CHARACTERISATION OF TRIPLE NEGATIVE BREAST CARCINOMA AND IDENTIFICATION OF THE VALUE OF IMMUNOHISTOCHEMICAL MARKERS AND STROMAL TUMOUR INFILTRATING LYMPHOCYTES

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PRESENTATIONS ARISING FROM THIS THESIS

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- Reproducibility and predictive value of scoring stromal tumour infiltrating lymphocytes in triple-negative breast cancer: a multi-institutional study. O'Loughlin M, Andreu X, Bianchi S, Chemielik E, Cordoba A, Cserni G, Figueiredo P, Floris G, Foschini MP, Heikkilä P, Kulka J, Liepniece-Karele IL, Regitnig P, Reiner A, Ryska A, Sapino A, Shalaby A, Stovgaard AS, Quinn C, Walsh EM, Zolota V, Glynn SA, Callagy G. Breast Cancer Res Treat; 2018, doi: 10.1007/s10549-018-4825-8.

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- Stromal tumour lymphocyte infiltration as a prognostic tool in triple negative breast cancer. 29th European Congress of Pathology, Amsterdam, The Netherlands (September 2017). Aliaa Shalaby, Elaine Walsh, Sharon Glynn, Grace Callagy. Virchows Arch., 2017; 471 (Suppl1): S57.

- Association between stromal tumour lymphocyte infiltration in the needle core biopsies and paired post-neoadjuvant chemotherapy excisional resection specimens in triple negative breast cancer. 29th European Congress of Pathology, Amsterdam, The Netherlands (September 2017). Aliaa Shalaby, Sharon Glynn, Grace Callagy. Virchows Arch., 2017; 471 (Suppl1): S62.

- Stromal tumour lymphocyte infiltration as a prognostic marker in triple negative breast cancer. Breast Cancer Res Treat, 2018; 167:337.

Poster presentations

- Elevated cyclooxygenase (COX-2) expression correlates with low grade hormone-dependent breast cancer in Irish female. Postgraduate research day, NUIG, Galway, Republic of Ireland (May 2016). Aliaa Shalaby, Celine Inderhaug, Laura S. Murillo, Mark Webber, Helen Ingoldsby, Sharon Glynn, Grace Callagy.

- Triple negative breast carcinoma arising in Microglandular Adenosis (MGA). 28th European Congress of Pathology, Cologne, Germany, (September 2016). Aliaa Shalaby, Sine Phelan.

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- Stromal tumour lymphocyte infiltration as a prognostic tool in triple negative breast cancer. 29th European Congress of Pathology, Amsterdam, The Netherlands (September 2017). Aliaa Shalaby, Elaine Walsh, Sharon Glynn, Grace Callagy.

- Association between stromal tumour lymphocyte infiltration in the needle core biopsies and paired post-neoadjuvant chemotherapy excisional resection specimens in triple negative breast cancer. 29th European Congress of

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ABBREVIATIONS AND SYMBOLS

ACC	adenoid cystic carcinoma
ADH	atypical ductal hyperplasia
ALH	atypical lobular hyperplasia
AR	androgen receptor
ATM	ataxia-telangiectasia mutated
bACC	breast-derived adenoid cystic carcinoma
BC	breast cancer
Bcl-2	B cell lymphoma 2 protein
BCL-2	B cell lymphoma 2 gene
BCSC	breast cancer stem cells
BCSS	Breast cancer specific survival
BL1, BL2	basal-like 1, 2
BLIA	basal-like immune-activated
BLIS	basal-like immunosuppressed
BMI	body mass index
BRCA 1, 2	breast cancer gene 1, 2
BRIP1	BRCA1-interacting protein 1
CCC	columnar cell change
CCH	columnar cell hyperplasia
CDH1	cadherin-1
cILC	classical invasive lobular carcinoma
CK 5/6, 7, 8, 14, 18, 19	cytokeratin 5/6, 7, 8, 14, 18, 19
cLCIS	classical lobular carcinoma in situ
cNOS	constitutive NOSs
COX-2	cyclooxygenase-2
DC	dendritic cell
DCIS	ductal carcinoma in situ
DFS	disease-free survival
EGFR	epidermal growth factor
eNOS/NOS3	endothelial nitric oxide synthase
ER	oestrogen receptor
FEA	flat epithelial atypia

FFPE	formalin fixed paraffin embedded
GUH	Galway University Hospital
H & E	haematoxylin and eosin
HER-2	human epidermal growth factor receptor 2
HG	high grade
HIF1α	hypoxia-inducible factor 1α
HR	hazard ratio
IARC	International Agency for Research on Cancer
IDC	invasive ductal carcinoma
IDC-NST	invasive ductal carcinoma of no special type
IFNβ	interferon beta
IHC	immunohistochemistry
IL-2, 10	Interleukin-2, 10
ILC	invasive lobular carcinoma
IM	immunomodulatory
iNOS, NOS2	inducible nitric oxide synthase
IPC	invasive papillary carcinoma
LAR	luminal AR
LCIS	lobular carcinoma in situ
LG	low grade
LN	lobular neoplasia
M	mesenchymal
M1, 2	type 1, 2 macrophage
MFS	metastases free survival
MGA	microglandular adenosis
MMP/s	matrix metalloproteinase/s
MSL	mesenchymal stem-like
mTOR	mammalian target of rapamycin
MYC	myelocytomatosis gene
NACT	neoadjuvant chemotherapy
NCB	needle-core biopsies
NF-κB	nuclear factor kappa-light-chain-enhancer
nNOS, NOS1	neuronal nitric oxide synthase

NO	nitric oxide
NOS	nitric oxide synthase family
NPI	Nottingham Prognostic Index
NSAID	non-steroidal anti-inflammatory drug
NUIG	National University of Ireland, Galway
OS	overall survival
P53	protein 53
PALB2	partner and localizer of BRCA2
PARP	poly (ADP-ribose) polymerase
pCR	pathologic complete response
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PgR	progesterone receptor protein
PI3K	Phosphatidylinositol 3-kinase
pILC	pleomorphic invasive lobular carcinoma
pLCIS	pleomorphic lobular carcinoma in situ.
pN	nodal stage
PTEN	Phosphatase and tensin homologue
RCSI	Royal College of Surgeons of Ireland
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC FNAC	supraclavicular fine needle aspiration cytology
sTILs	stromal tumour infiltrative lymphocytes
STAT3	signal transducer and activator of transcription 3
SLN excision	sentinel lymph node excision
SLNB	sentinel lymph node excisional biopsy
TGF-β	transforming growth factor beta
THE	therapeutic resections
Th1	T helper 1 cells
TILs	tumour infiltrative lymphocytes
TMA	tissue microarray
TN	triple negative
TNBC	triple negative invasive breast cancer

VEGF	vascular endothelial growth factor
WHO	World Health Organization
WOI BC	west of Ireland breast cancer

COMPANY ABBREVIATIONS

Abcam/LabVision	Abcam plc, 330, Cambridge, CB4 OFL, UK.
BD Transduction Laboartories	BD Transduction Laboratories, BD Biosciences, The Danby Building, Edmund Halley Rd., Oxford Science Pk., OX4 4DQ Oxford, UK
BioGenex	BioGenex Life Sciences Pvt Ltd 49026 Milmont Drive Fremont, CA 94538, California,USA
Cayman	Cayman Chemical 1180 East Ellsworth Road Ann Arbor, Michigan 48108 USA
Dako	Dako Ltd., 16, Manor Courtyard, Hughenden Ave., High Wycombe, HP13 5RE, UK.
Leica	Leica Microsystems, Pamaron House, Ballybin Rd., Ashbourne, Ireland.
Neomarkers	Lab Vision Products, Thermo Fisher Scientific, 46360, Fremont Blvd., Fremont, California 94538, USA.
Novocastra	Novocastra, Leica Biosystems Newcastle Ltd., Balliol Business Park West, Benton Lane, Newcastle Upon Tyne NE12 8EW, UK Kingdom.

Roche	Roche Ireland Ltd., Clarecastle, Co. Clare, Ireland.
Santa Cruz	Santa Cruz Biotechnology, Inc. 2145, Delaware Ave., Santa Cruz, California 95060, USA.
Thermo Scientific	Thermo Scientific, Gerhard Menzel GmbH, Saarbrückener Str. 248, D-38116 Braunschweig, Deutschland.
Zymed/Fisher Scientific	Zymed Laboratories, Inc. 561 Eccles Avenue South San Francisco, California 94080, USA.

ABSTRACT

Breast cancer is a complex disease that encompasses multiple molecular subtypes that have different clinical behavior. The behavior can be influenced by both the characteristics of the cancer cells as well as the surrounding microenvironment. Triple negative breast cancer (TNBC), is a breast cancer subgroup with important clinical implications, as its treatment remains problematic despite the recent advances in cancer therapy. The pathological, molecular and clinical diversity within this subgroup has hindered standardization of a targeted therapy and prognostication of its outcome as an entire group. Therefore, the aim of this work is to review and characterize TNBCs diagnosed and managed in Galway University Hospital over a 15 year period with reference to outcome, clinicopathological features and candidate markers with a view to identify any determinants of outcome.

A database of 355 TNBC patients managed at Galway University Hospital was constructed with detailed clinicopathological and outcome data. After a follow up of 42 months, 22% of patients experienced a recurrence of disease and 21% died from their disease. Only the traditional parameters of nodal status and tumour size were independent predictors of disease free, metastases free and breast cancer specific survival. In patients treated with neoadjuvant chemotherapy, a complete pathological response (pCR) was an independent predictor of outcome. Tumour type, tumour grade, age at diagnosis, breast cancer gene (BRCA) mutation status, basal status, androgen receptor status and other markers were not independent predictors of any outcome endpoint. Only B cell lymphoma-2 (Bcl-2) showed a potential independent prognostic role for metastases free and breast cancer specific survival; TNBC tumours with high expression of Bcl-2 were associated with longer survival rates on univariate and multivariate analysis, but not with nodal status or tumour size were included in the model.

A detailed evaluation of stromal tumour infiltrating lymphocytes (sTILs) was performed. sTILs was an independent predictor of disease free survival (DFS) and overall survival (OS) on univariate and multivariate analysis. The strongest

association with outcome was observed when sTILs was tested in 10% increments and was dichotomized with a cut-off of 25%.

The expression of two inflammation associated enzymes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), was evaluated in a consecutive series of invasive breast cancers that included hormone receptor positive and negative tumours. Expression of COX-2 and iNOS was associated with well-differentiated tumours and with oestrogen and progesterone receptors positivity but not with outcome either in the entire series or in the hormone receptor positive or negative groups.

TNBC is a biologically diverse group of cancers with poor outcomes. Traditional parameters of nodal status, tumour size and pCR, where neoadjuvant chemotherapy (NACT) has been given, are independent determinants of outcome. This work highlights a potential independent prognostic role for sTILs in TNBCs. Further work is required to validate sTILs as a robust biomarker for prognostication of TNBC.

1. INTRODUCTION

1.1. Breast cancer incidence / epidemiology

The breast is the most frequent non-cutaneous site diagnosed with primary malignant neoplasm among females worldwide. Approximately 1.68 million breast cancer (BC) cases were diagnosed in 2012 globally. Of all new cancers diagnosed among females, one in every four women suffers from BC.[1] Similar to the global trend, the incidence rates of BC among European and American females are superior to any other cancer.[2] The highest frequency of BC among European countries was recorded in Belgium (148/100,000 women), while the lowest frequency was recorded in Bosnia. With identical incidence rates, Ireland and Germany rank the seventh highest in Europe (122/100,000 women).[3] Approximately 3000 Irish women are diagnosed with invasive breast carcinoma annually.[4] Despite the introduction of breast screening programs in many countries including Ireland, BC remains the second most frequent cause of cancer-related deaths in women worldwide and in Ireland, following lung cancer. In 2012, it was responsible for the death of 521,900 women worldwide and 690 Irish women.[1,4]

1.2. BC pathogenesis

BC is a complex and heterogeneous disease that retains various differences regarding its histological and molecular features. These variables can be used as predictors and/or prognostic indicators,[5] while a number of variables also reflect the cell of origin of the BC.[6] The mammary gland is made up of a mixture of epithelial and mesenchymal tissue with different proportions influenced by personal differences, age and hormonal factors. The mesenchymal component is formed from the interlobular stroma constituted from fibrovascular and fatty tissue, while the epithelial components form the main functional unit of the breast defined as the terminal ducto-lobular unit, lined by two layers of cells. The inner luminal layer comprises of ductal or lobular cells resting on a layer of basal/myoepithelial cells.[7]

The cell layer populations can be recognized by their location, immunophenotype, and global gene expression profile.[8, 9] The luminal cells and the luminal cancer tumours express the oestrogen receptor (ER) protein, along with low molecular

weight cytokeratins (CKs), CK7, CK8, CK18 and CK19, the progesterone receptor protein (PgR), and other ER-induced genes. The resting layer of basal/myoepithelial cells is named as such for displaying features of epithelial and smooth muscle cells simultaneously.[10] The myoepithelial cells have distinct transcriptomes,[11] and proteomes,[12] when compared with the luminal layer of cells, and express high molecular weight CKs including CK 5/6 and CK14, in addition to other markers such as alpha-smooth muscle actin which is related to the myoid apparatus.[8, 9] Although BCs that express basal CKs have been named as such, their actual cell-of-origin is still debatable. Some authors suggest that they arise from the basal/myoepithelial cells[13, 14] while others indicate that BC is driven by cells that exhibit stem cell properties, such as in BC stem cells (BCSC). These cells can occur in multiple states, both epithelial and mesenchymal. The epithelial-like state is associated with expression of epithelial markers, establishment of cell polarity, and extensive proliferation. The mesenchymal-like state is associated with expression of mesenchymal markers, a state of cell dormancy, and a capacity to be highly invasive.[15]

Significant differences between the BC cells are noted. Heterogeneity within populations of tumour cells can be explained using two models: the clonal evolution model and the hierarchical model. In the former model, arbitrary mutations produce subpopulations, allowing particular subpopulations to be more invasive.[16, 17] Figure 1.1. shows the hierarchical relationship between stem cells and BC types. The self-repeating BCSC, the mammary stem cell, gives rise to a common progenitor. This progenitor then follows a structure to each cell subtype, either luminal or basal. The quantity of the cell types in the mammary gland dictates the frequency of the BC subtypes.

