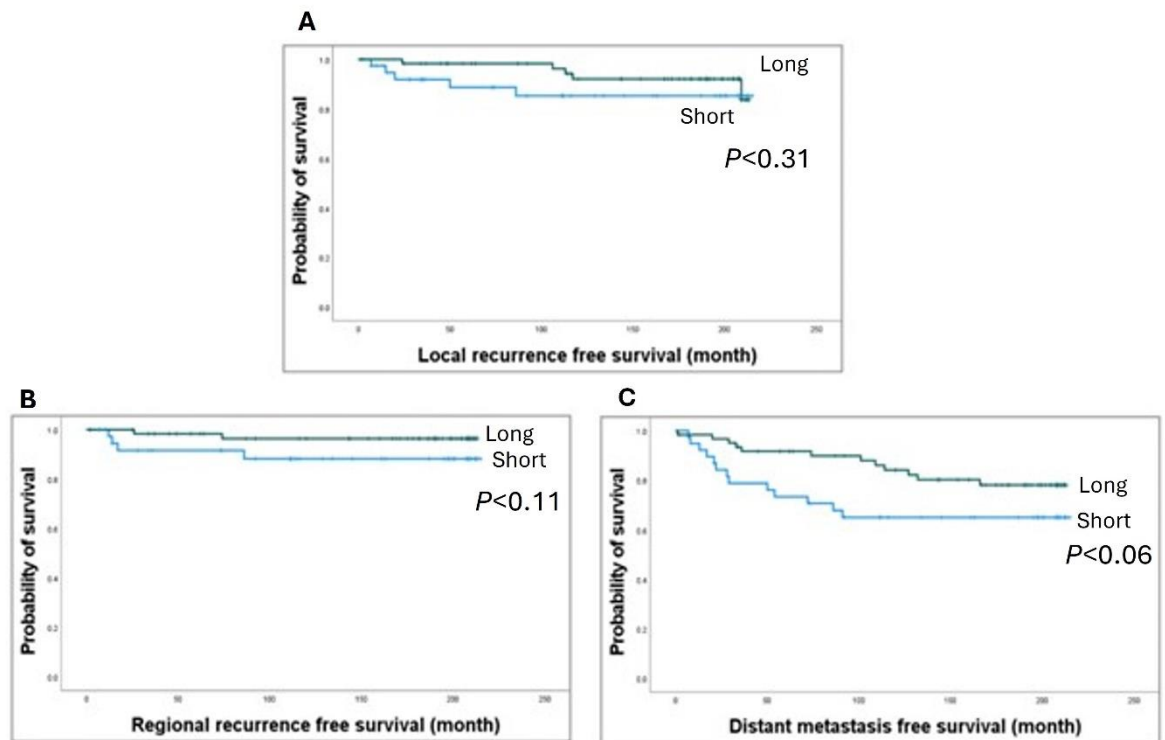


Figure 5-13: Kaplan Meier survival plots showing survival outcomes of cases with fibre length.

(A) Local recurrence free survival (LRFS; $p<0.31$). **(B)** Regional recurrence free survival (RRFS; $p<0.11$). **(C)** Distant metastasis free survival (DMFS; $p<0.06$).

Higher fibre density also correlated with shorter BCSS (HR=0.045, 95% CI= 0.006 to 0.334, $p=0.001$), shorter RRFS (HR=0.092, 95% CI= 0.012to 0.730, $p=0.06$) and also with shorter DMFS (HR=0.069, 95% CI= 0.016 to 0.259, $p=0.01$; **Figure 5-14**).

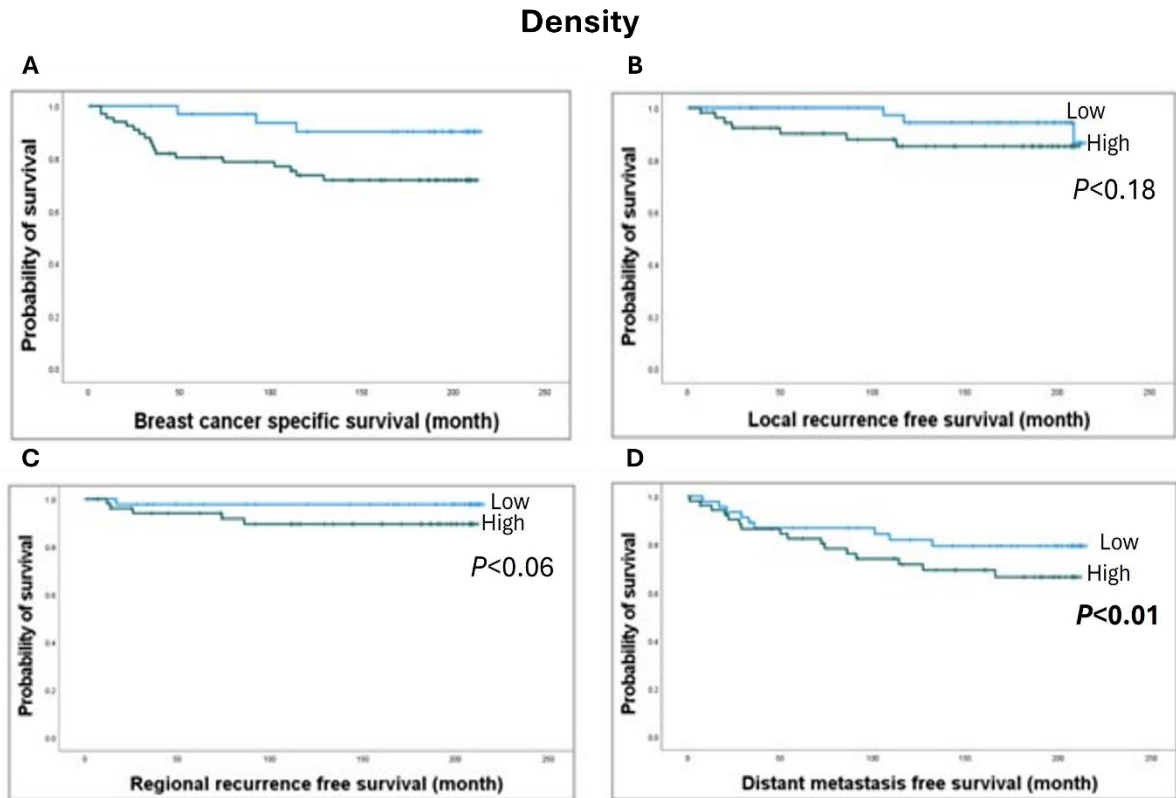


Figure 5-14: Kaplan Meier survival plots showing survival outcomes of cases with fibre density.

(A) Breast cancer specific survival ($p < 0.001$). **(B)** Local recurrence free survival ($p < 0.18$), **(C)** Regional recurrence free survival ($p < 0.06$). **(D)** Distant metastasis free survival, ($p < 0.19$).

Multivariate analyses revealed that the fibre alignment, fibre density, fibre length and fibre width were independent prognostic indicators for BCSS ($p = 0.03$, $p = 0.003$, $p = 0.01$ and $p = 0.002$ respectively) independent of tumour size, LVI, lymph node status and molecular classes. Regarding molecular subclasses, in luminal A, fibre width ($p = 0.02$), and fibre straightness ($p = 0.001$) were an independent prognostic indicator for BCSS while in TNBC, fibre alignment ($p = 0.04$) was an independent prognostic indicator for BCSS tumour size, LVI and lymph node status.

5.4 Discussion

The observations from the present study found that collagen fibre density was larger in invasive BC compared to the stroma of DCIS, however, the collagen fibres were straighter, thinner, more disorganised (less alignment) and more perpendicular in the invasive BC group compared to DCIS. Despite these morphological differences, the fibre length was not significantly statistically different between both groups. These results support a previous study by Lee and colleagues (527). Their work supported proof of concept data that changes concerning the organisation of the collagen network in the stroma play a key role in cancer progression and malignancy by stimulating a tumour-permissive microenvironment, especially for invasive BC.

Although the comparison of collagen fibre characteristics between the two cohorts, DCIS and the invasive groups showed significant variances in all the collagen parameters except the fibre length, the correlation between collagen fibre characteristics and patient outcome differed in both groups. This could be explained by 1- the disease nature as DCIS is a non-invasive lesion with no direct contact of neoplastic cells to the stroma however BC cohort is an invasive tumour and neoplastic cells invade the stroma, 2-the clinical events in the DCIS group are rare which may affect the analysis of outcome in this cohort. 3- the role of stroma could be different in DCIS and invasive tumours. In DCIS, tumour cells are separated from the surrounding stroma by a basement membrane and a layer of myoepithelial cells while in invasive tumour the tumour cells are in direct contact with the surrounding stromal elements and direct communication and interplay are more likely to occur.

In DCIS, the intraductal neoplastic cells at the sites of potential invasion may alter the adjacent stromal composition and structure to be ready for such process via direct or paracrine effect and the surrounding stroma may facilitate or avoid this early invasion process. In invasive tumours, the stroma plays a key role not only in the invasive tumour cells motility but also in the tumour progression and metastasis by many mechanisms

including the interplay and crosstalk between both components providing an appropriate microenvironment for the tumour to acquire more or less aggressive and invasiveness features.

DIC microscopy is a relatively simple technique that does not require any tissue staining which could potentially highlight collagen fibre features with the assistance of AI that could become an easy technique to be used in practice. The power of DIC microscopy is that it does not need extensive processing of the specimen such as fixation, dehydration and staining which entails an artefact risk in each step which may result in underestimation or overestimation of analysis (524). Although microscopy AFM microscopy has the same advantage, its resolution is affected by the irregular surfaces. For instance, high interfibrillar collagen fibre porosity does not let the individual collagen fibrils be imaged and requires special sectioning and extra surface finishing techniques (528). However, SHG microscopy is an expensive method with less accuracy and some limitations as it is commonly used for unipolar fibrils with constant collagen fibre density. SHG microscopy gives strong signals for only collagen types I and II but does not detect collagen types IV and V (466, 529). The signals of SHG microscopy also depend on the fibre angles which may cause inaccuracies in the quantitative studies (530), especially given the differing orientation angles observed in the present study. Multiphoton microscopy has the same advantages but has diffraction limits and resolution limitations. Therefore, a super-resolution detector needs to image collagen fibre that surpasses the diffraction which reflects a complex process (531).

The study results showed differences in the collagen fibre characteristics between invasive and DCIS groups, meaning that collagen fibres are mainstem in the remodelling process of the stroma for the transition from non-invasive to an invasive tumour. The collagen fibres in the stroma of the invasive group showed thinner, straight, and disorganised fibres, helping the cells to invade the stroma and metastasis. These collagen fibre characteristics were associated with clinicopathological data, especially with aggressive behaviour in both DCIS and invasive cohorts

which may suggest the role of collagen fibre in the invasion process and the probability to have prognostic and predictive roles in BC.

Our study results showed the correlation of collagen fibre parameters with the outcome in the invasive cohort however there was not any significant association with the outcome in the DCIS cohort which advocates that collagen organisation stimulates tumour progression (348, 349). Therefore, collagen may have a predictive and prognostic role in the invasive group rather than DCIS which is characterised by its non-invasive nature. Notably in this study, the DCIS cases were pure DCIS without any invasive components, differing results may be observed in cases with invasive elements.

Regarding fibre orientation and alignment, the observations in the present study displayed that the fibres were more perpendicular to the duct border in the stroma of invasive BC. These results were consistent with a previous study, which defined the morphological modifications of collagen fibre arrangement and deposition that are known as TACs reflecting that collagen fibres with a wider perpendicular orientation angle were responsible for building highways for tumour cell migration (278). Another study evaluated collagen fibres in human luminal BC using SHG and analysed with ImageJ reported that disorganization and uniformity of stromal collagen fibres were associated with poor outcome (254). This finding was consistent with the present study results showing a significant correlation between a wide orientation angle (90 degrees) with worse outcomes in BC ($p=0.042$). Additionally, there was a correlation between poor fibre alignment with worse BC outcomes ($p=0.001$). Radial orientation of collagen fibres was hypothesized to result from morphogenic and contractile processes from tumour boundaries, to restructure ECM for local invasion and metastasis (278).

In normal breast tissue, collagen fibres of the stroma are wavy and anisotropic, however in neoplastic tissue, they show progressive fibre straightness and thickening (532). However, non-invasive lesions such

as DCIS and benign tumours have a mixed collagen fibre similar to both normal and malignant fibres (533).

Concerning straightness, this study reported that collagen fibres were straighter in the invasive cohort compared to DCIS, where the collagen fibres appeared more curved. These results agreed with a study by Arwert and colleagues which informed that neoplastic cells migrated along unidirectional straighter collagen fibres towards blood vessels during the invasion process (469). In addition, realignment and/or straightening of collagen fibres has been correlated with local invasion, metastasis, and poor patient outcome in BC (277). This study showed consistent results as there were statistically significant associations between poor fibre alignment and high fibre straightness with worse BC outcome. Straightening of the collagen fibres may lead to ECM porosity alterations which enhance the neoplastic cell's requirements to leave the tumour by invading the adjacent tissues (470). This hypothesis was supported by research relating to collagen structure analysis within regions of invasion (278). In addition, the present study revealed that collagen fibre alignment was an independent indicator for invasive BC prognosis. This was consistent with a previous study evaluating collagen alignment using polarized microscopy that sought to distinguish low and high Oncotype DX risk groups, which reported that poor alignment significantly correlated with the high-risk group (534).

Regarding fibre density, the study also showed that collagen fibre density was greater in the invasive cohort compared to DCIS, this aligned with previously published studies assessing ECM stiffness in normal, benign, and invasive BC. The previous study showed that BC aggressiveness was correlated with an incremental increase in collagen fibre deposition and density (461). Several studies have shown that increased collagen density was associated with metastasis and poor outcome in human BC (462-465) this was consistent with the study results showed high density was correlated with worse outcomes. Other studies in animals have been consistent with the observations from our study, as increased collagen

density correlated with poor survival rates in canine and feline mammary tumours (438, 535).

A better understanding of the unique functionality of distinct collagen fibre networks may better inform the identification of BC subgroups. Indeed papillary, mucinous, and medullary BC tumours, which generally exhibit indolent clinicopathologic behaviours, have been shown to exhibit distinct collagen fibre characteristics as compared to more aggressive invasive tubular and lobular carcinomas (536, 537), consistent with results reported here which confirm an association between low-density, organised, well-aligned fibres with the special histological subtype **(Tables 5-11 & 5-12)**. Moreover, this is consistent with other studies which used different types of microscopies (polarized, SHG, Two-photon, multiphoton and AFM) to compare the collagen architecture in distinct molecular subtypes which showed that highly aggressive BC subtypes (including TNBC, HER2 positive and luminal B) had higher collagen density, thicker and straighter compared to less aggressive subtypes (538, 539) which is consistent with our results.

Regarding fibre width and length, It has been previously reported that fibre length was a significant prognostic factor for overall survival and disease free survival in a TNBC cohort (540). Kullage and colleagues reported a correlation between tumour differentiation and fibre width, as well-differentiated tumours showed thicker fibres and poorly differentiated tumours had thinner fibres (450). This observation is consistent with the study results that showed thinner collagen fibres were associated with invasive tumours. In addition, a study looking at canine mammary tumours showed that increased collagen fibre length was associated with poorer survival (535). However, a feline mammary tumour study which revealed that cats with thicker collagen fibres had a poorer prognosis was consistent with our results (438). Furthermore, it was proven that collagen fibre width was the most powerful influential parameter in predicting 5-year overall survival as increased fibre width has been associated with poor prognosis and outcome (468).

A previous study of stromal collagen fibre characteristics in non-malignant/benign and malignant breast lesions using SHG (466) reported an inverse correlation between stromal collagen density, fibre width and lesion severity as density and fibre thickness were lower in normal/benign relative to carcinoma (466). These findings were consistent with our study. One important distinction between these studies was the method used to image collagen fibres. In the present research, DIC was used, in contrast to the previous work using SHG microscopy (466).

The present study developed a diagnostic model to differentiate DCIS from invasive BC based on the most significant stromal collagen fibre characteristics between the two disease stages. This model granted an accuracy of 97% with high sensitivity and specificity supporting its diagnostic role, especially in the preoperative core needle biopsy. There was no significant difference between the neoplastic cells of DCIS and grade-matched invasive BC cells apart from the demonstration of the MEC and/or BM at the tumour stroma interface. Most of the collagen parameters showed differences between DCIS and invasive stroma and were correlated with outcome however, we observed that these parameters could be used in the model to differentiate between both BC lesions. The model includes all collagen fibre parameters except the fibre length and straightness as there was no significant difference in length between both cohorts and no overlapping in straightness. The straightness range was 0.90-0.94 in DCIS whereas, in the invasive cohort, it was 0.95-0.98, therefore, although there was no overlapping between both cohorts, it could be used as a single item to differentiate between both groups, so it was excluded. This study reported the ability of DIC microscopy utilising collagen fibre characteristics of the stroma to differentiate between DCIS and invasive BC which could empower pathologists to predict which DCIS cases are likely to be associated with invasive lesion. However, these results need further validation using a larger cohort with a larger number of cases and analysing the stroma

around DCIS in both pure DCIS and DCIS associated with invasive disease.

Digital image analysis and machine learning have been used to develop algorithms that could identify stromal changes in both benign and malignant BCs in addition to grading in situ lesions based on some stromal characteristics to predict invasion and malignancy (417). Computational algorithm evaluation for collagen fibre distribution is still a challenge for the clinical utility of image-based biomarkers of collagen fibre as collagen fibre matrices such as orientation, alignment, density, individual fibre measurements and their relation to tumour gland could have a diagnostic and prognostic value (420). However, Keikhosravi *et al.* developed a model which extracted fibre characteristics from H&E sections based on a trained neural network of SHG data, but their clinical value still needs further validation (421). Therefore, this study used DIC microscopy as it visualises collagen fibres without the need for staining which could provide additional information which helps further in validation.

These measurements could help to differentiate between DCIS and invasive tumours in the clinical setting. In addition, collagen plays an important role in cancer therapy resistance. For example, high collagen density was associated with chemotherapy resistance in oesophageal cancer (360), pancreatic cancer (361). Collagen density measurement could help us in therapy decisions.

The potential limitation of this study is the small sample size and low event rate in the DCIS group; however, we aimed the study to find a signature of collagen fibre characteristics that could differentiate between DCIS and invasive BC and its diagnostic application, regardless of predictive and prognostic value. Another limitation is the use of TMA due to limited tissue representation, which may miss tumour heterogeneity and core loss during the process; however, using a variation of ROIS to represent the sample. The model has not been tested to diagnose challenging cases as micro glandular adenosis due to the unavailability

of cases, in addition to the low number of cases with microinvasion, as the validation cohort was selected randomly. The study did not investigate the role of collagen in therapy response.

5.5 Conclusion

In conclusion, this study aimed to ascertain whether distinct stromal collagen fibre characteristics could be related to functional variations in the tumour microenvironment, molecular phenotypes and outcomes in BC.

Collagen fibre density was larger in invasive BC compared to the stroma of DCIS, however, the collagen fibres were straighter, thinner, more disorganised (less alignment) and more perpendicular in the invasive BC group compared to DCIS.

. These differences in stromal collagen fibre features could be used as useful diagnostic tool in differentiating between *in situ* and invasive lesions through the use of image analysis except for length which is not significantly different between both disease stages. Fibre showed a predictive value in patients diagnosed with invasive BC.

For invasive BC, fibre width and density could be used as a predictive indicator for BC in addition to the fibre's alignment for BCSS and risk of recurrence. Highlighting the role of collagen fibre length as a predictor factor which could be used in BC. Future work investigating the mechanisms behind these collagen fibre signatures could further discriminate between tumour types helping in patient outcomes predictions and developing new therapies.

6 Chapter 6: Identification of potential diagnostic and prognostic markers in breast cancer

6.1 Introduction

Identification of new clinical biomarkers in BC is critical to further prediction of prognosis, outcomes and therapeutic responses (541-543). The biomarkers of BC include proteins, macromolecules, and intact cells (542, 544). Novel biomarker identification may explain the reasons and mechanisms behind therapy resistance which is a challenging obstacle in BC management (542, 544). Moreover, due to the increasing proportions of patients who are demanding personalised management, there is a societal importance attached to developing new biomarkers for both the diagnostic and prognostic methods (545, 546).

Carcinogenesis is a complex process resulting from several cellular, genetic, and epigenetic changes (547). The TME plays a key role in the carcinogenesis process via ECM remodelling (548, 549). Tumour progression is promoted by ECM stiffness which is mediated by collagen cross-linking (274, 550). The ECM is a vital component of stromal cellular interactions which consequently triggers intracellular signalling cascades which are associated with tumour invasion (551). Tumours need to first penetrate their BM locally to invade the adjacent stroma or penetrate the BMs of blood vessels to disseminate haematogenous to distant organs (552, 553). The *in-situ* stage is commonly presented in invasive BC at the time of detection and approximately half of all invasive BC was estimated to be associated with DCIS (280, 554). This indicates the need for the concomitant study of ECM changes which precede invasion, such as those where a well-formed continuous BM converts to variably fragmented and even lost in the malignancy progression (329, 554).

There are two characterised, non-fibrillar collagens, XV and XIX, which have been identified in the BM zone (BMZ) of some normal human tissues (555-558). Type XV is a 200–250 kDa core protein which characterised by interrupted collagenous regions, heparan sulphate glycosaminoglycan or chondroitin chains that are attached to the non-collagenous amino-termini, and anti-angiogenic fragments which are

cleaved from the globular carboxy domains (559, 560). In contrast, Type XIX collagen is very distinctively 165 kDa containing a non-glycosylated α chain that includes five different collagenous subdomains producing the major molecule portion. The non-collagenous amino terminus and carboxy peptide are distinguished by an array of cysteines (561, 562). Collagens XV and XIX are exhibited in most epithelial BMZ, and in all BMZ of both striated and smooth muscle, peripheral nerve, and endothelial cells (555-558). The presence of these collagens with the BMZ raises questions about their expression in malignancy (173, 237).

Type XV collagen was reported to be present in trace amounts at the periphery of the invasive gland in adenocarcinomas of the colon which suggested that this collagen may be lost through changes in the invasiveness process (563). Interestingly, collagen XIX was elevated in the serum in lung cancer as compared to healthy controls (564).

During the progression process of invasive BC, there are various precancerous stages, such as the *in-situ* stage, which are crucial when detailing hypoxia. Notably, DCIS has been accompanied by the presence of large central necrosis. These lesions are high-grade and poorly differentiated *in-situ* lesions associated with a general hypoxic phenotype (565). The corresponding invasive lesion presents with similar hypoxic regions which are associated with aggressive clinicopathologic behaviour which is known by the old definition “comedo cancer” (566, 567).

Hypoxic microenvironments favour tumour growth, progression and metastasis development (568). The factor known to be produced in hypoxic microenvironments is hypoxia-inducible factor (HIF) which is a helix transcription factor involved in the carcinogenesis process and tumour growth through the regulation of genes involved in metabolic activation, angiogenesis and tumour invasion by ECM degradation (569). Approximately 25–40 % of BC tumours exhibit hypoxic regions (570). Cellular responses to hypoxia are mainly mediated by hypoxia-inducible

factors (HIF) activation which is composed of oxygen-regulated subunits (HIF-1 α & HIF-2 α) and a constitutively expressed subunit (HIF-1 β) (571)

Clinical studies showed that HIF-1 α is overexpressed in several primary and metastatic cancers and has been correlated with survival outcomes (572-575) despite the conflicting data in BC. Schindl and coauthors demonstrated that HIF-1 α was an independent prognostic factor for overall survival in BC patients with lymph node-positive (576). Conversely, Gruber and colleagues stated that high HIF-1 α expression was significantly correlated with DFS but not overall survival (577). In addition, Huang *et al.*, reported that there was not any association between HIF-1 α expression and BC outcomes (578). Expression of HIF-1 α is associated with aggressive tumour behaviour such as large size, high proliferation, high grade, lack of and hormone-receptor negativity. However, it is noted that marked HIF-1 α positivity in small tumours despite the link between tumour size and hypoxia (569). Several genes and transcription factors were proved to be elicited in hypoxia conditions by HIF-1, one of them is Lysyl oxidase (LOX) (579, 580).

LOX is a copper-containing amine oxidase which has a crucial role in ECM remodelling thereby facilitating tumour invasion and metastasis (581). LOX catalyses the lysine-derived cross-links in collagen and elastic fibres resulting in ECM stabilisation (582). This is supported by data that links the expression of LOX dysregulation with metastasis and survival rates in various cancers (579, 583-585). Leight *et al.*, proved that increased ECM stiffness stimulated tumour TGF- β signalling which is a potent inducer of EMT (586). Hypoxia-driven overexpression of LOX enhances tumour desmoplasia and ECM stiffness, which, in turn, promotes tumour invasiveness metastasis (587, 588).

Erler and coauthors revealed that LOX has a vital role in pre-metastatic niche formation which supports the idea of targeting LOX for metastatic prevention and treatment (589). The current therapeutic methods to treat

advanced breast cancer (BC) with distant metastasis are not considered to be effective which results in poor patient outcomes (590). Inhibition of LOX has been recommended as a therapeutic strategy to prevent BC metastasis. Numerous compounds have been exploited with different inhibitory actions against LOX (591).

To our knowledge, the expression of LOX has only been evaluated in the TNBC molecular subtype within a group of EMT markers (592). However, there have been no previous reports investigating the relationships between LOX expression and metastasis in BC patients (593). Additionally, the expression of collagen XV and XIX were reported to precede the loss of collagen IV in invasive BC but their expression in different types of BC has not been investigated (272). The predictive value of HIF-1 α in BC remains unclear and may be explained by the diversity of methodological approaches used (594).

6.1.1 Aims and hypothesis

This research in this chapter hypothesised that collagen XV and collagen XIX could be potential diagnostic biomarkers to distinguish invasive and non-invasive BC lesions, . In addition, HIF-1 α and LOX could provide predictive tool for BC prognosis.

Therefore, the aims of this chapter were:

- A) To compare the expression of both collagen XV and XIX between normal, *in-situ* and invasive BC, to understand their roles in the process of invasion that makes *in-situ* act as an intermediate stage between normal tissue and invasive carcinoma
- B) To evaluate the clinical significance of HIF-1 α in BC and more specifically within different BC molecular classes.
- C) To assess the expression of LOX and explore its clinical value in BC.

6.2 Patients and Methods

6.2.1 Proteomic analysis

For the assessment of collagen XV and XIX, 100 cases were used which included both normal and neoplastic breast tissue from patients who presented to Nottingham City Hospital, Nottingham, UK between 1999 and 2006. The cases were divided into four groups, each containing 25 patients. The four groups included normal breast tissue, DCIS, EPC, and invasive carcinoma (INV) characterised by the presence of BM-like material. Freshly cut, 4µm thick, full-face sections for this cohort were used. Two sections from each block were utilised for each study, one was stained with H&E for histological review and the other underwent IHC. Full and detailed clinicopathological and outcome data was covered in Chapter 3.

For the assessment of HIF-1α and LOX protein expression, a large well-characterised cohort of primary operable BC (n=876) from patients who presented at Nottingham City Hospital, Nottingham, UK was utilised. Tissue samples were prepared as a TMA as previously described in Chapter 2 (595). Full and detailed clinicopathological and outcome data are available in **Table 6-1**.

The mean patient follow-up was 144 months. Outcome data including BCSS and DMFS were calculated.

Table 6-1: Clinicopathological characteristics of the study cohort

Parameters	Invasive cohort cases (%) N=876
Patient Age	
≤ 50 years	277 (31.6%)
> 50 years	599 (68.4%)
Size	
≤ 2cm	519 (59.2%)
> 2cm	357 (40.8%)
Estrogen receptor	
Positive	574 (65.5%)
Negative	296 (33.8%)
Progesterone receptor	
Positive	574 (65.5%)
Negative	296 (33.8%)
Missing	6 (0.7%)
HER2 status	
Positive	91 (10.4%)
Negative	785 (89.6%)
Ki67 score	
High	363 (41.4%)
Low	340 (38.8 %)
Missing	173 (19.7%)
Molecular subtypes	
Luminal A	309 (35.3%)
Luminal B	218 (24.9%)
Her2 enriched.	90 (10.3%)
Triple-negative	154 (17.6%)
Tumour Grade	
Grade I	122 (13.9%)
Grade II	353 (40.3%)
Grade III	401 (45.8%)
Histological type	
ductal NST	584 (66.7%)
Lobular	62 (7.1%)
Metaplastic	2 (0.2%)
Special types	34 (3.9%)
Mixed	194 (22.1%)
Lymph vascular invasion (LVI)	
Definite	258 (29.5%)
Negative	618 (70.5%)
LN metastasis	
Negative	539 (61.5%)
Positive	336 (38.4%)
Nottingham prognostic index	
Good	289 (33%)
Moderate	432 (49.3%)
Poor	155 (17.7%)

6.2.2 Transcriptomic analysis

HIF-1 α and LOX mRNA expression were analysed at the transcriptomic level using publicly available cohorts the Kaplan Meier (KM) Plotter online dataset for both BCSS (n=4929) and DMFS (n=2765), in the whole

cohort and molecular subtypes (<https://kmplot.com/analysis/>) (596) and the bc-GenExMiner database (all DNA microarray data) for LOX and HIF-1 α for both BCSS (n=8921,n=9030 respectively) and DMFS (n=6514,n=6699 respectively). In addition, the expression of both proteins according to histological subtypes and molecular subtypes (597).

6.2.3 Collagen XV and XIX immunostaining

The specificity of the anti-collagen XV rabbit polyclonal antibody (DF13358, Affinity Biosciences, UK), and anti-collagen XIX rabbit polyclonal antibody (AF9044, Affinity Biosciences, UK) were validated by peptide block competition I using collagen XV peptide block (DF13358-BP, Affinity Biosciences, UK) and collagen XIX peptide block (AF9044BP, Affinity Biosciences, UK) according to the validation protocol (598) by determining the optimal concentration of antibody for IHC according to the data sheet and optimisation process (1:100). Then the blocking peptide was added at a ratio of 5:1 to 10:1 to the antibody in PBS, and incubated overnight at 4°C. The IHC staining protocol on identical samples of normal breast tissue, negative and positive control, staining one sample set with the blocked antibody and the other with the antibody was also conducted. The patterns of staining between both the unblocked and blocked antibodies were compared (599).

6.2.4 HIF-1 α and LOX immunostaining

The specificity of anti-HIF-1 α mouse monoclonal antibody (# MA1-516, Invitrogen, Thermo Fisher Scientific and UK), anti-LOX rabbit polyclonal antibody (# PA1-16953, Thermo Fisher Scientific and UK) were validated by WB using cell lysates of MCF7, MCF10, BT589, MDA-MB-231, SKBR3, and ZR-751 human BC cell lines obtained from American Type Culture Collection (Rockville, MD, USA). The HIF-1 α primary antibody was used at a 1:500 dilution. While LOX primary antibodies were used at a 1:1000 dilution. Anti- β -actin primary antibody (Sigma-Aldrich) was used as a loading control.

Three series of full-face sections of BC cases (n=14) representative of several molecular subtypes were used to evaluate the distribution of HIF-1 α and LOX expression. The tissue sections were stained using the Novocastra Novolink™ Polymer as described in detail in Chapter 2. HIF-1 α antibody was diluted at 1:50 and incubated for one hour at room temperature. LOX was diluted at 1:200 and incubated for 30 min at room temperature. The IHC-stained slides were digitally scanned using a high-throughput scanner (Panoramic 250 Flash III; 3D-Histech, Budapest, Hungary), and the WSIs were viewed with CaseViewer software (version 2.2.0.85; 3D-Histech).

6.2.5 Collagen XV and collagen XIX protein expression evaluation

The expressions of collagen XV and XIX were determined by the presence of a thin, dense linear deposition at the BM/BMZ. The intense vascular reactivity was utilised as an internal control in each case.

Slides were examined using light microscopy and categorised as 0= no positive reaction, 1=trace of positive reaction, 2=Mild positive reaction, 3=Moderate positive reaction, 4=strong positive reactions based on the staining intensity compared to the control tissue (normal breast tissue) in each slide.

6.2.6 HIF-1 α and LOX protein expression evaluation

HIF-1 α showed nuclear expression and LOX showed cytoplasmic expression, and the percentages of positive tumour cells were calculated. A semi-quantitative evaluation was used as both the percentage of positive cells (0–100%) and the staining intensity (0=negative, 1=weak, 2=moderate and 3=strong) were considered to calculate a modified histochemical score (H-score). as follows: H-score=Intensity (0–3) $\times$ per cent of expression (0–100) giving a final score of 0–300 (600-603). The scoring was validated by a second observer (Shorouk Makhoulf) who scored 20% of the cohort, and the

inter-observer concordance was calculated. A H-score of 180 was considered the optimal cutoff of HIF-1 α expression, While an H-score of 160 was considered the optimal cutoff of LOX expression using the X tile to categorise their expression into low and high groups. Then the expression was correlated with clinicopathological parameters and patient outcome.

6.2.7 Statistical analysis

Statistical Package for the Social Sciences software v.27.0 (SPSS, Chicago, IL, USA) and GraphPad Prism were used for statistical analysis. One-way ANOVA and chi-square tests were used to compare the groups in (collagen XV and XIX expression). Results are expressed as the mean $\pm$ SD of the mean throughout unless otherwise stated.