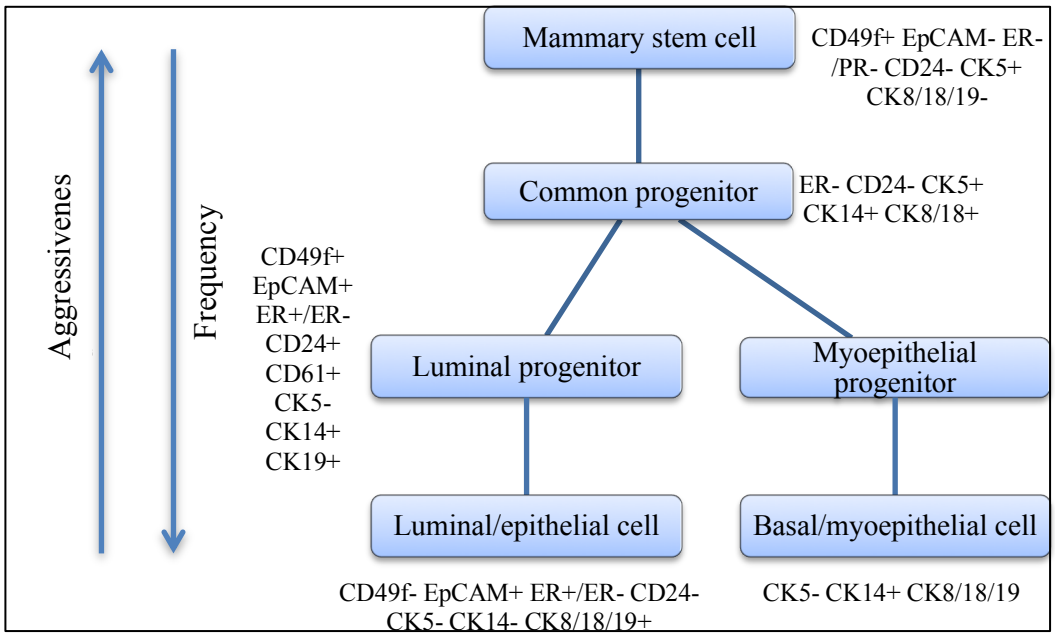
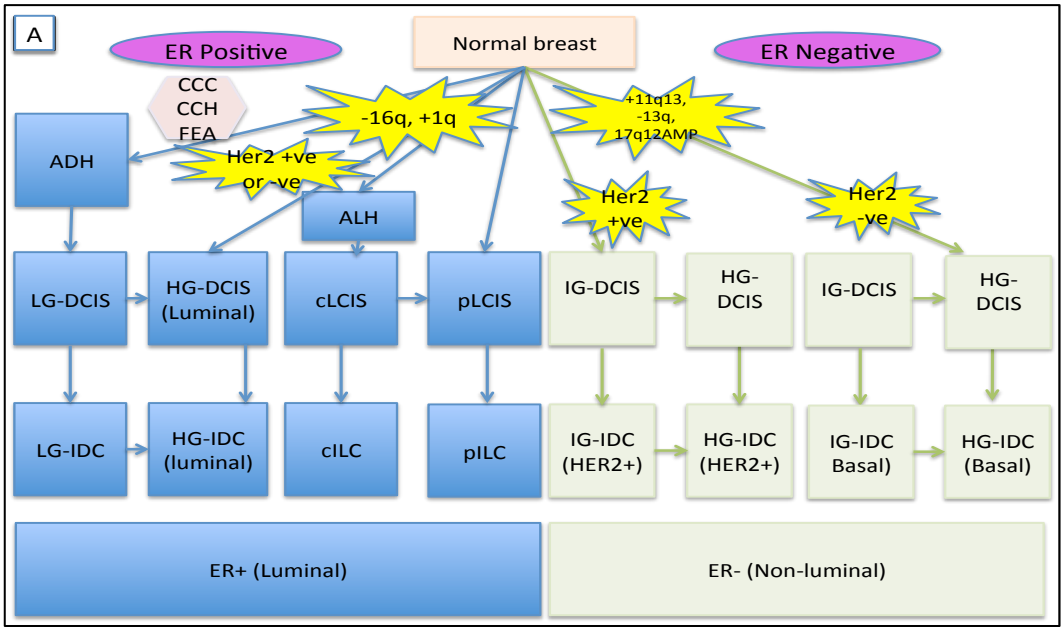


Figure 1.1. Hierarchical relationship between stem cells and BC types (adapted from Lim et al.[17])

Various hypotheses have been proposed with regard to the evolution of BC. Previously, the standard model of step-wise tumour initiation and progression due to sequential accumulation of genetic mutations was adopted in BC,[18] similar to that described in other types of cancer.[19] In the breast, this was represented by epithelial hyperplasia, atypical hyperplasia, in-situ carcinoma and invasive carcinoma in successive sequence.[18] There is a general consensus that the aggressiveness of the invasive BC corresponds to the grade of the pre-invasive lesion only, with differing carcinogenesis pathways experienced by low-grade and high grade tumours.[20-22] Some authors still debate that a proportion of lower-grade breast tumours can progress and de-differentiate into higher grade types.[23-25] Figure 1.2. illustrates the two above hypotheses.



B

		Precursor lesions	In situ carcinoma	Invasive BC histologic type	Invasive BC molecular subtype
Normal tissue	Oestrogen stimulation 16q loss 1q gain CDH1 loss	ADH FEA ALH	LG DCIS LN	Ductal, grade 1 Tubular Mucinous Lobular	Luminal A
	➔			➔	
	HER-2 gene amplification 11q13 amplification		Pleomorphic LCIS IG DCIS	Pleomorphic lobular Apocrine Ductal, grade 2	Luminal B
	➔			➔	
	17q12 amplification BRCA 1 dysfunction PTEN mutations P13K, p53		HG DCIS	Ductal, grade 3 Medullary Metaplastic	HER-2 enriched Basal-like Claudin-low

Figure 1.2. Different hypothesis for BC development (A adapted from Bombonati[25], B adapted from Masuda[22]) BC: breast cancer; CDH1: cadherin-1; ADH: atypical ductal hyperplasia: FEA: flat epithelial atypia; ALH: atypical lobular hyperplasia; DCIS: ductal carcinoma in situ (LG- low grade, HG- high grade); LN: lobular neoplasia; HER-2: human epidermal growth factor receptor 2; LCIS: lobular carcinoma in situ; PTEN: phosphatase and tensin homolog; CCC: columnar cell change; CCH: columnar cell hyperplasia; cILC: classical invasive lobular carcinoma; cLCIS: classical lobular carcinoma in situ; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; pILC: pleomorphic invasive lobular carcinoma; pLCIS: pleomorphic lobular carcinoma in situ

1.3. Aetiology and risk factors

1.3.1. Genetic factors

BC, similar to other tumours, is a polygenic disorder caused by an accumulation of genetic alterations (mutations) with subsequent activation of oncogenes, incapacitation of tumour suppressor genes and/or deregulation of DNA repair genes or cell-cycle genes.[26, 27] These genetic mutations can occur at any level and can be either inherited or acquired (sporadic) after exposure to causative factors.[26] Inherited BC with germline mutation is suspected to be responsible for up to a quarter of BC cases,[28] although the inherited susceptibility genes breast cancer 1 (BRCA1) and 2 (BRCA2) are identified in 10% of BC cases.[29]

BRCA1 and BRCA2

BRCA1 and BRCA2 account for 80% of highly invasive hereditary BC.[30] Families with mutations in both the BRCA1 and BRCA2 genes have an increased risk of BC of 85% over their lifetime.[30, 31] According to the National Comprehensive Cancer Network, to merit further examination, particular features must be present in the familial history: early-age-onset (<50 years) including both invasive or insitu BC; two breast primaries or breast and ovarian/fallopian tube/primary peritoneal cancer in a single individual, or two or more breast primaries or breast and ovarian/fallopian tube/primary peritoneal cancers in close (first-, second-, and third-degree) relative(s).[32]

BRCA1-type tumours often display poor differentiation histologically and frequently exhibit the features of the medullary subtype.[33] Moreover, there is an increased probability of BRCA1 type tumours being ER-negative, PgR-negative, and a decreased probability that they display human epidermal growth factor receptor 2 (HER-2)/neu overexpression. Therefore, they are categorized as triple-negative (TN).[34] Moreover, studies indicate that BRCA1 BCs are more likely to originate from the basal epithelial layer of the mammary gland and thus usually stain positive for CK5/6.[35, 36] In patients with versus patients without a BRCA1 mutation, a number of similarities exist with regard to morphology, immunophenotype and molecular makeup.[33] Contrastingly, there is a dearth of published data with regard to BRCA2 tumours; however they do not seem to

display a distinctive histopathology and may appear as sporadic BC. BRCA2 mutated BCs are often associated with ER-positive tumours.[37, 38]

BRCA1 is located on the long arm of Chromosome 17 at 17q21.[39, 40] BRCA1 is involved in a number of cellular pathways, including cell cycle regulatory checking gene transcription regulation and DNA damage repair.[39, 40] Studies indicated that BRCA1 and p53 together maintain genomic integrity. The loss of this function during BRCA1 dysfunction or p53 mutation, allows other abnormalities to manifest as cancer growth. The dysfunction of BRCA1 results in missing or non-functional proteins, which translates into defects in DNA repair, dysregulation of the cell cycle checkpoints and chromosome damage.[41, 42]

BRCA2 is located on the long arm of the Chromosome 13 at 13q12.3 and contains 27 coding exons. As mentioned, BRCA2 does not seem to display a distinctive histopathology, however does share a number of functional similarities to BRCA1. Similar to BRCA1, BRCA2 maintains genomic integrity.[43] BRCA2 appears to be involved in the DNA repair process; therefore cells lacking BRCA2 are unable to repair the DNA breaks.[42] Previous studies show that lobular carcinoma, tubular carcinoma, tubulolobular carcinomas and pleomorphic lobular carcinomas are associated with BRCA2.[44-46] However, larger cohort studies have not supported this finding.[47, 48]

Other Genes

Other genes have also been proven to be associated with inherited BC such as p53, phosphatase and tensin homolog (PTEN), ataxia-telangiectasia mutated (ATM), BRCA1-interacting protein 1 (BRIP1), partner and localizer of BRCA2 (PALB2) and many others. These genes show different penetrance levels.[49, 50] The presence of inheritable faulty genes of those stated above is typically associated with a strong family history of BC or vice-versa. The relative risk of BC is almost doubled in women who have a first-degree relative affected with BC, and tripled if the relative was diagnosed prior to menopause with unilateral disease. The relative risk of BC increases nine-fold if the relative was diagnosed premenopausal with bilateral BC.[51]

Many other genes are involved in tumourigenesis of sporadic BC, in addition to

those involved with inherited BC. For instance, the amplification of genes such as HER-2, myelocytomatosis (MYC), and cyclin D1 have been well documented.[26] The p53 gene is a type of tumour suppressor protein, and is located on the short arm of chromosome 17.[52] As a tumour suppressor, it is involved in cell cycle regulation. Additionally, it provokes apoptosis or autophagy when unrepairable cell damage is existent.[26, 53] Inherited mutated p53 leads to a rare cancer predisposition syndrome/disorder associated with the development of several types of neoplasms and named 'Li-Fraumeni syndrome'.[54] Nearly one in every two affected women with this syndrome will develop BC by the age of 60.[52] p53 mutations are more common in high-grade, large-size, node-positive cases and in both ER-negative and PgR-negative tumours,[55] and HER-2-positive tumours.[52] p53 mutations are low in well-differentiated and hormone-receptor-positive luminal subtype A tumours.[56]

1.3.2. Multifactorial causes

Previous studies have proven that various risk factors are associated with increased incidence for development of BC, such as advanced age, excessive or prolonged exposure to oestrogens, reduced parity and exposure to ionising radiation.[5, 57, 58] Behavioural and lifestyle factors exemplified in diet, body mass index (BMI), physical inactivity, alcohol intake and smoking have also been suggested to be modifiable risk factors for BC.[59, 60] Postmenopausal diabetic women with a higher body weight are at an increased BC risk.[60] After menopause, oestrogen biosynthesis occurs via enzymatic conversion of androstenedione, and occurs mainly in adipose tissue. Dietary-induced chronic inflammation in breast adipose tissue in obese women associated with the elevated aromatase activity and greater production of oestrogen, increase the BC risk.[61, 62]

Other reports have also indicated that the presence of certain benign epithelial proliferative and non-proliferative lesions within the breast is an additional predisposing factor for developing BC with variable degrees of risk.[58, 63] Previous studies demonstrated that women with a proliferative benign condition, including usual epithelial hyperplasia and radial scar, have a 1.5-1.9 fold increased risk of BC, while women with atypical hyperplasia (lobular or ductal) have a relative risk of 4-6 fold increase of developing BC.[63-66] Interestingly, several studies

documented that microglandular adenosis (MGA), a rare benign glandular lesion, is associated with invasive carcinoma, specifically of the TN phenotype.[66-68]

1.4. Inflammation and cancer (tumour microenvironment)

Tumour development and subsequent progression depends on both the genes that control cell proliferation, and non-tumour cells surrounding the tumour called the tumour microenvironment.[69, 70] Inflammation occurs in response to a site of infection or injury. At this site inflammatory mediators are released such as growth factors, cytokines, reactive nitrogen species (RNS), and reactive oxygen species (ROS).[71, 72] Numerous studies have noted a correlation between specific cancers and chronic inflammation, resulting from infectious agents or inflammatory conditions. This observation was first hypothesised by Rudolf Virchow in the mid-19th century,[73, 74] but only recently researchers provided a scientific basis to this theory.[74] An inflammatory process has been observed as a causative factor in up to 25% of cancers worldwide.[75] This has been proven in colon cancer caused by long-term ulcerative colitis,[76] and hepatocellular carcinoma caused by viral hepatitis.[77] Contrariwise, generation of tumour cell derived antigens aids in stimulation of the natural killer (NK) cells and the cytotoxic T-cells to attack the tumour cells.[78, 79, 71] Currently, there is an agreement that the inflammatory microenvironment can play pro-tumourigenic and anti-tumourigenic roles.