X-tile bioinformatics software version 3.6.1 (School of Medicine, Yale University, New Haven, CT, USA) (604) was used to define the optimum cutoff for HIF-1 α and LOX markers based on BCSS. The mRNA levels were categorised using the optimal splitting criterion in the online website. Chi-square tests were used to assess the correlations between categorical variables. The degree of inter-observer concordance ICC in the scoring was assessed statistically using the intercorrelation coefficient (ICC). Kaplan Meier curves with log-rank test were used to assess the correlation with outcomes. Cox regression models for the multivariate analysis with estimation of hazard ratios (HR) and confidence intervals (CI). For all tests, $p < 0.05$ (two-tailed) was considered statistically significant. Graphs and tables were constructed as applicable using GraphPad Prism10.

6.3 Results

6.3.1 Collagen XV and XIX expression in BC

6.3.1.1 Collagen XV and XIX protein expression

The staining patterns of both collagen XV and XIX were compared between the unblocked and peptide-blocked antibodies. In collagen XV, the positive control (placenta) showed IHC positive brown expression located on the BM in the unblocked antibodies sections while there was no expression in the peptide-blocked antibodies sections, **Figure 6-1**.

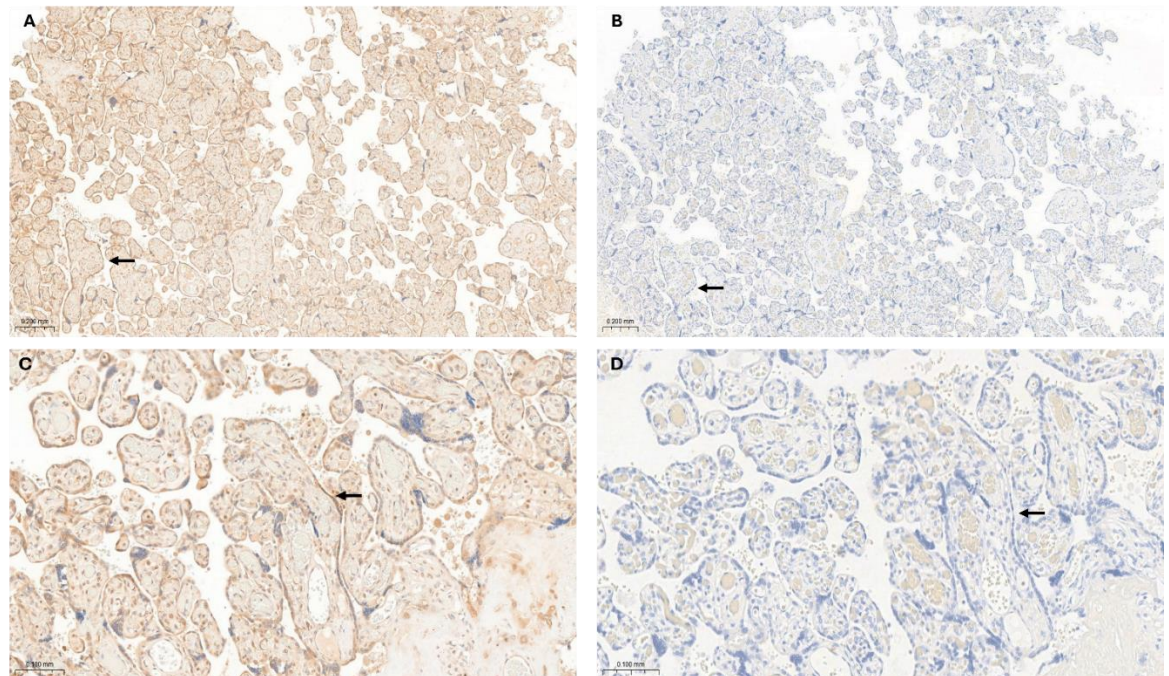


Figure 6-1: Expression of collagen XV in the placenta (positive control).

A) Positive expression of collagen XV (arrow) in unblocked antibody sections was observed in the BMs of the trophoblasts in the placenta (low power field), scale bar 200mm. **B)** Negative expression of collagen XV (arrow) in peptide-blocked antibodies in the BMs of the trophoblasts of the placenta (low power field), scale bar 200mm. **C)** Positive expression of collagen XV (arrow) in unblocked antibody sections was observed in the BMs of the trophoblasts in the placenta (high power field), scale bar 100mm. **D)** Negative expression of collagen XV (arrow) in peptide-blocked antibodies in the BMs of the trophoblasts of the placenta (low power field), scale bar 100mm.

Similarly, collagen XIX showed positive expression in the BMs of the positive control section (testis) in the unblocked antibodies sections while

there was no expression in the peptide-blocked antibodies sections (Figure 6-2).

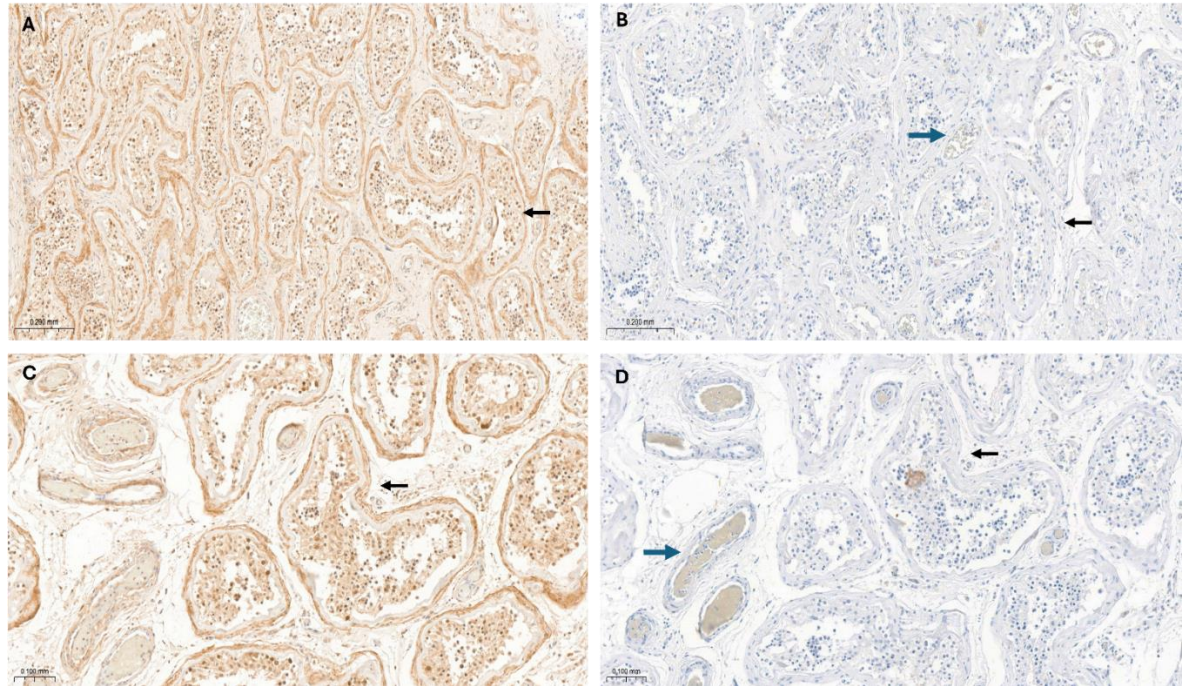


Figure 6-2: expression of collagen XIX in the testis (positive control).

A) Positive expression of collagen XIX (arrow) in unblocked antibody sections was observed in the BMs of the seminiferous tubules of the testis (low power field), scale bar 200mm. **B)** Negative expression of collagen XIX (black arrow) in peptide-blocked antibodies in the BMs of seminiferous tubules of the testis however positive reaction appears in the lumen of the blood vessels, not in the BMs (blue arrow; low power field), scale bar 200mm. **C)** Positive expression of collagen XIX (arrow) in unblocked antibody sections was observed in the BMs of seminiferous tubules of the testis (high power field), scale bar 100mm. **D)** Negative expression of collagen XIX (black arrow) in peptide-blocked antibodies in the BMs of the seminiferous tubules of the testis while, a positive reaction appeared in the lumen of blood vessels, but not in the BMs (blue arrow; low power field), scale bar 100mm.

Negative controls were used for both types of collagen. The collagen XV negative control was tonsil tissue while the collagen XIX negative control was skin (Figure 6-3).

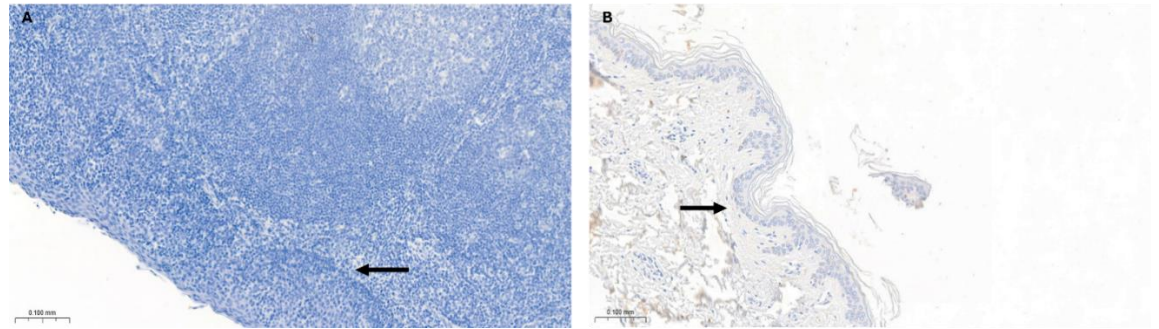


Figure 6-3: the expression of collagen XV and XIX in negative control tissues.

A) The tonsil with negative expression of collagen XV in the epithelial BM (arrow). **B)** Skin showing negative expression of collagen XIX in BM of the epidermis (arrow).

6.3.1.2 Comparison of collagen XV expression in BC

All the cases of both groups of normal breast tissue and DCIS showed positive expression of collagen XV in the BM with different intensities however, all the cases of EPC showed negative expression of collagen XV. Conversely, most of the cases (84%) of the invasive group showed positive expression of collagen XV ranging between weak and mild intensity.

There was a significant difference in collagen XV expression in the BM-like material of the invasive group (1.20 ± 0.76 ; $p < 0.001$) compared to the BMs in the normal breast group (3.84 ± 0.37). However, there was no significant difference in collagen XV expression between the DCIS BMs and normal breast tissue BMs ($p < 0.237$). There was a difference in the expression between BM of the DCIS and the other groups ($p < 0.001$) as well as between the EPC capsule and BM-like material of the invasive group. One-way ANOVA, $F=140.2$, $p < 0.001$, (**Table 6-2, Figure 6-4 & Figure 6-5**).

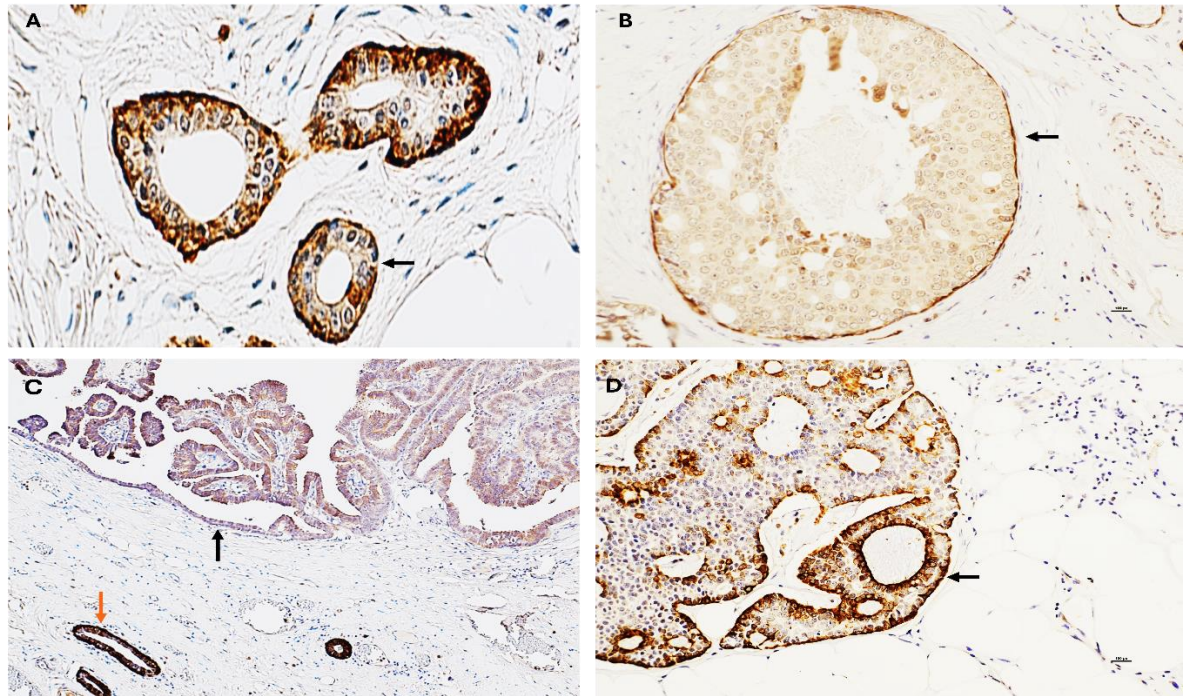


Figure 6-4: collagen XV expression in breast tissue.

A) Normal breast tissue section showing strong expression of collagen XV in the BM of breast ducts (arrow). **B)** Ductal carcinoma in situ (DCIS) section showing positive expression of collagen XV in the BMs (arrow). **C)** Encapsulated papillary carcinoma (EPC) section showing negative expression of collagen XV in the capsule (black arrow) while the positive expression of collagen XV appeared in the blood vessels BMs (red arrow). **D)** A special type of breast cancer (adenoid cystic carcinoma) showed positive expression of Collagen XV in the BM-like material surrounding the tumour. Magnification x20.

Table 6-2. Collagen XV within breast tissues basement membranes

Group	Mean intensity	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	3.84	0.37	0.75	DCIS	0.237
				EPC	0.001*
				Invasive	0.001*
DCIS	3.60	0.50	0.10	EPC	0.001*
				Invasive	0.001*
EPC	0	0	0	Invasive	0.001*
Invasive	1.20	0.76	0.15	-	

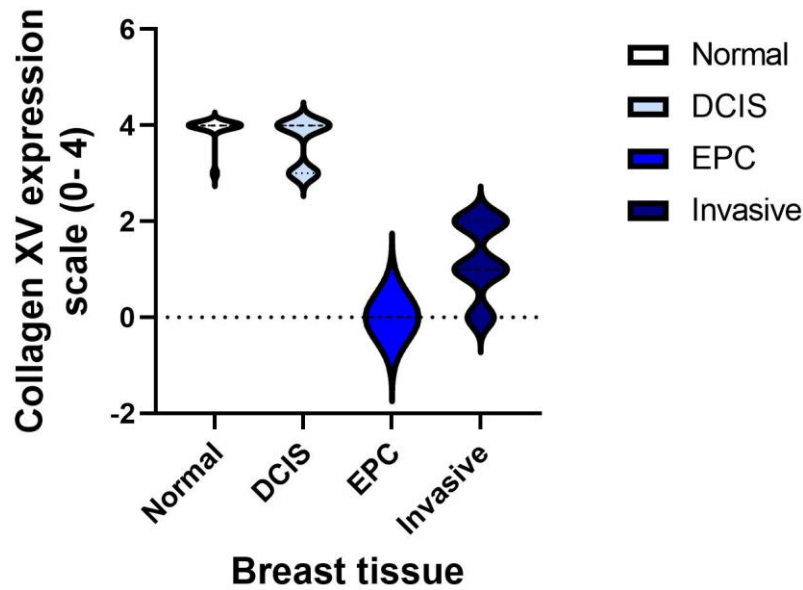


Figure 6-5: Collagen XV expression in the breast basement membrane (BM) on a scale of 0-4.

6.3.1.3 Comparison of collagen XIX expression in BC

All the cases of both groups of normal breast tissue and DCIS showed positive expression of collagen XIX in the BM with various intensities conversely, all the cases in the EPC and the invasive groups had negative expression of collagen XIX.

There was no significant difference in collagen XIX expression between the DCIS BMs and the normal breast tissue BMs ($p < 1$). There was a difference in the expression between BMs of the DCIS and the other groups ($p < 0.001$). One-way ANOVA, $F=141.6$, $p < 0.001$, (**Table 6-3**, **Figure 6-6** & **Figure 6-7**).

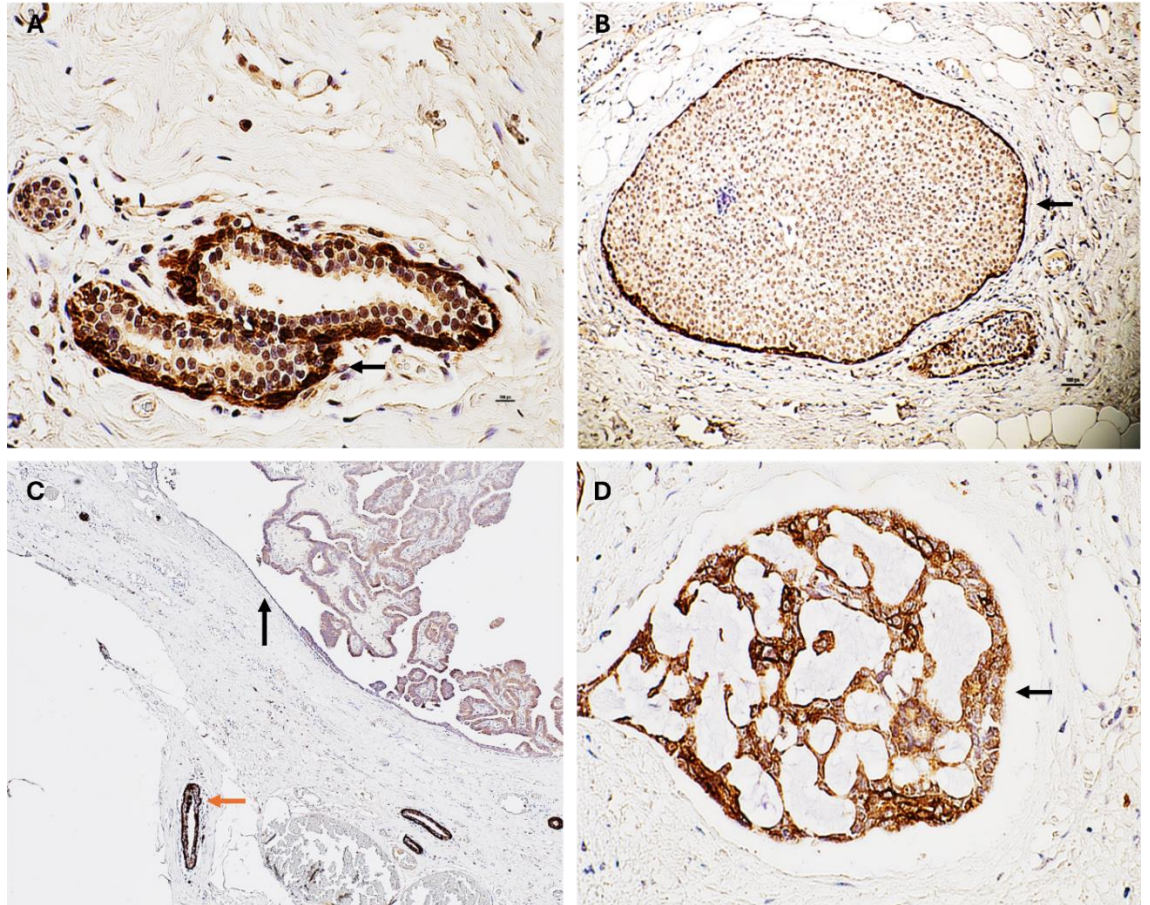


Figure 6-6: collagen XIX expression in breast tissue.

A) Normal breast tissue section showing strong expression of collagen XIX in the BMs of breast ducts (arrow). **B)** Ductal carcinoma in situ (DCIS) section showing positive expression of collagen XIX in the BMs (arrow). **C)** Encapsulated papillary carcinoma (EPC) section showing negative expression of collagen XIX in the capsule (black arrow) while the positive expression of collagen XV appeared in the blood vessels BMs (red arrow). **D)** A special type of breast cancer (adenoid cystic carcinoma) showed negative expression of collagen XIX in the BM-like material surrounding the tumour. Magnification x20.

Table 6-3: Collagen XIX within breast tissue basement membranes.

Group	Mean intensity	Standard deviation (SD)	Standard error of mean (SEM)	Tissue comparisons	<i>P</i> value (*= <i>significance</i> = <i>p</i> <0.05)
Normal	3.80	0.33	0.06	DCIS	1
				EPC	0.001*
				Invasive	0.001*
DCIS	3.60	0.50	0.1	EPC	0.001*
				Invasive	0.001*
EPC	0	0	0	Invasive	0.001*
Invasive	0	0	0	-	

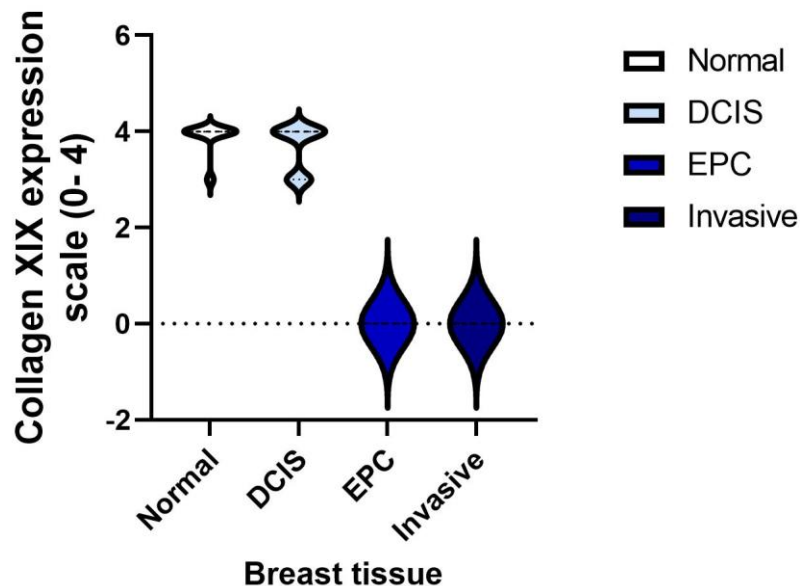


Figure 6-7: Collagen XV expression in the breast basement membrane on a scale of 0-4.

6.3.2 HIF-1 α expression in BC

6.3.2.1 HIF-1 α mRNA expression

High HIF-1 α mRNA levels were significantly associated with poor outcome in the whole cohort in terms of BCSS (HR 1.52, 95%CI 1.37-1.68; $p=3.2e-16$) and DMFS (HR 1.32, 95%CI 1.13-1.54; $p<0.0004$), (Figure 6-8).

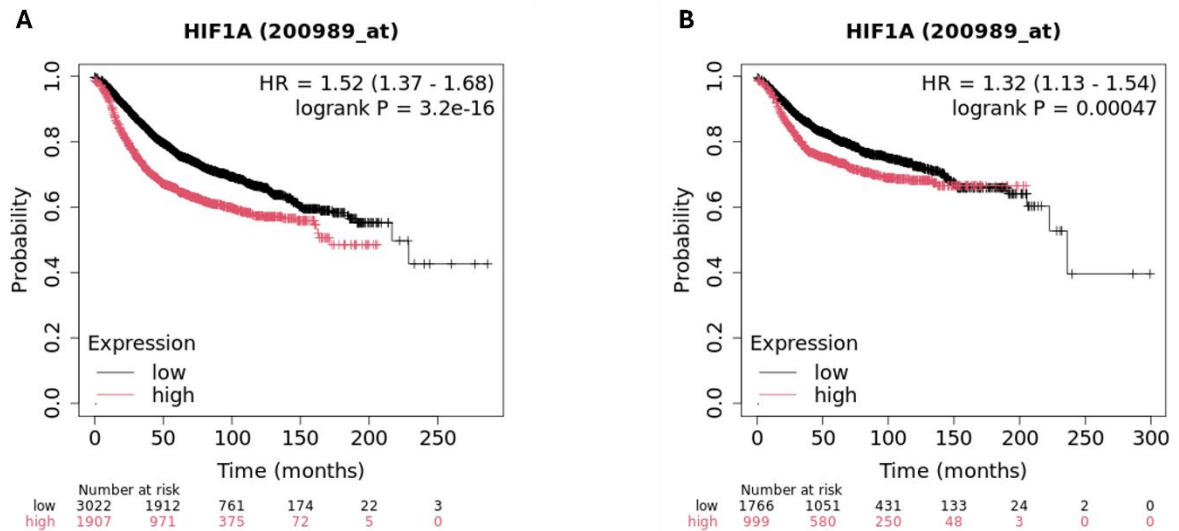


Figure 6-8: Kaplan Meier curves show significant associations between high HIF-1 α mRNA expression and poor outcomes in the whole cohort.

(A) Breast cancer specific survival (BCSS) and **(B)** Distant metastasis free survival (DMFS).

Regarding molecular subtypes, high HIF-1 α mRNA levels were significantly associated with shorter BCSS in all molecular subtypes, luminal BC (HR 1.24, 95%CI 1.05-1.46; $p=0.013$), HER2 positive (HR 1.29, 95%CI 1.03-1.62; $p=0.024$) and TNBC (HR 1.54, 95%CI 1.16-12.04; $p=0.0024$) (**Figure 6-9**).

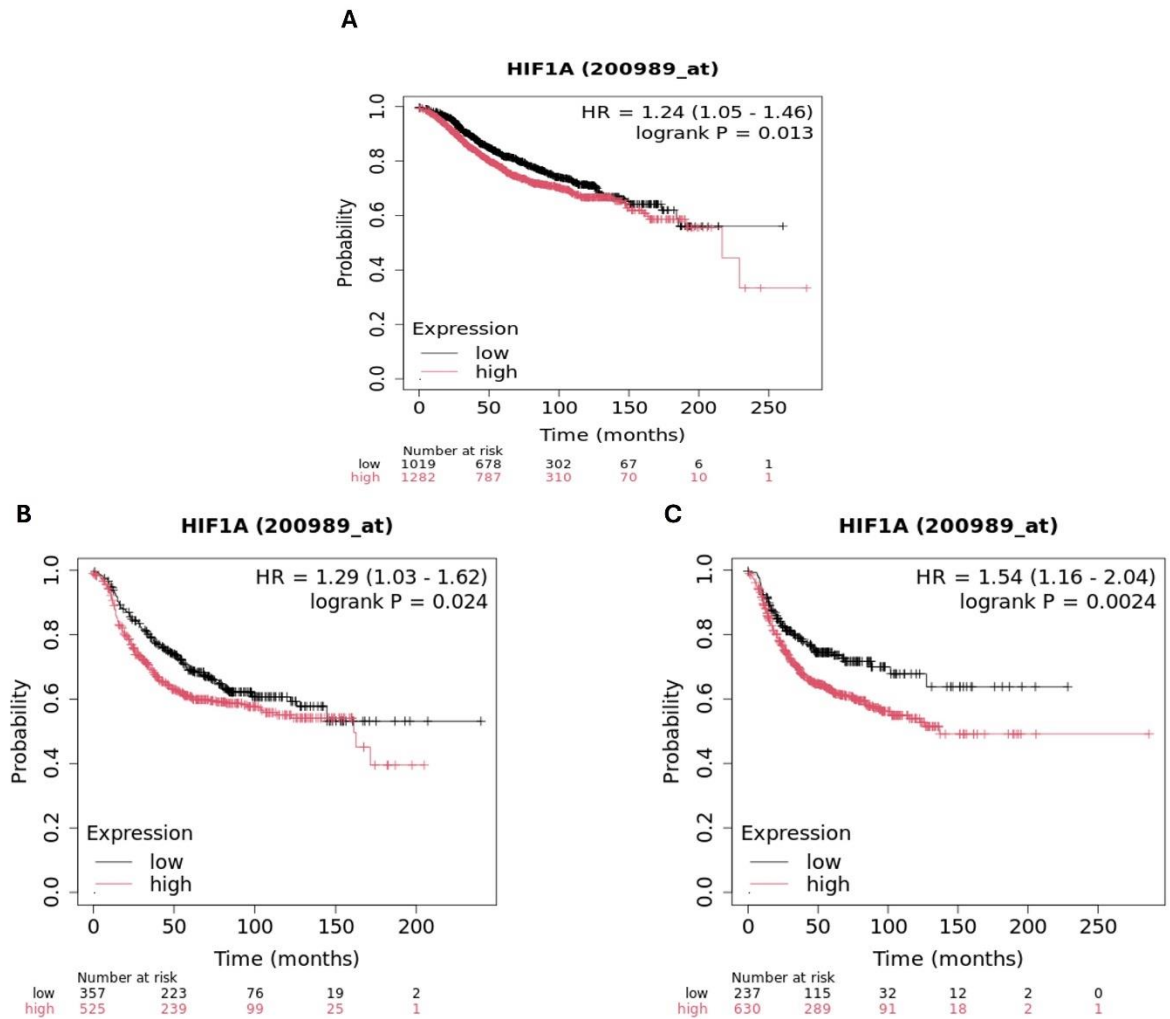


Figure 6-9: Kaplan Meier curves showed significant associations between high HIF-1 α mRNA expression and poor outcome in molecular subtypes.

A) Luminal BC, **B)** HER2 positive and **C)** TNBC.

Based on the adjuvant therapy, high HIF-1 α mRNA expression showed a significant association with shorter BCSS in patients who were on hormonal therapy (HR1.35, 95%CI 1.14-1.60; $p=0.0005$) and chemotherapy (HR 1.40, 95%CI 1.18-1.65; $p=9.5e-05$), (**Figure 6-10**).

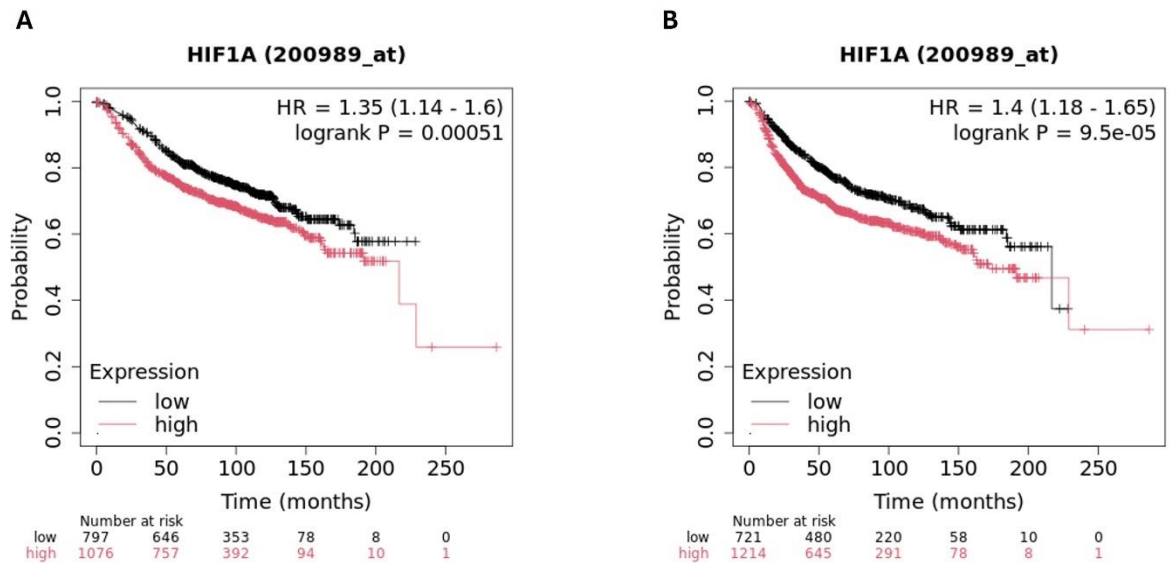


Figure 6-10: Kaplan Meier curves showed significant associations between high HIF-1 α mRNA expression and adjuvant therapy

(A) Endocrine therapy and (B) Chemotherapy.

Regarding bc-GenExMiner data, there was a significant association between high HIF-1 α mRNA levels and NST tumour type ($p < 0.0003$), and HER2 positive and basal-like BC ($p < 0.0001$) (**Figure 6-11**). Additionally, high HIF-1 α mRNA levels were significantly associated with poor outcomes in the whole cohort of bc-GenExMiner data either BCSS (HR: 1.19; 95%CI 1.11-1.27; $0 < 0.0001$) OR DMFS (HR: 1.25; 95%CI 1.15-1.38; $0 < 0.0001$), (**Figure 6-12**).

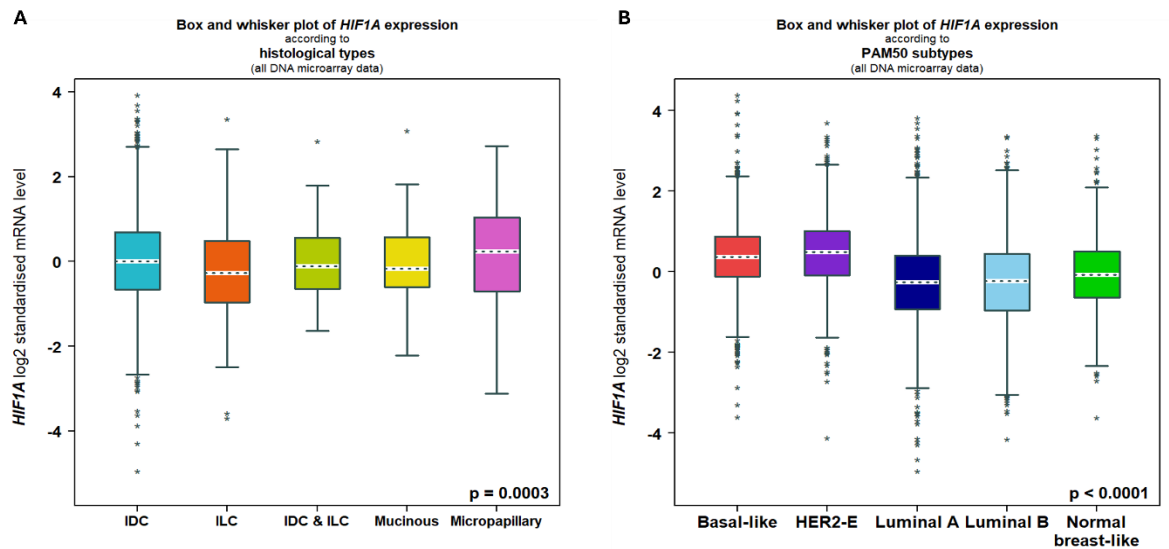


Figure 6-11: Box and whisker plots showed a significant association between high HIF-1 α mRNA expression histological and PAM50 subtypes.

(A) Association high HIF-1 α mRNA with NST tumour type. **(B)** Association of HIF-1 α mRNA levels with HER2 + and Basal-like BC.

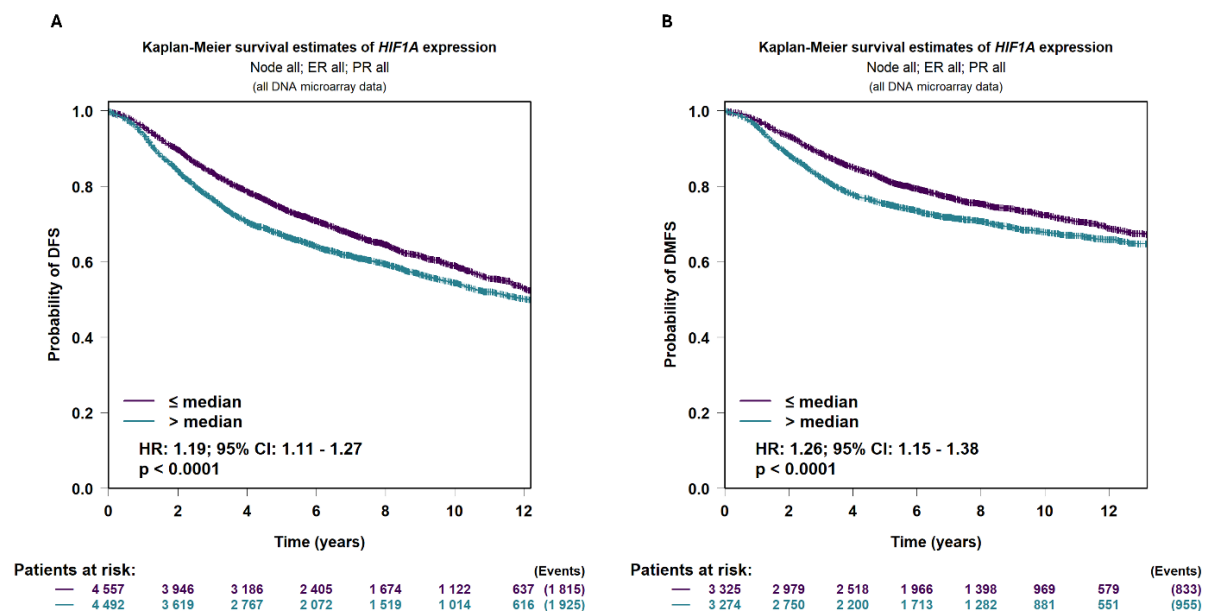


Figure 6-12: Kaplan Meier curves showed significant associations between high HIF-1 α mRNA expression and poor outcome in the whole bc-GenExMiner cohort.

(A) BCSS (HR: 1.19; 95%CI 1.11-1.27; $p < 0.0001$). **(B)** DMFS (HR: 1.25; 95%CI 1.15-1.38; $p < 0.0001$).

6.3.2.2 HIF-1 α protein expression

A specific light band for HIF-1 α was observed, using western blot, at the predicted molecular weight, 95 KDa confirming the specificity of the HIF-1 α antibody, **Figure 6-13**.

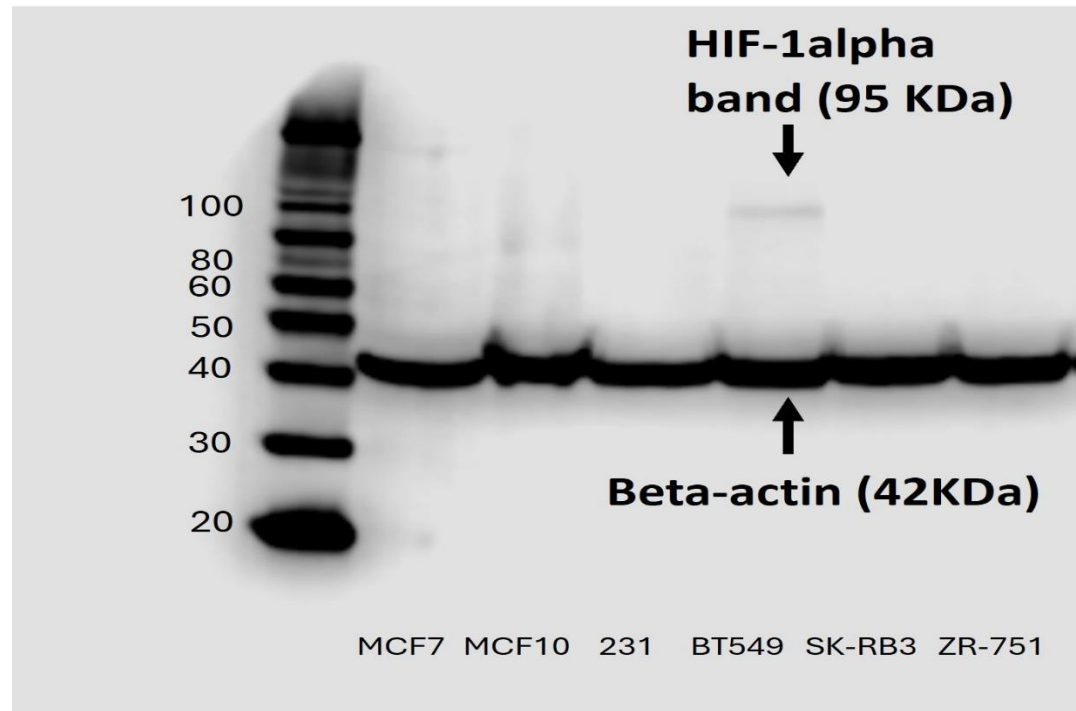


Figure 6-13: Western blot analysis of human inducible factor 1-alpha (HIF-1 α) in breast cancer cell lines.

HIF-1 α expression showed both nuclear and nucleolar subcellular localisation (**Figure 6-14 & Figure 6-15**)

There was good interobserver agreement in the scoring of HIF-1 α (ICC=0.7). The mean $\pm$ SD of H. score of HIF-1 α was 103.44 $\pm$ 98.59, the median was 60 and ranged from 0-300. High HIF-1 α expression (H-score >180) was observed in 23.4% (205/876) of the patients.

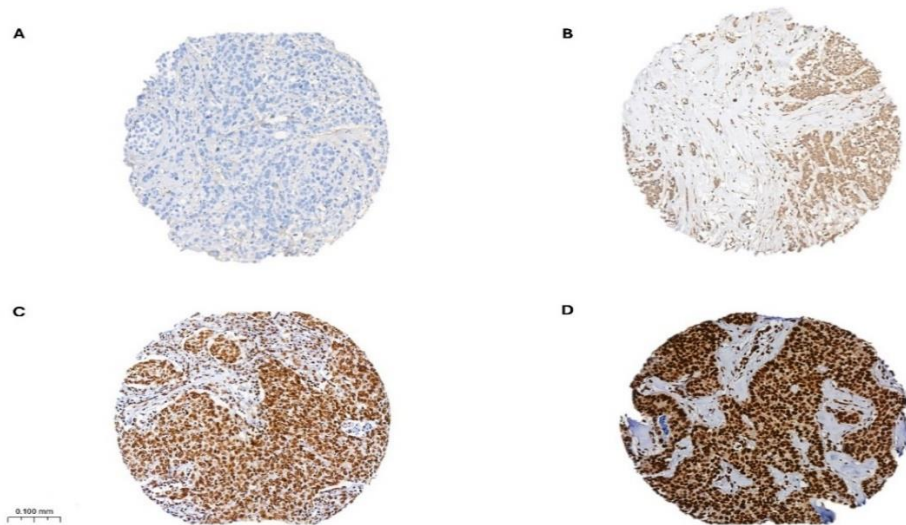


Figure 6-14: Expression of HIF-1 α in tissue microarrays (TMAs) in a three-tiered scoring system for intensity in a low-power view.

A) Negative expression. Expression scores: **B)** Score 1 (weak), **C)** Score 2 (moderate), **D)** score 3 (strong).

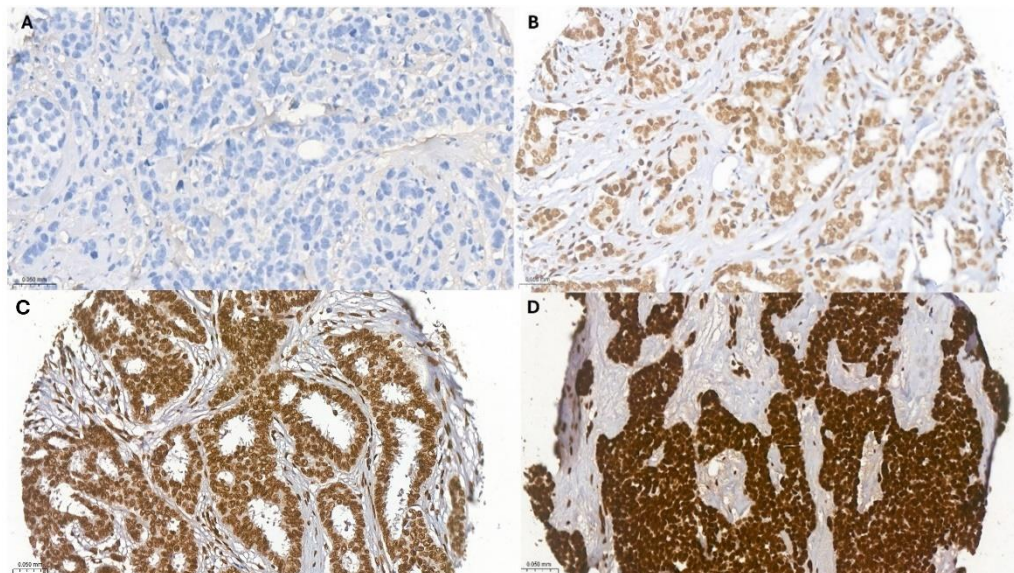


Figure 6-15: Expression of HIF-1 α in tissue microarrays (TMAs) in a three-tiered scoring system for intensity in a high-power view.

A) Negative expression. Expression scores: **B)** Score 1 (weak), **C)** Score 2 (moderate), **D)** score 3 (strong).

6.3.2.3 Association of HIF-1 α expression with clinicopathological data

High HIF-1 α expression was significantly associated with features characteristic of aggressive behaviour including larger tumour size, higher tumour grade, poor NPI, HER2 status, high Ki67 index, molecular subtypes HER2 positive and TNBC (All, $p=0.001$; **Table 6-4**).

Table 6-4: The association of clinicopathological data with HIF-1 α expression.

Parameters	Low HIF-1 α expression (n, %)	High HIF-1 α expression (n, %)	P value Significance $p < 0.05$
Patient Age ≤ 50 years > 50 years	195 (70.4%) 476 (79.5%)	82 (29.6%) 123 (20.5%)	$P < 0.5$
Size ≤ 2cm > 2cm	440 (84.8%) 231 (64.7%)	79 (15.2%) 126 (35.3%)	$P < 0.001$
Estrogen receptor Positive Negative	524 (82.9%) 147 (60.2%)	108 (17.1%) 97 (39.8%)	$P < 0.001$
Progesterone receptor Positive Negative	477 (83.1%) 191 (64.5%)	97 (16.9%) 105 (35.5%)	$P < 0.001$
HER2 status Positive Negative	58 (63.7%) 613 (78.1%)	33 (36.3%) 172 (21.9%)	$P < 0.001$
Ki67 score High Low	238 (65.6%) 307 (90.3%)	125 (34.4%) 33 (9.7%)	$P < 0.001$
Molecular subtypes Luminal A Luminal B Her2 enriched. Triple-negative	291 (94.2%) 153 (70.2%) 58 (64.4%) 89 (57.8%)	18 (5.8%) 65 (29.8%) 32 (35.6%) 65 (42.2%)	$P < 0.001$
Tumour Grade Grade I Grade II Grade III	118 (96.7%) 302 (85.6%) 251 (62.6%)	4 (3.3%) 51 (14.4%) 150 (37.4%)	$P < 0.001$
Histological type NST Lobular Metaplastic Special types Mixed	421 (72.1%) 52 (83.9%) 2 (100%) 33 (97.1%) 163 (84%)	163 (27.9%) 10 (16.1%) 0 (0%) 1 (2.9%) 31 (16%)	$P < 0.001$
Lymph vascular invasion (LVI) Definite Negative	168 (65.1%) 503 (81.4%)	90 (34.9%) 115 (18.6%)	$P < 0.001$
LN metastasis Negative Positive	441 (81.8%) 230 (68.5%)	98 (18.2%) 106 (31.5%)	$P < 0.001$
Nottingham prognostic index (NPI) Good Moderate Poor	274 (94.8%) 313 (72.5%) 84 (54.5%)	15 (5.2%) 119 (27.5%) 70 (45.5%)	$P < 0.001$

6.3.2.4 HIF-1 α protein expression and patient's outcome

In univariate analysis, BC patients with high HIF-1 α expression showed a significantly poor outcome in terms of shorter BCSS (HR 1.05, 95%CI 1.03-2.08; $p<0.001$) and shorter DMFS (HR 1.09, 95%CI 1.07-2.17; $p<0.001$), **Figure 6-16**.

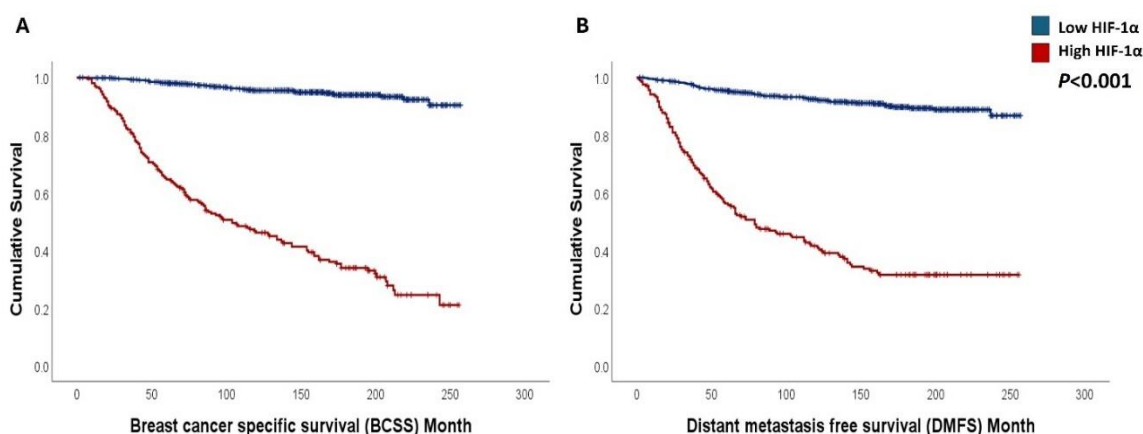


Figure 6-16: Kaplan Meier survival plots showing the correlation of HIF-1 α expression on survival outcomes, cut-off is 180.

(A) breast cancer-specific survival (BCSS) (HR 1.05, 95%CI 1.03-2.08; $p<0.001$). **(B)** Distant metastasis free survival (DMFS) (HR 1.09, 95%CI 1.07-2.17; $p<0.001$).

When cases were classified according to the intrinsic molecular subtypes, low HIF-1 α expression was associated with longer survival in luminal A (BCSS: HR 0.09, 95%CI 0.07-0.12; $p<0.001$ and DMFS: HR 0.02, 95%CI 0.01-0.05; $p<0.001$) and luminal B (BCSS: HR 0.22, 95%CI 0.12-0.40; $p<0.001$ and DMFS: HR 0.24, 95%CI 0.14-0.42; $p<0.001$). Regarding HER2 positive, low HIF-1 α expression was associated with favourable outcomes (BCSS: HR 0.02, 95%CI 0.006-0.07; $p<0.001$ and DMFS: HR 0.05, 95%CI 0.02-0.12; $p<0.001$). Similarly, in TNBC, low HIF-1 α expression was associated with better outcomes (BCSS: HR 0.05, 95%CI 0.02- 0.16; $p<0.001$ and DMFS: HR 0.01, 95%CI 0.002-0.09; $p<0.001$) (**Figure 6-17 & Figure 6-18**).

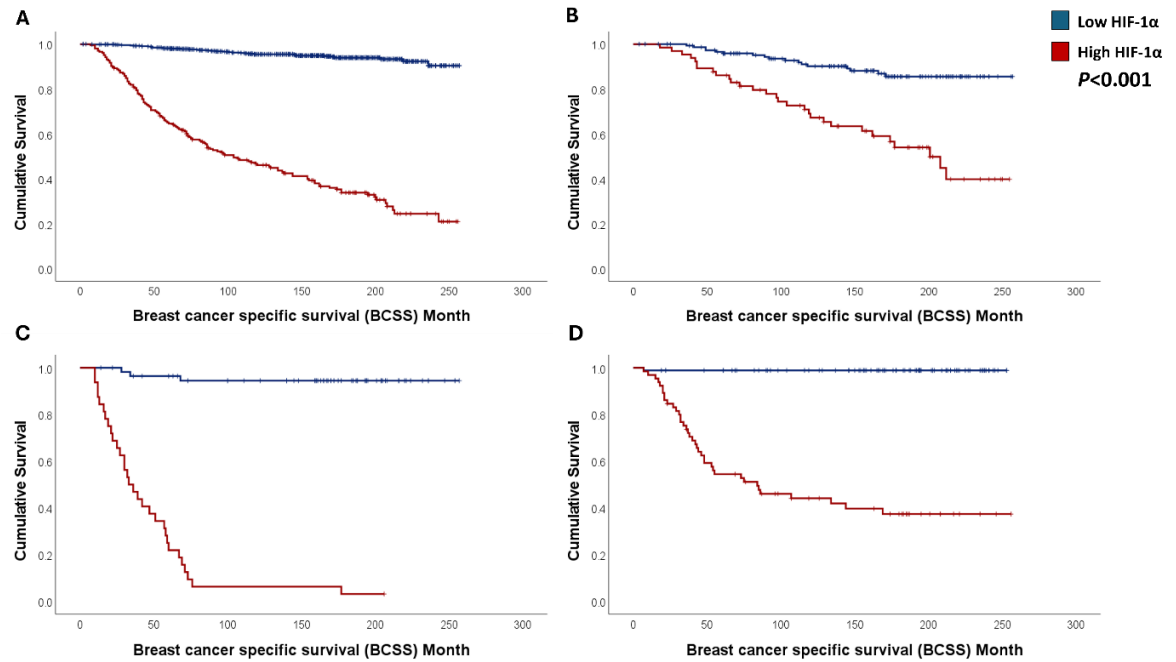


Figure 6-17. Kaplan Meier survival plots showing the correlation of HIF-1α expression on breast cancer-specific survival (BCSS) in different molecular subtypes of BC, cut-off is 180..

(A) Luminal A (HR 0.09, 95%CI 0.07-0.12; $p < 0.001$). **(B)** Luminal B (HR 0.22, 95%CI 0.12-0.40; $p < 0.001$). **(C)** HER2+ (HR 0.02, 95%CI 0.006-0.07; $p < 0.001$; $p < 0.001$). **(D)** TNBC (HR 0.05, 95%CI 0.02- 0.16; $p < 0.001$).

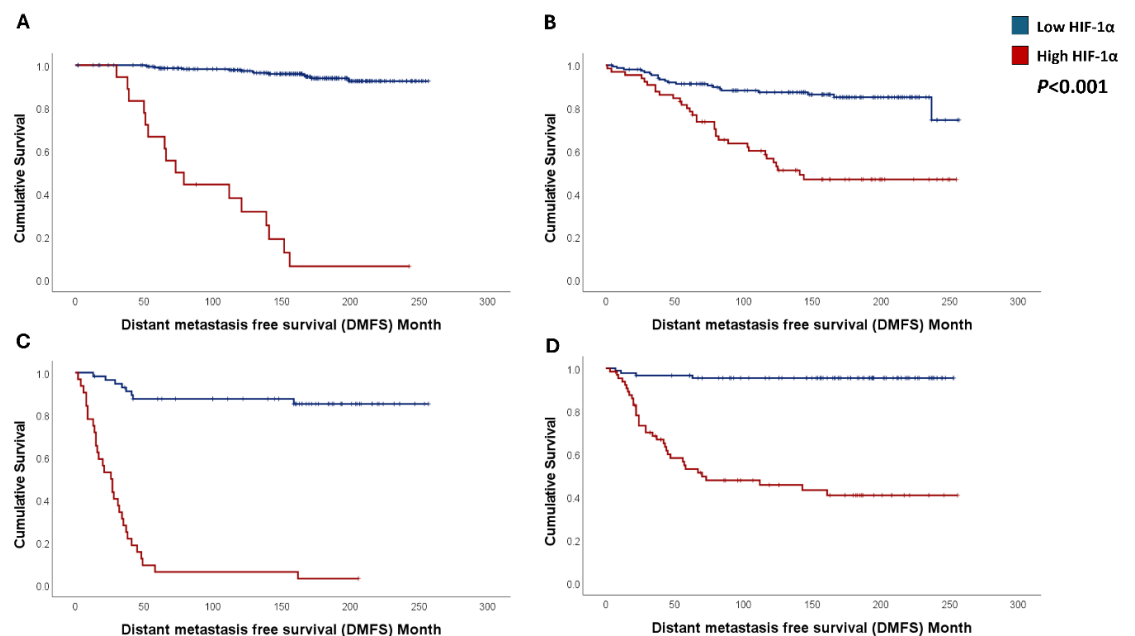


Figure 6-18: Kaplan Meier survival plots showing the correlation of HIF-1α expression on Distant metastasis free survival (DMFS) in different molecular subtypes of BC, cut-off is 180..

(A) Luminal A (HR 0.02, 95%CI 0.01-0.05; $p<0.001$). (B) Luminal B (HR 0.22, 95%CI 0.12-0.40; $p<0.001$; $p<0.001$). (C) HER2+ (HR 0.05, 95%CI 0.02-0.12; $p<0.001$; $p<0.001$). (D) TNBC (HR 0.01, 95%CI 0.002-0.09; $p<0.001$).

When the cohort was stratified based on the adjuvant therapy, high HIF-1 α expression showed a significant association with shorter BCSS in patients who were on hormonal therapy (HR1.06, 95%CI 1.04-1.08; $p<0.001$) and chemotherapy (HR 1.07, 95%CI 1.04-1.1; $p<0.001$). Similarly, high HIF-1 α expression was associated with shorter DMFS in patients receiving hormone treatment (HR 1.09, 95%CI 1.07-2.13; $p<0.001$), as well as chemotherapy (HR 1.11, 95%CI 1.08-1.16; $p<0.001$), (**Figure 6-19**).

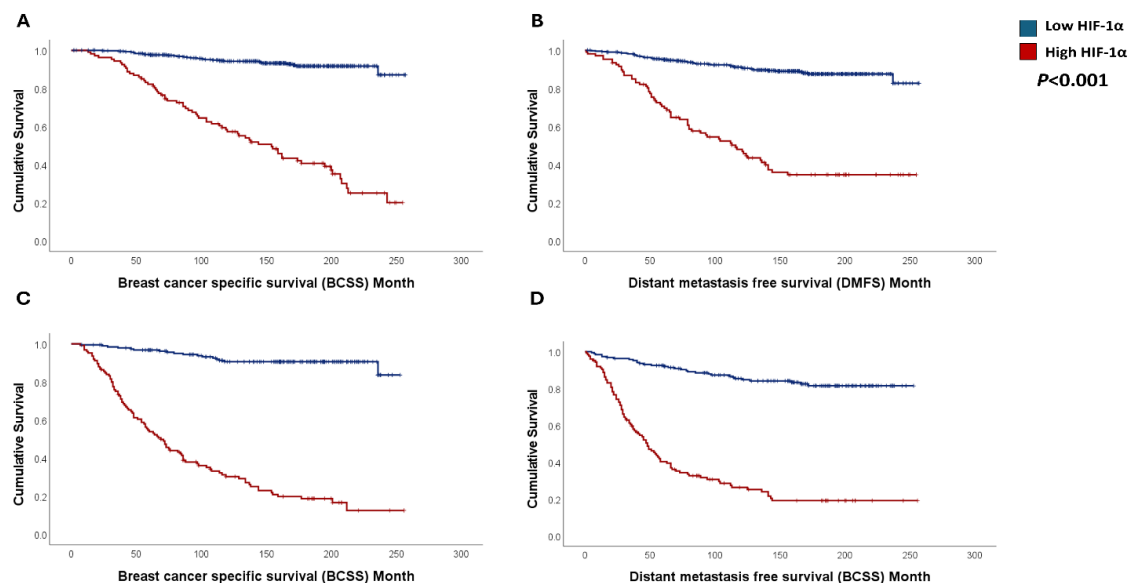


Figure 6-19: Kaplan Meier survival plots showing the correlation of HIF-1 α expression on adjuvant therapy, cut-off is 180..

(A) Association of HIF-1 α expression on patients who received endocrine therapy on breast cancer-specific survival (BCSS) (HR1.06, 95%CI 1.04-1.08; $p<0.001$; $p<0.001$) as well as (B) Distant metastasis-free survival (DMFS) (HR 1.09, 95%CI 1.07-2.13; $p<0.001$). (C) Association of HIF-1 α expression on patients who received chemotherapy on BCSS (HR 1.07, 95%CI 1.04-1.1; $p<0.001$) as well as (D) DMFS (HR 1.11, 95%CI 1.08-1.16; $p<0.001$).

In the multivariate Cox regression model for the whole cohort including other prognostic covariates (grade, tumour size, LVI invasion, lymph node status and Ki67), HIF-1 α expression was an independent predictor of shorter BCSS (HR 1.9, 95% CI 1.28-2.87, $p<0.001$) as well, as shorter DMFS (HR 1.05, 95% CI 1.02-1.15, $p<0.001$) (**Table 6-5**).

Table 6-5: Multivariate analysis of HIF-1 α in breast cancer

Parameters	Breast cancer specific survival (BCSS)			Distant metastasis free survival (DMFS)		
	Hazard ratio	95% (CI)	P-value	Hazard ratio	95% (CI)	P-value
HIF-1 α	0.07	0.4-0.1	0.001	0.18	0.12-0.28	0.001
Tumour size	0.67	0.47-0.94	0.02	1.35	1.30-2.2	0.2
Grade	0.36	0.12-0.98	0.04	0.45	0.19-0.91	0.04
LN status	0.8	0.1-0.92	0.001	0.12	0.01-0.91	<0.001
LVI	0.63	0.45-0.89	0.01	0.64	0.47-0.88	0.006
Ki67	0.7	0.45-1.18	0.20	0.76	0.68-1.15	0.23

6.3.3 Lox expression in BC

6.3.3.1 Lox mRNA expression

High LOX mRNA levels were significantly associated with poor outcomes in the whole cohort in terms of BCSS (HR 1.21, 95%CI 1.08-1.35; $p=0.0009$) and DMFS (HR 1.39, 95%CI 1.16-1.67; $p<0.0003$), (**Figure 6-20**).

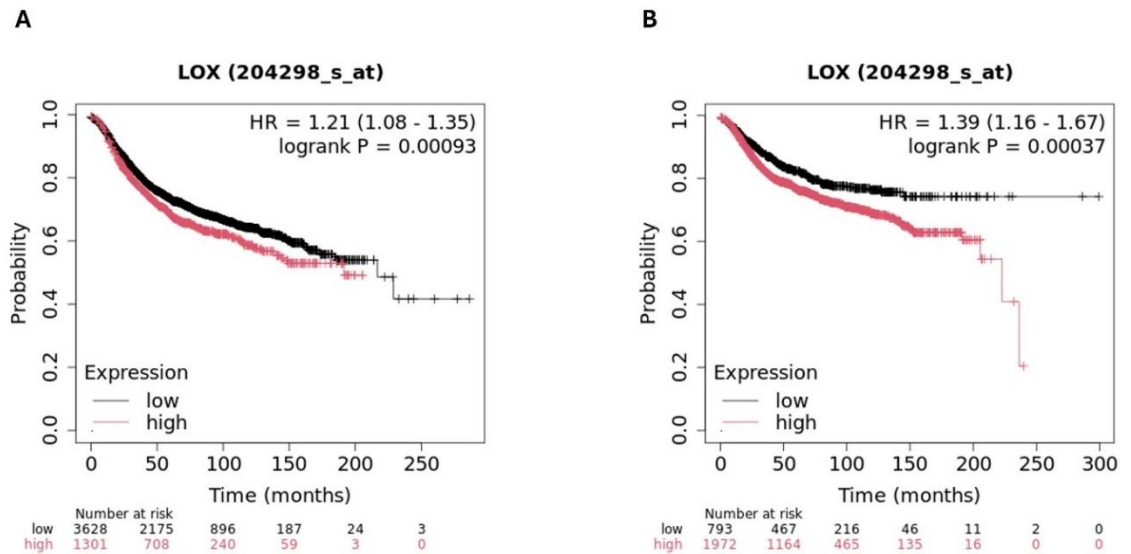


Figure 6-20: Kaplan Meier curves showing significant associations of high LOX mRNA expression and poor outcome in the whole cohort.

(A) Breast cancer specific survival (BCSS) and **(B)** Distant metastasis free survival (DMFS).

Regarding molecular subtypes, high HIF-1 α mRNA levels were significantly associated with shorter BCSS in all molecular subtypes, luminal BC (HR 1.74, 95%CI 1.03-2.93; $p=0.036$), HER2 positive (HR 2.38, 95%CI 1.21-4.71; $p=0.01$) and TNBC (HR 1.95, 95%CI 1.01-3.78; $p=0.044$), (**Figure 6-21**).

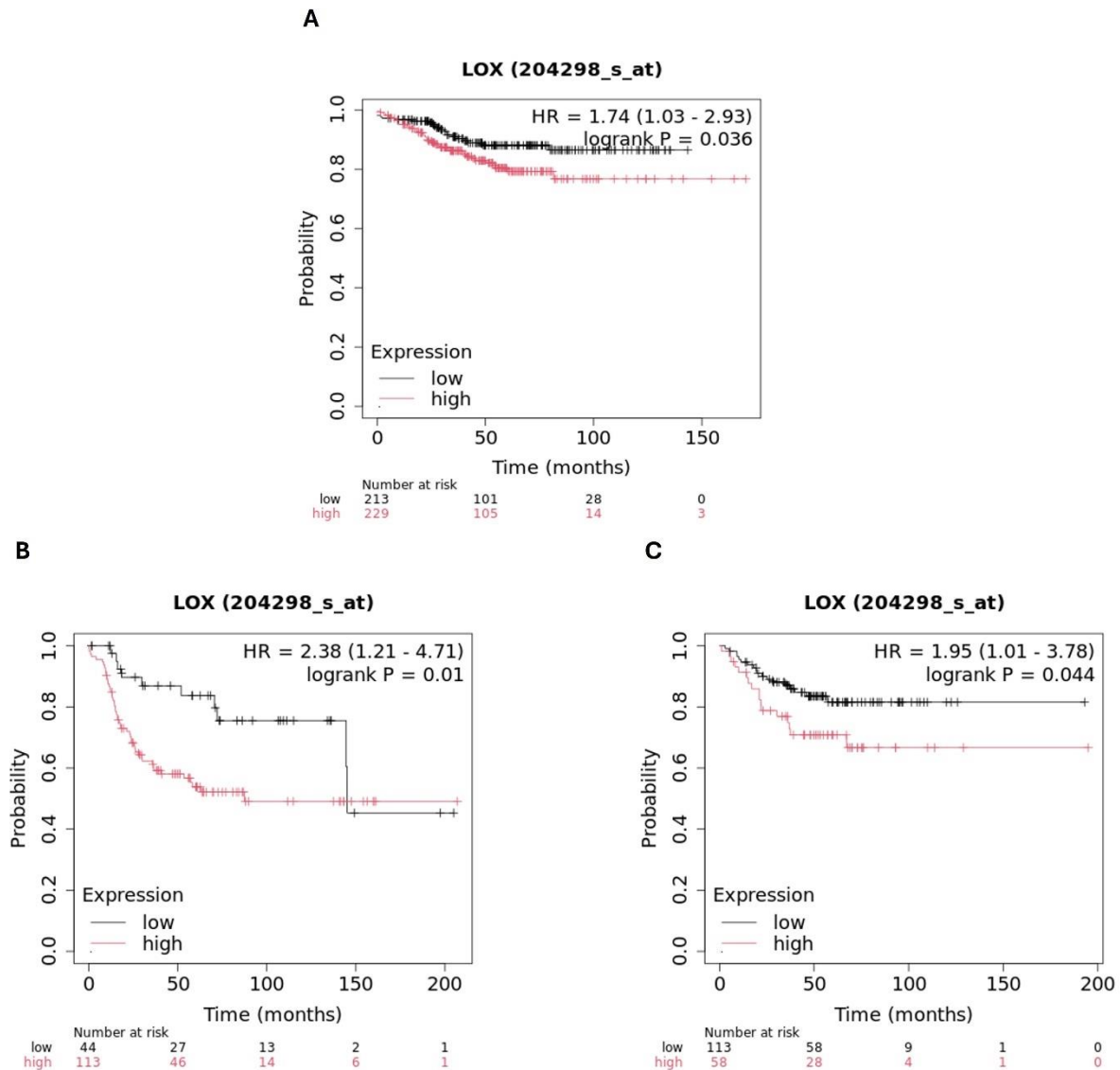


Figure 6-21: Kaplan Meier curves showing significant associations between high LOX mRNA expression and poor outcome in molecular subtypes.