1.4.1. Pro-tumourigenic effect of inflammation

Inflammation and cancer are linked by two pathways (Figure 1.3.): an extrinsic pathway driven by inflammatory conditions, and an intrinsic pathway driven by oncogenes.[74]

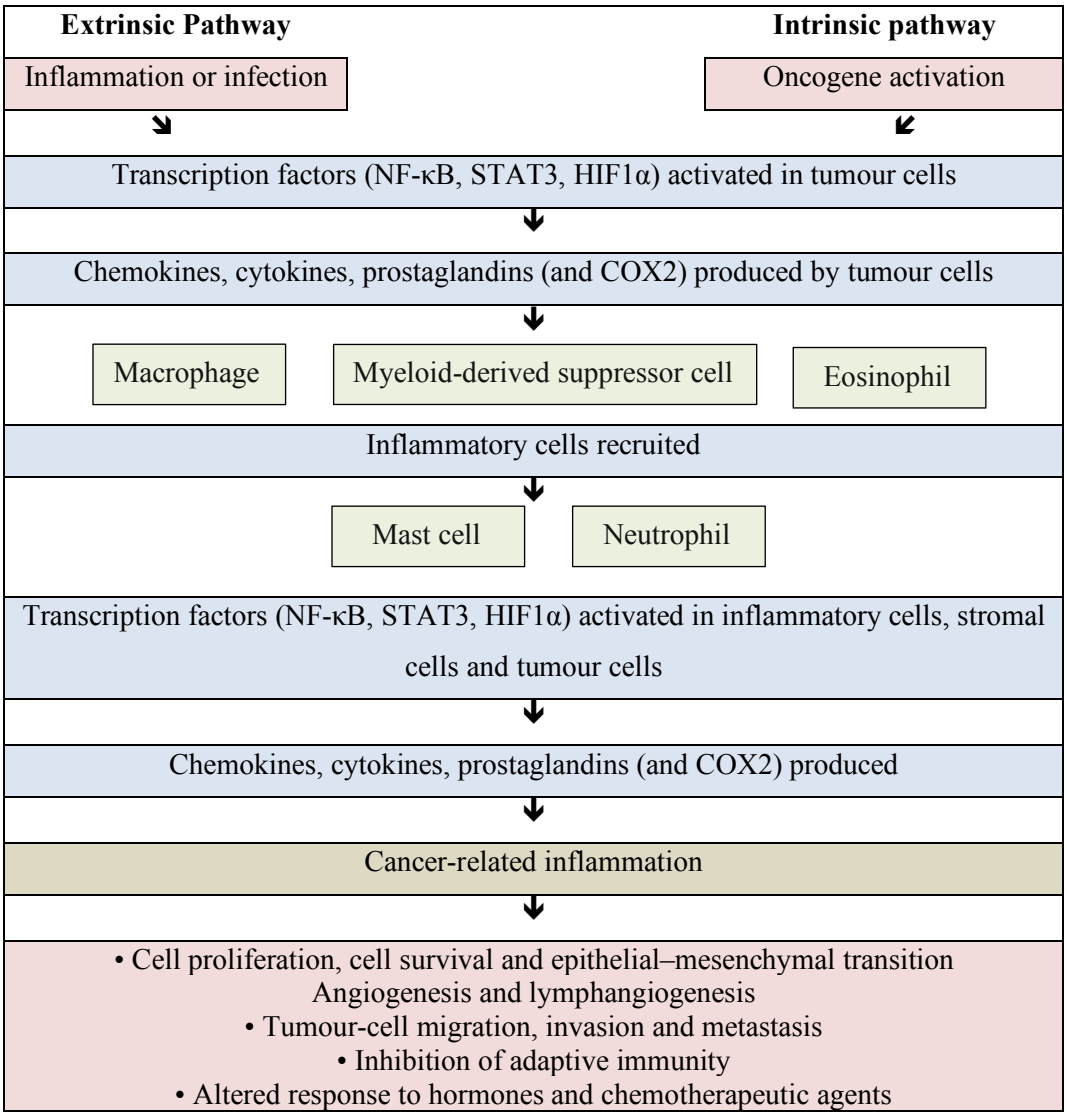


Figure 1.3. Pathways that connect inflammation and cancer (adapted from Mantovani et al.[74])

Both the extrinsic and intrinsic pathways result in transcription factor activation i.e., nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α) in tumour cells. These factors regulate pro-inflammatory factors in tumour cells, such as prostaglandins, chemokines, and cytokines. Pro-inflammatory factors also activate transcription factors (NF-κB, STAT3, HIF1α) in cells of the tumour microenvironment (stromal cells, leucocytes and tumour cells) that will result in increased pro-inflammatory signalling. In BC, the NF-κB pathway has been shown to promote the invasion of BC cells by regulating the expression of matrix metalloproteinases.[80] Cross-talk between the ER and NF-κB, which is regulated by estradiol and the inflammatory

cytokine TNF-α, stimulates BC cells to become aggressive, and ER-positive but antioestrogen-resistant, with activation of NF-κB and a susceptibility for distant metastasis.[81] NF-κB regulates immune cell function and promotes inflammation, expressing cytokines, chemokines, and their receptors, and inhibits cell death by stimulating the transcription of antiapoptotic genes.[82] NF-κB may also cause cell cycle arrest. In normal epidermal cells, NF-κB and oncogenic Ras trigger cell cycle arrest, whereas inhibition of NF- κB results in malignant alterations in normal cells.[83]

Within the tumour microenvironment different types of inflammatory and immune cells are present including neutrophils, macrophages and T cells.[71, 84, 85] Neutrophils show pro-tumoural activity by releasing ROS and RNS, induce genetic instability, and promote angiogenesis.[71, 84] There are two types of macrophages that differentiate from monocytes, through activation by different stimuli.[85] Type 2 macrophages (M2) contribute to tumour progression and growth by releasing chemokines, cytokines, growth and transcriptional factors such as Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and interferon beta.[71] VEGFs, interleukin-8, and cyclooxygenase-2 (COX-2), among others, promote angiogenesis and hence support the tumour vascularity.[86]

Different functional and phenotypical types of T-cells are detailed in the literature. Among these, T regulatory cells ($CD4^+$ $CD25^+$ T_{reg} cells) which promote immunosuppression through effector elements such as Interleukin-10 (IL-10), transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2) and others promote immunosuppression via T_{reg} cells.[87, 88]

Chronic inflammation can occur in adipose tissue in the breast, with augmented production of cytokine by associated macrophages.[89, 90] Chronic inflammatory conditions can be subtle in the breast but commonly associated with obesity and diabetes. Also systemic inflammatory conditions can influence the microenvironment within the breast by increasing cytokine production.[91]

1.4.2. Anti-tumourigenic effect of inflammation

Conversely, inflammation may also be antitumourigenic. Previous studies have shown that nod-like receptor inflammasomes aid resistance to colon and colorectal cancer development.[92, 93] APR-associated neutrophilia may also be antitumourigenic and anti-metastatic, where neutrophils generate tumour cell derived antigens, influence dendritic cell (DC) maturation, and secrete alarmins, cytokines, and chemokines.[78, 79] Type 1 macrophages (M1) assist in the war against the tumour mainly through production of IL-12 which in turn stimulates the NK cells and the cytotoxic T-cells.[71]

Tumour-promoting inflammation and antitumour immunity can exist concomitantly during tumour progression.[94, 95] Endoplasmic reticulum stress-induced inflammation influences tumour-promoting cytokine production.[94, 95] However, other processes activated by endoplasmic reticulum stress may have a greater influence on antitumour immunity. ROS and endoplasmic reticulum stress together can reinforce antitumour immunity via immunogenic apoptosis induction in the cancer cells. However apoptosis may become inflammatory if there is a delayed clearance of apoptotic cells.[96-98] The type of inflammation produced i.e. cancer-provoking or cancer-preventing is dependent upon the target cell type, the stage of the disease and the type of endoplasmic reticulum stressor.

1.4.3. Tumour-infiltrating lymphocytes

The tumour microenvironment consists of both pro-tumourigenic and immunosuppressive abilities, which provide tumour cell survival, proliferation, and a simultaneous hostile environment for immune cells. Antitumour activity is encouraged via cytotoxic T cells (such as CD8+ T cells), $CD4^+$ T helper 1 (Th1) cells, NK cells, M1 macrophages, and dendritic cells while M2 macrophages and T_{reg} cells ($CD4^+$ forkhead box P3 ($FOXP3^+$)), $CD4^+$ Th2 cells and myeloid-derived suppressor cells suppress antitumour activity.[99, 100] Therapies that augment immune cell activity strengthen antitumour reaction or prevent inhibitory activity. The effect these therapies have via immune cell penetration has a prognostic indicator,[101] such as measuring various types of tumour infiltrating lymphocytes (TILs).[101, 102] TILs are present either within the stroma (stromal TILs: sTILs) or

within the tumour cell islets (intratumoural TILs: iTILs).[103] It is important to note that TILs include all mononuclear inflammatory cells and are not exclusively restricted to lymphocytes. The major components (75%) of TILs are T lymphocytes, either CD4+ or CD8+ or NK cells, with smaller percentages of B lymphocytes and monocytes.[104-106] In general, compared to normal breast tissue, DCIS and invasive BC show higher levels of lymphocytes. This was highlighted in a study by DeNardo et al. using CD45 staining.[88] Recent studies suggest that the different molecular subtypes of BC have different quantities and types of TILs.[107-109] Moreover, the percentage of TILs has been proposed to reflect an association with the outcome for BC patients. This prognostic importance has been reported for patients with triple negative BC (TNBC)[110] and HER-2-positive tumours.[111] Moreover, a predictive role for neoadjuvant chemotherapy (NACT) has been linked to the existence of TILs in preoperative BC biopsies. This has been validated by Denkert et al. in their study of 1,058 specimens.[112]

T-Lymphocytes

In the thymus, T cells mature and differentiate to express either CD4 or CD8 glycoprotein on their surface, and are hence named CD8+ and CD4+ T cells. CD4+ T cells comprise different subsets with opposing functions and cytokine profiles: including Th1, Th2, T_{reg} and others. Naïve CD4+ cells differentiate into Th1 upon immunogenic stimulation by IL-12 and IFN-γ[113, 114] which in turn augment the anti-tumourigenic environment by activation of M1 macrophages, NK and CD8+ cells.[71] On the contrary, Th2 cells which emerge under the effect of IL-4 on the naïve CD4+ cell,[113, 114] subdue the cytotoxic CD8+ cells through IL-4, IL-10 and IL-13 hence diminishing the antitumour response.[88] Furthermore, T_{reg} are also generated from the immature CD4+ cells when stimulated by TGF-β and IL-2.[113] In addition to expression of CD4 on the T_{reg} cells surface, they also express CD25, CTLA4 and FOXP3. They attain immunosuppressive characteristics through the existence of the transcription factor FOXP3, and secretion of various substances including IL-10, IL-35 and TGF-β. Of interest, direct cellular interaction through ligation of CTLA4 molecule on the surface of $T_{reg,}$ CD86 and CD80 on the dendritic cells (DC) leads to cessation of the maturation of the DC.[115, 116, 117] The diverse activities of various types of T-

lymphocytes are illustrated in Figure 1.4. Studies show that a build-up of TILs, particularly T_{reg} cells, is associated with progressive tumour growth and a subsequent poor prognosis,[118] while those characterized by cytotoxic T cells can improve prognosis.[100]

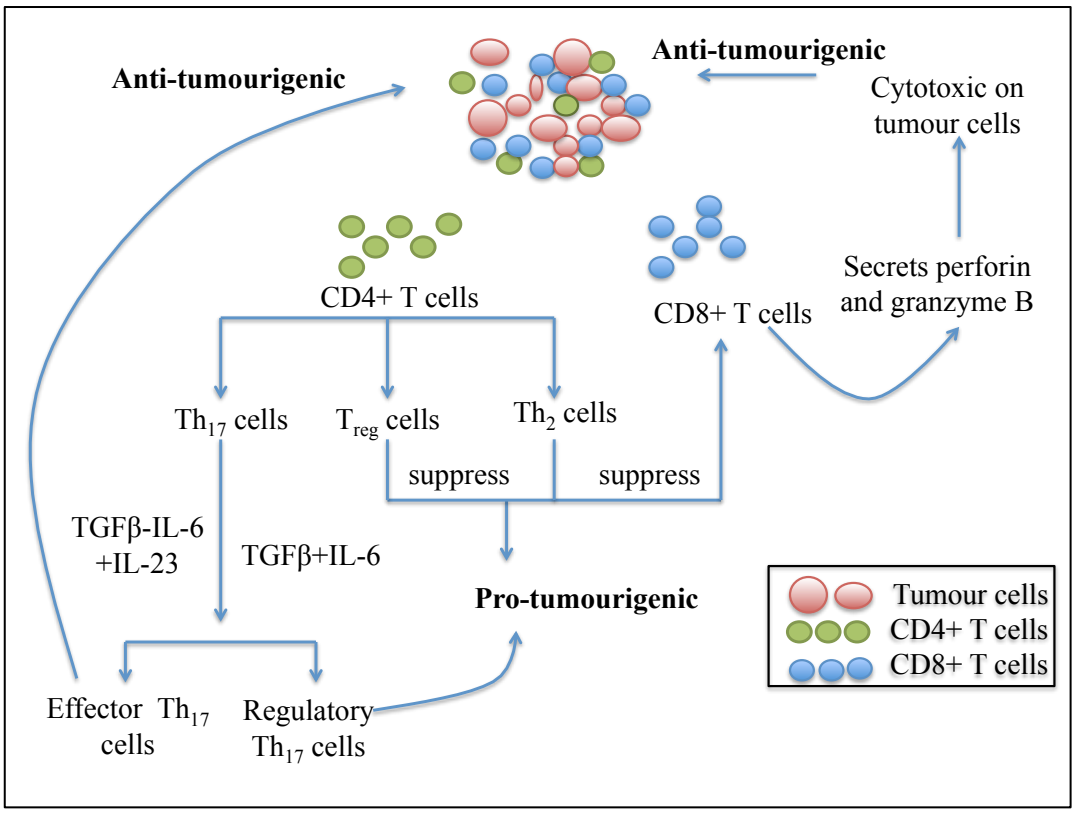


Figure 1.4. Pro- and anti-tumourigenic roles in T-cell lymphocytes (adapted from Sharma et al.[100])

B-Lymphocytes

The role of B-lymphocytes is still debatable as well as changeable during the course of the disease, with both anti-tumour and pro-tumour roles reported. Regarding their tumour-suppressive action, plasma cells (differentiated B-lymphocytes) fight the cancer cells through secretion of antigen-specific immunoglobulins, with subsequent immune cell activation for dendritic cells (DCs) and CD8+ T cells. Paradoxically, their tumour-promoting action emerges through secretion of IL-10 and expression of activated IgA on the surface of B-cells under the effect of certain stimulants such as TGF-β (Figure 1.5).[119]

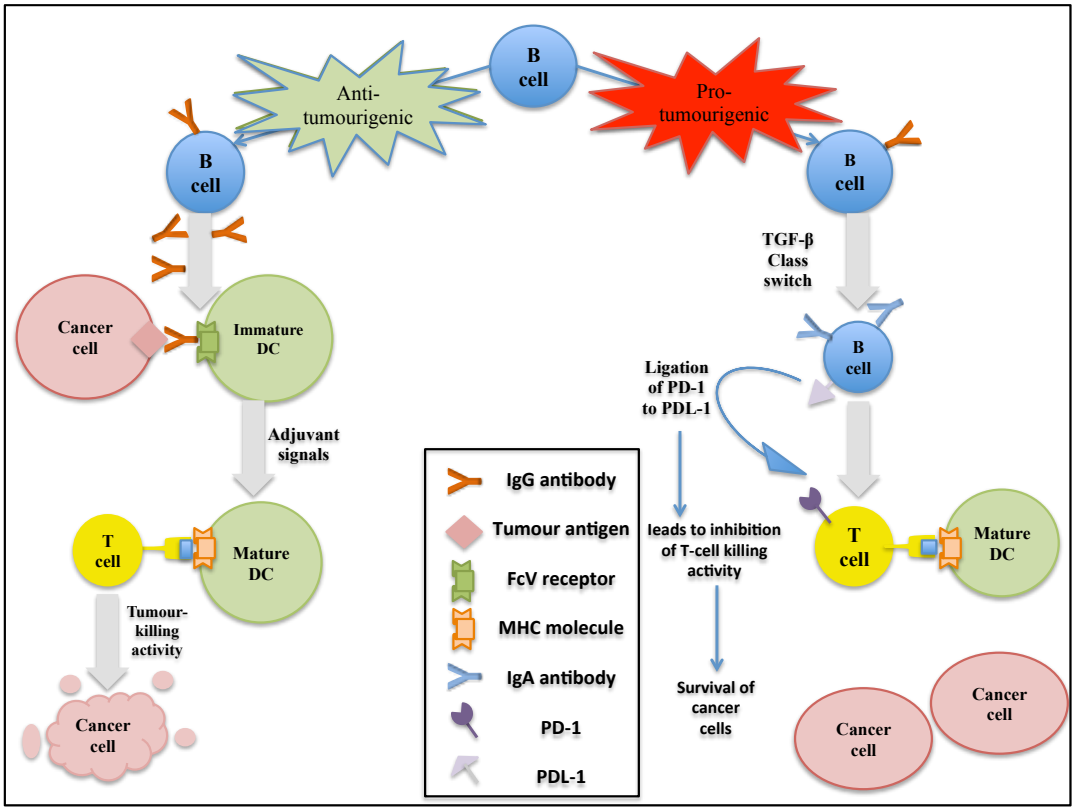


Figure 1.5. Pro- and anti-tumourigenic roles in B-cell lymphocytes (adapted from Zitvogel and Kroemer[119])

Few reports have evaluated the tumour infiltrating B-lymphocytes in BC. They have been reported to be present in 24%[120, 121] up to 55% of BCs.[122] Some authors have noted that B-lymphocytes can infiltrate the tumour and display a nodular aggregate configuration surrounding the vessels[123] while others have noted a diffuse pattern of infiltration.[122] In response to the recognition of tumour autoantigens, antagonist autoantibodies produced by mature B-cells (plasma cells) can be identified in the serum of less than half of cancer patients.[124] Antibodies directed against HER-2 have been detected in 20%[125] and 40% of the HER-2-positive patients.[126] Promising research has been done on animal models, to support the anti-tumourigenic role of the autoantibodies produced by the B-cells. Immunization of experimental mice with HER-2 antigen preparation prevented the development[127] and halted the progression of established HER-2-positive BC.[128] A recent study assessed CD19+ B-lymphocytes and investigated their relationship to PDL1 within 134 cases of BC and 31 cases of fibroadenomas by immunohistochemistry (IHC), immunofluorescence and flow cytometric analysis.