(A) Luminal BC, **(B)** HER2 positive, and **(C)** TNBC.

Based on the adjuvant therapy, high LOX mRNA expression showed a significant association with shorter BCSS in patients who were on chemotherapy (HR 1.18, 95%CI 1.0-1.4; $p=0.047$). Conversely, there was no association between LOX and outcome in patients who were treated with hormonal therapy ($p=0.2$) (**Figure 6-22**).

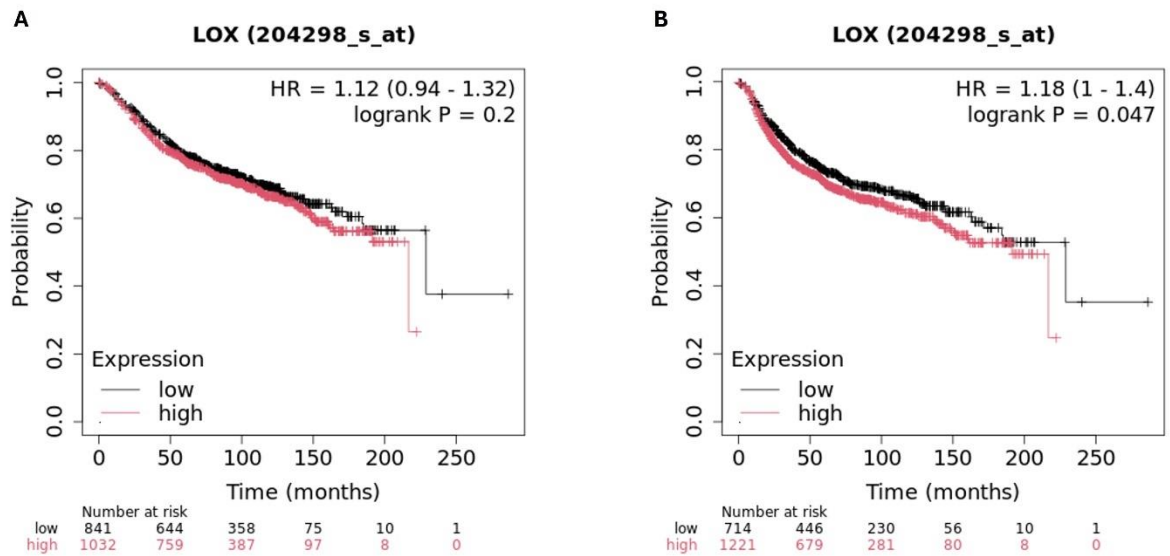


Figure 6-22: Kaplan Meier curves showing associations between high LOX mRNA expression and adjuvant therapy.

(A) Endocrine therapy and **(B)** Chemotherapy.

Regarding the bc-GenExMiner data, there was a significant association between high LOX mRNA levels and NST tumour type, and basal-like BC (Both $p < 0.0001$; **Figure 6-23**). Additionally, high LOX mRNA levels were significantly associated with poor outcomes in the whole cohort of the bc-GenExMiner data in terms of both BCSS (HR: 1.12; 95%CI 1.05-1.19; $p < 0.0007$) and DMFS (HR: 1.25; 95%CI 1.13-1.387 ; $p < 0.0001$; **Figure 6-24**).

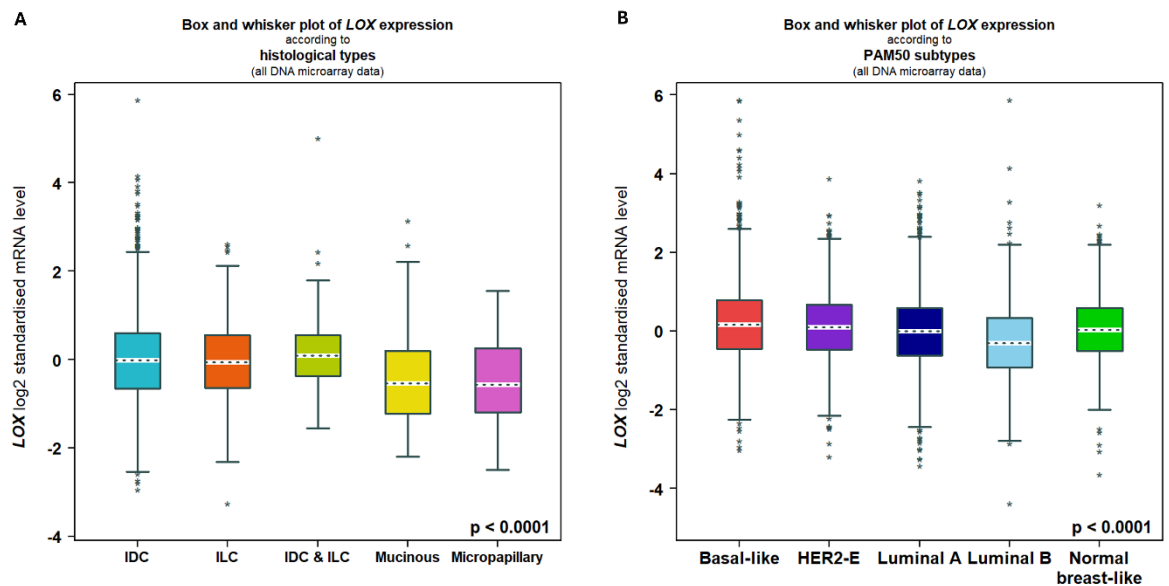


Figure 6-23: Box and whisker plots showing significant associations between high LOX mRNA expression histological and PAM50 subtypes.

(A) Association high LOX mRNA with NST tumour type. **(B)** Association of LOX mRNA levels with Basal-like BC.

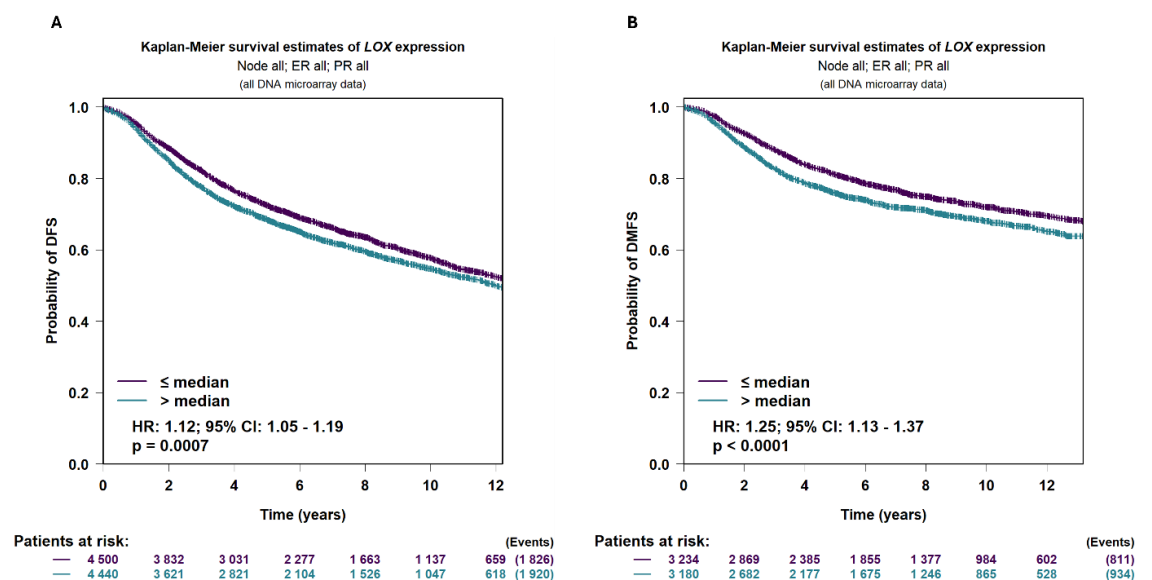


Figure 6-24: Kaplan Meier curves show significant associations of high LOX mRNA expression and poor outcome in the whole bc-GenExMiner cohort.

(A) BCSS ($HR: 1.12; 95\%CI 1.05-1.19; p < 0.0007$). **(B)** DMFS ($HR: 1.25; 95\%CI 1.13-1.387; p < 0.0001$).

6.3.3.2 Lox protein expression

Specific bands for LOX were observed following western blot, which were present at the predicted molecular weight of 47 KDa confirming the specificity of the LOX antibody (**Figure 6-25**).

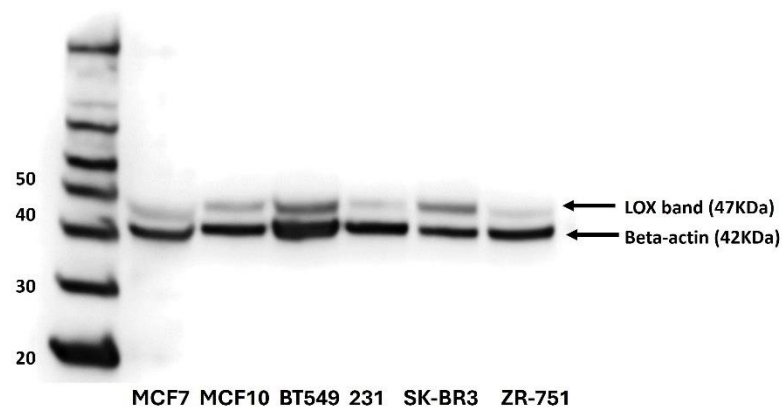


Figure 6-25: Western blot analysis lysyl oxidase (LOX) in breast cancer cell lines.

LOX expression showed cytoplasmic localisation (**Figure 6-26 & Figure 6-27**). There was good interobserver agreement in the scoring of LOX (ICC=0.8). The mean $\pm$ SD of the H. score of LOX was 120.91 ± 74.94 , the median was 100 and they ranged from 0-300. High LOX expression (H-score >160) was observed in 28.2% (247/876) of the patients.

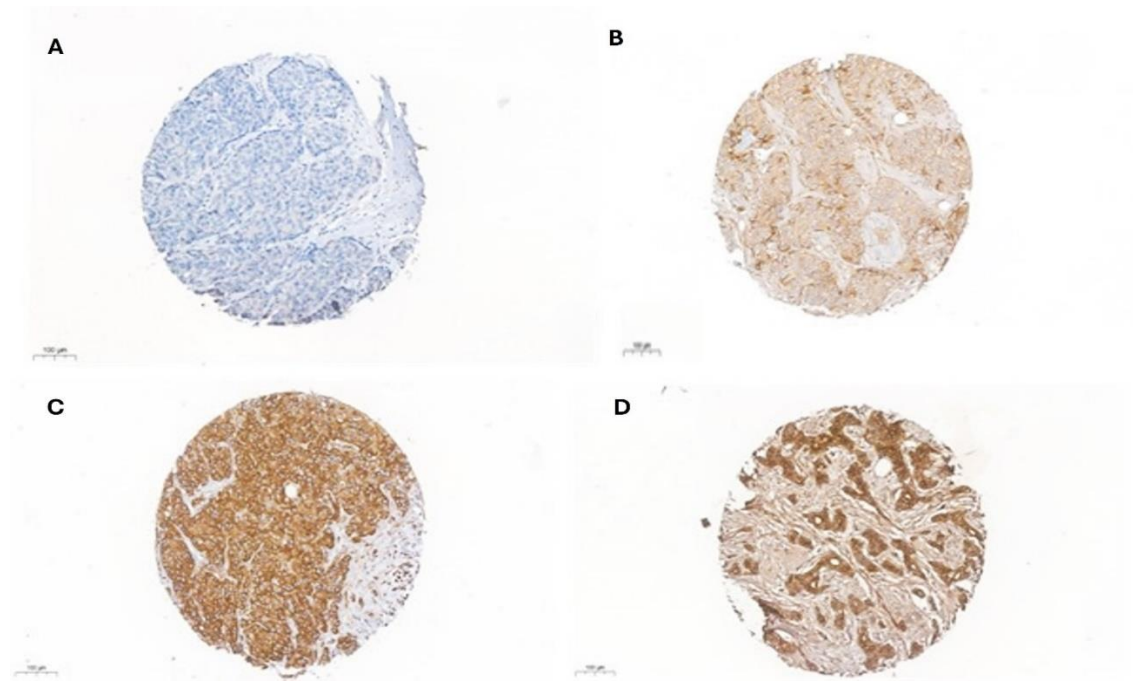


Figure 6-26: Expression of LOX in tissue microarrays (TMAs) in a three-tiered scoring system for intensity in a low-power view.

(A) negative expression. **(B)** Score 1 (weak) expression. **(C)** Score 2 (moderate) expression. **(D)** score 3 (strong) expression.

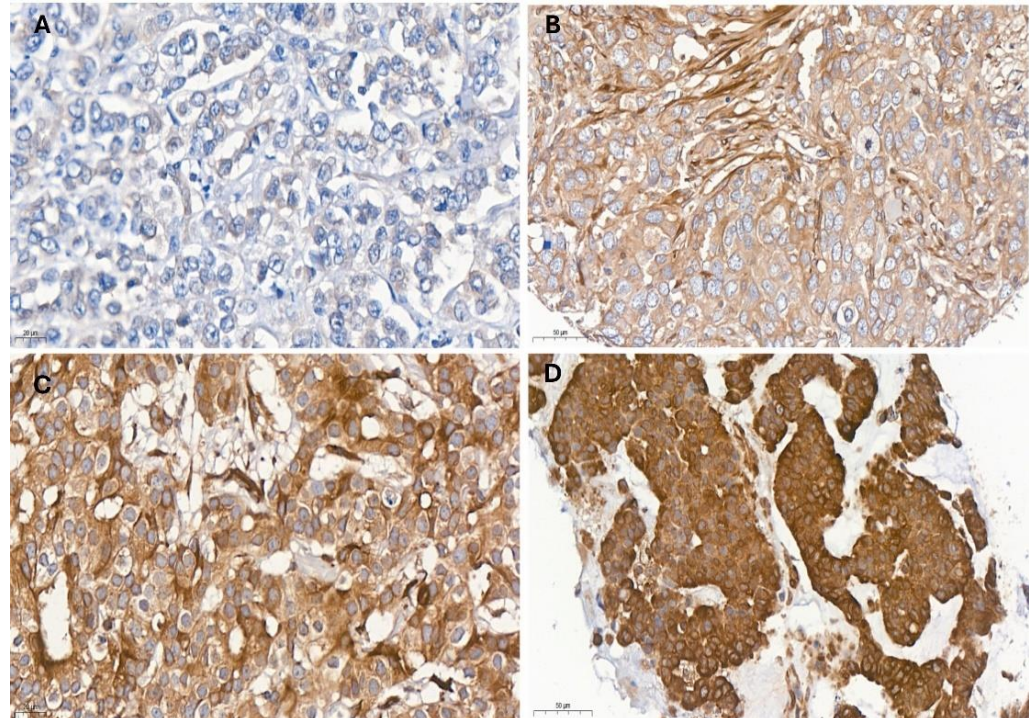


Figure 6-27: Expression of LOX in tissue microarrays (TMAs) in a three-tiered scoring system for intensity in a high-power view.

(A) negative expression. **(B)** Score 1 (weak) expression. **(C)** Score 2 (moderate) expression. **(D)** score 3 (strong) expression.

6.3.3.3 Association of LOX expression with clinicopathological data

High LOX expression was significantly associated with features characteristic of aggressive behaviour including larger tumour size, higher tumour grade, poor NPI, high Ki67 index, molecular subtypes HER2 positive and TNBC (All, $p=0.001$), (**Table 6-6**).

Table 6-6: Associations between clinicopathological data and LOX expression

Parameters	Low LOX expression (n, %)	High LOX expression (n, %)	P value Significance $p < 0.05$
Patient Age ≤ 50 years > 50 years	189 (68.2%) 440 (73.5%)	88 (31.8%) 159 (26.5%)	$P < 0.11$
Size ≤ 2cm > 2cm	405 (64.4%) 114 (46.2%)	224 (35.6%) 133 (53.8%)	$P < 0.001$
Estrogen receptor Positive Negative	490 (77.5%) 139 (57%)	142 (22.5%) 105 (43%)	$P < 0.001$
Progesterone receptor Positive Negative	444 (77.4%) 184 (62.2%)	130 (22.6%) 112 (37.8%)	$P < 0.001$
HER2 status Positive Negative	59 (64.8%) 570 (72.6%)	32 (35.2%) 215 (27.4%)	$P < 0.11$
Ki67 score High Low	221 (60.9%) 286 (84.1%)	142 (39.1%) 54 (15.9%)	$P < 0.001$
Molecular subtypes Luminal A Luminal B Her2 enriched Triple-negative	265 (85.8%) 145 (66.5%) 58 (64.4%) 73 (52.6%)	44 (14.2%) 73 (33.5%) 32 (35.6%) 81 (47.4%)	$P < 0.001$
Tumour Grade Grade I Grade II Grade III	111 (91%) 296 (83.9%) 222 (55.4%)	11 (9%) 57 (16.1%) 179 (44.6%)	$P < 0.001$
Histological type NST Lobular Metaplastic Special types Mixed	390 (66.8%) 50 (80.6%) 2 (100%) 30 (88.2%) 157 (80.9%)	194 (33.2%) 12 (19.4%) 0 (0%) 4 (11.8%) 37 (19.1%)	$P < 0.001$
Lymph vascular invasion (LVI) Definite Negative	160 (62%) 469 (75.9%)	98 (38%) 149 (24.1%)	$P < 0.001$
LN metastasis Negative Positive	401 (74.4%) 228 (67.6%)	138 (25.6%) 109 (32.4%)	$P < 0.07$
Nottingham prognostic index Good Moderate Poor	256 (88.6%) 290 (67.1%) 83 (53.2%)	33 (11.4%) 142 (32.9%) 72 (46.8%)	$P < 0.001$

6.3.3.4 LOX protein expression and patient's outcome

In univariate analysis, BC patients with high LOX expression showed a significantly poorer outcome in terms of shorter BCSS (HR 1.46, 95%CI 1.17-1.99; $p<0.001$) and shorter DMFS (HR 1.75, 95%CI 1.5-3.7; $p<0.000$) (**Figure 6-28**).

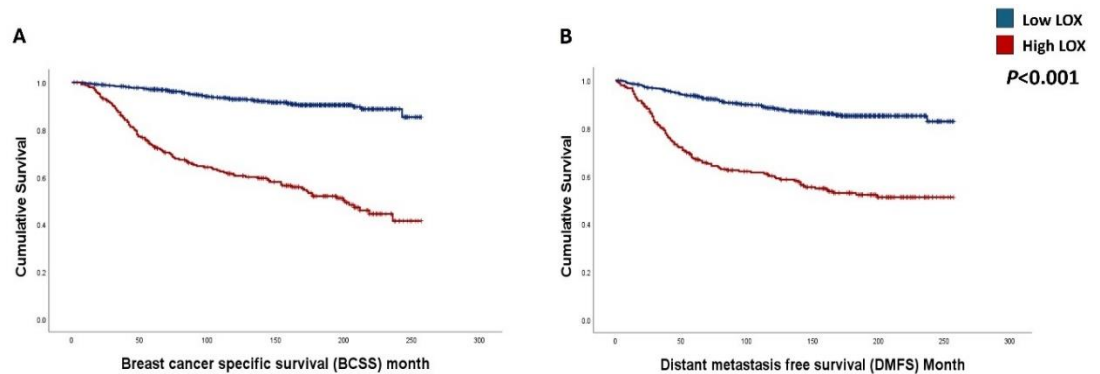


Figure 6-28: Kaplan Meier survival plots showing the correlation of LOX expression on survival outcomes.

(A) breast cancer-specific survival (BCSS) (HR 1.46, 95%CI 1.17-1.99, $p<0.001$). **(B)** Distant metastasis free survival (DMFS) (HR 1.75, 95%CI 1.5-3.7; $p<0.001$).

When cases were classified according to the intrinsic molecular subtypes, low LOX expression was associated with longer survival in luminal A (BCSS: HR 0.12, 95%CI 0.05-0.29; $p<0.001$ and DMFS: HR 0.26, 95%CI 0.12-0.55; $p<0.001$) and luminal B (BCSS: HR 0.28, 95%CI 0.15-0.51; $p<0.001$ and DMFS: HR 0.36, 95%CI 0.21-0.62; $p<0.001$). With regards to the HER2 positive, low LOX expression was associated with favourable outcomes (BCSS: HR 0.16, 95%CI 0.08-0.33; $p<0.001$ and DMFS: HR 0.1, 95%CI 0.04-0.2; $p<0.001$). Similarly, in TNBC, low LOX expression was associated with better outcomes (BCSS: HR 0.16, 95%CI 0.07- 0.38; $p<0.001$ and DMFS: HR 0.3, 95%CI 0.15-0.6; $p<0.001$), (**Figure 6-29 & Figure 6-30**).

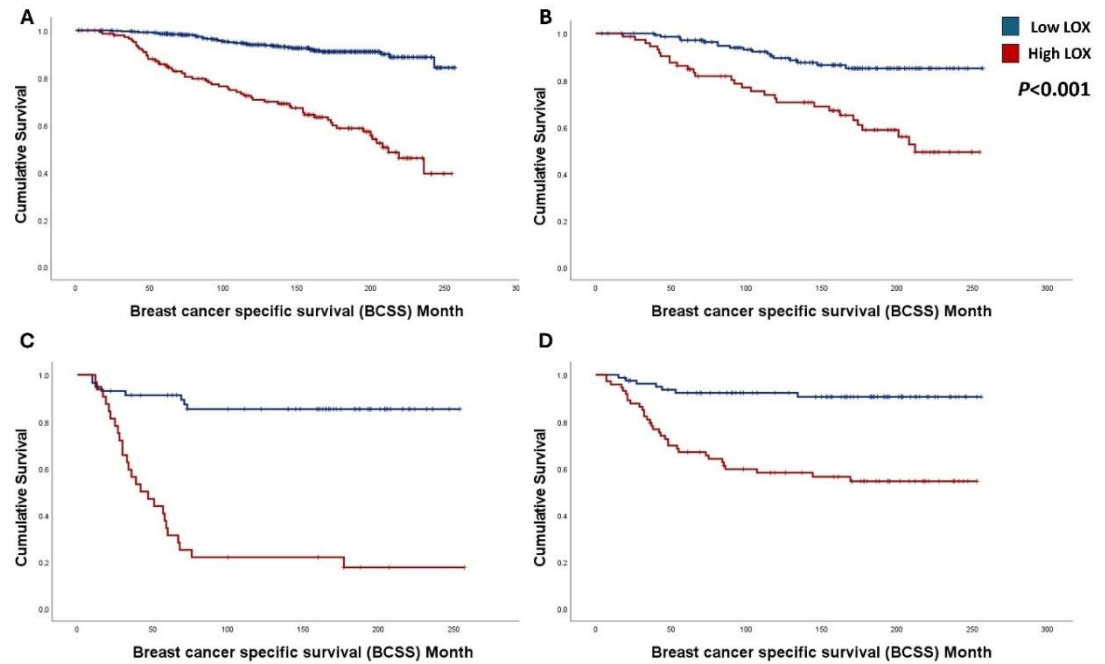


Figure 6-29: Kaplan Meier survival plots showing the correlation of LOX expression on breast cancer-specific survival (BCSS) in different molecular subtypes of BC.

(A) Luminal A (HR 0.12, 95%CI 0.05-0.29; $p < 0.001$). **(B)** Luminal B (HR 0.28, 95%CI 0.15-0.51; $p < 0.001$). **(C)** HER2+ (HR 0.16, 95%CI 0.08-0.33; $p < 0.001$). **(D)** TNBC (HR 0.16, 95%CI 0.07-0.38; $p < 0.001$).

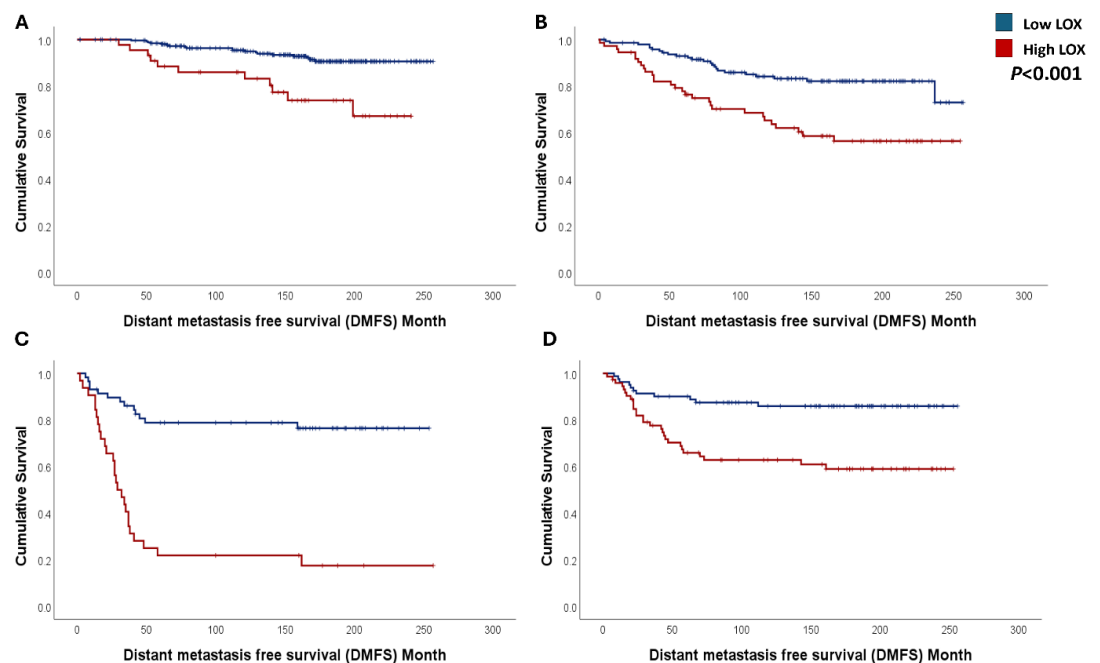


Figure 6-30: Kaplan Meier survival plots showing the correlation of LOX expression on Distant metastasis free survival (DMFS) in different molecular subtypes of BC.

(A) Luminal A (HR 0.26, 95%CI 0.12-0.55; $p < 0.001$). **(B)** Luminal B (HR 0.36, 95%CI 0.21-0.62; $p < 0.001$). **(C)** HER2+ (HR 0.1, 95%CI 0.04-0.2; $p < 0.001$). **(D)** TNBC (HR 0.3, 95%CI 0.15-0.6; $p < 0.001$).

When the cohort was stratified based on the adjuvant therapy, high LOX expression showed a significant association with shorter BCSS in patients who were on hormonal therapy (HR1.52, 95%CI 1.1-2.11; $p<0.001$) and chemotherapy (HR 1.8, 95%CI 1.3-2.5; $p<0.001$). Similarly, high LOX expression was associated with shorter DMFS in patients receiving hormone treatment (HR 2.4, 95%CI 1.8-3.2; $p<0.001$), as well as chemotherapy (HR 2.9, 95%CI 2.1-3.9; $p<0.001$), (**Figure 6-31**).

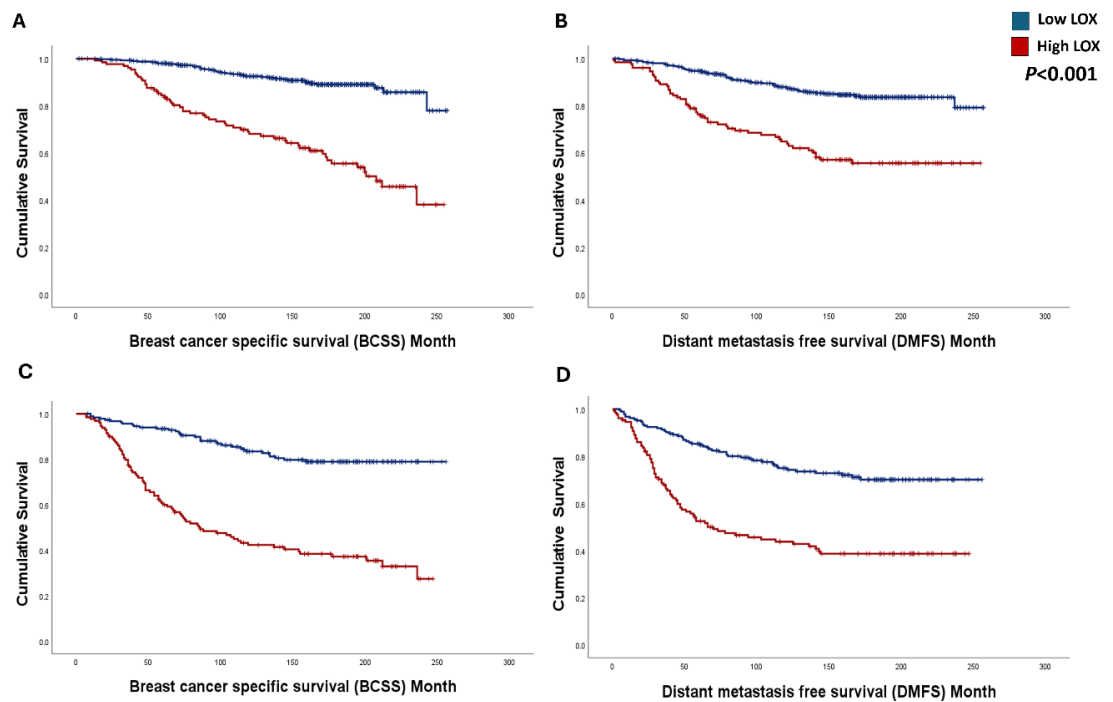


Figure 6-31: Kaplan Meier survival plots showing the correlation of LOX expression on adjuvant therapy.

(A) Association of LOX expression on patients who receive endocrine therapy on breast cancer-specific survival (BCSS; HR1.52, 95%CI 1.1-2.11; $p<0.001$) as well as (B) Distant metastasis-free survival (DMFS; HR 2.4, 95%CI 1.8-3.2; $p<0.001$) (C) Association of LOX expression on patients who receive chemotherapy on BCSS (HR 1.8, 95%CI 1.3-2.5; $p<0.001$) as well as (D) DMFS (HR 2.9, 95%CI 2.1-3.9; $p<0.001$).

In the multivariate Cox regression model in the whole cohort including other prognostic covariates (grade, tumour size, LVI invasion, lymph node status and Ki67), LOX expression was an independent predictor of shorter BCSS (HR 1.9, 95% CI 1.28-2.87, $p<0.001$) as well, as shorter DMFS (HR 1.98, 95% CI 1.1-4.3, $p<0.001$) (**Table 6-7**).

Table 6-7: Multivariate analysis of LOX in Breast cancer

Parameters	Breast cancer specific survival (BCSS)			Distant metastasis free survival (DMFS)		
	Hazard ratio	95% (CI)	P-value	Hazard ratio	95% (CI)	P-value
LOX	0.28	0.2-0.86	0.001	0.17	0.11-0.26	0.001
Tumour size	1.82	1.28-2.58	0.004	1.35	1.30-2.2	0.02
Grade	1.86	1.21-1.69	0.38	1.25	1-2.20	0.37
LN status	1.003	1-1.005	0.003	1.1	1-2.15	<0.001
LVI	2.1	1.5-3.02	0.024	1.5	1.12-2.10	0.001
KI67	1.33	0.88-2.22	0.17	0.76	0.68-1.15	0.17

6.3.4 Correlation between HIF-1 α and LOX

There was a strong positive correlation between HIF-1 α and LOX ($r=0.7$, $p<0.0001$).

6.4 Discussion

The usual traditional biomarkers for BC prognosis and prediction have enabled progress in the overall survival of this disease (605-607). Still, to deliver additional profits from precision medicine, there is a continuous and necessary demand for the identification of robust biomarkers capable of predicting the prognosis of BC and therapy responses (608). Identifying biomarkers could also help address the issue of drug resistance, a significant challenge in BC treatment (542).