A higher proportion of BC expressed PDL1 paired with infiltration by CD19+ B-lymphocytes compared with the fibroadenomas. Hence the authors proposed a link between PDL1, CD19+ B lymphocytes and immune system escape leading to BC.[129] Taken together, this evidence suggests a link between BC and the immune response elicited by B-lymphocytes. However, there are still missing chains within this link that warrant more exploratory studies.

There are other factors to consider, especially in the context of developing different cell-targeted treatments for BC. The microenvironment also consists of type-2 tumour-associated macrophages (M2), myeloid-derived suppressor cells, and T_{regs}, all of which support a pro-tumourigenic environment consistent with growth.[71, 87, 88] Therefore therapies must be specifically targeted to combine both anti-tumour mediators that ensure TIL infiltration and activate specific cell cytotoxicity.

The immune suppressive properties of M2 are used to infiltrate a tumour where they support metastasis by remodelling and forming blood vessels.[71, 130, 131] During tumour growth, various types of cells are used to trigger immune system activation such as M1. However, depending on microenvironmental factors, macrophages can be reprogrammed to differentiate into different types of tumour associated macrophages, effectively circumventing the immune system.[71]

1.4.4. COX-2

Cyclooxygenase enzymes

There are three types of COX enzyme: COX-1, COX-2 and COX-3.[132-136] However, COX-3 is merely a spliced variant of COX-1 and lacks any enzymatic activity.[135] Therefore the COX family is mainly classified into two groups: constitutive COX (COX-1) and inducible COX (COX-2). Ultrastructural localization of the COX enzymes indicates their presence on the endoplasmic reticulum membrane as well as on the nuclear envelope membranes.[137] While the COX-1 is expressed constitutively in almost all normal tissues at a constant and low concentration, COX-2 is not normally expressed in tissues and is tightly regulated. COX-2 is a highly inducible enzyme. Pro-inflammatory cells, cytokines, growth factors, bacterial endotoxins and tumour promoters induce

transient expression of COX-2.[134, 136] Also, it had been reported that COX-2 production can be stimulated through specific genetic mutations in Wnt, Ras and HER-2/neu.[138, 139]

COX-1 and COX-2 act on the arachidonic acid that is liberated from the membrane phospholipids upon their stimulation by phospholipase A2. They produce different types of prostanoids as an end-product of a multi-stepped reaction.[134, 140] COX-1 is predominantly involved in physiological reactions and has a substantial role in protection of gastric mucosa and maintaining homeostasis via prostaglandin levels.[141] On the other hand, the up-regulation of COX-2 directly influences tumourigenesis.[72, 132]

Carcinogenic role of COX-2

The tumourigenic effect of COX-2 is complex and multifaceted. Studies showed that COX-2 upregulation, as well as other inflammation-related genes, lead to activation of a survival pathway regulated by NF-κB transcription factor. The inappropriate activation of NF-κB subsequently results in alterations in many genes. Of importance, the dysregulated NF-κB has a positive regulatory effect on anti-apopototic genes such as BCL-2 and a negative regulatory effect on apoptosis-inducing genes such as p53.[72, 142] Therefore, COX-2 inhibits programmed cell death through several pathways. Additionally, COX-2 activates macrophages to secrete several cytokines that help in the development of cancer, including BC, as shown in Figure 1.6.[143] Of emphasis, the COX-2 induced PGE2 triggers the expression of vascular endothelial growth factor (VEGF) and beta fibroblast growth factor (bFGF) which turn on neovascularization needed for the tumour growth.[72, 144] Moreover, some studies described that COX-2 can induce increased cell proliferation through activation of either the EGFR/AKT pathway or the Src kinase/STAT3/cyclin D1.[145] It was proposed that there is an actual cause effect relationship between COX-2 and metastases. Yin and Toker showed that induction of COX-2 promoted cell migration in BC cells in vitro.[146] Selective blockage of COX-2 was associated with suppressed metastases in colorectal cancer cells, both in vitro[147] and in vivo on experimental rodent models.[148] There is evidence that the decreased production of matrix metalloproteinase enzymes and/or upregulation of E-cadherin were the mechanisms by which the COX-2

expressing cells acquire their invasive capabilities.[149, 150]

Its precise role in promoting the development and progression of neoplasia was best demonstrated in the gastrointestinal tract. COX-2 expression is increased in the carcinomatous as well as the adenomatous colorectal lesions.[72, 151] Similarly, COX-2 predisposes different organs including the mammary gland to carcinogenesis. [72, 143]

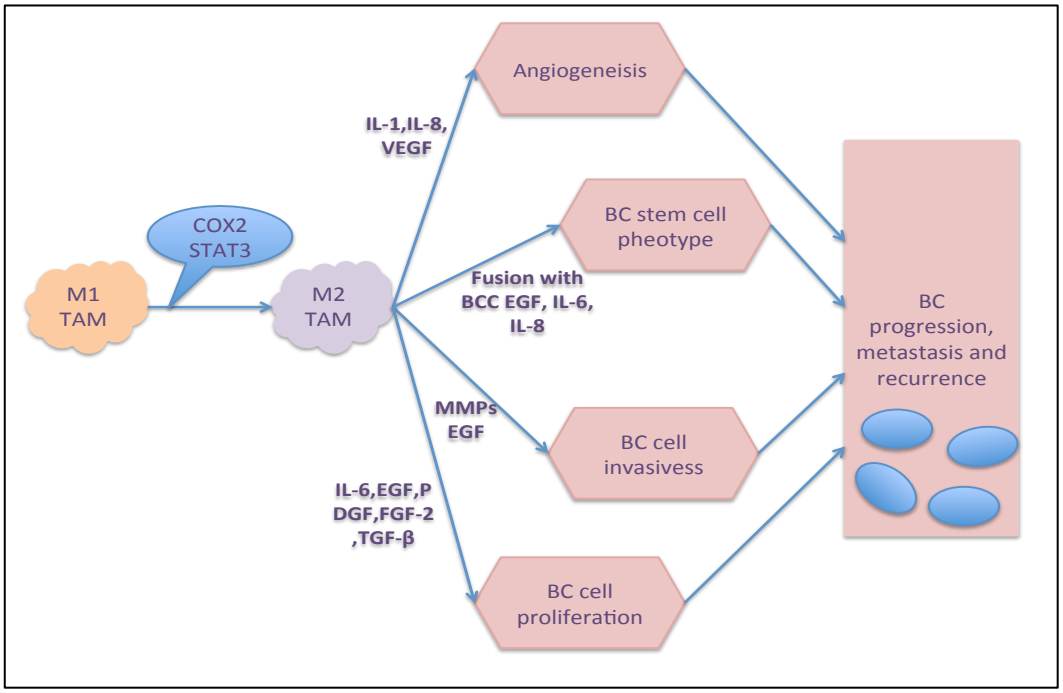


Figure 1.6. COX-2 and BC pathogenesis (adapted from Jiang[143])

COX-2 and BC

Liu et al. studied transgenic mice that overexpressed the human COX-2 gene in the mammary glands. The authors demonstrated that enhanced COX-2 expression induced mammary gland tumourigenesis via decreased apoptosis of mammary epithelial cells and enhanced synthesis of PGE2, prostaglandin-D and prostaglandin-F.[152] This was also demonstrated by Rozic et al.[153] and Mayoral et al.[154] Moreover, a close link was observed, in ER-negative and HER-2-positive breast tumours, between COX-2 expression and AKT phosphorylation with subsequent suppression of apoptosis and induction of tumour vasculature.

There is inconsistency in data found in previous studies with regard to the prevalence of the expression of COX-2 in BC.[155-157] Holmes et al. reported that

positive expression of COX-2 was detected in 28% of the studied 2001 BC samples.[157] Conversely, Nassar et al. found that 95% of the breast carcinomas showed COX-2 expression.[156] In in vitro studies, a gradual increase in COX-2 protein quantity was observed, using western blot, with progression from benign breast cells (MCF-10F) to insitu and then to invasive ER-positive BC cells (MCF-7).[158]

Studies are contradictory regarding the relationship of COX-2 and hormone receptor expression in BC. Holmes et al. and Mirson et al. perceived that the positivity of BCs for COX-2 was parallel to positivity for ER and PR.[157, 159] However, others have described an association between COX-2 expression and ER negativity.[160, 161] Additional studies found no correlation between COX-2 expression and hormone receptor status.[156, 162, 163] Of interest, an increased mRNA expression in hormone receptor positive BC and BC with positive progesterone receptors has also been established.[164, 165] It has been described that there is a significant correlation between COX-2 and aromatase expression, responsible for the synthesis of oestrogen.[166-168] Generally, the aromatase enzyme is found in many tissues including adipose tissue, particularly in postmenopausal women. Stimulation of aromatase enzyme occurs under the effect of different motivators including PGE2 that is produced from COX-2. Active aromatase enzyme is responsible for the following aromatization of androgens into oestrogens.[169] COX-2/aromatase/oestrogen positive BC connection is illustrated in Figure 1.7.

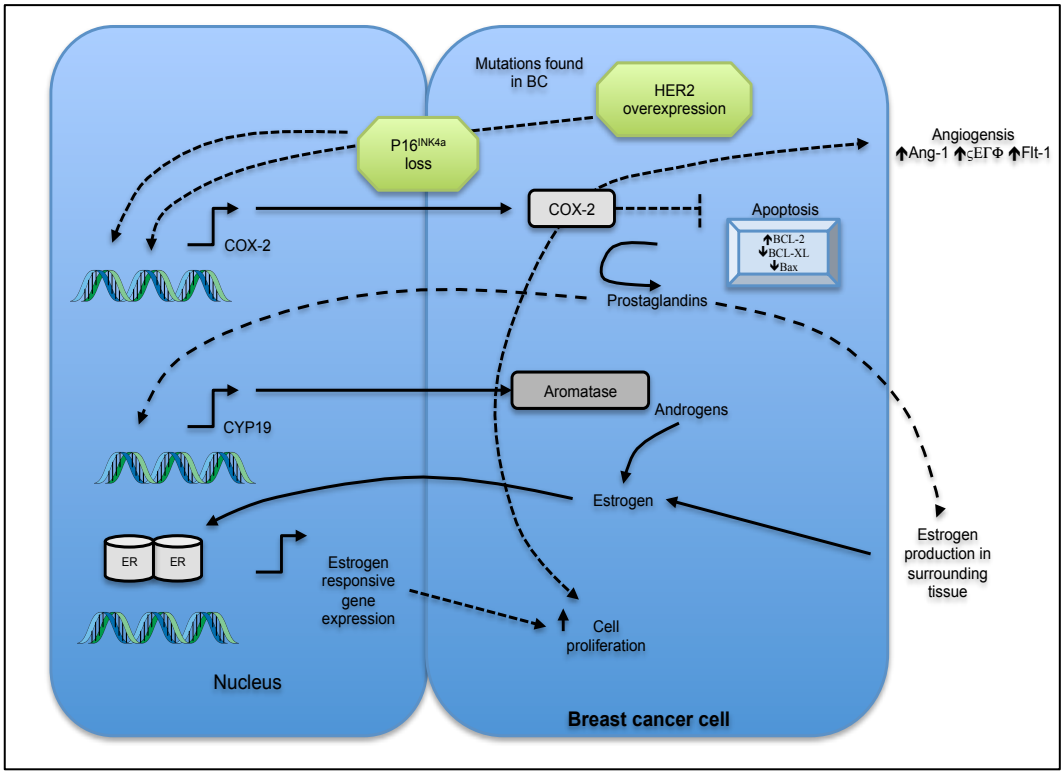


Figure 1.7. COX-2/aromatase/oestrogen relationship (adapted from Falandry et al.[170])

Blocking or hindering aromatase expression in breast tumours can therefore accomplish suppression of oestrogen synthesis. Aromatase synthesis also occurs in normal breast tissue. O'Neill et al. provided evidence of a significant relationship between breast adipose tissue and BC.[171] While previous studies have suggested that aromatase expression in breast tumours showed an increased response to aromatase inhibitors,[172, 173] they have yet to be proven in the prevention of BC, and additional studies are necessary to establish reproducible evidence. Selective ER modulators have been developed which selectively target the ER, blocking production in the breast, and hence blocking COX-2 expression. Due to their relationship, COX-2 inhibitors therefore reduce aromatase expression with subsequent prevention of development of oestrogen dependant BC.[174]

COX-2 inhibitors are a type of non-steroidal anti-inflammatory drug (NSAID). There is a direct relationship between their use and decreased incidence of cancer, including breast,[175, 176] which also supports the tumourigenic role of the COX-2. In contrast, the evidence suggesting a link between COX-2 inhibitors and their effect

on aromatase expression may lead to promising results with regard to BC development.[177] Masferrer et al. evaluated the anti-angiogenic activity of celecoxib, a type of COX-2 inhibitor, in the rat corneal model of angiogenesis, and found that it was a very potent inhibitor of angiogenesis. However they also noted that this potency was very much dose dependent.[178] Forced COX-2 expression subsequently treated with a highly selective COX-2 inhibitor (SC-58125) decreased the growth of human colonic cancer xenografts.[179] Prevention of BC in women using COX-2 inhibitors is therefore dependent on a combination of apoptosis promotion, down-regulation of COX-2 to reduce tumourigenisis, and inhibition of oestrogen synthesis.