BC progression proceeds through different stages starting from *in situ*, micro-invasive, invasive, and metastatic (231, 329, 552, 554, 609). Extensive characterisation of BM continuity to better define the interactions between tumour cells and TME, clarifying the mechanism of local infiltration, is required. Analyses of IHCs have consistently reported the presence of collagen IV and laminin surrounding the *in-situ* carcinomas proving intact BM presence and their lack in the malignant glands (173, 237, 610, 611). However, fragmentation or complete absence of BM is found in invasive BC (237). There is evidence that some invasive BCs can show variable retention of the BM structure

at the epithelial stroma interface (428, 429). Some special types of BC, such as ACC, show thickened BMs in comparison to normal, benign or in situ lesions. However, data on the nature of BM materials associated with invasive tumour foci remains to be elucidated. Tumour-associated BM materials and structure are likely to be different from the native BM present around normal parenchymal structure in the breast and around benign and in situ lesions (431). Therefore, the identification of new proteins in the BM may develop a greater insight into the process of tumour invasion.

Collagen XV is a non-fibrillar collagen type presented in the BMZ of the normal breast tissue, Its role is to help restrict epithelial cells to their BM, which contributes to ECM integrity and stability (551). Similarly, collagen XIX is present in normal breast tissue's BMZ and supports ECM integrity and epithelial layer growth and maintenance (272). A previous study reported the presence of collagens XV and XIX in the BMZ of DCIS however, levels started to diminish which suggested that the BM is still intact and acts as a barrier to invasion. However, loss of collagen XV could be an early sign of BM loss, indicating the beginning of ECM remodelling and invasion behaviour (272).

This was consistent with our study results as the IHC showed that collagens XV and XIX were observed surrounding the DCIS group conversely their expression was absent in EPC. Collagen XV was expressed in the BM-like material in the ACC which may explain the presence of BM-like material with altered function. This loss disrupts the physical and molecular barrier provided by the BM, allowing tumour cells to penetrate and metastasize to distant sites. Moreover, previous studies have shown that collagen XV is a tumour suppressor. Overexpression of collagen XV was associated with tumorigenicity suppression which suggests that collagen XV is essential for tumour progression prevention. In contrast, the loss of collagen XV is associated with metastatic potential (551, 612). This may explain the good prognosis of ACC. Conversely, in the present study, collagen XIX lost its expression in ACC which could be

used as a diagnostic marker to differentiate between DCIS and BM-like material in some invasive lesions. Additionally, collagen XIX plays a key role as anti-tumour and anti-angiogenic (613). The levels of circulating collagen XIX showed a significant rise in BC patients' serum compared to healthy controls suggesting that it could be a promising biomarker for BC (564).

Notably, there was been a significant number of women diagnosed with in situ carcinoma who subsequently developed metastatic disease (614). This could be a preliminary clarification of unrecognized molecular events that happened at the interface of stroma and *in-situ*, lesions which presented histologically as micro invasion. These BMZ collagens could act as a method to speculate the micro invasion process. Understanding the dynamics of collagen types XV and XIX expression and their interaction with other BMZ components may provide valuable insights into the mechanisms of BC progression and metastasis (551, 564, 612).

Tumour fibrosis has been recognized as an essential factor which influences tumour progression and metastasis (582). The molecular mechanisms that contribute to BC invasion and metastasis are still poorly understood (615). Recent studies have supported that LOX is implicated in creating a fibrotic microenvironment and fibrosis-enhanced cancer metastasis (582, 616, 617). Lox expression in tumour cells is induced by hypoxia that contributes to fibrogenesis promotion and tumour growth and invasion through the EMT process (618). Tumour hypoxia is considered an adaptive mechanism caused by tumour cells can survive in the oxygen-deprived TME to support tumour progression (619). In our study, we analysed a large cohort of BC and compared their expression with the patient outcomes in different molecular subtypes. The results suggested that HIF-1 α and LOX overexpression conveys part of the aggressiveness in BC.

Studies have shown that high HIF-1 α levels correlate with more aggressive tumour behaviour. For example, patients with high HIF-1 α levels presented with advanced-stage disease and a higher tendency of

metastasis, indicating that HIF-1 α may serve as a marker of tumour aggressiveness (620, 621). In addition, high HIF-1 α expression is significantly associated with poorer outcomes. A meta-analysis reported that high HIF-1 α levels were correlated to a hazard ratio of 1.46 for OS and 1.91 for BCSS, suggesting that patients with high HIF-1 α have about 50% higher risk of death or recurrence compared to those with lower levels (621). Unfortunately, the studies included in this meta-analysis had a small cohort with heterogeneous HIF-1 α expression classification. In addition, HIF-1 α has been associated with therapy resistance to either chemotherapy or hormonal (622). This could be explained by the role of high HIF-1 α levels in angiogenesis via the upregulation of vascular endothelial growth factor (VEGF) which enhances tumour vascularization and promotes metastasis (623, 624).

TCGA data showed that HIF-1 α was not significantly associated with overall survival (OS) in BC however, it was significantly associated with recurrence (625). Although several studies support the prognostic value of HIF-1 α in BC, other research has described inconsistent associations, especially regarding specific molecular BC subtypes such as ER-positive tumours. Therefore, this heterogeneity may suggest the diverse roles of HIF-1 α depending on the TME and molecular characteristics which needs further investigation (619).

Levels of HIF-1 α expression varied among BC molecular subtypes, TNBC showed the highest levels, while luminal A BC had the lowest (624). Previous studies have also shown that there was an inverse correlation between ER expression and HIF-1 α expression (565, 623). Few studies have investigated the association of HIF-1 α in luminal BC. Some studies reported an association between high HIF-1 α expression and shorter BCSS in ER-positive, but no significant association was reported in ER-negative patients (626, 627). Conversely, many studies showed associations between HIF-1 α and TNBC where there was an association with bad outcomes (628-630). Moreover, few data about the association of HIF-1 α in HER2 positive BC are available but the upregulation of HIF-1 α was observed with HER2/erb2 and was

associated with poor survival (631). The present study's results showed that the high levels of HIF-1 α had poor outcomes in all molecular subtypes.

These inconsistent results may result from different cut-offs used for scoring HIF-1 α expression therefore, a distinct definition of HIF-1 α overexpression should be based on an international standardised practice. For example the College of American Pathologists, the clinical practice guidelines from the American Society of Clinical Oncology, and Association for Molecular Pathology may need to standardise HIF-1 α expression guidance (625).

The present study results showed that a high HIF-1 α level was associated with therapy-resistance, either for hormonal therapy or chemotherapy, in all molecular subtypes. However, the mechanisms responsible for therapy resistance in both chemotherapy and hormonal therapy remain to be clarified (632). This finding was consistent with Generali *et al.*, who reported that HIF-1 α expression was a predictive marker of poor therapy response either hormonal or chemotherapy especially in ER+ patients (633). Both in vitro and in vivo experiments proved that hypoxia declines the effects of specific cytotoxic agents specifically anthracyclines (626, 634). This may be an indication that erythropoietin administration increases drug efficacy (635). Several clinical and preclinical trials targeted HIF-1 α to overcome therapy resistance through inhibition of HIF-1 α transcription and translation or degradation promotion (636-641).

LOX and HIF-1 α expression levels are positively correlated in many cancers. Both proteins are upregulated under hypoxic conditions which contribute to tumour progression and invasion (642). In hypoxic conditions, HIF-1 α regulates LOX transcription of LOX by binding to hypoxia response elements in the LOX promoter resulting in an increase in its expression (643). LOX can influence HIF-1 α levels. In contrast, LOX produces reactive oxygen species (ROS) to stabilize HIF-1 α enhances its activity and induces further LOX expression to create a self-timing

regulatory circuit (643). Regarding BC, hypoxia-induced LOX expression stimulates focal adhesion and integrin signalling which contribute to chemotherapy resistance (644).

Chan and coauthors demonstrated an association between LOX expression and the outcome in ER-negative BC, as high LOX expression showed shorter DMFS and BCSS (645), but in contrast, there is also a study that showed no association of LOX in the outcome of TNBC (592). The results published by Chan were consistent with our study's results that proved an association between high LOX and bad outcomes in TNBC and HER2 positive BC.

An in vivo study showed that LOX induces bone metastasis and bone destruction in TNBC cells by robust interleukin 6 (IL6) expression which stimulates receptor activator of nuclear factor kappa-B ligand (RANKL) expression in bone cells and promotes tumour growth and bone metastasis through osteoclast differentiation and activation (646). Leo and his colleagues demonstrated that strong LOX expression was significantly higher in TNBC and was stronger in poorly differentiated high grade cases (592) which was in agreement of our results which that showed that LOX overexpression was present in high grade tumours in all molecular subtypes. Moreover, there was up-regulation LOX expression in highly invasive/metastatic BC cell lines (Hs578T and MDA-MB-231) compared with a poorly invasive/nonmetastatic cell line (MCF7) (615).

LOX activation was identified in the mechanism of chemotherapy resistance (644). LOX inhibition was also proved to reduce extracellular collagen cross-linking and fibronectin deposition resulting in enhancing the drug penetration and reversal of chemotherapy resistance in TNBC cell lines. Interestingly, targeting LOX downstream resulted in strong sensitization of cancer cells to the chemotherapy (644). These findings agreed with our results that showed poor prognosis of LOX overexpression in patients who receive chemotherapy.

The role of LOX in BC invasiveness was proved by the addition of LOX antisense oligonucleotides to invasive cell lines which resulted in invasion inhibition. In addition, treatment of invasive cell lines with beta-aminopropionitrile which is an irreversible inhibitor of LOX enzymatic activity caused declined invasive activity (615). These results are consistent with the present study hypothesis that LOX expression contributes to cellular ability of invasiveness and poor outcome. This could be explained by in vitro studies that showed the correlation between LOX expression and an interconverted phenotype in which the cells expose cytokeratin and vimentin intermediate filaments which have a metastatic potential (647-650).

Various pre-clinical research studies have described that LOX is responsible for tumour progression and metastasis promotion (550, 615, 651, 652). In addition, many clinical studies have also shown that LOX expression was correlated with poor outcomes in various cancers (593, 651, 653-655), therefore LOX has emerged as a therapeutic target in cancer treatment.

6.5 Conclusion

In conclusion, collagen XV and XIX are significantly involved in the structural and biological changes associated with BC. They may act as potential biomarkers, and therapeutic target offers promising avenues for early detection of invasion process and treatment strategies. Collagen XIX could be used as a diagnostic tool to differentiate between invasive and non-invasive lesions in challenging cases.

HIF-1 α could be a new biomarker for BC prognosis and therapy response. The HIF-1 α levels in BC may be used to prospectively stratify patients who received neoadjuvant therapy to have drugs that can increase oxygen delivery to the tumour or inhibit HIF-1 α .

LOX could be a new biomarker for prognosis and therapy response. LOX inhibitors could be a good choice with chemotherapy for effective management. LOX could be a good therapeutic target specifically for metastasis prevention as beta-aminopropionitrile which is an irreversible inhibitor of LOX enzymatic activity caused decline in invasive activity in cell lines

LOX and HIF-1 α are closely related via a positive feedback loop which enhances their expression and activity leading to tumour growth promotion and therapy response resistance. This relationship underscores the potential of targeting the LOX/HIF-1 α axis in cancer therapy by using LOX inhibitors combined with HIF-1 α inhibitors.

7 Chapter 7: The conflicting prognostic role of the stroma-ratio in breast cancer

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7.1 Introduction

BC stroma is a crucial component of the tumour tissue, with a significant role in the promotion and progression of BC, therefore it has gained a wide research attention (657). The stroma-tumour ratio (STR), which is defined as the amount of tumour-associated stroma in relation to the volume of neoplastic cells, has been described as a prognostic factor in various tumours (16, 658-672). Several studies have evaluated the association between STR and BC outcomes, however, the results were conflicting (673). BCs with high stroma have shown poor survival outcomes in some studies (259, 418, 662, 669-672, 674-676), while it was associated with better survival outcomes in others (677, 678). Such variation in the prognostic significance between studies can be related not only to the molecular subtypes of BC but also to the different composition of the stroma. In luminal (ER+/HER2-) BCs, tumours encompassing a high STR were reported to have a better prognosis (663, 679), although other studies did not find the same association (658, 659, 665). In triple negative BC (TNBC), high STR showed an association with poor prognosis (259, 659, 669) but not in all studies (671, 672). The few reports that evaluated the role of stroma in HER2-positive BC showed unfavourable prognoses associated with stroma-rich tumours (680, 681).

The variable prognostic significance of STR can be related to the different mechanisms underlying the formation and composition of the stroma in each molecular subtype. Therefore, we aimed to explore the molecular mechanisms associated with the pathogenesis of the stroma in the various molecular subtypes of BC and assess the impact of contribution of the main components of the stroma utilizing large cohorts of early-stage BC. We hypothesized that the mechanisms driving STR, and its immune composition, are different in BC subtypes, which would provide an explanation to the conflicting prognostic role of the stroma. This could further provide a morphology-based novel stratification prognostic tool.

7.1.1 Aims and hypothesis

In this chapter, it was hypothesised that the mechanisms driving stroma, and its immune composition, are different in BC subtypes which could explain the conflicting roles of the prognostic value of stroma ratio in different molecular subtypes.

We aimed to evaluate the mechanisms of stroma formation and composition in different molecular subtypes which could explain the different prognostic values.

7.2 MATERIALS AND METHODS

7.2.1 Study cohorts

7.2.1.1 Nottingham cohort

A large, well-characterised cohort of primary operable BC patients presented to Nottingham City Hospital (n=822) was investigated.

Full and detailed clinicopathological and outcome data were available. These included patient age at diagnosis, tumour size, grade, histological tumour type, the NPI, hormonal (ER and PR) and HER2 status. Outcome data in the form of BCSS were evaluated. The mean patient follow-up was 372 months. **Table 7-1.**

7.2.1.2 The Cancer Genome Atlas BC (TCGA) dataset

A large BC cohort (n=978) was studied. Clinical data was accessed from <https://www.cbioportal.org/>, WSI was downloaded from <https://portal.gdc.cancer.gov/> (682). Characteristics of the cohorts are presented in Error! Reference source not found.. The mean patient follow-up was 40 months. **Table 7-1.**

Table 7-1: Clinicopathological characteristics of the study cohorts

Parameters	Nottingham cohort (n=822) Cases (%)	TCGA cohort (n=978) Cases (%)
Patient Age ≤ 50 years > 50 years Missing	243 (29.6%) 579 (70.4%)	260 (26.6%) 713 (72.9%) 5 (0.5%)
Size ≤ 2cm > 2cm Missing	502 (61.1%) 320 (38.9%)	162 (16.6%) 546 (55.8%) 270 (27.6%)
Estrogen receptor Positive (≥1%) Negative (<1%) Missing	627 (76.3%) 195 (23.7%)	729 (74.5%) 205 (21%) 44 (4.5)
Progesterone receptor Positive (≥1%) Negative (<1%) Missing	440 (53.5%) 349 (42.5%) 33 (4%)	625 (63.9%) 304 (31.1%) 49 (5%)
HER2 status Positive Negative Missing	207 (25%) 614 (73.5%) 13 (1.5%)	147 (15%) 663 (67.7%) 168 (17.3)
Ki67 score High (>14%) Low (<14%) Missing	186 (22.6%) 307 (37.3%) 329 (40%)	-
Molecular subtypes Luminal HER2 positive Triple-negative Missing	489 (59.6%) 207 (25.1%) 126 (15.3%)	618 (63.2%) 147 (15%) 127 (13%) 86 (8.8%)
Tumor grade Grade 1 Grade 2 Grade 3 Missing	214 (26%) 304 (37%) 304 (37%)	82 (8%) 159 (16%) 180 (18.4%) 557 (57%)
Lymph node stage Stage I Stage II Stage III Missing	543 (66.1%) 212(25.8%) 66 (8%) 1 (0.1%)	
Nottingham Prognostic Index (NPI) Good Intermediate Poor Missing	345 (42%) 365 (44.4%) 111 (13.5%) 1 (0.1%)	
Lymph vascular invasion (LVI) Definite Negative Missing	187 (22.7%) 635 (77.3%)	274 (28%) 500 (51.1%) 204 (20.9%)
Lymph node status Positive Negative Missing	278 (33.8%) 543 (66.1%) 1 (0.1%)	800 (81.8%) 19 (1.9%) 159 (16.3%)

7.2.2 Scoring methods

Using the digitalised H&E-stained tissue sections, STRs were scored using a standardised method for quantifying STR (683). STR was scored in a total of 1800 cases (Nottingham plus TCGA cohorts), where a proposed method by Hagennars et al., (683) and Pelt et al.,(684) was followed. An initial scanning on low power for the WSI to determine the most abundant tumour-associated stromal area was carried out. Then a circular field annotation measuring 3.46 mm² around the most abundant stroma surrounded by tumour tissue in four directions was performed in each WSI. The field size was selected to be equivalent to a 10× objective image field on the most frequent ocular sizes of conventional microscopes used in clinical practice (ranging from 1.8 mm to 2.2 mm) (683, 684).

This methodology provides an accessible time-saving method that can be easily replicated in clinical practice. However, to validate this method, double scoring using STR field assessment and WSI assessment on 822 cases from the Nottingham cohort was carried out to avoid biased results caused by intra-tumoural heterogeneity. An excellent agreement (ICC=0.9) was revealed when both methods were compared. Stratifying the STR to low and high groups showed 98% agreement in the low STR group and 85% agreement in the high group. The discordant cases (n=80) with high STR in the field scoring and categorised as low STR with the WSI scoring were reviewed and 21 % (n=17) of the discordant cases had a scoring close to 50% (≤10% difference), while wide scoring differences were noted in 23% of them which had a heterogenous stromal distribution (NST and ILC).

In the Nottingham cohort, one to five slides per case containing tumour tissue were scanned into high-resolution WSIs. All WSIs were examined for each case, and the best single image representative of abundant tumour tissue, and hence tumour-associated stroma, was selected for scoring. Evaluation of STR was performed on full face WSIs that were taken from the most representative tumour blocks. For each tumour, we

chose the WSI with the most abundant amount of stroma to assess the STR, and for WSIs that were heterogeneous, the average STR percentage was estimated. The same WSI was used for both STR and TILs scoring. STR was scored by selecting the most abundant stromal area (3.46 mm²) surrounded by tumour tissue from all sides as the standard method to ensure stroma associated tumour is examined as previously proposed (683, 684). Nonetheless, this study utilised all available WSI for TILs scoring, where the average calculated expression of stromal TILs (sTILS) was measured following TILs working group guidelines in all tumour-associated stromal areas.

The type of tumour-associated stroma was also assessed and classified as cellular desmoplastic and non-cellular stroma types. In general, desmoplasia is the growth of fibrous CT featuring increased stromal cellularity with larger stromal cells (myofibroblasts) and increased extracellular fibres. In this study, desmoplastic cellular stroma was defined as the predominance of cellular fibrous tissue rich in fibroblasts/myofibroblasts (more than 30 cells per one HPF) and this stroma type should comprise more than 60% of the stromal area (685). Other stromal types including collagenised (hypocellular) desmoplastic stroma, fibro elastotic stroma (rich in elastic fibres) and mucinous type stroma were considered as non-cellular stroma.

TILs were scored by the same pathologist (Suzan Ghannam) as a continuous parameter following the standardised scoring method as described by the TILs Working Group (686). This consisted of scoring only sTILs within the tumour borders excluding those infiltrating the tumour cells, areas of necrosis or tissue artefacts, and TILs around normal breast ducts or *in-situ* carcinoma. The scoring of both STR and TILs was validated by another certified pathologist (Shorouk Makhlof), in 10% of the cases, with a high concordance (ICC=0.7).

7.2.3 Combined STR and TILs scores

Each STR and TILs scores were dichotomised into low and high groups for outcome analysis using x-tile software version 3.6.1 (Yale University, New Haven, CT) (604) based on BCSS. STR was divided as either low ($\leq 50\%$) or high ($> 50\%$) stromal percentages, and the correlations with patient outcomes were tested. TILs scores were also dichotomised into low ($\leq 50\%$) and high TILs ($> 50\%$). Considering TILs as a vital component of TME, additional analysis was carried out using a combination of TME scores (STR and TILs), where each cohort was stratified into four groups as follows:

- 1- Low STR/Low TILs (LS/LT)
- 2- Low STR/High TILs (LS/HT)
- 3- High STR/High TILs (HS/HT)
- 4- High STR/Low TILs (HS/LT)

7.2.4 Differential gene expression and pathway enrichment analysis

Differential gene expression (DGE) analysis was conducted using the TCGA BC cohort (n=978, **Table 7-2**) Gene expression data expressed as RNA sequencing counts were accessed for female primary BC tumour specimens obtained from the NCI Genomics Data Commons data portal. Cases were stratified on the basis of STR and differential gene expression analysis between tumours with high STR and low STR was completed using DESeq2 (687). Pathway enrichment analysis was performed using WebGestalt (WEB-based Gene Set Analysis Toolkit). The gene expression pattern data of the DEGs involved in a specific KEGG (36) (Kyoto Encyclopedia of Genes and Genomes) pathway were shown. Log2 fold change ($\geq \pm 1$) and false discovery rate (FDR=0.05, Adjusted *p* value) was set for significance.

Table 7-2 : Number of cases used in differential gene expression

Molecular subtype	Number of cases	Number of differentially expressed genes	Number of upregulated genes	Number of down regulated genes
Luminal BC	618	1647	1025	622
HER2+	147	1396	720	676
TNBC	127	4882	3844	1038

7.2.5 Statistical analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (IBM SPSS) v.28 (Chicago, USA). Chi-square tests were used to assess the correlations between categorical variables. The degree of inter-observer concordance in the scoring was statistically evaluated using the ICC. Kaplan Meier curves with log-rank test were used to assess the correlation with outcomes in the whole cohort and the various BC molecular subtypes. Cox-regression analysis was calculated. For all tests, $p < 0.05$ (two-tailed) was considered statistically significant.

7.3 RESULTS

In the Nottingham Cohort, high STR showed an association with favourable tumour behaviour (**Table 7-3**). For the whole Nottingham cohort, patients with high STR showed significantly favourable BCSS compared to low STR (HR=0.96, 95% CI= 0.95 to 0.97, $p < 0.001$).

Cases with high STR within the luminal group showed significantly longer BCSS compared to those with low STR (HR=0.63, 95% CI= 0.4 to 0.85, $p < 0.001$). On the other hand, tumours showing high STR were associated with significantly shorter BCSS compared to low STR in the TNBC group (HR=1.03, 95% CI= 0.99 to 1.06, $p < 0.026$). However, there was no significant outcome difference in HER2 positive cases between high and low STR (**Figure 7-1**).

**Table 7-3: The association of STR with clinicopathological data in
the study cohorts**

Parameter	Nottingham cohort			TCGA cohort		
	Low STR	High STR	p-value	Low STR	High STR	p-value
Patient Age ≤50 years >50 years	96 (33.3%) 192 (66.7%)	147 (27.5%) 387 (72.55)	<i>P</i> <0.08	127 (26.1%) 360 (73.9%)	133 (27.4%) 353 (72.6%)	<i>P</i> <0.65
Tumor Size ≤2cm >2cm	164 (56.9%) 124 (43.1%)	338 (63.3%) 196 (36.7%)	<i>P</i> <0.075	77 (22.1%) 272 (77.9%)	85 (23.7%) 274 (76.3%)	<i>P</i> <0.6
Estrogen receptor Positive Negative	188 (66%) 97 (34%)	439 (82.7%) 92 (17.3%)	<i>P</i> <0.01	46 (9.9%) 159 (34%)	420 (90.1%) 309 (66%)	<i>P</i> <0.001
Progesterone receptor Positive Negative	128 (46%) 150 (54%)	312 (61.1%) 199 (38.9%)	<i>P</i> <0.001	262 (56.5%) 202 (43.5%)	363 (78.1%) 102 (21.9%)	<i>P</i> <0.001
HER2 status Positive Negative	123 (44.1%) 156 (55.9%)	80 (15.3%) 443 (84.7%)	<i>P</i> <0.01	73 (18.6%) 320 (81.4%)	74(17.7%) 343 (82.3%)	<i>P</i> <0.76
Ki67 score High Low	68 (51.9%) 63 (48.1%)	123 (34%) 239 (77.9%)	<i>P</i> <0.004			
Molecular subtypes Luminal HER2 positive Triple-negative	115 (37.3%) 121 (39.2%) 72 (23.3%)	374 (72.8%) 86 (16.7%) 54 (10.5%)	<i>P</i> <0.001	262 (60.2%) 73 (16.8%) 100 (23%)	356 (77.9%) 74 (16.2%) 27 (5.9%)	<i>P</i> <0.001
Histological subtypes NST Lobular Metaplastic Special Mixed	153 (53.1%) 48 (16.7%) 8 (2.8%) 66 (22.9%) 13 (4.5%)	169 (31.6%) 208 (39%) 4 (0.7%) 117 (21.9%) 36 (6.7%)	<i>P</i> <0.001	348 (73.3%) 77 (16.2%) 6 (1.3%) 44 (9.3%) 0 (0%)	356 (75.6%) 103 (21.9%) 1 (0.2%) 11 (2.3%) 0 (0%)	<i>P</i> <0.001
Tumor grade Grade 1 Grade 2 Grade 3	58 (20.2%) 83 (28.8%) 147 (51%)	156 (29.2%) 221 (41.4%) 157 (29.4%)	<i>P</i> <0.001	30 (13.7%) 77 (35.2%) 112 (51.1%)	52 (25.7%) 82 (40.6%) 68 (33.7%)	<i>P</i> <0.001
Lymph node stage Stage I Stage II Stage III	180 (62.7%) 70 (24.4%) 37 (12.9%)	363 (68%) 142 (26.6%) 29 (5.4%)	<i>P</i> <0.001			
Nottingham Prognostic Index (NPI) Good Intermediate Poor	96 (33.5%) 137 (47.7%) 54 (18.8%)	249 (46.6%) 228 (42.7%) 57 (10.7%)	<i>P</i> <0.001			

Lymph vascular invasion (LVI)						
Definite	67 (23.3%)	120 (22.5%)	$P<0.79$	126 (34.4%)	148 (36.3%)	$P<0.59$
Negative	221 (76.7%)	414 (77.5%)		240 (65.6%)	260 (63.7%)	
LN status						
Positive	107 (37.3%)	171 (32%)	$P<0.12$	15 (3.7%)	4 (1%)	$P<0.01$
Negative	180 (62.7%)	363 (68%)		393 (96.3%)	407 (99%)	

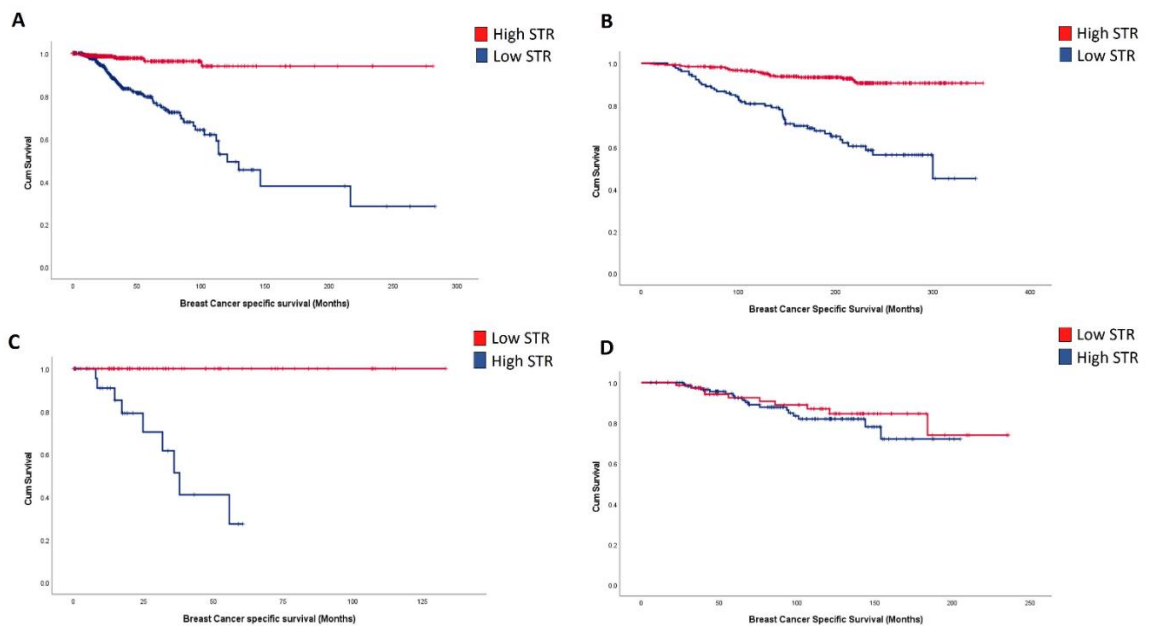


Figure 7-1: Kaplan Meier survival plots showing the correlation of STR with breast cancer-specific survival (BCSS).

(A) The whole cohort, $n=822$ (HR=0.96, 95% CI= 0.95 to 0.97, $p<0.001$). **(B)** Luminal BC, $n=489$ (HR=0.63, 95% CI= 0.4 to 0.85, $p<0.001$). **(C)** TNBC, $n=126$ (HR=1.03, 95% CI= 0.99 to 1.06, $p<0.026$). **(D)** HER2+, $n=207$ ($P<0.27$).

Multivariate Cox regression analysis adjusted for patient age, tumour grade, LN metastasis, and LVI, revealed that STR is an independent predictor of outcome in the whole Nottingham cohort (HR=0.6, 95% CI= 0.1 to 0.8, $p<0.02$) as well as in the luminal BC class (HR=0.5, 95% CI= 0.2 to 0.76, $p<0.002$) (**Table 7-4**).

Table 7-4: Multivariate Cox regression analysis of STR in BC

Variables	p-value			
	Whole cohort	Luminal BC	TNBC	HER2+
STR	0.002	0.001	0.45	0.42
Age	0.99	0.01	0.84	0.08
Grade	0.13	0.67	0.24	0.96
LN metastasis	0.01	0.13	0.01	0.02
Lymph-vascular invasion	0.001	0.19	0.01	0.06

LN – lymph node, STR - stroma-tumour ratio.

Similar results were obtained when the validation TCGA cohort was tested, where high STR showed an association with longer BCSS compared to low STR cases in the whole TCGA cohort (HR=0.98, 95% CI= 0.98 to 0.99, $p<0.001$) and in the luminal BC (HR=0.98, 95% CI= 0.96 to 0.99, $p<0.001$). In TNBC, patients with high STR showed shorter BCSS (HR=1.24, 95% CI= 1.1 to 3.9, $p<0.018$). In HER2 positive BC patients, there was no significant outcome difference between high and low STR cases.

7.3.1 Differential gene expression (DGE) analysis

DGE analysis was carried out to identify genes and enriched pathways differentially expressed between BCs with high and low amounts of tumour associated stroma in each BC subtype.

In the luminal tumours, a total of 1647 DEGs were identified, where 1025 genes were commonly up-regulated, and 622 genes were commonly down-regulated in tumours with high STR (**Figure 7-2**). Pathway enrichment analysis of the up-regulated genes unravelled involvement in specific pathways related to myofibroblast differentiation and extracellular matrix formation as notch signalling, Wnt signalling, proteoglycans, and focal adhesions, **Figure 7-2 & Table 7-5**.

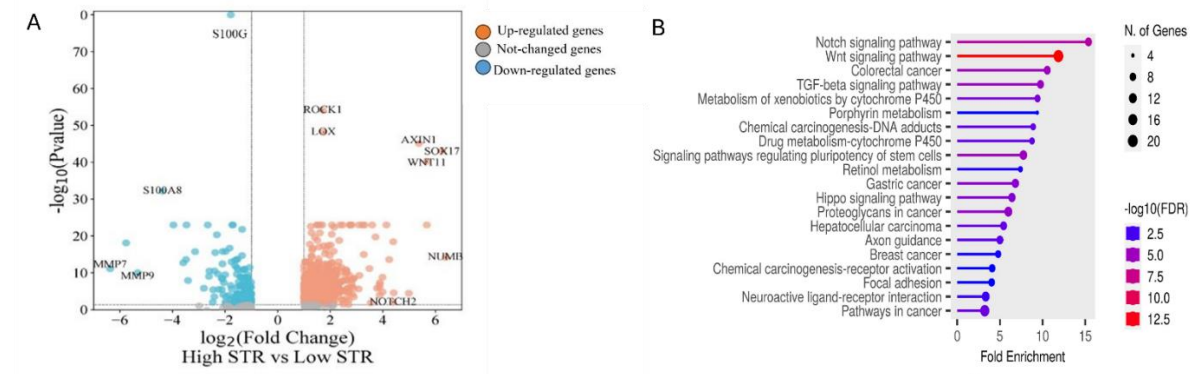


Figure 7-2: Differential gene expression (DGE) analysis for luminal BC

$\log_2$ fold change (how much expression changes between high and low STR), red colour are genes that are upregulated, and blue colour are genes downregulated, while grey colour genes do not change in expression between both conditions

(A) Volcano plots showing up-regulated and down-regulated genes in BC. **(B)** Gene Set Enrichment Analysis (GSEA).