1.4.5 Nitric oxide synthease 2 (NOS2/iNOS)

Three isoenzymes make up the nitric oxide synthase (NOS) family: neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial (eNOS, NOS3). These isoenzymes catalyse the hydroxylation and oxidation of L-Arginine to produce citrulline and nitric oxide (NO).[180] The NOS family are classified into two groups: constitutive NOSs (cNOS) and inducible NOS (iNOS). Unlike the cNOS, iNOS activity is not dependent on intracellular calcium concentration but on the presence of certain inflammatory cytokines and bacterial wall constituents.[181]

NO regulates blood flow, iron homeostasis and neurotransmission at lower concentrations, while it has a cytotoxic effect at high concentrations.[182] Its cytotoxic effects can target bacteria, viruses[183] and tumour cells.[184, 185] Reactive nitrogen species of NO can also result in gene mutation, loss of protein function, necrosis and apoptosis.[186] Depending on the concentration of NO, proliferation of cells may be stimulated or inhibited. NO has been shown to inhibit tumour growth at higher concentrations, while at lower concentrations it can stimulate cell proliferation.[187, 188] NO concentration has been found in higher quantities associated with infection-related cancers, such as HPV-related cervical carcinoma and H-pylori related gastric cancer.[189, 190] Previous studies show that iNOS has a prognostic value and is a predictor of poor survival in ER-negative breast tumours, and is associated with vascularisation, p53 mutations, and activated EGFR.[191] A number of studies on NO have revealed contrasting results. For example, some studies propose that biosynthesis of NO originating in the tissue, hinders tumour

growth and metastases,[192, 193] and that biosynthesised NO encourages cells such as macrophages and endothelial cells to destroy tumour cells.[194, 195] On the other hand, other authors have shown that the presence of iNOS in tumour cells has been associated with tumour grading and metastasis in some cancers.[196, 197] Therefore NOS inhibitors may moderate tumour size and its ability to metastasise.[197, 198]

In summary, abnormal production of COX-2 and iNOS, in turn leads to overproduction of pro-inflammatory mediators, prostaglandins and NO and are found in many precancerous and malignant lesions.[199] Previous animal studies on both COX-2 and iNOS have revealed that targeted inhibition of COX-2 and iNOS can prevent inflammation-associated carcinogenesis.[200-202]

1.5. Histo-morphological types

Histopathological typing of BC has important implications for management. Currently, according to the 2012 WHO classification, twenty histological subtypes, are recognized,[203] reliant on the tumour's cytomorphological and architectural appearance.[204] The most frequent subtypes of BC are invasive ductal carcinomas of no special type (IDC-NST),[203] followed by invasive lobular carcinoma (ILC), which accounts for 40-80% and 5-15% of diagnosed tumours respectively in most of the published literature[205-207] and they show similar clinical outcomes.[208]

The special invasive tumour types are less common and knowledge related to their prognostic significance has been based on studies including a relatively small patient cohort compared to IDC-NST studies. Diverse clinical outcomes are linked to different special subtypes of invasive breast carcinomas. For instance, favourable prognosis has been conferred to many uncommon and rare subtypes, which include mucinous, tubular, medullary, cribriform, secretory and adenoid cystic carcinomas.[207, 209-214] In contrast, a morphological variant that is constantly reported to be associated with aggressive behaviour and poor prognosis is metaplastic carcinoma.[215, 216] Uncertainty of the prognostic value is still questioned for the invasive micropapillary carcinoma and carcinomas with neuroendocrine features. Earlier studies conducted on small cohorts of invasive micropapillary

carcinoma highlighted its poor prognosis.[217, 218] However, a more recent study of 624 patients showed that it is associated with similar outcomes to that of IDC-NST.[219] A characteristic feature that was consistently described with this subtype is angio-invasion with a high propensity for lymph node metastases.[217-219] Similarly, reported prognosis of carcinomas with neuroendocrine features varies, [220, 221] and exclusively depends on tumour grade and stage and not on the neuroendocrine differentiation itself.[220, 222] Furthermore, no specific prognostic significance was linked to invasive papillary carcinoma.[222, 223]

"Pure" invasive apocrine carcinoma is rare, and characterized as a special form with an incidence as low as 0.5%.[224] According to Japaze et al.,[225] criteria for its diagnosis consisting of 75% of cells with apocrine features, large cells with eosinophilic granular cytoplasm, a nucleus to cytoplasmic ratio of 1:2 or a large round nucleus with pleomorphic vesicles, and sharply defined borders. While apocrine carcinomas are usually ER and PgR negative, and androgen receptor (AR) positive,[225] they can inconsistently express HER-2 and/or EGFR.[226] The prognosis for IAC is similar to IDC-NST, with no statistical significance found in one over the other.[224, 225]

Case reports for exceptionally unusual special types conveyed further subtypes such as polymorphous carcinoma, oncocytic carcinoma, sebaceous carcinoma of the breast, lipid-rich carcinoma, glycogen-rich clear cell carcinoma, and acinic cell carcinoma.[203, 222]

Clinical data on specialist types of BC has not facilitated specialist targeted therapy,[227] however they respond differently to NACT, e.g. various sub-types of BC were reduced with treatment (mucinous carcinoma, ILC and apocrine carcinoma) and increased despite treatment (metaplastic carcinoma subcategories squamous carcinoma and spindle cell carcinoma).[203, 222]

1.6. Grading

BC is graded using the Nottingham grading system (Elston-Ellis modification of Scarff-Bloom-Richardson grading system). This system has validated a prognostic significance. Mitotic index, tubular differentiation, and nuclear pleomorphism are evaluated individually on a scale from 1 to 3, then a composite total score for the results from the 3 categories is calculated.[228] Finally, the composite score is allocated to the tumour grade, with scores 3-5 assigned to grade 1 (well differentiated); scores 6-7 assigned to grade 2 (moderately differentiated); and scores 8-9 assigned to grade 3 (poorly differentiated).[228]

1.7. Staging and other prognostic factors

Tumour stage is an accepted prognostic indicator in BC. The American Joint Committee on Cancer (AJCC) stages cancer according to tumour extent/size, lymph node status and distant metastases.

Tumour size used for TNM staging refers to the invasive tumour size, which is one of the most extremely influential prognostic factors of BC.[229] Studies confirmed that adverse outcomes are associated with larger invasive tumours.[229-231] Whole tumour size refers to the size of the invasive tumour in addition to the size of extra-tumoural extension of DCIS.[232] However, it is reported that the whole tumour size is inferior to the invasive tumour size as a predictive factor for BC.[233] Lymph node status is proven to be superior to any other histological factor in the prediction of disease-free and overall survival in BC patients,[234-236] even after neo-adjuvant systemic therapy.[237] Lymph node metastasis leads to a decrease in five-year survival from 98.8% to 85.2%.[234] The survival rates have been linked to the absolute number of the involved axillary lymph nodes,[238, 239] as well as the size of the metastatic tumour.[240, 241] Nodal involvement of four lymph nodes was used in various studies as a cut-off to predict poorer outcomes.[236-238] Lymphovascular invasion is an additional independent prognostic factor.[242, 243] The summary of the TNM staging system (7th edition) is shown in Appendix I.[203]

Nottingham Prognostic Index

Some institutions use other prognostic indices e.g. the Nottingham Prognostic Index (NPI) in the management of BC. NPI incorporates three prognostic factors: lymph node status, tumour size and histological grade into a single index that gives a numerical score for each patient that separates patients into three and potentially five prognostic groups. The prognostic power of NPI has been validated.[244]

1.8. Molecular Classification of BC

For many decades, clinical decisions relied on the categorization of BC into ER-positive and ER-negative subgroups by utilizing hormone receptor expression.[245, 246] At least 1% of the tumour cells show nuclear staining for ER protein to be assigned to the ER-positive subgroup, which represents nearly 75% of BC tumours.[247, 248] More recently, studies on the prediction of therapeutic response based on molecular analysis are being conducted to examine its potential to provide additional useful information. Perou et al.[249] were the first group to propose the initial taxonomy of BC based on gene expression pattern. Complementary DNA microarray experiments involving 8,102 genes were carried out on 65 breast specimens from 42 patients. These included 38 invasive carcinomas (36 IDC and 2 ILC), 1 ductal carcinoma in situ, 1 fibroadenoma and 3 normal breast samples. A hierarchical clustering algorithm was used to group the samples based on the homogeneity of their gene expression patterns. Four major BC subtypes were defined (Figure 1.8). The relationship between the BC genetic subtypes and patient outcome has also been studied, and uncovered heterogeneous outcomes among the major subtypes and among the luminal subtype.[250, 251]

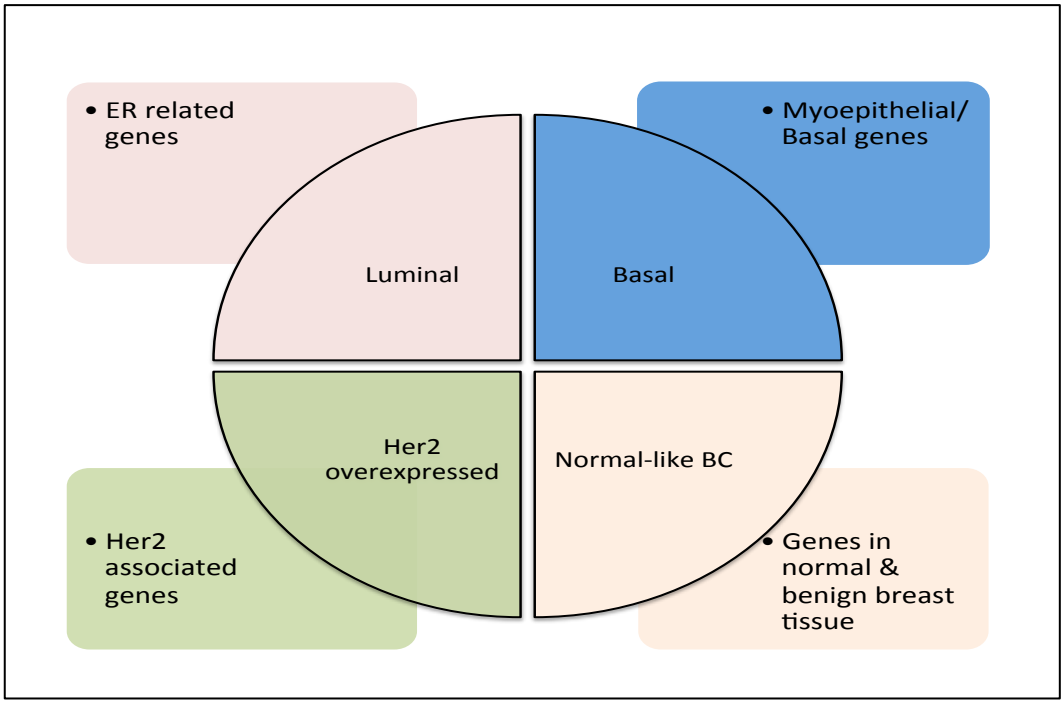


Figure 1.8. Initial molecular classification of invasive BC (Adapted from Perou et al.[249])

1.8.1. Luminal Subtypes

Luminal subtype is the most prevalent form of BC within the classification, and is predominantly characterised by hormone receptor expression.[250, 249] Further analysis by Sorlie et al. identified a further subdivision of the luminal subtype into luminal A, luminal B and luminal C (Figure 1.9).[250] The existence of luminal C is controversial. Few studies have successfully identified it,[250, 252] while others failed to do so.[253, 254] Luminal A and luminal B are inherent subtypes of BC, which are distinctive in both baseline prognosis and sensitivity to cytotoxic therapies.[255] Initially, differentiation of luminal B tumours was defined by a higher expression of proliferative genes,[256-258] with suggested cut-offs for Ki67 proliferation index dichotomisation at 13.25%,[14] 14%,[257] and 20%.[258] Other studies subsequently observed that nearly a third of the luminal neoplasms showed amplification of HER-2-associated genes, and proposed separation of the luminal B subgroup according to HER-2 overexpression in association with ER positivity.[259, 260] Blockage of HER-2 pathways in addition to oestrogen deprivation was recommended for their treatment.[261]

Luminal B tumours express greater numbers of cell markers for proliferation such as Ki67 and aurora kinase A, and lower numbers of luminal cell related markers such as PgR and Forkhead box protein A1 (FOXA1), but not the ER.[255] Luminal A tumours show an overall lower number of mutations and chromosomal copy-number changes compared to luminal B tumours.[253] Luminal A tumours have a significantly better prognosis than luminal B tumours.[253] This may be related to the overexpression of the HER-2 related genes, the faster proliferation rate translated with the high Ki67 expression or endocrine treatment resistance. Green et al.[262] used gene expression profiling and a panel of 25 specific immunohistochemical BC markers to identify a further luminal subtype known as luminal N. Luminal N subtype is characterized by high levels of ER and PgR, and negative/low expression of the HER (HER3 and HER4). When compared, both luminal A and luminal N demonstrated good prognostic factors. Luminal A and luminal B tumours showed high expression of CK7/8, ER, HER3 and HER4 but were distinguished by lower levels of PgR expression in luminal B compared with luminal A tumours. In contrast, luminal N tumours showed differential expression of HER3 and HER4. Luminal A and N had the lowest frequency of BC-specific death.[262] Over a period of 20 years, luminal B and HER-2-positive/ER-positive tumours showed the worst BC severity score. The use of immunohistochemical BC markers as a means of distinguishing luminal A and B tumours is inherently flawed. However, a definition of luminal A tumours as hormone-receptor-positive/HER-2-negative/Ki-67 <14% and PgR >20% will support patient classification to specific therapy modalities.[254]

1.8.2. HER-2 positive subtype

HER-2 positive BC, characterized by a deficiency in expression of luminal/ER-related genes and HER-2 overexpression, is an aggressive type due to a high copy number of genes, has a poor response to treatment and a poor prognosis.[263, 264] The HER-2 gene is located on chromosome 17q12 and encodes the tyrosine kinase receptor of EGFR. HER-2 is overexpressed in 15-30% of BC.[265] The amount of overexpression of HER-2 is determined via fluorescence in situ hybridization (FISH), or via IHC, which detects specific markers for overexpression. Previous studies propose that overexpression of HER-2 occurs during the initial onset of

invasive carcinomas and is maintained during progression.[266] It is generally high-grade with an elevated number of p53 mutations,[13] and typically metastasises to the bone and brain.[267]

Up until recently, overexpression of HER-2 was associated with poor survival. Newer therapies such as trastuzumab, pertuzumab and lapatinib, which act by interfering with the HER-2/neu receptor or inhibiting tyrosine kinase improves prognosis, however there is continuous debate regarding its concomitant use with chemotherapy, the duration of its use and continuous use over time.[268]

In some instances, HER-2 overexpression is not detected in HER-2 overexpressing tumours. These subtypes are known as HER-2 enriched,[249] and characterized by the high expression of HER-2 related proliferation-related genes and proteins, intermediate expression of luminal-related genes and proteins, and a low expression of basal-related genes and proteins.[269]

1.8.3. Normal Breast-like subtype

Normal breast-like subtypes have a gene expression profile that is analogous to normal breast tissue such as elevated expression of adipose cells and non-epithelial cells.[249, 270] It is thought however that this subtype does not actually exist.[270, 271]

1.8.4. Basal-like subtype

Basal-like subtypes account for 15% of BC.[272] They are not characteristic of oestrogen or HER-2 overexpression, are typically high grade, highly proliferative,[206, 272] with frequent pushing margins and areas of geographic necrosis or fibrosis.[273] They occur in younger women, often of African-American origin.[206, 260] They are associated with poor survival and frequently metastasise to the lungs and brain.[267, 270, 274] Basal-like carcinomas express basal CKs such as CK5 and CK5/6, and P-cadherin markers.[275] The majority of BRCA1 related BC are TN, and exhibit basal-like profiles.[276] As shown in Figure 1.1., basal-like refers to the basal/myoepithelial cells. CK5 and CK17 are fundamental markers of basal-like BC.[249, 250, 277] CK17 is not sufficiently expressed unless CK14 and CK5 are absent.[278] The expression of these basal-like genes augments cellular

proliferation, apoptosis, extracellular remodelling, cell migration, and invasion.[279-281] Similar to HER-2-related BC, basal-like tumours have an elevated number of mutations across the genome (i.e., p53 and PIK3CA). Basal-like BCS also have elevated levels of EGFR/HER1 (18-60%).[282, 283]

There has been some confusion in defining basal-like cancer (i.e., basal-like versus TN; ER-negative/PgR-negative/HER-2 negative).[284, 285] One of the reasons for this might have been the use of different methodologies for defining each of these subtypes.[286] The recent extensive review and research highlighted a continuous controversy and debate about the two subtypes. The basal subtype has been segregated through initial gene expression profiling segregated studies[249] and also through immunohistochemical studies.[287] However, studies show a discrepancy of up to 30% between the stated methods of assessment.[284, 285] According to Banerjee et al.[274] and Cheang et al.,[287] immunohistochemical detection of basal-like BC includes expression of CK5/6, CK14, or CK17, in isolation or together; absence of expression of ER, PgR, and HER-2 (i.e., TN phenotype); concomitant absence of expression of ER and HER-2 with expression of CK5/6 and/or EGFR; and concomitant absence of expression of ER, PgR, and HER-2 with expression of CK5/6 and/or EGFR. Further studies show that not all basal subtypes that are detected using gene expression profiling lack RNA for ER, PgR and HER-2, while all TNBC do not display a basal-like phenotype using immunohistochemical marker panels.[13, 34, 280, 285, 288-291] In-fact TN tumours are more varied, and can consist of both basal and non-basal subtypes.[292] More recently, TNBC has been classified into four subtypes, basal-like immune suppressed, basal-like immune-activated, mesenchymal and apocrine.

Three terms have been introduced by Gazinska et al.[286] who examined 142 invasive TNBC tumours for histological characteristics, immunohistochemical staining for basal markers (CK 5/6,CK14 and/or EGFR) and gene expression, and designated them as ‘Path-Basal’, ‘Core-Basal’ and ‘PAM50-Basal’ respectively. Less than 10% of the cases shared the assignment to the three categories. Only 64.7% of the ‘Core-basal’ tumours displayed the gene signature of basal tumours ‘PAM50-Basal’, with poor sensitivity (59%) between the two methods of assessment. Up to 50% of TNBC can be classified as ‘core basal’ with expression

of EGFR and/or CK 5/6[287] and/or CK14,[286] and are associated with considerably worse treatment outcomes.[287] In summary, there is an overlap between the two types but they are not identical.

Based on the observation that the expression of basal markers is not restricted to the TNBC type, the Cambridge research group proposed that basal tumours are dispersed within other major BC molecular subtypes (Figure 1.9).[293] This illustrates the highly distinctive molecular mechanisms and biological heterogeneity of basal-like tumours.

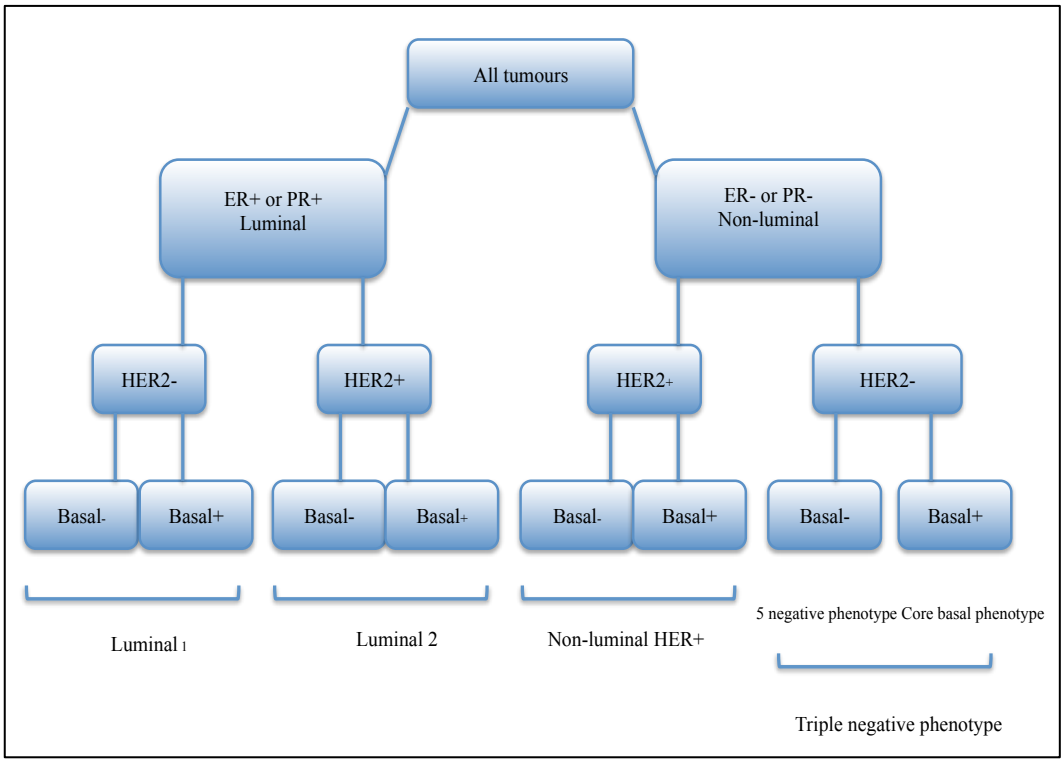


Figure 1.9. Classification of BC subtypes according to immunohistochemical marker profile (adapted from Blows et al.[293])

1.9. TNBC

1.9.1. Epidemiology and risk factors

BC is considered to be TN when there is no positive immunohistochemical expression of ER, PgR and HER-2. This term was first characterised in the medical literature in 2005.[294] Nearly one in every 5 to 7 women who develop BCs are victims of this aggressive phenotype, which is known by its unfortunate outcome and scarcity of effective targeted treatment.[295, 296] Women of African ethnicity and African-Americans showed the highest incident rates of TN tumours, at 82%, 28.5% and 26.4% within Ghanaians, African-Americans and Egyptians respectively.[297, 298] TNBC unusually presents in premenopausal females and has a unique pattern of disease with higher frequencies of disease progression and BC-related mortality 3 to 5 years after diagnosis, when compared to other BC types.[295] The vast majority of women who had no evidence of disease progression after 8 years of TNBC did not have any recurrence thereafter.[299, 300]

There are a number of risk factors associated with the development of TNBC. Studies show that patients aged 42 years or less and those with a body mass index (BMI) greater than 32.4 kg/m^2 have a greater incidence of developing TNBC.[301] Multiparity (>4 children) and younger age at which first pregnancy occurred increase the risk of TNBC, while breastfeeding decreases the risk of TNBC.[302-304] Other factors such as oral contraceptives and their duration of use have shown a 2.5-fold increase in risk of development of TNBC.[305] Unusually, a history of alcohol intake decreased the risk of development of TNBC when compared with non-alcoholics.[306] Moreover, a relationship between metabolic syndromes such as insulin resistance and TNBC has also been reported.[307, 308] Studies point at MGA as a probable precursor lesion for TNBC, with evidence of chromosomal alterations developing within MGA to progress to atypical MGA and then into invasive carcinoma.[66-68] However, due to the infrequency of occurrence of the MGA, it is not likely to be the precursor for the majority of the TNBC.

Genetic impairment of the BRCA1 function is often detected in TNBC. A study of 1,824 patients with TNBC showed mutations of BRCA1 (8.5 %) and BRCA2 (2.7 %).[309] Additional high number of molecular alterations has been identified in

TNBC. Some of these alterations involved the loss of negative regulators such as PTEN and/or mutations within oncogenes such as PIK3CA and AKT.[310] As mentioned before, BRCA1 mutant BCs are often associated with high-grade BC types and commonly classified as basal subtype within the TNBC due to expression of CK 5/6, one of the basal cellular markers,[34, 35,36] while BRCA2 mutant BCs are often associated with ER-positive tumours.[37, 38] Several additional chromosomal abnormalities are also detected with BRCA mutated tumours, including p53 mutation and c-myc amplification.[311]

1.9.2. Clinical features of TNBC phenotype

TNBCs are typically aggressive, with poor clinical outcomes reported in the literature.[295, 296, 312] It has also been associated with pulmonary, hepatic and cerebral metastases, local recurrence and younger age of onset.[267, 270, 274, 313] Commonly, the patients diagnosed with TNBC present with a palpable mass.[314] Of particular note, that TNBC is less frequently detected through mammogram or an ultrasound than with clinical examination.[295, 315, 316] Among women who are part of a BC screening programme, TNBC are more likely to present between screening intervals (between two mammography screening rounds) known as 'Interval cancer'.[314, 315] This might be a reflection of their rapid growth pattern.[316] Unfortunately, TNBC have been noticed to present with a larger tumour size and at a higher stage.[317] These factors, beside the lack of the targeted therapy, had contributed to a worse outcome than ER-positive tumours. In TNBC, there is a somewhat confusing relationship between tumour size development and respective lymph node involvement, and TNBC favours dispersion via the blood.[314, 318] Initially, TNBC responds positively to various types of chemotherapies, but nonetheless has a low disease-free survival.[319]

1.9.3. Histopathological features of TNBC phenotype

Approximately 90% of TNBC tumours are high-grade and classified as unifocal IDC-NST, with the remaining 10% classified as medullary, secretory, apocrine, adenoid cystic, metaplastic, ILC and those that develop from MGA.[314]

TNBC is often accompanied by central necrosis, acellular fibrous zones and/or lymphocytic infiltration.[320] TNBC is also characterised by its high metabolic activity resulting from poor differentiation and tumour necrosis.[34, 321] On a cellular level, cells show noticeable nuclear pleomorphism and mitotic activity.[322] Those with successful pathological response to NACT, assessed on the therapeutic excision, with full eradication of the entire tumour tissue have an associated positive prognosis, while those with residual cancer have a worse prognosis.[323]

Metaplastic breast carcinoma

Up to 1% of all BC are metaplastic type, evident by neoplastic epithelium differentiating into squamous cell and/or mesenchymal-appearing elements.[324] The World Health Organization (WHO) defines metaplastic carcinoma as a heterogeneous neoplasm with many distinctive subtypes, that demonstrates admixture of epithelial and mesenchymal components or can be completely epithelial or mesenchymal in nature.[203] Where metaplastic carcinoma is purely epithelial it exhibits the morphology of squamous cell carcinoma, adenocarcinoma with spindle cells or adenosquamous carcinoma. Mesenchymal-looking elements e.g. chondroid or osseous matrix production are also often present.[203] Due to its rarity, it was scarcely studied through several case reports and research studies on small numbers of cases, to be finally classified by the WHO as a distinct entity in 2000.

On the molecular level, metaplastic carcinoma demonstrates TN in over 90% of cases,[325] and seemingly resembles the claudin-low type.[326, 327] High-grade metaplastic BC can be resistant to chemotherapy with a poor prognosis, while lower-grade fibromatosis-like metaplastic BCs have relatively better prognosis. [328] It has a specific gene profile including downregulation of genes involved in chemotherapy reaction, and upregulation of genes involved in adhesion and migration.[329] The up/down regulation of these specific genes in metaplastic BC

could be responsible for the commonly poor prognosis in this type of cancer.

Medullary carcinoma

Medullary carcinoma accounts for less than 5% of all invasive BCs [211, 330], and most medullary carcinomas are TNBC.[211] There are three recognised current subsets of this special BC subtype; typical (classic) medullary carcinoma, atypical medullary carcinoma and carcinoma with medullary features.[331] Their classification depends on the presence of all or some of the following histological features: a predominant syncytial growth pattern, with defined margins that pushes towards the surrounding breast tissue, nuclear pleomorphism, a high mitotic rate, lymphoid infiltration of the tumour, and deficiency of glandular features or an in situ component.[332, 333] Medullary carcinomas have an improved prognosis with a 10-year survival ranging from 61% to 92%.[332, 333] Moreover, they have high radiosensitivity that further improves their outcome, even in incidences without adjuvant chemotherapy.[334] Medullary BC has a specific gene profile and shows upregulation of specific immune response and tumour necrosis genes, and downregulation of genes involved in invasiveness and adhesion.[335, 336] The up/down regulation of these specific genes in medullary carcinomas could be responsible for increased cytotoxic activity, and overall improved prognosis in this type of cancer. Additionally, significant proportions of medullary carcinomas immunohistochemically express one or more of the basal markers (CK14, CK5/6 and/or EGFR).[335]

Apocrine carcinoma

Less than 0.5% of all BCs are pure apocrine carcinoma type.[224, 337, 338] This BC special subtype has an imprecise WHO definition. Vranic *et al.* proposed strict criteria to demarcate apocrine carcinoma, with combined morphologic, immunohistochemical and molecular characteristics. Apocrine carcinoma can demonstrate growth patterns of invasive ductal carcinoma of no special type. It is diagnosed via the presence of apocrine-type epithelial cells which constitute >90% of the tumour. Apocrine-type epithelial cells are characterized by having well-defined sharp borders, abundant eosinophilic granular to foamy vacuolated cytoplasm, round nuclei and conspicuous nucleoli.[338] Sometimes the cells have

apical decapitation secretion pattern known as apical snouting. Apocrine carcinomas are hormone-receptor (ER and PgR) negative and this together with the expression of both gross cystic disease fluid protein 15 (GCDFP-15) and AR can be used to aid diagnosis of apocrine breast carcinoma.[225, 339] However, apocrine carcinomas inconsistently express HER-2 and/or EGFR.[226] Currently, there is no standardised diagnostic criteria for apocrine carcinoma, [203] which creates poor interobserver agreement for this subtype.