Table 7-5: KEGG pathways significantly associated with high STR in luminal tumours

Gene set	Pathway	Overlap	Adjusted p-value	Gene symbol
hsa04310	Wnt signalling pathway	15	4.4866e-9	RHOA, GSK3B, WNT11, DAAM1, AXIN1, DVL1, INVS, ROCK2, RAC1, MYC, MMP7, LEF1, SENP2, SOX17, AXIN2
hsa-04350	TGF-beta signalling pathway	6	0.001	RHOA, ROCK1, TGFB1, ACVR1, MYC, NODAL,
hsa04330	Notch signalling pathway	5	0.0007	DVL1, NUMB, NOTCH2, ADAM17, NCSTN
hsa-04024	cAMP signalling pathway	9	0.003	RHOA, ROCK1, CREB3, CREB1, NPY, ROCK2, RAC1, GABBR2, GRIA1
hsa-05205	Proteoglycans in cancer	10	0.0009	RHOA, ROCK21, WNT11, MMP9, TGFB1, VTN, SOS1, ROCK2, RAC1, MYC
hsa05224	Breast cancer	10	0.00007	FGF4, GSK3B, WNT11, AXIN1, DVL1, SOS1, NOTCH2, MYC, LEF1, AXIN2
hsa-04390	Hippo signalling pathway	9	0.0005	LEF1, GSK3B, WNT11, AXIN1, DVL1, MYC, TGFB1, YAP, AXIN2
hsa04510	Focal adhesion	9	0.003	ITG3, RHOA, ROCK1, GSK3B, VTN, SOS1, ROCK2, RAC1, COL2A1
hsa05200	Pathways in cancer	17	0.002	ITGA3, RHOA, TGFB1, ROCK1, FGF4, GSK3B, WNT11, MMP9, AXIN1, DVL1, SOS1, NOTCH2, ROCK2, RAC1, MYC, LEF1, AXIN2

KEGG=Kyoto Encyclopaedia of Genes and Genomes, has=homo sapiens

In the TNBC subtype, 4882 differentially expressed genes were identified, where 3844 genes were commonly up-regulated, and 1038 genes were commonly down-regulated (**Figure 7-3**). The genes that showed higher expression in cases with high STR were involved in specific pathways related to cell immunity, hypoxia and necrosis, including the TNF signalling pathway, IL-17 signalling pathway, HIF-1 signalling pathway, NF-kappa B signalling pathway, Toll-like receptor signalling pathway, NOD-like receptor signalling pathway, JAK-STAT

signalling pathway, C-type lectin receptor signalling pathway, Cytokine-cytokine receptor interaction, (Figure 7-3 & Table 7-6).

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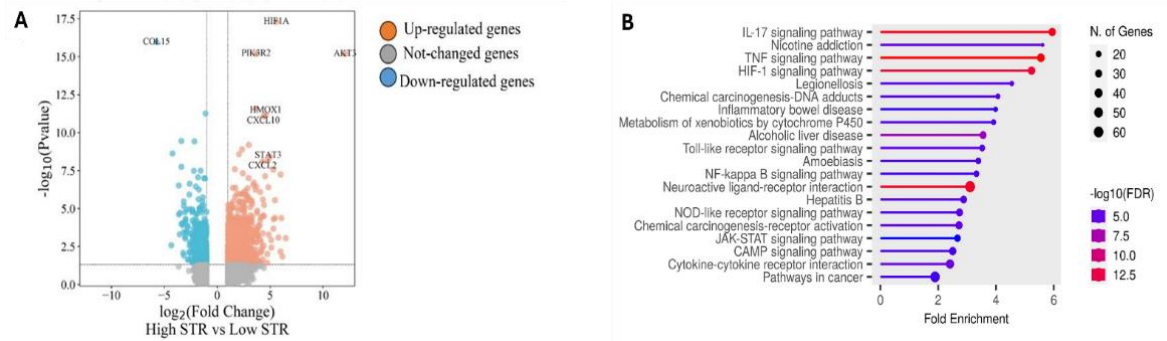


Figure 7-3: Differential gene expression (DGE) analysis for TNBC,

$\log_2$ fold change (how much expression changes between high and low STR), red colour are genes that are upregulated, and blue colour are genes downregulated, while grey colour genes do not change in expression between both conditions (A) Volcano plots showing up-regulated and down-regulated genes in BC. (B) Gene Set Enrichment Analysis (GSEA).

**Table 7-6: KEGG pathways significantly associated with high STR
in TNBC tumours.**

Gene set	Pathway	Overlap	Adjusted p-value	Gene symbol
hsa-04066	HIF-1 signalling pathway	33	4.9738e-14	CREBBP, ENO1, EDN1, HMOX1, RBX1, HIF1A, PGK1, TIMP1, AKT2, PIK3R2, CUL2, NFKB1, CDKN1B, IFNG, VEGFA, AKT3, CDKN1A, PDHA1, VHL, IL6, TLR4, RPS6, EGF, PFKL, AKT1, ARNT, PDK1, IL6R, PDHA2, PDHB, STAT3, EIF4E1B, MTOR
hsa-04668	TNF signalling pathway	37	6.6613e-16	CASP3, CASP7, MLKL, CXCL10, IFNB1, CREB3L2, PGAM5, RIPK3, IL6, AKT1, CREB5, VEGFC, CREB3L1, TAB3, CXCL1, CXCL3, CXCL5, MMP9, TRADD, AKT2, PIK3R2, MAP2K6, NFKB1, CCL20, AKT3, CREB1, TRAF2, CASP10, TAB2, TRAF1, CASP8, PTGS2, EDN1, ITCH, CXCL2, TRAF5, TAB1
hsa04657	IL-17 signalling pathway	31	2.1805e-13	TAB2, TRAF3IP2, CASP8, PTGS2, TRAF4, CXCL2, TRAF5, MMP9, TRADD, CCL17, CSF3, NFKB1, IFNG, IL17A, IL17F, CCL20, IL17C, CXCL6, FOSB, TRAF2, IL6, S100A7, S100A8, TAB3, S100A9, CXCL1, CXCL3, CXCL5, CXCL10, FOSL1
hsa04064	NF-kappa B signalling pathway	17	0.0005	TRAF2, LBP, TLR4, CCL21, BCL2A1, TAB3, LTB, TAB2, TRAF1, PTSG2, NFKB2, CXCL2, TRAF5, TAB1, XIAP, TRADD, NFKB1
hsa-04630	JAK-STAT signalling pathway	25	0.0003	CREBBP, AKT2, PIK3R2, CSF3, IFNG, STAT1, AKT3, IL13RA2, CDKN1A, IL6, STAT4, IL12, EGF, AKT1, IFNK, IL6R, IL22RA2, STAT6, STAT3, IL12A, STAT2, IFNB1, PRL, IL20RB, MTOR
hsa04060	Cytokine-cytokine receptor interaction	42	0.00002	CXCL2, CCL17, CSF3, IL26, IFNG, IL17A, IL17F, CCL20, IL1R2, IL13RA2, IL17C, CXCL6, TNFSF9, IL6, IL36G, IL36A, IL36B, IL1F10, CCL21, IL21, BMPR1B, CXCL9, INHBE, CXCL14, IFNK, BMP3, CXCL13, IL6R, CXCL3, CXCL5, PPBP, PF4, CXCL1, TNFRSF11B, IL12A, CXCL10, IFNB1, PRL, IL20RB, TNFSF15, LTB, CXCL17
hsa-04024	cAMP signalling pathway	28	0.0006	ADORA1, NPY1R, HTR1E, DRD5, HTR1A, CREB3L2, GRIN2A, CNGA2, NPY, PPP1R1B, SSTR1, AKT1, CNGA3, CREB5, ENDRA, ADCY8, ATP2B2, CREB3L1, CFTR, CREBBP, ATP2B3, ATP1B4, AKT2, PIK3R2, NFKB1, AKT3, CREB1, GRIA2
hsa04620	Toll-like receptor signalling pathway	17	0.001	IL6, TLR4, CXCL9, AKT1, IL12A, CXCL10, IFNB1, TAB2, CASP8, TAB1, AKT2, PIK3R2, MAP2K6, NFKB1, STAT1, AKT3, LBP
hsa04621	NOD-like receptor signalling pathway	27	0.00009	TAB2, CASP8, CXCL2, TRAF5, TAB1, XIAP, NFKB1, STAT1, TRAF2, RIPK3, IL6, TLR4, CASP1, GBP5, TAB3, GBP4, AIM2, CXCL1, CXCL3, CAMP, NLRP7, PYDC1, STAT2, IFNB1, GPRC6A, GBP7, CARD18,

hsa05200	Pathways in cancer	57	0.001	RBX1, HIF1A, MMP9, BIRC7, XIAP, AKT2, FGF21, PIK3R2, FGF8, CUL8, CREBBP, TRAF1, CASP8, CTNNA2, PTGS2, FGF4, TRAF4, NFKB2, TRAF5, HMOX1, CDKN1B, FZD10, IFNG, VEGFA, STAT1, AKT3, FLT3, CDKN1A, TRAF2, VHL, GSTM1, EDNRB, IL6, STAT4, EGF, AKT1, KLK3, RXRG, ARNT, VEGFC, EDNRA, WNT3A, ADCY8, IL6R, CASP3, CASP7, STAT3, STAT6, IL12A, STAT2, ARNT2, GSTA2, FZD9, MTOR, GSTA1, GSTA2

KEGG=Kyoto Encyclopaedia of Genes and Genomes, has=homo sapiens

In the HER2 positive group, 1396 genes were identified, 720 genes were up-regulated, and 676 genes were down-regulated (**Figure 7-4**). However, they were involved in stroma non-specific pathways (**Figure 7-4 & Table 7-7**).

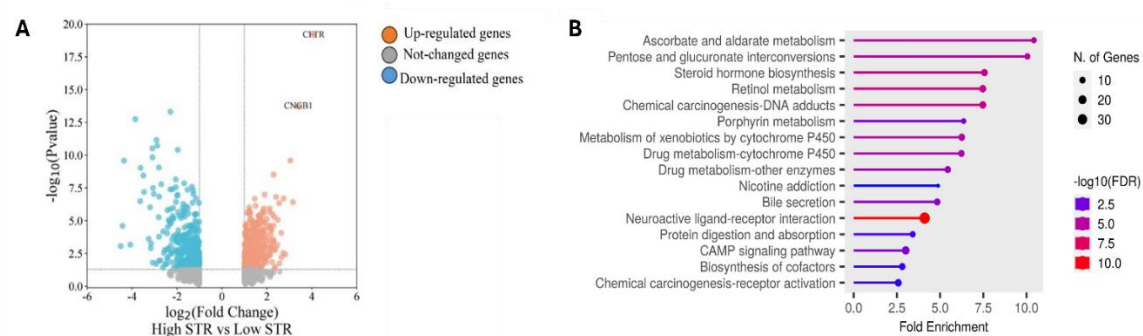


Figure 7-4: Differential gene expression (DGE) analysis for HER2 positive

$\log_2$ fold change (how much expression changes between high and low STR), red colour are genes that are upregulated, and blue colour are genes downregulated, while grey colour genes do not change in expression between both conditions (**A**) Volcano plots showing up-regulated and down-regulated genes in BC. (**B**) Gene Set Enrichment Analysis (GSEA).

Table 7-7: KEGG pathways associated with high STR in HER2+ tumours

Gene set	Pathway	Overlap	Adjusted p-value	Gene symbol
hsa-04024	cAMP signalling pathway	11	0.0002	CFTR, CNGB1, GRIA2, GABBR2, CHRM1, CNGB3, CHRM2, HCAR2, CNGA2, DRD1

KEGG=Kyoto Encyclopaedia of Genes and Genomes, has=homo sapiens

7.3.2 Combination of immune status with STR and their effect on patient outcome in BC subtypes

As a crucial component of stroma, we aimed to further analyse the contribution and prognostic impact of TILs in different molecular subtypes of BC.

For the overall cohorts, there was a negative, statistically significant correlation between STR and TILs ($r = -0.24$, $p < 0.001$). After stratification according to molecular subtypes, no significant correlation between STR and TILs ($r = 0.05$, $p < 0.1$) in luminal BCs was found, but there was a negative correlation in the TNBC ($r = -0.405$, $p < 0.001$) and HER2 positive cohorts ($r = -0.237$, $p < 0.004$).

In the whole Nottingham cohort, patients with low TILs showed shorter BCSS (HR=3.09, 95% CI= 1.34 to 7.12, $p < 0.012$; **Figure 7-5**) than high TILs.

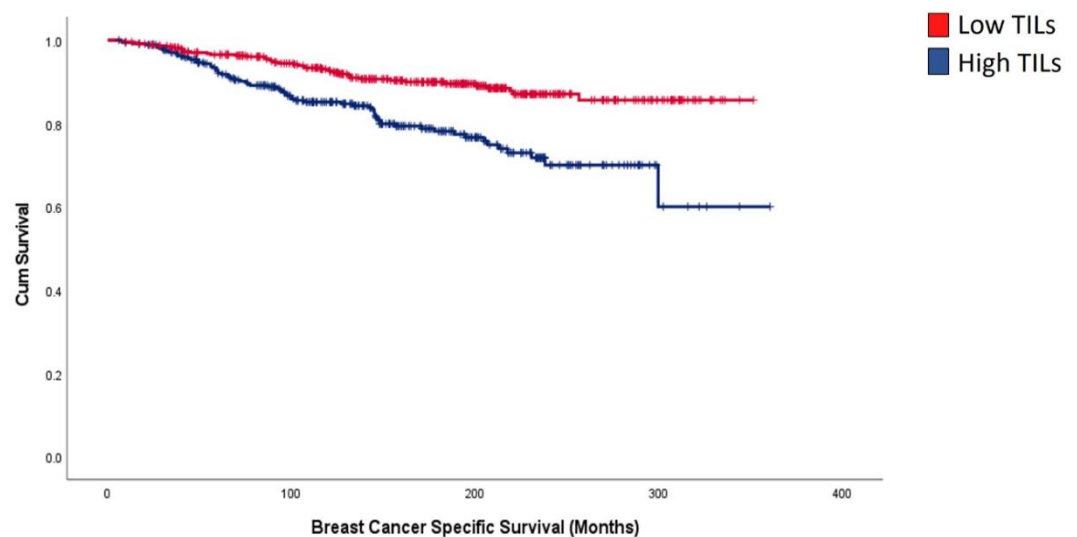


Figure 7-5: Kaplan Meier survival plots showing the correlation of TILs with breast cancer-specific survival (BCSS). in The whole cohort, N=822 (HR=3.09, 95% CI= 1.34 to 7.12, $p < 0.012$).

With regards to the combined groups, the best prognostic group was HS/HT, which showed longer BCSS ($p < 0.001$) compared to the other groups, while the worst prognostic group was LS/LT (**Figure 7-6**). When

TILs combined with STR showed that comparing LS/LT and LS/HT, the LS/LT group showed worse BCSS (HR=5.01, 95% CI= 2.38 to 10.55, $p<0.001$).

There was a significantly shorter BCSS within the LS/LT group compared to HS/LT (HR=11.62, 95% CI= 5.92 to 22.79, $p<0.001$). While there were no significant differences between either HS/HT or HS/LT.

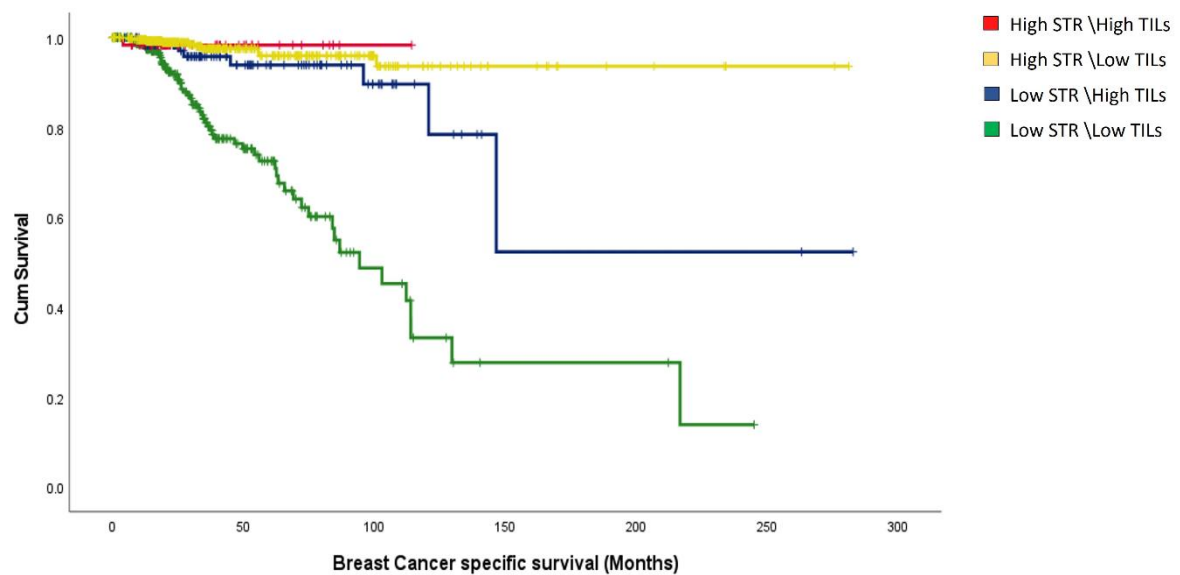


Figure 7-6: Kaplan Meier survival plots showing breast cancer-specific survival (BCSS) of cases within combined groups in the whole cohort, ($p<0.001$).

7.3.2.1 Luminal subtype

Luminal BC cases with high TILs showed no significant outcome difference compared to low TILs cases (HR=1.3, 95% CI= 0.59 to 2.83, $p=0.50$, **Figure 7-7**). The validation TCGA data for the luminal group also showed no association between TILs and outcome.

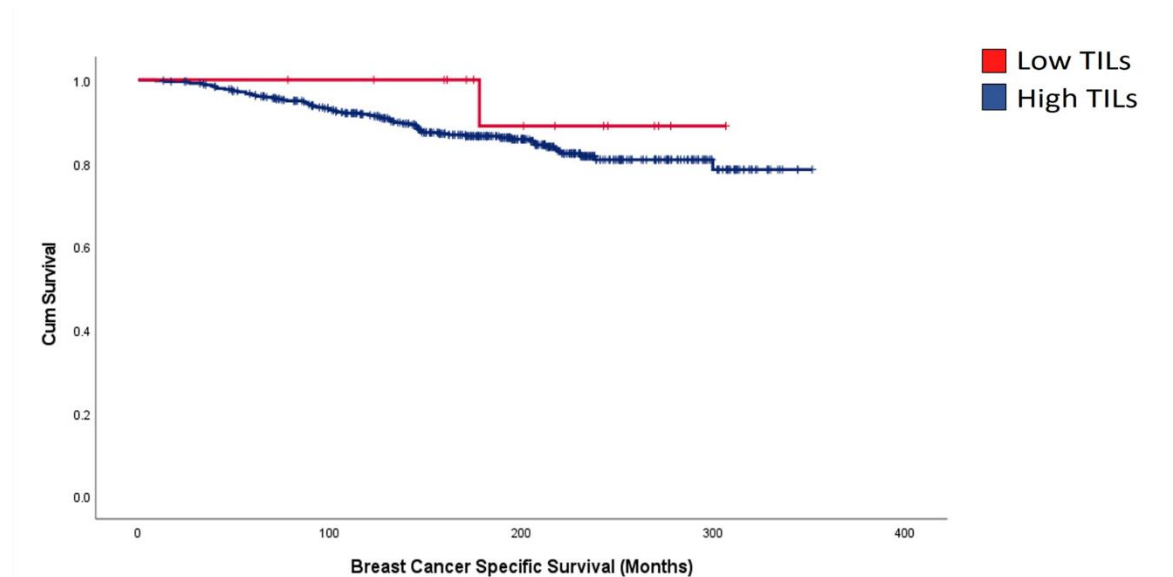


Figure 7-7: Kaplan Meier survival plots showing the correlation of TILs with breast cancer-specific survival (BCSS). in Luminal BC, (HR=1.3, 95% CI= 0.59 to 2.83, $p<0.50$).

When the combination of TILs with STR was evaluated, the best prognostic group was HS/LT which exhibited better BCSS (HR=0.13, 95% CI= 0.05 to 0.72, $p<0.001$) compared to the other groups. In contrast, the worst prognostic group was LS/HT (**Figure 7-8**). Additionally, the validation TCGA data also showed that when STR was combined with TILs, HS/LT was the best prognostic group compared to the other groups ($p<0.001$), while the worst prognostic group was LS/HT.

In Nottingham cohort, HS/LT group showed better BCSS compared to the LS/LT group (HR=0.56, 95% CI= 0.03 to 0.90, $p<0.0001$). The LS/HT group showed shorter BCSS compared to the HS/HT (HR=5.2, 95% CI= 1.8 to 10.2, $p<0.02$) group. There were no significant differences between the LS/LT and LS/HT groups or between the HS/HT and HS/LT groups.

In the validation TCGA data, within the high STR groups, low TILs tumours showed longer BCSS compared to the LS/LT group (HR=0.64, 95% CI= 0.29 to 0.73, $p<0.001$). The LS/HT group showed shorter BCSS compared to the HS/HT (HR=5.37, 95% CI= 3.2 to 7.5, $p<0.001$) group. As with the Nottingham cohort data, in the validation TCGA cohort, there

were no significant differences between the LS/LT and LS/HT groups ($p<0.62$) or between the HS/HT and HS/LT groups ($p<0.11$) which determined that the stroma has the prognostic role and not affected with the immune status.

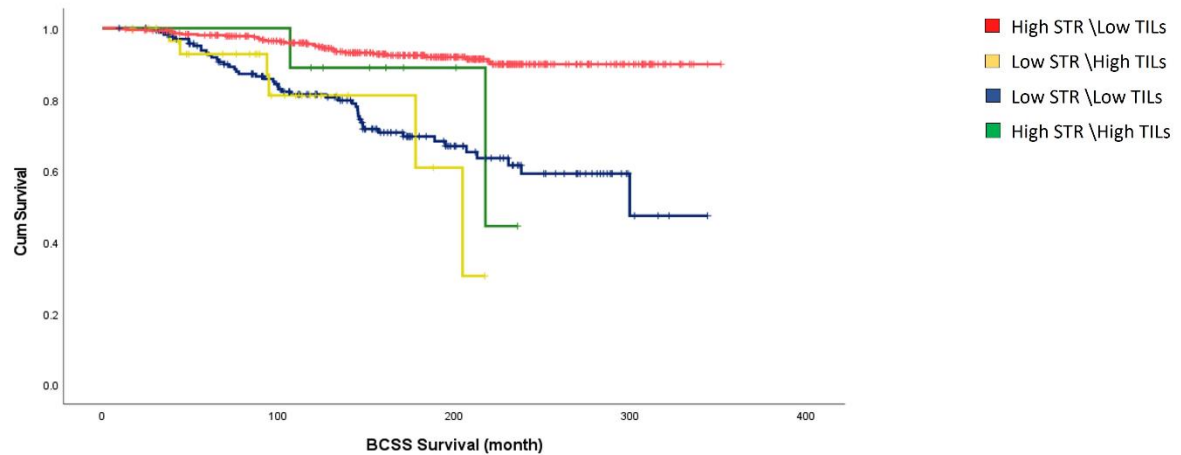


Figure 7-8: Kaplan Meier survival plots showing breast cancer-specific survival (BCSS) of cases within combined groups in luminal BC, ($p<0.001$).

7.3.2.2 TNBC subtype

TNBC patients with high TILs showed better BCSS compared to low TILs (HR=0.91, 95% CI= 0.86 to 0.97, $p<0.001$, **Figure 7-9**).

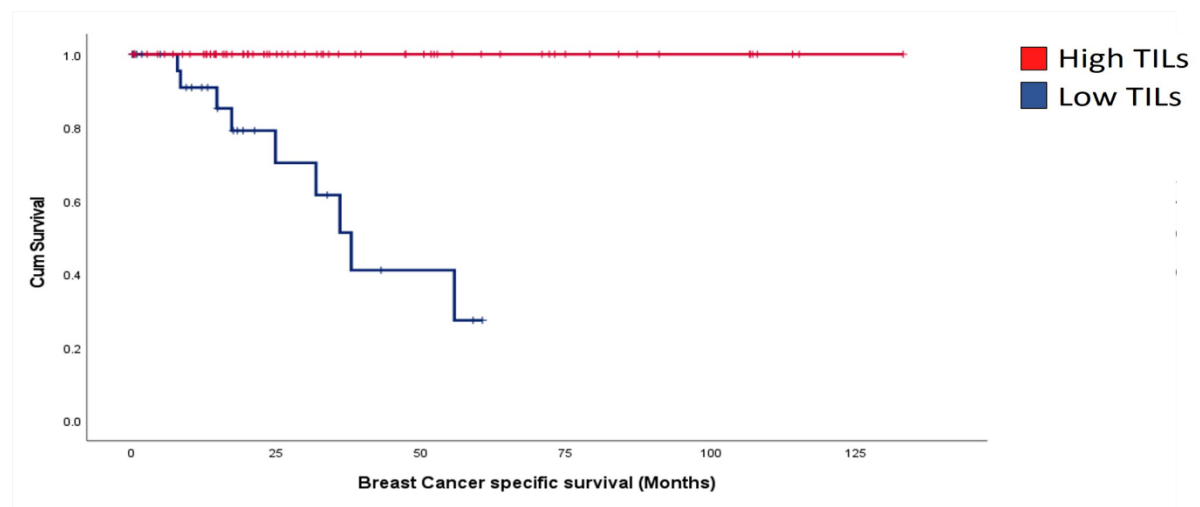


Figure 7-9: Kaplan Meier survival plots showing the correlation of TILs with breast cancer-specific survival (BCSS). TNBC, (HR=0.91, 95% CI= 0.86 to 97, $p<0.001$).

In the validation TCGA data in the TNBC group, patients with high TILs also showed significantly favourable BCSS compared to low TILs (HR=0.13, 95% CI= 0.03 to 0.59, $p<0.001$).

When TILs scores were combined with STR, LS/HT and HS/HT showed better BCSS compared to the LS/LT and HS/LT groups ($p<0.001$). LS/LT showed shorter BCSS compared to LS/HT (HR=2.3, 95% CI= 1.2 to 3.33, $p<0.001$). In addition, HS/HT showed better BCSS compared to HS/LT (HR=0.5, 95% CI= 0.23 to 0.95, $p<0.04$). While there were no significant differences between both the HS/HT and LS/HT, and the HS/LT and LS/LT groups, indicating that the TILs have predominant prognostic effects more than observed for the stroma in TNBC patients (**Figure 7-10**).

The validation TCGA data mirrored the Nottingham cohort data, when STR was combined with TILs, LS/HT was the best prognostic group and HS/LT was the worst prognostic group ($p<0.001$). In addition, LS/LT showed shorter BCSS compared to LS/HT (HR=4.87, 95% CI= 0.03 to 7.87, $p<0.04$). The validation TCGA data for HS/HT also showed better BCSS compared to HS/LT (HR=0.04, 95% CI= 0.12 to 0.95, $p<0.02$). As with the Nottingham cohort data, there were no significant differences between either the LS/HT and HS/HS groups, or between the HS/LT and LS/LT in the validation TCGA data.

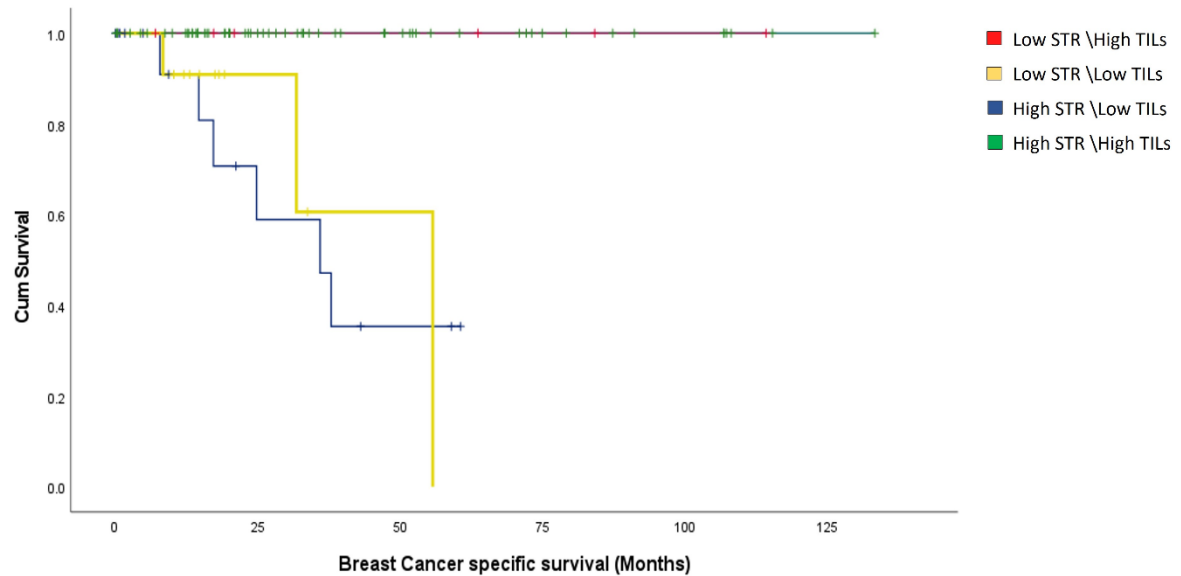


Figure 7-10: Kaplan Meier survival plots showing breast cancer-specific survival (BCSS) of cases within combined groups in TNBC, ($p < 0.001$).

7.3.2.3 HER2 positive group

The high TILs cases showed better BCSS compared to low TILs cases (HR=0.19, 95% CI= 0.1 to 0.87, $p < 0.01$, **Figure 7-11**). However, there were no significant differences either between high or low STR cases or after combination with TILs scores. Similar results were obtained when the validation cohort was tested, cases with high TILs showed better BCSS compared to low TILs (HR=0.08, 95% CI= 0.07 to 0.1, $p < 0.03$).

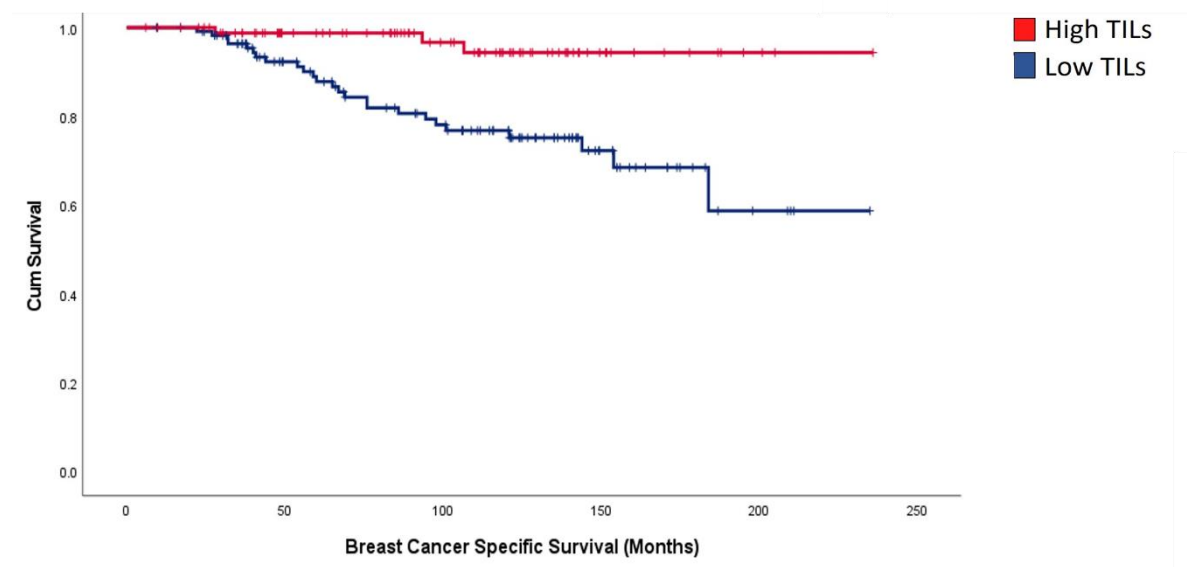


Figure 7-11: Kaplan Meier survival plots showing the correlation of TILs with breast cancer-specific survival (BCSS). HER2+, (HR=0.19, 95% CI= 0.1 to 0.87, $p<0.01$).

7.3.3 Multivariate analysis

In the whole cohort, TNBC and HER2+, multivariate Cox regression analysis of all significant features, adjusted for patient age, tumour grade, LN metastasis, LVI, revealed that TILs are an independent predictor of outcome (**Table 7-8**).

Table 7-8: Multivariate Cox regression analysis of TILs in BC

Variables	p-value			
	Whole cohort	Luminal BC	TNBC	HER2+
TILs	0.018	0.52	0.02	0.03
Age	0.96	0.20	0.15	0.06
Grade	0.002	0.91	0.001	0.99
LN metastasis	0.015	0.10	0.49	0.11
Lymph-vascular invasion	0.002	0.02	0.01	0.02

TILs - tumour-infiltrating lymphocytes

7.3.4 Effect of cellular desmoplasia of the stroma on the outcome

Desmoplastic cellular stroma in luminal BC showed better outcomes compared to cases with no desmoplastic cellular stroma (HR=0.02, 95%CI= 0 to 0.7, $p<0.01$). However, no significant difference was shown either in TNBC or HER2+ BC (**Figure 7-12**).