Invasive lobular carcinoma

ILC constitute 5-15% of all BCs.[205-207] Although ILC have typically intermediate grade and ER positivity,[340, 341] a small proportion of ILC demonstrate TN phenotype.[342] Compared to invasive lobular non-TNBC, ILC-TNBC is characterized by being higher grade with increased expression of galectin-3, a biomarker involved in inflammatory and fibrotic pathways.[342] Both types of ILC share the low frequency of expression of basal markers such as CK5, CK5/6 and EGFR.[342]

Salivary gland-type neoplasms

Salivary gland-type neoplasms are rare, constituting approximately 2% of all BC.[343] Although they are characteristically TN, they are low-grade.[343] Some salivary gland tumours also have genetic malfunctions similar to some types of BC.[324, 344] Adenoid cystic carcinoma (ACC) is a common cancer of the salivary glands, and consists of differentiated basal/myoepithelial and luminal cells.[345] As mentioned, this particular type of cancer can also occur in the breast, known as breast-derived adenoid cystic carcinoma (bACC),[344, 346] and constitutes roughly 0.1% of all BC.[347] Although bACC expresses markers of TNBC, bACC in contrast to aggressive TNBC has a 100% 5-year survival rate.[346] bACC is thought to downregulate specific genes involved in migration and proliferation, leading to an improved overall survival.[348] In recent years, the advent of specifically targeted hormone and/or receptor therapies has changed the face of BC, i.e. oestrogen, progesterone and HER-2 receptors. This allows specific classification of BC and hence specifically targeted treatment and prognosis.[349]

Secretory breast carcinomas are a rare subtype of BC, are often slow-growing, mimic benign lesions, and occur generally in a wider range of ages, with improved prognosis.[350] Secretory BC contain a large amount of intracellular and extracellular, eosinophilic secretion material and stain negative for TN associated receptors. Secretory BC consists of an intraductal component, which can consist of comedo necrotic cells in rare cases.[350]

Dreyer *et al.* analysed the clinicopathological features of TNBC according to the histological subtypes in 476 tumours. Apocrine or lobular carcinomas were found to present in older age when compared to other tumour types. Metaplastic carcinoma showed a characteristic poor prognosis although lymph node metastases were seen only in 29.4% of cases.[215]

1.9.4. TNBC stratification and reconstruction

Recent research unveiled that it should not be considered as a single disease as it shows molecular and clinical heterogeneity.[326, 351] Efforts to subclassify TNBC have been attempted using gene expression, with the aim of defining prognostic and therapeutic targets for the diverse subsets within this group.[326, 351] Studies by Perou *et al.* have identified three TNBC subtypes, which originate from epithelial cells at different stages of the cell development.[272] The two main studied subsets are the basal and claudin-low which are illustrated in the Table 1.1. The third subtype is the HER-2 enriched (HER-2 enriched but not HER-2 amplified), which comprise a small proportion of TNBC.[272] There is minimal knowledge about this minor subgroup within the literature.

Table 1.1. Characteristics of Basal-like and Claudin-low BC subtypes
(summarized from Perou, 2010)[272]

	Basal	**Claudin-low**
Percentage within TNBC	Up to 75%	<10%
Gene expression	Genes enriched with myoepithelial/basal cells p53 mutations RB loss	-Low expression of cell-adhesion proteins -Tumour-initiating (stem-cell) genes (ZEB1, TWIST,SNAIL)
	Hypoxia signature genes (+/- benefit from angiogenesis inhibitors)	
IHC	CK 5,6,17 Ki67,PCNA	E-cadherin & Claudin 3,4,7 –ve CD44+, CD24-
Other features		Intense immune response Epithelial-Mesenchymal transition

In 2011, Lehmann *et al.* analyzed 3,247 genes for 587 TNBCs through examining 21 invasive BC data sets and concluded that TNBC can be subcategorized into six unique subtypes which might be have prognostic and therapeutic implications: basal-like (BL1 and BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal AR (LAR).[326] Each subtype is characterized by enrichment in certain cellular pathways and genes (Table 1.2.). The Basal subtypes and the M subtype express higher levels of basal CKs, while LAR expresses higher levels of luminal CKs and lacks basal CKs expression.[326] By using gene expression, specific markers were identified with the aim of developing targeted therapies, e.g. cisplatin has a significant effect on BL1 and BL2 subtypes, dual Phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and Src inhibitors are sensitive to mesenchymal and mesenchymal stem-like subtypes, and AR antagonists had a significant effect on LAR subtypes.[282]

Table 1.2. Characteristics of the TNBC subtypes (summarized from Lehmann *et al.*)[326]

Subset	Pathway enriched	Genes commonly expressed	Other features
BL1	Cell cycle, cell division	*AURKA*, *AURKB*, *CENPA*, *CENPF*, *BUB1*, *TTK*, *CCNA2*, *PRC1*, *MYC*, *NRAS*,	
	DNA damage response (ATR/BRCA) pathways	*CHEK1*, *FANCA*, *FANCG*, *RAD54BP*, *RAD51*, *NBN*, *EXO1*, *MSH2*, *MCM10*, *RAD21*, *MDC1*	
BL2	Growth factor signalling (EGF pathway, NGF pathway, MET path- way, Wnt/β-catenin & IGF1R pathway)	*EGFR*, *MET*, and *EPHA2* P63, MME(CD10)	
	Glycolysis & gluconeogenesis		
IM	Immune cell signalling (CTLA4, IL2, IL7 pathways) Cytokine signalling		Similar gene signature as medullary BC
M	Cell differentiation pathways		
MSL	Growth factor signalling	EGFR, PDGF	
	EMT-associated genes	*TGFB1L1*, *BGN SMAD6*, *SMAD7*, *NOTCH1,* *MMP2*, *ACTA2*, *SNAI2*, *TWIST1*, *ZEB1*, *COL3A1*, *COL5A2*, *ZEB2* & decreased E-cadherin	Low levels of cell-cell adhesion genes(E-Cadherin, Claudin 3,4&7) Low levels of proliferation genes
	Angiogenesis genes	VEGFR2, TIE1	
LAR	AR & downstream genes	AR, FOXA1, KRT18, XBP1	

In contrast, studies by Burstein *et al.* identified only four subtypes; including LAR and mesenchymal, and in addition basal-like immunosuppressed (BLIS), and basal-like immune-activated (BLIA) (Figure 1.10.).[351] Data regarding LAR and mesenchymal subtypes were more consistent and stable in the two classification systems. The basal subtype was further subgrouped according to immune-modulatory gene expression, into BLIS and BLIA. This classification drew a prognostic value, in which the BLIS was significantly associated with earlier relapse, followed by LAR and mesenchymal, while BLIA had the best prognosis with the longest disease free survival.[351] A recent study by Lehmann group[352] failed to reproduce the results from their original classification and adapted a classification which is consistent with Burstein classification of TNBC.[351, 352]

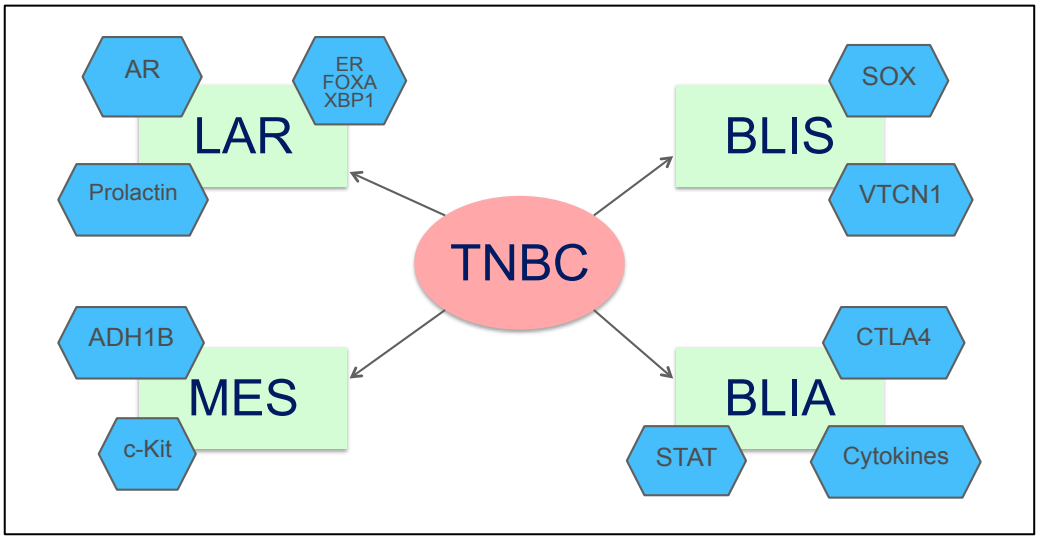


Figure 1.10. TNBC subtypes as identified by genomic profiling[351]

The most popular form of TNBC is the basal subtype, constituting 15-20% of all types of BC[249] and 75% of TNBC using gene expression analysis (Table 1.1.).[353] As explained before in section 1.2.1., basal-like BC originates in the basal layer of normal breast tissue, from basal myoepithelial cells and expresses CK5/6, CK14, CK17, vimentin, p-cadherin, alpha B crystalline, caveolins 1 and 2 and EGFR genes. CD44+/CD24- are also sometimes expressed in basal-like BC,[354] which are used to characterise claudin-low BC along with triple-negativity. Basal-like BC which is TN is identified by ER, PgR, and HER-2 negativity, CK5/6-positivity, and/or high amounts of expression of EGFR, known as the five-marker method as

defined by Nielsen *et al.*[282] However, classification of TNBC has shown via immunohistochemical methods that not all TNBC can be characterised as basal-like, with non-TNBC being not basal-like the majority of the time.[355] Studies show that almost 50% of BL1 BC patients who received chemotherapy achieved a pathological complete response (pCR), when compared to BL2 BC patients.[352] BL1 BC is more responsive to chemotherapy due to characteristic DNA signalling and repair function.[352] Although previous studies had linked the basal subtype with unfavourable prognosis,[206, 282] there is recent evidence that some of the BL tumours which are associated with activated immune response have a good prognosis.[351]

Claudin-low BC is usually TN, with very low levels or no expression of luminal cells, and high levels of epithelial-to-mesenchymal cells.[327] The proteins and genes involved in tight junction and cell-cell adhesion are downregulated in this subtype, with low expression of claudin 3, 4 and 7,[356] and E-cadherin[327]; hence the name 'Claudin-low'. It also has characteristics of cancer stem cells, with high amounts of medullary differentiation.[269, 326] The possible origination from stem cells is illustrated with their exhibition of epithelial mesenchymal transition traits as the lack of cell-cell adhesion and expression of E- cadherin transcriptional repressors such as Zeb1/2, Twist1/2 and Snail 1/2.[269, 357]

In summary, TNBC can also be classified as either basal-like types, molecular apocrine (LAR) or mesenchymal types.[351] Women with TNBC have poorer survival than other BC, irrespective of stage at diagnosis.[295, 317] A previous study by Dent *et al.* on survival showed that patients with TN tumours had a higher risk of metastases, and associated deaths, when compared to those with non-TN tumours.[295] Recent studies based on gene expression subclassified BL cancers into 2 distinctive groups, BL1 and BL2, which project opposite outcomes.[351] Particularly, it has been revealed that non-Hispanic black women with TNBC diagnosed at late-stage, have the poorest survival with a 5-year survival of 14%.[317]

1.9.5. Prognostic markers in TNBC

In other BC subtypes, molecular assays and the tumour stage and size represent imperial markers for survival. However, these markers are not as useful in TNBC.[358, 359] Moreover, the majority of TNBCs are high-grade tumours and as a result, the prognostic value of grade is lessened.[464] However, lymph node metastases and pathological response to NACT[360] retained their prognostic value in TNBC. Moreover, it is suggested that sTILs play an important role with an emerging prognostic impact and a promising predictive value for immunotherapy.[102] Some authors observed a potential association between outcome and histological subtype in TNBC.

1.9.5.1. Lymph node metastasis

Spread of the malignant cells into the lymph nodes is considered an important prognostic factor in the management of the BC patients.[235, 236] Unlike other BC subtypes that show a positive correlation between the primary tumour size and rate of the lymph node positivity, the nodal status in TNBC is not directly correlated to the local extent of the tumour.[295, 318] Nevertheless, similar to other BC subtypes, cases with lymph node involvement in TNBC have a significantly worse outcome, with higher incidence of recurrences and death,[234, 361] as opposed to those with negative lymph nodes.[362] Surprisingly, some authors suggest that the prognostic value of the nodal status in TNBC is independent of the number of the affected nodes.[361, 363] Noteworthy, the increased recurrence and death risks remain associated with the nodal status after treatment in the neoadjuvant setting.[364, 365]

1.9.5.2. Response to the NACT

At the present time, TNBC is primarily treated with NACT[366] as will be detailed later. Several authors had published grading systems for the response to NACT, with different approaches and thresholds for defining the categories of the pathological response to NACT.

pCR has been shown to be greatest when there is an absence of infiltrating residual disease in the breast and axillary lymph nodes.[365] It had been suggested that hormone negativity is the strongest predictor of pCR in BC,[367] with the better response in the TN.[368] However, not all TNBC are chemosensitive as a recent meta-analysis noticed that the rate of pCR ranges from 14.3% to 67%.[369] Predictability of pCR in TN tumours is one of the most powerful prognostic factors in TN cancer.[368, 370-372] Those who achieve pCR have a relatively good prognosis, with survival rates similar to those of other BC subtypes.[323] On the other hand, the remainder of the TNBC patients have a subpopulation of chemoresistant tumour cells that hinder the achievement of pCR. Studies showed that those who fail to achieve pCR have a worse prognosis, compared to those who attained pCR.[369] Denkert *et al.* showed that elevated TILs, in pre-treatment tumour can predict pCR in TNBC.[373] When TILs were occupying more than 60% of the tumour stroma or intratumoural area, the pCR rate was 74%. In contrast, with lesser amounts of TILs the pCR rate was 46%.[373]

1.9.5.3. TILs

There is evidence that the presence of high levels of TILs is an useful prognostic marker in different malignancies including cancers of the colon,[374, 375] ovary,[376] lung[377] and in malignant melanomas.[378, 379] As described first in melanoma, specific T-cell growth factors stimulate the immune system to fight the tumour cells and lead to tumour regression[380, 381] and better survival.[382, 383] Several studies confirmed that a prominent inflammatory response in BC was associated with better prognosis.[384-386] It was noted that elevated TILs are more frequently associated with highly proliferative, poorly differentiated and more aggressive BCs.[107, 110, 386-393] As BC is not a single disease, Stanton *et al.* analyzed the pooled data for sTILs in 13,914 BCs with special consideration on the BC molecular

subtypes.[394] The median sTILs tended to be higher in TNBC and HER-2-positive BC than in ER-positive tumours (20% and 16% versus 6% respectively).[394]

1.9.5.4. Histological subtype

Histological type has prognostic implication in TNBC.[295, 324, 332, 346, 350, 328] The majority of the TNBCs are IDC-NST and a smaller percentage constitute a variety of the other histological types that includes rare ones.[395] These rare types, i.e. secretory, adenoid cystic and medullary carcinomas are associated with an excellent outcome and represent a form of 'low grade' TNBC,[295, 332, 346, 350] while poorer outcome is seen with metaplastic carcinomas that are known to have poor response to chemotherapy.[328]

1.9.5.5. Other biomarkers

TNBC expresses greater amounts of the proliferation marker Ki67 and p53 mutation marker, when compared to non-TNBC. Nodal metastasis and p53 overexpression have been linked, which may contribute to the worse survival.[396] [55] A high pCR has been linked to Ki67 expression, showing Ki67 to be an independent predictor for pCR, overall survival, and distant disease free survival in BC patients concomitantly treated with a NACT.[397]

In the basal-like TNBC, amplified EGFR, CK 5/6 and diminished AR are proposed to be associated with nodal and distant metastasis, highlighting that anti-EGFR target specific therapies may be used.[398]

Rakha *et al.*[359] proposed that the absence of AR expression in TNBC is linked with higher histological grades, development of recurrences and distant metastasis. Higher histological grade was in turn linked with negative E-cadherin expression and positive expression of P-cadherin and p53.[359] Significantly, Rakha *et al.*[359] concluded that assessment of the AR and basal phenotype, along with lymph node status and tumour size, can be used to classify high-risk and low-risk tumours and hence specific treatment regimes.