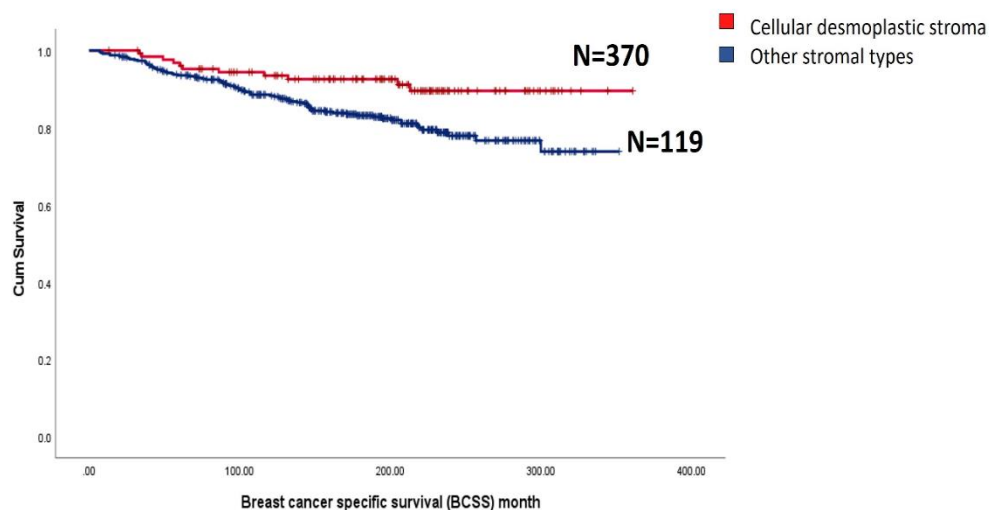


Figure 7-12: Kaplan Meier survival plots showing breast cancer-specific survival (BCSS) of cases with desmoplastic cellular stroma compared to others in luminal BC ($p<0.01$).

7.4 Discussion

BC is a highly heterogeneous disease with distinct molecular subtypes. The prognostic role of tumour-associated stroma has shown variability across different studies and molecular subtypes. STR was evaluated in the breast using different methodologies, including different assessment platforms (e.g., visual assessment, image analysis or artificial intelligence) or different tumour areas utilising whole slide images (683), glass slides of surgical specimens (661) or tissue microarray cores (688). Such lack of standardisation in the determination of STR has contributed to the variable results observed in the literature. However, different

results were also obtained using the same methodologies (661, 683). Hence, we hypothesised that each molecular subtype has a different mechanism for stroma remodelling that explains this conflicting role.

As a vital component of TME, the level of TILs, was also associated with patient outcomes in various tumour types (689). TILs play a favourable prognostic role in TNBC (690-695) and HER2-positive BCs (696, 697), while in luminal BC, the prognostic role of TILs remains unclear. There have also been contradictory results, as some studies reported an association of TILs with poor prognosis, while others have failed to prove any prognostic significance in luminal BC (698, 699). The combination of stromal abundance and the immune response has been proposed, however, it is not yet fully elucidated (700-703). Moreover, the stromal responses of BCs defined as desmoplasia show that the stromal response histologically can vary from being predominantly cellular (fibroblasts/myofibroblasts) with little collagen tissue, to dense collagen or mucinous type stroma with few stromal cells (704).

There is rising evidence that tumour stroma and host immunity responses might be crucial parameters in patient stratification for optimal treatment decisions. The relationship between stroma and immunity as part of TME has been evaluated in several studies. Gujam and colleagues found that a high stroma level was consistently correlated with lower immunity (671), which was consistent with our result that showed a negative correlation between STR and TILS. In the present study, STR with the combination of TILs is a prognostic factor in different molecular subtypes. This combination of both parameters even strengthens each other's effect. This was consistent with a previous colon cancer study showing that combined STR with peritumoral lymphoid inflammation and other TME features in colon cancer is a strong predictor of survival outcomes (705). In addition, the relationship between STR and immunity was evaluated using IHC, as the combination of STR and six different cellular immune cells, classified into three immune status subgroups, demonstrated that both combined were

independent prognostic factors with a high correlation between stroma and Human leukocyte antigens (HLA) class I (706). Another study proved that the combination of TILs and STR could significantly improve the prediction performance for the pathological complete response (pCR) in BC and could be a novel candidate to better stratify BC patients (707).

Despite the importance of stroma in tumour promotion and progression, its relationship with other TME components has yet to be fully characterised. A few studies have described that a high stroma was associated with low immunity in colon cancer (708, 709). This is consistent with the present study in BC which showed an inverse significant correlation between stroma ratio and TILs. In our study, high STR with low TILs showed a better prognosis in luminal BC whereas the same group showed a worse prognosis in TNBC. A recent study that recognised a score for TME in BC composed of TSR, TILs and tumour budding revealed that a high score was associated with aggressive behaviour and poor prognosis (710). However, that study was limited with regards to estimating the score in different molecular subtypes. Millar and colleagues studied the effect of combined stroma with TILs in TNBC and showed that the best prognostic group was low STR/high TILs which is consistent with the present study results (418).

In this study, DGE analysis identified that a high stromal component in TNBC tumours was associated with genes involved in pathways and mechanisms related to hypoxia and necrosis, in addition to immune cell signalling pathways such as the NF κ B and JAK/STAT pathways. These findings are consistent with other studies linking TNBC tumours to these pathways (711-713). On the other hand, DGE analysis also identified that genes in the high stroma of luminal tumours were associated with proliferation, growth, and apoptosis, which is in agreement with a BC study published by Eroles and colleagues (714).

TNBC tumours are aggressive tumours which are associated with hypoxia compared to other types. HIF-1 α regulates the biological processes in TNBC, including glycolysis, invasion, angiogenesis,

metastasis, and immune escape, to promote growth and survival (715). Hypoxia has a negative impact on the patient outcome and is associated with aggressive tumour behaviour and resistance to both chemotherapy and immunotherapy (570). Previous studies have shown that tumour necrosis factor (TNF) is highly expressed in TNBC compared to other molecular subtypes, suggesting that TNF-induced EMT may be specific to TNBC (716, 717). This may explain the poor prognostic effect of high STR in TNBC tumours.

One of the pathways identified in our study was the JAK-STAT signalling pathway which is responsible for tumour development, and chemotherapy resistance (718). It has been shown that STAT3, which is a member of the JAK-STAT signalling pathway, is highly expressed in TNBC and is responsible for tumour occurrence and development, and chemotherapy resistance (718). The NF- κ B signalling pathway has been identified to be activated in TNBC which is responsible for cell proliferation, growth, angiogenesis, and apoptosis evasion (719).

Immune pathways were related to high STR TNBC, especially the IL-17 signalling pathway, which is known to stimulate tumour development and aggressiveness (720). In addition to this, there were some pathways related to inflammation, such as the toll-like receptor pathway, responsible for the synthesis and release of inflammatory chemokines and cytokines, which trigger the inflammatory response (721).

In contrast, DGE analysis in luminal tumours showed other microenvironmental EMT signalling pathways and fibrosis pathways as Wnt signalling, Notch signalling TGF- β signalling pathways (722).

The Wnt pathway stimulates the differentiation of the resting fibroblasts into myofibroblasts, which increases the release of extracellular matrix components and induces fibrosis and desmoplasia (723). It plays a key role in cell proliferation and oncogenesis through stabilising β -catenin, which in turn enhances cell-cell adhesion to bind to cadherin complexes in adherents junctions and activates EMT by stimulating the expression

of the intracellular protein Axin2 to stabilise Snail. In addition to these, it activates the epidermal growth factor receptor (723). The Notch signalling pathway maintains a balance between both cell proliferation and apoptosis. Moreover, it is also involved in myofibroblast differentiation and fibrosis development (724). The Focal adhesion pathway stimulates fibroblast proliferation, fibroblast migration, and collagen synthesis and induces fibrosis, and it also enhances EMT and inflammation (725, 726). This was consistent with our results that showed the association between cellular desmoplasia (increased fibroblasts/myofibroblasts) and better outcomes within luminal BC. Ahn and colleagues reported that patients with fibroblast-dominant stroma had longer overall and disease-free survival time outcomes compared to collagen-dominant stroma in breast cancer (727). The Hippo pathway regulates tissue development and homeostasis and controls the most important cellular processes such as cell division and growth (728). The Hippo pathway includes a transcriptional coactivator with PDZ-binding motif which is an important factor for regulating breast cancer stem cells (729). TGF- β is the primary inducer of EMT, as it is a tumour suppressor, but with tumour development, the ability to arrest growth decreases whereas the EMT response increases (730).

7.5 Conclusion

In conclusion, the prognostic role of STR differs between the molecular subtypes of BC due to the different involved pathways. Hypoxia or necrosis-induced and immune mechanisms are the main driving pathways in TNBC in contrast to fibrosis-induced mechanisms in Luminal BC. The immunity status had a key prognostic role in the stroma of TNBC. The combination of STR and TILs could be a promising histological parameter to improve patient stratification. A scoring system composed of STR combined with TILs could empower pathologists to routinely evaluate TME features which may serve as a robust, simply assessed prognostic and predictive biomarker.

8 Chapter 8: Characterisation of stroma morphology and types in breast cancer

8.1 Introduction

In 2020, BC surpassed lung cancer ranking fifth for cancer-related mortality (16). The diversity of BC at the morphological level is determined by assessing particular features, mainly based on histological tumour type and grade, which are dependent predominantly on tumour neoplastic cell features. In addition, the stroma of BC is also highly heterogenous which affects diagnosis and influences tumour behaviour (1). The 5th edition of the 2019 WHO Blue Book describes stroma structure in NST tumour type as extremely variable, with some associated with densely hard stroma while other are soft with minimal stromal reaction (731). Other histological types of BC show distinct stromal features. For example, invasive lobular carcinomas usually present with minimal host reaction and few stromal architecture disturbances, making radiological diagnosis and macroscopical identification relatively difficult, (732). Meanwhile, other specific histological types, such as mucinous, invasive micropapillary, tubular, matrix-producing metaplastic and medullary-like carcinomas, display unique features within the stroma. For example, mucinous carcinomas contain floating cell clusters in mucin lakes separated by delicate fibrous septa and micropapillary carcinomas show spongy stroma (731).

Tumour-associated stroma is considered a distinct microenvironment which critically regulates the neoplasms' growth and behaviour. The regions of stroma can be classified according to their maturity. In BC, TME undergoes several dynamic changes, such as increased fibrous tissue production with abundant fibroblasts (desmoplasia), which is defined as immature stroma (733). Other types of cancers do not produce this desmoplastic reaction, so their stroma primarily has mature collagen fibres known as mature stroma (733, 734).

The assessment of stroma could be valuable in BC diagnosis. For example, tubular BC may show similar characteristics to benign lesions as radical scar therefore, the presence of immature stroma could be a

valuable feature to confirm malignancy (735). Ueno et al. established a histological classification for fibrotic stroma in rectal cancer to reflect the tumour's biological behaviour (736). the stroma was divided into three classifications: immature, intermediate and mature. Mature is defined as fine collagen fibres and stratified with fibrocytes in layers while intermediate show the presence of broad collagen bands with eosinophilic hyalinisation similar to keloids and immature are classified as haphazardly oriented keloid-like collagen fibres with loose stroma (736).

Stromal myxoid changes are another stromal reaction observed in BC which is composed of basophilic or amphophilic vacuolated material among collagen fibres (737). Histological features of stroma in DCIS, such as myxoid and inflammatory changes, have been associated with RFS (738, 739). Several genetic studies have reported distinct gene expression signatures of the stroma in the BC which could be a valuable tool to stratify patients and predict outcomes independently of the other clinical predictive and prognostic factors (734, 740, 741).

Many studies have systematically described different histopathological stromal changes seen; however, no studies have described the types of BC stroma and their impact(s) on outcome.

8.1.1 Aims and hypothesis

In this chapter, it was hypothesised that the interplay between cancer cells and TME is exemplified by the morphological changes observed in the stroma, could have a prognostic role in BC. therefore, this chapter aimed to characterise stromal changes and stroma types in BC and investigate their impact on patient prognosis and outcome.

8.2 Patients and Methods

8.2.1 Study cohort

A large, well-characterised, cohort of primary operable BC patients presenting to Nottingham City Hospital (n=822) was investigated. Clinicopathological and follow-up data were available. Slides were scanned yielding WSI, **Table 8-1**.

Table 8-1: Clinicopathological characteristics of the study cohort

Parameters	Nottingham cohort (n=822) Cases (%)
Patient age	
≤50 years	243 (29.6%)
>50 years	579 (70.4%)
Size	
≤2cm	502 (61.1%)
>2cm	320 (38.9%)
Estrogen receptor	
Positive	629 (76.5%)
Negative	193 (23.5%)
Progesterone receptor	
Positive	440 (53.5%)
Negative	349 (42.5%)
Missing	33 (4%)
HER2 status	
Positive	203 (24.7%)
Negative	637 (75.3%)
Ki67 score	
High (>14%)	186 (22.6%)
Low (<14%)	307 (37.3%)
Missing	22 (2.7%)
Molecular subtypes	
Luminal	630 (76.6%)
HER2 positive	79 (9.6%)
Triple-negative	113 (13.7%)
Histologic subtypes	
NST	322 (39.2%)
Lobular	256 (31.1%)
Metaplastic	12 (1.5%)
Special types	183 (22.3%)
Mixed type	49 (6%)
Tumor grade	
Grade I	214 (26%)
Grade II	304 (37%)
Grade III	304 (37%)
Lymph node stage	
Stage I	544 (66.2%)
Stage II	212 (25.8%)
Stage III	66 (8%)

Nottingham Prognostic Index (NPI)	
Good	345 (42%)
Intermediate	365 (44.4%)
Poor	111 (13.5%)
Missing	1 (0.1%)
Lymph vascular invasion (LVI)	
Definite	187 (22.7%)
Negative	635 (77.3%)
Lymph node status	
Positive	279 (33.8%)
Negative	543 (66.1%)

8.2.2 Scoring method

For each case, stroma was scored in BCs for 16 features (**Table 8-2**). In addition, the presence of congestion and vascularity were scored. Features were given a percentage score for the total tumour-associated stroma. Double scoring was carried out with a pathologist (Shorouk). An excellent agreement (ICC=0.9) was revealed.

The type of tumour-associated stroma was also assessed and classified. Seven final stromal types were classified: desmoplastic sclerotic desmoplastic cellular, fibro elastic, myxoid, mucinous, and inflammatory.

To be considered one type of stroma within a lesion, more than 60% of the stromal area needed to be classified as that type of stroma. In this study, desmoplastic sclerotic was defined as fibrous tissue rich in broad bands of collagen fibres (less than 30 cells/HPF). Desmoplastic cellular stroma was defined as the predominance of cellular fibrous tissue rich in fibroblasts/myofibroblasts (more than 30 cells/HPF) (685). Inflammatory stroma was defined as stroma with the predominance of inflammatory cells mainly TILs (>300/HPF) (685). . Mixed type was defined when more than one type was presented equally in the stroma, **Figure 8-1**.

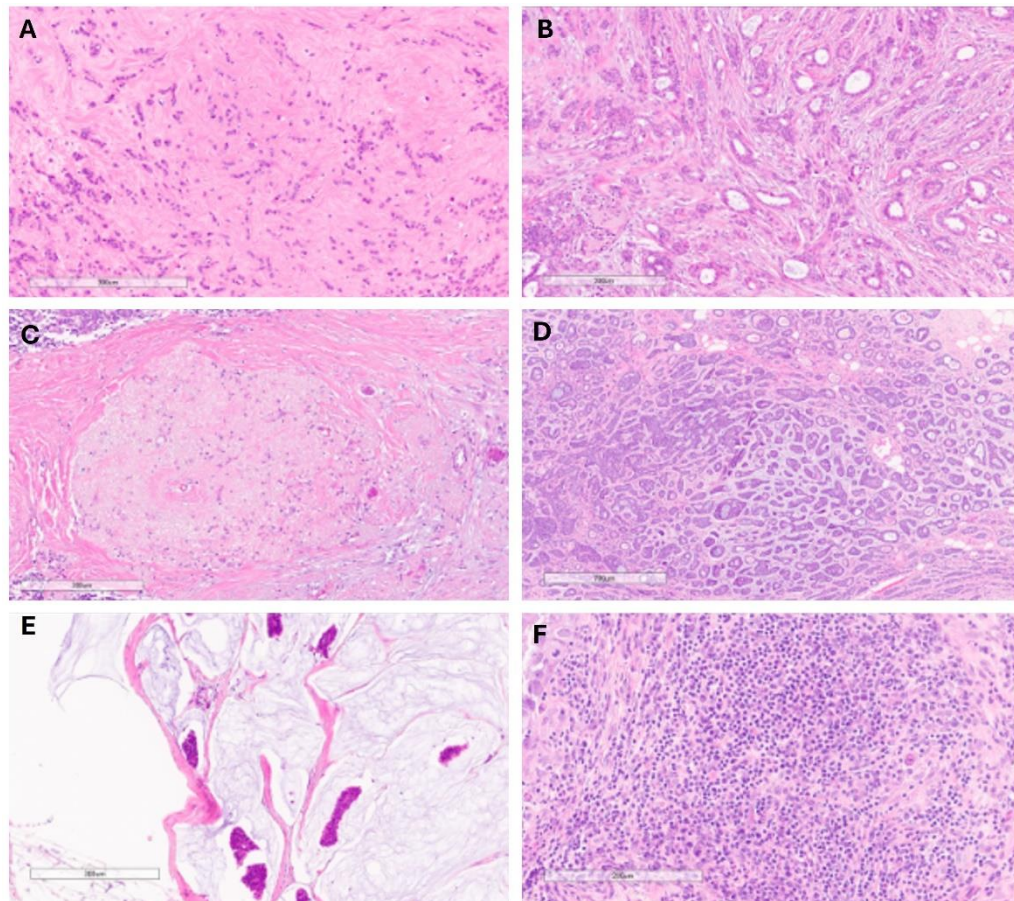
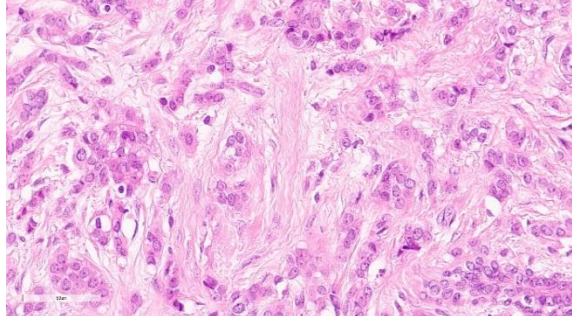
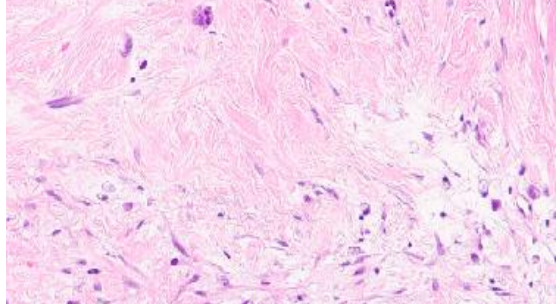
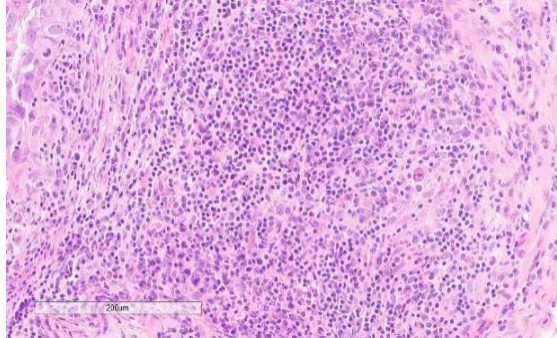
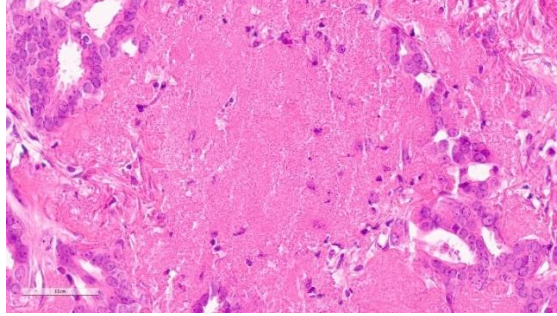


Figure 8-1: Stroma types

A) Desmoplastic sclerotic stroma. **B)** desmoplastic cellular stroma. **C)** Fibroelastic stroma. **D)** Myxoid stroma. **E)** Mucinous stroma. **F)** Inflammatory stroma.

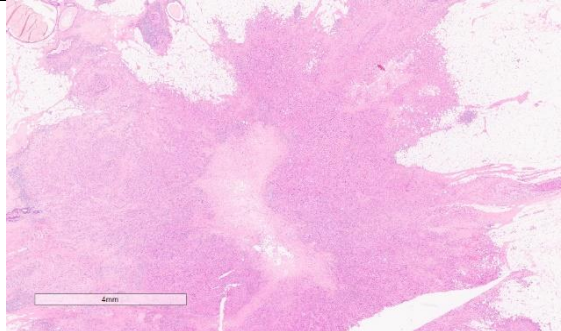
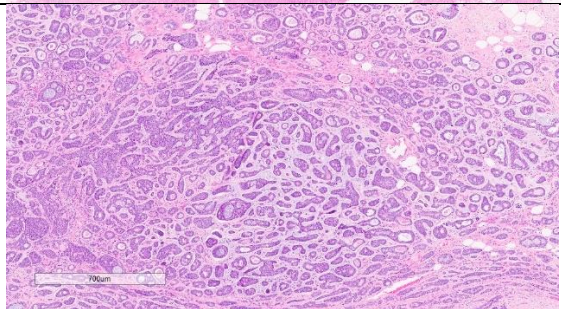
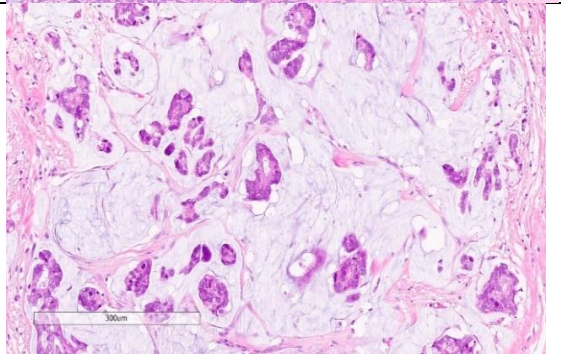
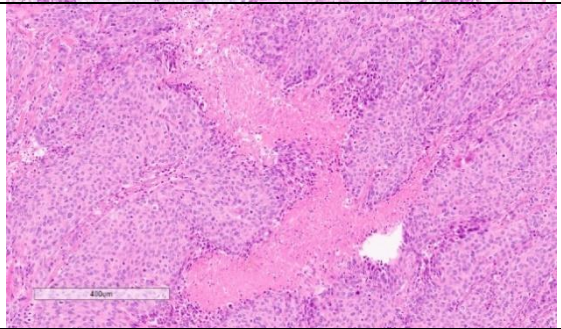
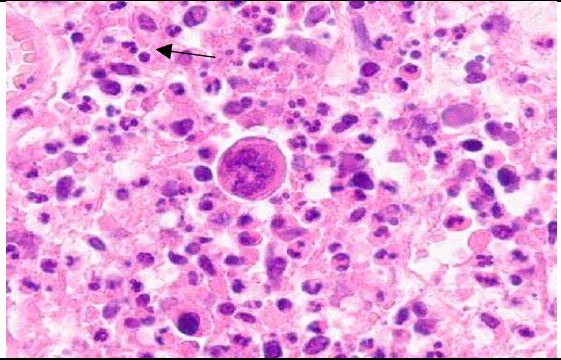
Intratumor heterogeneity was evaluated for all cases. Tumours exhibiting marked heterogeneity in stromal composition or variability in stromal geographical distribution were scored as heterogeneous stroma as stroma localised more centrally or peripherally.

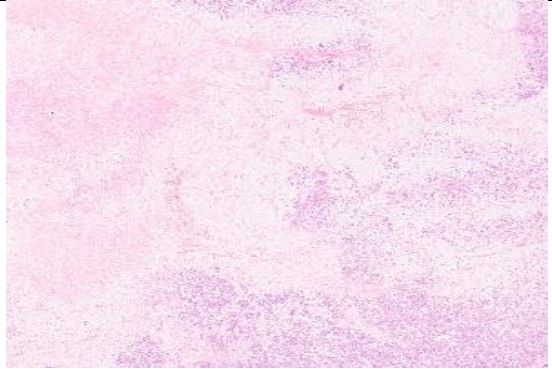
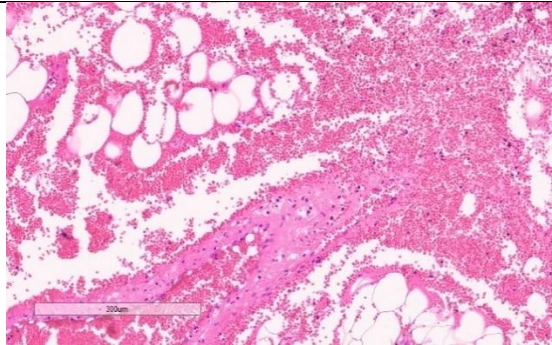
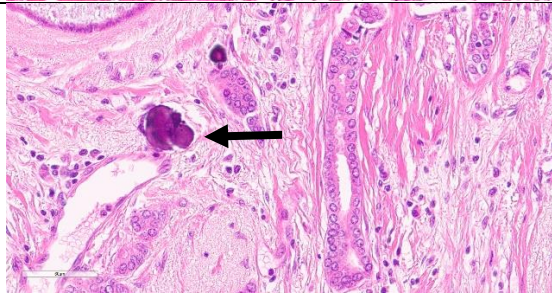
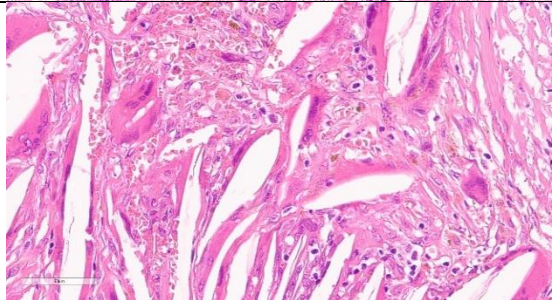
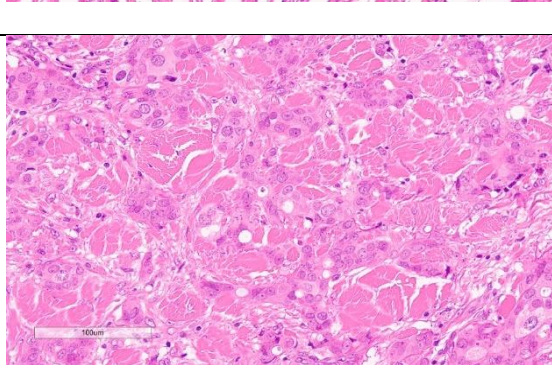
Table 8-2: Stromal features in BC

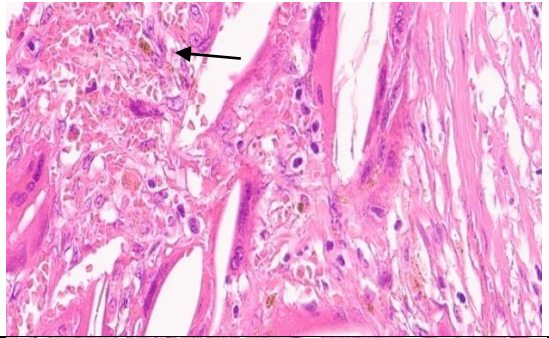
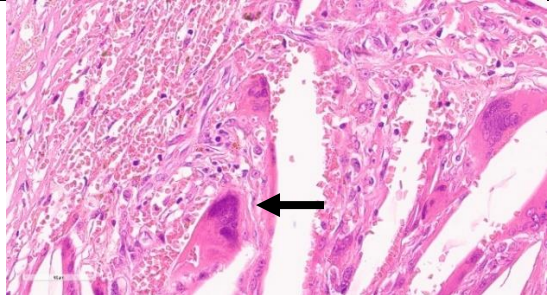
Stromal component	Definition	Image
Collagen	The fibre in ECM of CT was characterised as elongated and made up of collagen glycoproteins (742)	
Fibroblasts	A type of cell that contributes to the formation of connective tissue (742)	
Tumour infiltrating lymphocytes. (TILs)	Lymphocytes that directly surround tumour cells (302)	
Elastosis	The presence of clumps of elastic fibres thin short fibres (743)	

Chapter 8

Characterisation of stroma morphology and types in BC

Central scar (fibrotic focus)	A scar-like area in the centre of tumour surrounded by neoplastic cells (744)	
Myxoid changes	The presence of amorphous extracellular substances, which comprise an amphophilic or slightly basophilic material within the fibrous stroma (745)	
Mucin	Extensive glycosylation of the PTS domain (prolines, threonines and serines) (746)	
Necrosis	Irreversible cell injury and eventual cell death due to pathological processes, cells were eosinophilic, glassy, and vacuolated (747)	
Acute inflammation	Acute inflammatory cell infiltrate with tumour associated stroma	

Oedema	Excessive volume of fluid accumulated in the tissues, either within cells or within the collagen-mucopolysaccharide matrix distributed in the interstitial spaces (interstitial oedema)(748)	
Haemorrhage	Acute loss of blood from a damaged blood vessel (749)	
Calcifications	The abnormal deposition of calcium salts in tissues (750)	
Cholesterol clefts	Small open space filled with a type of fat called cholesterol (751)	
Keloids	A thick bundle of hypocellular collagen with eosinophilic hyalinization (752)	

Pigments	Endogenous pigments which are normal constituents of or are derived from blood (753)	
Giant cells (multinucleated)	Large cells characterised with multiple nuclei (754)	

8.2.3 Statistical analysis

Statistical analysis was carried out using Statistical Package for the Social Sciences (IBM SPSS) v.28 (Chicago, USA). Chi-square tests were used to assess the correlations between categorical variables. The degree of inter-observer concordance in the scoring was statistically evaluated using the ICC. Kaplan Meier curves with log-rank test were used to assess the correlation with outcomes in the whole cohort and the various BC molecular subtypes. Cox regression analysis with were calculated. For all tests, $p < 0.05$ (two-tailed) was considered statistically significant.

8.3 Results

8.3.1 Frequency of assessed stromal features in BC

The frequency of stromal features is shown in **Table 8-3**

Table 8-3: Frequency of stromal features in BC

Stromal feature	Frequency (%)
TILs	109 (13.3%)
Elastosis	236 (28.7%)
Central scar	80 (9.7%)
Myxoid	100 (12.2%)
Mucin	35 (4.3%)
Necrosis	
Mild	80 (9.7%)
Extensive	30 (3.6%)
Acute inflammation	52 (6.3%)
Oedema	405 (49.3%)
Haemorrhage	
Minimal	173 (21%)
extensive	22 (2.7%)
Calcifications	110 (13.4%)
Cholesterol cleft	3 (0.4%)
Keloids	183(22.3%)
Pigments	9 (1.1%)
Giant cell	1 (0.1%)

8.3.2 Frequency of predominant stroma types

Regarding stroma types, 66.1% were sclerotic desmoplastic, 18% desmoplastic cellular, 7.1% inflammatory, 4.6% mucinous, 2.6% myxoid, 1.7% fibro-elastic, and half of the cases (49.4%) showed stroma variability.

8.3.3 Distribution of stroma types within histological subtypes

Most cases of sclerotic desmoplastic stroma were lobular breast cancer (42.2%), 38.3% NST type, 11.9% special type, 6.4% mixed, and 1.3% metaplastic. In contrast, most of the cases of cellular desmoplastic stroma (47.3%) were special type, 34.5% NST type, both lobular and

mixed type had the same proportion (8.1%), while 2% of metaplastic cases had desmoplastic cellular stroma. Regarding fibro-elastic stroma, 42.9% of the cases were special type, 28.6% of cases were lobular, 21.4% were NST type and 7.1% were mixed type. 42.9% of myxoid stroma were shown in lobular BC while 28.6% were shown in special types, 19% of NST, and 9.5% metaplastic. 97.4% of mucinous stroma showed in special type while the remaining were in mixed type. Most of the inflammatory stroma was identified in NST type (98.3%) while 1.7% were shown in lobular, Chi-square ($\chi^2=340$, $p<0.001$).

8.3.4 Distribution of stroma types within molecular subtypes

Most of the desmoplastic sclerotic stroma were luminal BC type A (61.9%) however luminal type B (18.6%). 9.4% of desmoplastic sclerotic stroma were HER2 while 10.1% were TNBC. Regarding desmoplastic cellular stroma, 72.3% were luminal type A whereas 8.1% luminal type B had desmoplastic cellular stroma. 14.2% of desmoplastic cellular stroma were TNBC while 5.4% were HER2. 85.7% of fibro-elastic stroma were luminal type A and the remaining cases were typing B. 61.9% of myxoid stroma were luminal type A and 9.5% were HER2, whereas 28.6% of cases were luminal type B and TNBC equally (each 14.3%). Over half of the inflammatory stroma cases were TNBC (58.6%), 31% were HER2 and 10.3% were luminal type A. Chi-square ($\chi^2=191$, $p<0.001$).

8.3.5 Characterisation of molecular subtypes according to stroma types

Regarding molecular subtypes, 66.7% of Luminal type A cases showed desmoplastic sclerotic stroma while 21.2% showed desmoplastic cellular type and 7.1% were mucinous stroma. Fibro-elastic stroma was found in 2.4% of the cases. Few cases showed myxoid (2.6%), and no cases showed inflammatory stroma. In contrast, Type B luminal BC cases showed desmoplastic sclerotic stroma (80.2%) or desmoplastic cellular stroma (9.5%), 2.4% were myxoid stroma and fibro-elastic stroma

presented in 1.6%, only two cases had a mucinous stroma. Within HER2 cases, 64.6% had desmoplastic sclerotic stroma, 22.8% had inflammatory stroma, 10.1% with desmoplastic cellular stroma and 2.5% had myxoid stroma. No cases of HER2 showed fibro-elastic or mucinous stroma. 30.1% of TNBC cases had inflammatory stroma while 48.7% had desmoplastic sclerotic stroma. 18.6% of TNBC with desmoplastic cellular stroma while few cases showed myxoid (2.7%). $x=191$, $p<0.001$, (**Table 8-4**)

8.3.6 Characterisation of molecular subtypes according to stroma types

Regarding histological subtypes, most of the cases of NST types had a desmoplastic sclerotic stroma (61.3%) while 16.7% showed desmoplastic cellular stroma. 20% of NST cases showed inflammatory stroma however, few cases showed either fibro-elastic (1%) or myxoid stroma (1%). Lobular BC had desmoplastic sclerotic stroma (89.8%), while 4.7% of the cases showed desmoplastic cellular stroma, and few cases showed fibro-elastic or myxoid stroma (1.6%, 3.5% regularly). Only one lobular BC case showed inflammatory stroma. Approximately half of the metaplastic cases had desmoplastic sclerotic stroma (58.3%), while a quarter of the cases with desmoplastic cellular stroma and about 16.7% of myxoid stroma. 35% of special type had desmoplastic sclerotic stroma and 38.3% had desmoplastic cellular type. Few cases had either fibro-elastic or myxoid stroma (both 3.3%). 19.7% of the special type showed mucinous stroma however, only one case showed inflammatory stroma. Most of the mixed type showed desmoplastic sclerotic stroma (70.8%) while a quarter of the cases showed desmoplastic cellular stroma. Notably, a case of mixed type showed fibro-elastic stroma and only one case showed inflammatory stroma. Chi-square ($x=345$, $p<0.001$), **Table 8-4**.

Table 8-4: Frequency of stroma types within histological and molecular subtypes in breast cancer

	Desmoplastic sclerotic stroma	Desmoplastic cellular stroma	Fibro- elastic stroma	Myxoid stroma	Mucinous stroma	Inflammatory stroma
Histological subtypes						
- NST	208 (61.3%)	51 (16.7%)	3 (1%)	4 (1%)	0 (0%)	57 (20%)
- Lobular	229 (89.8%)	12 (4.7%)	4 (1.6%)	9 (3.5%)	0 (0%)	1 (0.4%)
- Metaplastic	7 (58.3%)	3 (25%)	0 (0%)	2 (16.7%)	0 (0%)	0 (0%)
- Special types	64 (35%)	70 (38.3%)	6 (3.3%)	6 (3.3%)	37 (20.2%)	0 (0%)
- Mixed stroma	35 (71.4%)	12 (24.5%)	1 (2%)	0 (0%)	1 (2%)	0 (0%)
1- NST and special	25 (73.5%)	8 (23.5%)	0 (0%)	0 (0%)	1 (3%)	0 (0%)
2- NST and Metaplastic	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3- Lobular and special	5 (62.5%)	3 (37.5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
4- Mixed Special	3 (60%)	1 (20%)	1 (20%)	0 (0%)	0 (0%)	0 (0%)
Molecular subtypes						
- Luminal A	336 (66.7%)	107 (21.2%)	12 (2.4%)	13 (2.6%)	36 (7.1%)	0 (0%)
- Luminal B	101 (80.2%)	12 (9.5%)	2 (1.6%)	3 (2.4%)	2 (1.6%)	6 (4.8%)
- TNBC	55 (48.7%)	21 (18.6%)	0 (0%)	3 (2.7%)	0 (0%)	34 (30.1%)
- HER2-enriched	51 (64.6%)	8 (10.1%)	0 (0%)	2 (2.5%)	0 (0%)	18 (22.8%)
Special types						
- Tubular	36 (40.9%)	46 (52.3%)	3 (3.4%)	3 (3.4%)	0 (0%)	0 (0%)
- Cribriform	10 (31.3%)	17 (53.1%)	3 (9.4%)	2 (6.3%)	0 (0%)	0 (0%)
- Mucinous	0 (0%)	0 (0%)	0 (0%)	0 (0%)	37 (100%)	0 (0%)
- Micropapillary	6 (54.5%)	4 (36.4%)	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)
- Papillary	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
- Solid papillary	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
- Adenoid cystic	6 (85.7%)	1 (14.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
- Apocrine	2 (66.7%)	1 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

8.3.7 Correlation of stroma types with clinicopathological data

The association of stroma types with clinicopathological data is summarised in **Table 8-5**

Table 8-5: Association of stroma types with clinicopathological data

Parameter	Desmoplastic sclerotic stroma	Desmoplastic cellular stroma	Fibro-elastic stroma	Myxoid stroma	Mucinous stroma	Inflammatory stroma	P-value
Patient Age <50 years > 50 years	150 (27.6%) 393 (72.4%)	57 (38.5%) 91 (61.5%)	0 (0%) 14 (100%)	4 (19%) 17 (81%)	9 (23.7%) 29 (76.3%)	23 (39.7%) 35 (60.3%)	0.04*
Tumour size <2cm >2cm	321 (59.1%) 222 (40.9%)	102 (68.9%) 46 (31.3%)	13 (92.9%) 1 (7.1%)	13 (61.9%) 8 (38.1%)	25 (65.8%) 13 (34.2%)	28 (48.3%) 30 (51.7%)	0.001*
Grade Grade I Grade II Grade III	89 (16.4%) 257 (47.3%) 197 (36.3%)	82 (55.4%) 19 (12.8%) 47 (31.8%)	9 (64.3%) 5 (35.7%) 0 (0%)	7 (33.3%) 8 (38.1%) 6 (28.6%)	27 (71.7%) 11 (28.9%) 0 (0%)	0 (0%) 4 (6.9%) 54 (93.1%)	0.001*
Stage Stage I Stage II Stage III	341 (62.9%) 154 (28.4%) 48 (8.9%)	109 (73.6%) 32 (21.6%) 7 (4.7%)	11 (78.6%) 3 (21.4%) 0 (0%)	11 (52.4%) 6 (28.6%) 4 (19%)	35 (92.1%) 3 (7.9%) 0 (0%)	36 (62.1%) 14 (24.1%) 8 (13.8%)	0.5
Estrogen receptor Positive Negative	439 (83.3%) 88 (16.7%)	114 (77%) 34 (23%)	14 (100%) 0 (0%)	15 (75%) 5 (25%)	33 (86.8) 5 (13.2%)	17 (30.9%) 38 (69.1%)	0.01*
Progesterone receptor Positive Negative Missing	300 (55.2%) 226 (41.6%) 17 (3.1%)	86 (58.1%) 52 (35.3%) 10 (6.8%)	8 (57.1%) 5 (35.7%) 1 (7.1%)	12 (57.1%) 9 (42.9%) 0 (0%)	32 (84.2%) 3 (7.9%) 3 (7.9%)	2 (3.4%) 54 (93.1%) 2 (3.4%)	0.8
HER2 status Positive Negative	152 (28%) 391 (72%)	20 (13.5%) 128 (86.5%)	0 (0%) 14 (100%)	5 (23.8%) 16 (76.2%)	2 (5.3%) 36 (94.7%)	24 (41.4%) 34 (58.6%)	0.003*
Ki67 score Low High Missing	198 (36.5%) 121 (22.3%) 224 (31.4%)	66 (44.6%) 32 (21.6%) 50 (33.8%)	8 (57.1%) 2 (14.3%) 4 (28.6%)	7 (33.3%) 4 (19%) 10 (47.6%)	23 (60.5%) 4 (10.5%) 11 (28.95)	5 (8.6%) 23 (39.7%) 30 (51.7%)	0.01*
Nottingham prognostic index (NPI) groups Good Moderate Poor	209 (38.5%) 253 (64.6%) 81 (14.9%)	84 (56.8%) 52 (35.1%) 12 (8.1%)	12 (85.7%) 2 (14.3%) 5 (8.6%)	8 (38.1%) 10 (47.6%) 3 (14.3%)	32 (84.25) 6 (15.8%) 0 (0%)	0 (0%) 42 (72.4%) 16 (27.6%)	0.002*
Lymph node status Negative Positive	342 (63%) 201 (37%)	109 (73.6%) 39 (26.4%)	11 (78.6%) 3 (21.4%)	11 (52.4%) 10 (47.6%)	35 (92.1%) 3 (7.9%)	36 (62.1%) 22 (37.9%)	0.07
Lymph vascular invasion (LVI) Definite Negative	134 (24.7%) 409 (75.3%)	32 (21.6%) 116 (78.4%)	0 (0%) 14 (100%)	7 (33.3%) 14 (66.7%)	6 (15.8%) 32 (84.25)	8 (13.8%) 50 (86.2%)	0.01*

8.3.8 Association of stroma types with outcomes

Within the stroma types of the cases, the fibro-elastic stroma subtype was associated with the longest BCSS while desmoplastic sclerotic stroma was associated with the shortest BCSS ($p<0.001$).

Desmoplastic cellular stroma showed better outcomes than desmoplastic sclerotic stroma type ($p<0.001$). In addition, desmoplastic sclerotic stroma showed shorter BCSS compared to other stroma types including fibroblastic stroma ($p<0.04$), mucinous stroma ($p<0.02$), and inflammatory stroma ($p<0.01$). There was no significant difference in the outcome between desmoplastic sclerotic stroma and myxoid stroma ($p<0.9$).

There were no significant differences in the outcomes between desmoplastic cellular stroma and fibro-elastic stroma ($p<0.28$) or myxoid stroma ($p<0.08$), mucinous stroma ($p<0.5$) or inflammatory stroma ($p<0.6$). In addition, no significant differences in the outcomes between fibro-elastic stroma and myxoid stroma ($p<0.09$), mucinous stroma ($p<0.3$), or inflammatory stroma ($p<0.3$). Similarly, myxoid stroma showed no significance in the outcomes compared to mucinous stroma ($p<0.07$) or inflammatory stroma ($p<0.08$), also inflammatory stroma showed no significant outcome compared to mucinous stroma ($p<0.9$).

8.3.9 Association of stroma types with outcomes in molecular subtypes

Within luminal type A, desmoplastic cellular stroma showed significantly longer BCSS compared to desmoplastic sclerotic stroma ($p<0.01$). Similarly, within luminal type B, desmoplastic cellular stroma showed a better outcome compared to desmoplastic sclerotic stroma ($p<0.004$).

In luminal type B, desmoplastic sclerotic stroma showed shorter BCSS compared to both fibro-elastic stroma ($p<0.04$), mucinous stroma ($p<0.01$) and inflammatory stroma ($p<0.01$). In addition, myxoid stroma

showed shorter BCSS compared to both mucinous stroma ($p<0.001$) and inflammatory stroma ($p<0.001$).

Regarding HER2 cases, desmoplastic cellular stroma showed better outcomes compared to desmoplastic sclerotic stroma ($p<0.001$). Desmoplastic sclerotic stroma showed shorter BCSS compared to both mucinous stroma ($p<0.001$), and inflammatory stroma ($p<0.01$). Inflammatory stroma showed longer BCSS compared to myxoid stroma ($p<0.003$).

In TNBC, desmoplastic sclerotic stroma showed shorter BCSS compared to desmoplastic cellular stroma ($p<0.001$), fibro-elastic stroma ($p<0.006$), mucinous stroma ($p<0.001$), and inflammatory stroma ($p<0.001$). Desmoplastic cellular stroma showed better outcome than myxoid stroma ($p<0.03$).

8.3.10 Association of stroma types with outcomes in molecular subtypes

In the NST subtype, the fibro-elastic stroma was associated with the best outcome while the myxoid stroma was associated with the worst outcome ($p<0.001$). Desmoplastic sclerotic stroma showed shorter BCSS compared to desmoplastic cellular stroma ($p<0.001$) and inflammatory stroma ($p<0.004$). Inflammatory stroma showed a favourable outcome compared to myxoid stroma ($p<0.001$).

Within lobular BC, desmoplastic sclerotic stroma showed no significant difference compared to desmoplastic cellular stroma ($p<0.07$).

Regarding the metaplastic subtype, desmoplastic sclerotic showed shorter BCSS compared to desmoplastic cellular stroma ($p<0.001$), fibro-elastic stroma ($p<0.001$), myxoid stroma ($p<0.001$) and inflammatory stroma ($p<0.001$).

In the special cases, desmoplastic cellular stroma showed better outcomes compared to myxoid stroma ($p<0.004$). Mucinous stroma

showed better outcome compared to desmoplastic sclerotic stroma ($p<0.001$).

In mixed type, desmoplastic sclerotic stroma showed shorter BCSS compared to desmoplastic cellular stroma ($p<0.001$), fibro-elastic stroma ($p<0.01$), and inflammatory stroma ($p<0.001$). Within cases of mixed NST with special type, desmoplastic sclerotic stroma showed shorter BCSS than desmoplastic cellular stroma ($p<0.001$), fibro-elastic stroma ($p<0.01$), and inflammatory stroma ($p<0.004$). In addition, both fibro-elastic ($p<0.02$) and inflammatory stroma ($p<0.006$) showed better outcomes than desmoplastic cellular stroma. However, within lobular mixed with special, there was no significant difference in the outcome between the stroma types (only desmoplastic sclerotic and desmoplastic cellular).

There was no significant difference in the outcome between tubular and cribriform groups. Within the tubular group, desmoplastic cellular stroma showed better outcomes compared to myxoid stroma ($p<0.01$).

8.3.11 Association of stromal features with outcome

The presence of a central scar showed shorter BCSS ($p<0.001$). Elastosis showed a better outcome ($p<0.02$). Cases associated with haemorrhage showed shorter BCSS ($p<0.02$). Similarly, cases associated with necrosis showed worse outcomes when either mild ($p<0.001$) or severe ($p<0.001$). Calcifications were associated with shorter BCSS ($p<0.004$). High vascularity cases showed a worse outcome than low vascularity ($p<0.02$). Stromal heterogeneity showed shorter BCSS ($p<0.001$), and peripheral distribution showed better outcomes than central stroma ($p<0.001$).

Within the desmoplastic sclerotic stroma, elastosis showed a favourable outcome ($p<0.02$), however, other types of stroma showed no significant difference in the outcome with the association with elastosis.

8.4 Discussion

The stromal reaction has attracted the attention of several types of carcinomas (755, 756). However, the stromal reaction in BC has not received enough attention (745). A study demonstrated that a dominant stroma type may be a significant prognostic factor in univariate and multivariate analyse (727).

This study evaluated stromal features to characterise the stroma types in BC. To summarise, the study results showed that desmoplastic sclerotic stroma showed worse outcomes compared to all other types except myxoid stroma as there was no significant difference. In general, there was no difference between desmoplastic cellular stroma and other types. Similarly, the remaining stroma types have no differences in outcome. Regarding sclerotic stroma, the presence of elastosis had better outcomes however, heterogeneity showed worse outcomes.

Within molecular subtypes, desmoplastic cellular stroma showed better BCSS compared to sclerotic stroma in all molecular subtypes, however, fibro-elastic stroma showed better outcomes. In luminal B, HER2 and TNBC, Myxoid stroma worse outcome than inflammatory stroma. Inflammatory stroma had better outcomes in HER2 and TNBC.

Within histological subtypes, desmoplastic sclerotic stroma showed worse outcomes than desmoplastic cellular stroma in NST but not in lobular carcinoma as there were no differences. In special types, stroma type has no effect except in the tubular group as desmoplastic cellular stroma showed better outcomes than myxoid stroma.

Ahn and colleagues categorised BC stroma into four types; collagen dominant, fibroblastic dominant, and lymphocytic dominant. They reported that patients with fibroblast-dominant stroma had longer overall and disease-free survival time outcomes compared to collagen-dominant stroma in BC. In addition, Lymphocytic dominant had longer outcomes (727). This was consistent with the study's results

Shadan et al. stated histological classification for BC. The stroma was divided into three categories: immature, intermediate and mature. Mature is defined as fine collagen fibres and stratified with fibrocytes in layers while intermediate show the presence of broad collagen bands with eosinophilic hyalinisation similar to keloids and immature are classified as haphazardly oriented keloid-like collagen fibres with loose stroma (757). A poor survival outcome was associated with immature stroma (736). On the other hand, it has been documented that a fibrotic focus, a fibrotic area of fibroblasts and collagen, surrounded by an infiltrating carcinoma zone has a worse prognostic influence in BC patients (758). This was consistent with the results.

The increases in collagen fibre density result in matrix stiffening which affects the nature and number of binding sites available for neoplastic cell migration. This phenomenon stimulates metastatic cells to enhance their contractile force and their ability to spread, which leads to reorganisations in their cytoskeletal structures and infiltration into the dense stroma. On the other hand, fibrosis results in hypoxia of the microenvironment, which triggers angiogenesis and lymph invasion (759). Studies have described the development of stromal desmoplasia in cancers as being similar to the processes in wound healing (383). It has been stated that tumours are “wounds with no healing” and it is known that chronic fibrosis is a risk factor for cancer (382). Chang highlighted that patients with wound response signatures (stromal fibrosis) had a poor prognosis (384).

Myxoid changes were associated with aggressive clinicopathological behaviour such as higher grade and LN status, therefore it may be a poor prognostic indicator in BC (704). However, myxoid changes and central scars were proved as independent poor prognostic indicators in TNBC (745). In addition, Wernicke et al. documented a significant association between myxoid stroma and LN metastasis in invasive BC (737).

Central scars showed worse outcomes in BC and could be considered a reflection of TNBC; however, in this study, about 20 cases of luminal BC showed central scars with worse outcomes. Similarly, as elastosis is remarkably associated with low-grade luminal BC, however, 5% of cases were HER2 positive and showed good outcomes.

Breast elastosis also correlates with good prognostic markers such as ER positivity, lower Ki-67, and HER2 negativity, and elastosis was increased in low-grade tumours compared to high-grade ones (392-394). On the other hand, other studies showing the value of elastosis in survival had contradictory results (760). In addition, researchers indicated that low stromal elastosis correlated with vascular invasion and metastasis (392). The elastosis absence stimulated an angiogenic microenvironment, which resulted in a high risk of lymph vascular or blood invasion and tumour metastasis (392). Some studies have demonstrated that elastosis assessment in BCs could be used in selecting patients for adjuvant endocrine therapy, as elastosis was combined with a complete response to endocrine therapy and a good prognosis (388, 761). This was consistent with the results.

STR has a prognostic role in BC, however, mucinous carcinoma showed a better prognosis although the stroma volume is low (septae) therefore the qualitative nature of the stroma is important. Stroma evaluation is not currently a standard practice for pathologists in clinical diagnosis, therefore idea of using this factor as a prospective feature for evaluating BC behaviour is needed (733).

8.5 Conclusion

The dominant stroma type could be a predictor factor for predicting outcomes.

desmoplastic sclerotic stroma showed worse outcomes compared to all other types except myxoid stroma as there was no significant difference.

Regarding sclerotic stroma, the presence of elastosis had better outcomes however, heterogeneity showed worse outcomes

Evaluation of tumour stroma types and features may be easily performed in routine clinic practice as a guide in stratifying patient risk.

9 Chapter 9: General Discussion

This study hypothesised that the morphological features of TME could be useful as diagnostic, prognostic, or predictive tools for routine clinical practice in BC. Therefore, this study integrates DP and AI to decipher these features in BC and identify novel biomarkers for improved BC prognosis.

9.1 Cardinal findings of the study

9.1.1 Chapter 3: Characterisation of basement membrane associated collagen morphology in breast cancer

To study TME, we started to investigate BM.

In this study, we hypothesised that distinguishing a native BM from a reactive BM surrounding an invasive lesion has significant clinical importance as it enables accurate diagnosis at an early stage and appropriate patient management when considering the implications of management *in-situ*.

The observations from this study found that DCIS's BM resembled normal breast BM except it is higher in density, straighter, disorganised and less aligned. However, EPC, invasive and special invasive groups had higher density, thicker fibre, more collagen I content, wider, shorter, straighter, less aligned and disorganised. The metastatic LN cases were surrounded by thin shorter fibres and their overall content was less than the thin/thick ratio of the normal BM. This is consistent with studies that explain tumourigenesis as mammary collagen fibres activate signalling pathways which result in stimulating the proliferation and progression of tumour cells (277). Many studies proved that collagen fibre organization and density influence tumorigenesis and tumour invasiveness (471, 472).

9.1.2 Chapter 4: Characterisation of encapsulated papillary carcinoma capsule in breast cancer

We observed in the previous study that the EPC capsule resembled invasive carcinoma and showed heterogeneity between its parts

therefore it was hypothesised that the deciphering of the EPC capsule could explain the nature of EPC behaviour.

The study results demonstrated that EPC is surrounded with BM-like material resembling those surrounding some invasive tumour types. The capsule of EPC has the characteristics of both in situ and invasive lesions based on the site of the capsule. The outer part of the capsule had significantly higher collagen density with more collagen I compared to the inner part. Outer capsule fibres were thicker, straighter and less aligned than the inner part with no differences in fibre length. EPC capsules showed heterogeneity between their inner and outer parts, the inner part exhibited the BM of DCIS only in fibre straightness while the outer part of EPC capsules showed no significant differences relating to fibre density, collagen distribution (I and III) and width compared to the invasive tumour. Compared with EPTC, EPC capsule showed no significant difference except more straighter fibres than EPTC capsule. This was consistent with that many tumours exhibit capsule with indolent behaviour (515-519).

The present study provides further evidence that EPC is an indolent invasive carcinoma surrounded by BM-like material with bio-pathological features between in situ and invasive carcinoma based on capsule characteristics which was consistent with many studies that described that EPCs are indolent invasive cancers with a small percentage of *in situ* (235, 490, 497).

9.1.3 Chapter 5: Geometric Characteristics of Stromal Collagen Fibres in Breast Cancer

Then we studied another part of TME, the stroma. This study hypothesised that stromal ultrastructure features of stromal collagen fibre could be a potential method of differentiating non-invasive and invasive lesions and may have a prognostic role in BC.

This study observed that the collagen fibres in the stroma of the invasive group showed thinner, straight, and disorganised fibres, helping the cells

to invade the stroma and metastasis. These collagen fibre characteristics were associated with clinicopathological data, especially with aggressive behaviour in both DCIS and invasive cohorts. A statistically significant association between fibre characteristics and patients' outcomes was observed in the invasive group but not in DCIS. This was consistent with studies which advocate that stromal collagen organisation stimulates tumour progression (348, 349). In addition, These results support a previous study that supported proof of concept data that changes concerning the organisation of the collagen network in the stroma plays a key role in cancer progression and malignancy by stimulating a tumour-permissive microenvironment, especially for invasive BC (527).

The present study developed a diagnostic model to differentiate DCIS from invasive BC based on the most significant stromal collagen fibre characteristics between the two disease stages. Keikhosravi *et al.* developed a model which extracted fibre characteristics from H&E sections based on a trained neural network of SHG data, but their clinical value still needs further validation (421). Therefore, this study used DIC microscopy as it visualises collagen fibres without the need for staining which could provide additional information which helps further in the validation.

9.1.4 Chapter 6: Identification of potential diagnostic and prognostic markers in breast cancer

This study hypothesised that collagen XV and collagen XIX may have a role in the progression from non-invasive to invasive stages of BC, therefore, they could potentially be good diagnostic tools. In addition, it was tested where HIF-1 α and LOX provide important clinical value and insights in BC and hypothesised that their roles would differ among the different BC molecular subtypes.

All the cases of both groups of normal breast tissue and DCIS showed positive expression of collagen XV in the BM with different intensities however, all the cases of EPC showed negative expression of collagen

XV. Conversely, most of the cases of the invasive group showed positive expression of collagen XV ranging between weak and mild intensity. All the cases of both groups of normal breast tissue and DCIS showed positive expression of collagen XIX in the BM with various intensities conversely, all the cases in the EPC and the invasive groups had negative expression of collagen XIX. A previous study reported the presence of collagens XV and XIX in the BMZ of DCIS however levels started to diminish which suggested that the BM is still intact and acts as a barrier to invasion. However, loss of collagen XV could be an early sign of BM loss indicating the beginning of ECM remodelling and invasion behaviour (272).

The study observed a strong correlation between LOX and HIF-1 α expression which was proven in many cancers (642). The study showed that high LOX levels correlate with more aggressive tumour behaviour therapy resistance and poor outcomes which was consistent with many studies (644). (593, 645, 651, 653-655). In addition, it has shown that high HIF-1 α levels correlate with more aggressive tumour behaviour, therapy resistance and poor outcomes (620-622).

LOX and HIF-1 α are closely related via a positive feedback loop which enhances their expression and activity leading to tumour growth promotion and therapy response resistance. This relationship underscores the potential of targeting the LOX/HIF-1 α axis in cancer therapy.

9.1.5 Chapter 7: The conflicting prognostic role of the stroma-ratio in breast cancer

In this study, it was hypothesised that the mechanisms driving Stroma, and its immune composition, are different in BC subtypes which could explain the conflicting roles of the prognostic value of stroma ratio in different molecular subtypes

The study results reported that high STR was associated with favourable patient outcomes in the whole cohort and the luminal subtype, whereas

high STR showed an association with poor outcomes in TNBC. No association with outcome was found in the HER2 positive BC which was agreed with many studies (259, 659, 663, 669, 679).

DGE analysis identified various pathways in luminal and TNBC subtypes, with immune upregulation and hypoxia pathways enriched in TNBC, and pathways related to fibrosis and stromal remodelling enriched in the luminal group instead. Low STR accompanied by high TILs was shown to carry the most favourable prognosis in TNBC. In line with the DGE results, TILs played a major prognostic role in the stroma of TNBC, but not in the luminal or HER2-enriched subtypes. This was consistent with the study of Gujam and colleagues which found that a high stroma level was consistently correlated with lower immunity (671). The study also showed that Low STR accompanied by high TILs was shown to carry the most favourable prognosis in TNBC, Millar and colleagues studied the effect of combined stroma with TILs in TNBC and showed that the best prognostic group was low STR/high TILs which is consistent with the present study results (418).

9.1.6 Chapter 8: Characterisation of stroma morphology and types in breast cancer The characterisation of stromal morphology and types in BC

The study results showed that desmoplastic sclerotic stroma showed worse outcomes compared to all other types except myxoid stroma as there was no significant difference. In general, there was no difference between desmoplastic cellular stroma and other types. Similarly, the remaining stroma types have no differences in outcome. Regarding sclerotic stroma, the presence of elastosis had better outcomes however, heterogeneity showed worse outcomes. The effect of stroma differs within molecular and histological subtypes. This was consistent with other studies (727).

9.2 Final conclusions

With the era of DP and AI, prognostic stratification of BC patients and prediction of survival for tailored therapy would be improved. several collagen fibres feature in the tumour microenvironment could help develop collagen signatures.

The study emphasises the importance of considering all morphological features of TME in tumour behaviour and prognosis. Collagen parameters of BM and stroma could be used as a diagnostic and prognostic tool to differentiate between invasive and non-invasive lesions, 3.5.

EPC is surrounded with BM-like material resembling those surrounding some invasive tumour types, but it is significantly distinctive from the native BM of normal breast and DCIS. EPC is an indolent invasive carcinoma which surrounded by BM-like material with bio-pathological features between in situ and invasive carcinoma based on capsule characteristics. Most papillary tumours in various organs which are surrounded with BM-like material show indolent behaviour with a good prognosis, 4.5.

The collagen parameters are predictive of outcomes in the invasive group including recurrence and survival. Collagen fibre characteristics are not predictive of outcome endpoints in DCIS. Moreover, fibre characteristics can differentiate DCIS from invasive diseases in the breast, 5.5.

Stromal and collagen related genes could be used as prognostic biomarkers for BC. Collagen XIX could be used as a diagnostic tool to differentiate between invasive and non-invasive lesions in challenging cases, 6.5.

HIF-1 α could be a new biomarker for BC prognosis and therapy response. The HIF-1 α levels in BC may be used to prospectively stratify patients who received neoadjuvant therapy. LOX could be a new

biomarker for prognosis and therapy response. LOX could be a good therapeutic target specifically for metastasis prevention, 6.5.

The underlying molecular mechanisms and composition of the stroma in BC are variable in the molecular subtypes. The prognostic role of STR differs between the molecular subtypes of BC due to the different involved pathways. Hypoxia or necrosis-induced and immune mechanisms are the main driving pathways in TNBC in contrast to fibrosis-induced mechanisms in Luminal BC. The immunity status had a key prognostic role in the stroma of TNBC. The combination of STR and TILs could be a promising histological parameter to improve patient stratification. A scoring system composed of STR combined with TILs could empower pathologists to routinely evaluate TME features which may serve as a robust, simply assessed prognostic and predictive biomarker, 7.5.

The dominant stroma type could be a predictor factor for predicting outcomes. Evaluation of tumour stroma types and features may be easily performed in routine clinic practice as a guide in stratifying patient risk, 8.5.

9.2.1Key finding list

- BM collagen fibres could distinguish between non-invasive and invasive BC
- -EPC is an indolent invasive tumour
- Stromal collagen fibres could differentiate between DCIS and invasive BC
- Stromal collagen fibres predict BC outcomes
- Collagen XV and XIX, new biomarkers, could be used to differentiate non-invasive and invasive BC
- LOX and HIF-1 alpha are biomarkers that could predict BC prognosis.
- STR combined with TIL could be a powerful predictive tool and vary depending on molecular subtype.
- The type of stroma could predict BC outcomes.

9.3 Clinical implications of the study

- 1- Collagen parameters of BM and stroma could be used as a diagnostic tool to differentiate between invasive and non-invasive lesion.
- 2- A model was built to be used to differentiate between invasive and non-invasive lesions based on stromal collagen fibres.
- 3- Provide evidence that EPC is indolent invasive carcinoma which surrounded by BM-like material.
- 4- Most papillary tumours in various organs which are surrounded by BM-like material show indolent behaviour with a good prognosis.
- 5- Collagen parameters are predictive of outcomes in invasive BC.
- 6- Collagen XIX could be used as a diagnostic tool to differentiate between invasive and non-invasive lesions in challenging cases.
- 7- Collagen XV could be used as a diagnostic tool in challenging cases of papillary lesions.
- 8- HIF-1 α could be a new biomarker for BC prognosis and therapy response.
- 9- LOX could be a biomarker for BC prognosis and therapy response.
- 10- LOX could be a good therapeutic target especially for metastasis prevention.
- 11- Combination between STR and TILs could be a promising histological parameter to improve patient stratification.
- 12- The dominant type of stroma could be a predictor factor for BC outcomes.

9.4 Strengths of the study

- 1- The study was designed to use a large cohort of BC patients. In this cohort, the clinicopathological parameters and clinical outcome data was available and prospectively maintained. Therefore, findings of observational studies and the studied tissue biomarkers were linked to clinical outcome and different molecular classes.

- 2- In this study, the antibodies were used with positive and negative controls in addition to WB for some of the studied antibodies.
- 3- The use of the publicly available mRNA cohorts to validate the results of proteomic results found in this study. The TCGA available digital images was used to assess the morphological features of tumours as validation methods
- 4- Different statistical approaches in the determination of the optimal cutoffs of staining positivity of the used markers, rather than using arbitrarily chosen ones.
- 5- Validation with another pathologist's eye approach in scoring with testing ICC
- 6- The use of data as both continuous and categorical was helpful in confirmation of the results.
- 7- Using bioinformatic analysis to study the association of the studied variables with biological and molecular pathways

9.5 Potential limitations

- 1- The data were only assessed in one institution and no multi-institutional validation.
- 2- Some of the biomarkers used in this study were performed on TMAs. However, a subset of full-face sections was used as training. However, TMA results have repeatedly been reported to show excellent concordance with full-face sections in BC.
- 3- Small sample size in some studies using full-face sections.
- 4- The small sample size and low event rate in the DCIS group in DIC microscopy.
- 5- Western blot for collagen markers was not available but another way for optimisation was used.

9.6 Future work

- 1- Studying the mechanisms behind these collagen signatures could further assist the patient outcome prediction and help develop novel therapies.
- 2- Investigate the mechanisms that could explain that the capsule of EPC has the characteristics of both in situ and invasive lesions based on the site of the capsule
- 3- Investigate mechanisms that explain the phenomenon of papillary tumours in various organs which are surrounded with BM-like material that show indolent behaviour with a good prognosis.
- 4- Analysis of the stroma around DCIS in both pure DCIS and DCIS associated with invasive disease.
- 5- Complete the study of novel biomarkers collagen IV alpha 5 and collagen IV alpha 6.
- 6- Validation of the model that differentiate between invasive and non-invasive lesions with a larger cohort.

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