1.9.6. TNBC outcome

TNBC does not behave clinically as a single disease. Recently, its heterogeneous nature with the discovery of several molecular subtypes had been studied and highlighted differences in response to treatment and outcomes. The majority of the TNBCs have an aggressive nature and generally TNBC have a worse outcome, with higher recurrence rates and shorter survival, than other BC subtypes. Recurrence of TN and basal BC is high initially but quickly decreases when compared to ER-positive BC.[249, 295, 399] Patients with TNBC have a high risk of developing locoregional recurrence within two years of initial diagnosis,[295] with a proclivity for metastasis into the brain and lungs.[267, 270, 274] Dent *et al.* observed that after the first recurrence, the median time survival time for TNBC was only 9 months.[295] Nearly two-thirds of the deaths from TNBC occur in the first five years following diagnosis.[295, 321] On the other hand, a small proportion of the TNBC patients who have specific rare histological morphology e.g. medullary carcinoma and carcinomas of salivary gland origin tend to have a good prognosis. [295, 332, 346, 350] Dent *et al.* defined the characteristics of TNBC as an interval cancer with a weak correlation between tumour size and node status, a high risk of recurrence, increased mortality rate in the first 5 years, and a fast progression from distant recurrence to death.[295]

1.9.7. Treatment of TNBC

To date, TNBC is still treated as one disease, with a combination of surgery, radiotherapy and chemotherapy.

1.9.7.1 Systemic treatments

Chemotherapy

At present, chemotherapy is the only approved systemic treatment for TNBC.[366] Chemotherapy can be administered as an adjuvant (after primary surgery) or as a neo-adjuvant (prior to the primary surgery) therapy.[400] In addition to the decrease of the tumour size to avert mastectomy, other advantages to the application of chemotherapy prior to primary surgery is that it allows assessment of the treatment/drug efficacy through measurement of the degree of response to the

applied therapy, both clinically and histologically.[401, 402] It has also been used as treatment of early micrometastatic nodal disease.[402] In TNBC, NACT has shown that pCR can be used as a marker for long-term response and survival.[306, 368, 370-372] Those who do not achieve pCR demonstrated higher rates of reoccurrence and shorter periods of disease free survival.[369, 403] Anthracycline and/or taxane-based therapies, cell proliferation inhibitors, are a part of the standard treatments for BC patients at present and are particularly effective in TNBC.[291, 404] With using these two drugs combined, TNBC demonstrates higher chemo-sensitivity than non-TNBC and has a greater probability of achieving pCR.[405, 406] Anthracycline or anthracycline and taxane chemotherapies show a 20% to 45% pCR in patients with TNBC, and an improved prognosis over those with non-TNBC, even when pCR is accomplished.[360, 370]

Moreover, platinum agents can inhibit tumour growth and causes cell death through induction of toxic breaks in the double-stranded DNA. Therefore, sensitivity to platinum-based drugs is more evident in BRCA-mutated cells that are deficient in DNA repair mechanisms.[404, 407] Upon addition of Platinum to neoadjuvant regimen consisting of anthracycline and taxane, higher pCR rates had been observed.[408]

Other specifically targeted treatments such as molecular-directed targeted therapies, therapies that target cytoskeleton, cell migration, vascular system and high proliferation gene pathways, immunotherapy and vaccines are currently under investigation.[400] Molecular-directed targeted therapy is used as a means of inhibiting specific molecular pathways of cancer development. These include the inhibition of vascular endothelial growth factor (VEGF), poly (ADP-ribose) polymerase (PARP), epidermal growth factor (EGFR), mammalian target of rapamycin (mTOR), AR, and others.[400]

PARP inhibitors

PARPs are enzymes involved in DNA repair and programmed cell death. An artificial fatal interaction is generated upon PARP1 inhibition with the loss of BRCA1, which causes inability of the tumour cells to buffer oncogene-induced replicative stress.[404, 409] PARP1 inhibitors, alone or combined with

chemotherapeutic agents, showed promising results in clinical trials enrolling BC patients with the BRCA mutation.[410, 411]

AR inhibitors

Some TNBC tumours express AR as proved by gene expression profile and immunohistochemical experiments.[326, 351, 352] Lehmann *et al.* identified the LAR subtype, characterised by a steroid hormone profile and high frequency of PIK3CA mutations.[326] Although the efficacy of the anti-androgenic treatment within LAR subtype was demonstrated by different researchers, conflicting results for its exclusive anti-proliferative effects on LAR cell-line were found.[159-161] Published favourable results from a clinical trial by Triana *et al.*, who treated 118 LAR+ TNBC patients with an AR inhibitor revealed 42% of patients achieved a clinical benefit at 16 weeks.[412]

EGFR inhibitor*s*

EGFR can be blocked by either a monoclonal antibody that competitively binds to extracellular domain or by tyrosine kinase inhibitors that target the intracellular domain.[413] A clinical trial on 173 metastatic TNBC patients using the monoclonal antibody cetuximab, an EGFR inhibitor, in addition to cisplatin demonstrated modest improvements in progression free survival from 1.5 months to 3.7 month.[414]

VEGF inhibitors

VEGF inhibitors, angiogenesis inhibitors, are used to slow the growth of newly formed blood vessels. They have been shown in a number of studies to strengthen both response rate and progression-free survival.[415-417] However, they are not routinely used currently outside the clinical trials setting.

mTOR inhibitors

mTOR is part of the PI3K family, which regulates cellular metabolism, growth, and proliferation. Mutations in BC affect PI3K leading to increased mTOR.[418] When combined with platinum agents, mTOR inhibitors produce a synergistic effect with significant inhibition of cell proliferation, and provoke apoptosis.[419] A

28% increase in response rate and 36% clinical benefit rate was shown in patients receiving everolimus, an mTor inhibitor, combined with carboplatin in stage IV TNBC.[419]

The advent of monoclonal antibodies which inhibit particular pathways in cancer development are under continuous investigation for treatment of TNBC.

1.9.7.2. Surgery

Factors such as patient age, tumour size and grade, and the patient's own preference impact surgical treatment choice of mastectomy versus lumpectomy.[420] Previous studies show that surgery which conserves the breast remains the gold standard in patients with clinically T1 and T2 cancers, while mastectomy is reserved for larger tumours, multifocal/multicentric tumours and in tumours with positive margins after conservative surgery.[370]

1.9.7.3. Radiotherapy

Current treatment guidelines for BC recommend breast conserving surgery and concomitant radiotherapy if the tumour is less than 4cm, with negative surgical margins.[370] With regard to TNBC, the specific radiotherapy treatment practice is directly correlated to the degree of surgery carried out and the lymph node status. Post-mastectomy, adjuvant radiotherapy is given in high risk patients as with tumours >5cm (pT3), metastatic axillary disease and/or presence of lymphovascular invasion.[421, 422] Studies by the Early Breast Cancer Trialists' Collaborative Group show that radiotherapy received after breast conserving surgery and/or mastectomy improved long term survival,[423] while a reduced rate of recurrence was found to be very much dose dependant.[424]

1.10. Roadmap of this thesis

1.10.1. Aims

The aims of this work were

(i) To perform a comprehensive review of the clinicopathological features, treatment and outcome of patients diagnosed with TNBC at GUH over 15 years.

(ii) To evaluate pathological features, sTILs and candidate biomarkers as prognostic factors in TNBC.

(iii) To evaluate the prognostic role of iNOS and COX-2 in BC.

1.10.2. Overview of the approach

1.10.2.1. Construction of a TNBC database and TMA

- Creation of TNBC database from cases managed in GUH from January 2000- December 2015 to include pathological, clinical, and outcome data.

- Construction of a TNBC TMA from formalin fixed paraffin embedded (FFPE) tissue.

- Validation of TNBC status by IHC on the TMA.

1.10.2.2. Evaluation of the prognostic role of sTILs and other biomarkers in TNBC

- Assessment of sTILs on full-face tumour sections from the therapeutic excisions of TNBC.

- Determination of the association between sTILs and clinicopathological features and outcome.

- Testing of the correlation between sTILs in TNBC assessed pre- and post-NACT.

- Examination of the associations between expression of other routine biomarkers and clinicopathological variables and outcome.

1.10.2.3. Characterization of COX-2 and iNOS expression in BC

- Optimisation of COX-2 and iNOS antibodies for immunohistochemical staining in BC.

- To evaluate association between COX-2 and iNOS and clinicopathological features and outcome in IBC, including in ER-negative BC.

2. MATERIALS AND METHODS

2.1. Patient materials

Our work is based on a retrospective observational study, held in the Discipline of Pathology Department in NUI Galway.

Two series of invasive BCs were studied in this work.

1. The first is TNBC series, comprising 376 tumours of invasive BCs, diagnosed in 355 patients, managed in GUH between January 2000- December 2015.

2. The second is West of Ireland Invasive BC (WOI BC) series, comprising 666 cases of invasive BCs, managed in GUH from 1999 to 2005.

GUH Ethics Committee granted ethical approval for collection of patient data and the use of patient material, approval references C.A.41, C.A 489 and C.A.1012. All patient data was handled anonymously and confidentially.

2.1.1. TNBC Series

2.1.1.1. Study cohort and data collection

A database of BC cases reported with negative results for ER, PgR and HER-2 on pathological (diagnostic and/or therapeutic) examination was created by Dr. Elaine Walsh and Dr. Aliaa Shalaby (AS). The patients were managed in GUH, Mayo University Hospital and The Galway Clinic, between January 2000-December 2015. The eligible patients were retrieved from a search of patients with BC in the Department of Anatomical Pathology, GUH, through the Laboratory information system (Apex) from (January 2000 - December 2015). This was cross-referenced to a database of BC patients held in the Discipline of Surgery, NUI Galway. In total 365 patients were deemed eligible for inclusion in the study. There were 389 primary tumours that belong to these 365 patients. Multiple primary tumours were recorded separately and linked to the same patient data entry.

All relevant clinicopathological data, neoadjuvant oncologic treatment and follow up information were collected from patient medical records including the patient charts and hospital information system (PAS: Patient Administration System) which is a computerized up-to-date system with patient demographic data and health history. The patient identities were anonymised. The last patient follow-up update was performed in December 2016. The follow-up for patients who received NACT (n = 97) was updated again in March 2017.

Demographic and epidemiological parameters collected included:

- Hospital number
- Age at diagnosis
- Ethnicity
- Menopausal status at diagnosis
- Reproductive history including age of menarche, age of menopause, age of first live birth, number of full term pregnancies, history of breastfeeding regarding the number of children breastfed and the duration of breastfeeding
- Smoking status and alcohol intake
- Previous medical history as well as history of any benign breast disease
- Medication in addition to use of oral contraceptive pills or hormone replacement therapy
- History of another cancer including previous BC
- Family history of cancer
- BRCA status
- Body weight, height and body mass index (BMI)

All pathological reports for all the surgical procedures for each patient were reviewed and the histopathological parameters were collected.

Where data was missing the slides for such cases were reviewed to generate data.

The pathological data was incomplete in many cases and, where possible, those cases were reviewed by (AS) and Prof. Grace Callagy (GC) and data was completed. Representative slides from all cases were also reviewed.

The Parameters collected from the histopathological reports included:

- Type of surgical procedure for the primary and axillary disease
- Tumour Site (laterality)
- Tumour histological type
- Tumour grade (using Elston and Ellis' modified Bloom Richardson system)[228]
- Tumour tubule formation score
- Tumour nuclear pleomorphism score
- Tumour mitotic count score
- Invasive tumour size
- Presence of in situ disease and its type, grade and percentage
- Whole tumour size
- Presence of lymphovascular invasion
- Nodal status including sentinel, axillary non-sentinel, intra-mammary and supraclavicular lymph nodes
- Pathological response to NACT within the tumour and the lymph nodes and if any reported change in tumour grade
- Presence of tumour necrosis or inflammatory infiltrate
- The TNM stage was recorded/modified according to the TNM classification of breast tumours (TNM: 7th edition)

Treatment and survival parameters:

- Details of NACT and clinical response.
- Details for adjuvant chemotherapy and radiotherapy.
- Any subsequent diagnoses of recurrence/metastasis and its date and site.
- Date of last follow-up
- Status at last follow-up

Disease-free survival (DFS) was calculated as the interval from the date of primary diagnosis to the first event whether it is a recurrence (loco-regional or distant) or development of a new primary TNBC. Metastases-free survival (MFS) was calculated as the interval from the date of primary diagnosis to the development of distant metastases. Overall survival (OS) was calculated as the interval from the date of primary diagnosis to until death. Breast cancer specific survival (BCSS) was calculated as the interval from the date of primary diagnosis to date of death due to BC. These parameters were measured in months.

2.1.1.2. Exclusion criteria

A total of 389 tumours from 365 patients were deemed eligible for this study. Immunohistochemical (IHC) positivity for ER/PgR and/or HER-2 or HER-2 positivity on fluorescence in situ hybridization (FISH) leads to exclusion of the tumour from the cohort. In total, fifteen tumours were excluded due to positivity of ER/PR/HER-2 either on IHC done on the tissue microarray (TMA) or on FISH for HER-2 done on whole tissue section. These cases were mostly diagnosed in earlier years where different IHC assays were used. Cases for which there was no material available for TMA were included in the database; the majority of these were diagnosed in recent years and/or had NACT, where pCR was achieved. The IHC was deemed to be reliable for these cases. Therefore the final TNBC database included 374 tumours from 355 patients. Four cases had bilateral synchronous TNBC, five patients had bilateral asynchronous TNBC, Seven cases had synchronous ipsilateral bifocal TNBC, one case had synchronous ipsilateral three foci of TNBC and one case had asynchronous ipsilateral TNBC primary tumours.

With regards to the inclusion of cases on the TMA, material from 300 cases of the cohort was available for TMA. The remainder of the cases could not be arrayed and these comprised cases that were unsuitable for punching (n=65), cases for which the therapeutic resection (THE) was unavailable (n=22), and cases with proven HER-2 positivity by FISH (n=2).

2.1.1.3. TMA construction

In our study, 300 archival TNBCs were assembled on TMA blocks according to Kononen *et al.*, 1998.[425]

The histology slides from the cases included in TNBC database (n=389), as described earlier were retrieved from the archives of the Department of Histopathology, GUH. For each case, all the slides were reviewed by AS, and the tumours were identified and marked. The corresponding areas on the FFPE tissue blocks were labelled and the availability of sufficient material for use in TMA creation was evaluated. Subsequent to the selection of the most appropriate representative (donor) block from each tumour, a map for each array was created and the co-ordinates for each core were recorded. To mark the position of the first sample on each TMA and ensure the precise positioning of the array, additional asymmetric sample spots were placed in the upper left hand corner of each recipient block. These assigned orientation cores were collected from normal multiple tissues.

Empty (recipient) paraffin wax blocks (34 x 23 x 4 mm) were constructed into which the donor cores were inserted. A tissue-arraying instrument (Beecher Instrument, Silver Springs) was utilized to extract cylindrical tissue cores from each tumour donor block (using a stainless steel needle-like tube) and inject it into the allocated pre-drilled hole on the mapped recipient TMA block. Cores at 1 mm in diameter (0.7857mm surface area) were punched. The choice of the core size was practical to maximize the number of tumour cells examined; minimize the possibility of cores devoid of cancer cells; with least possible damage to the tumour area on the donor block. As the donor blocks exhibit varying thicknesses, a depth stop was adjusted at 3mm height to increase the chance of height uniformity of the cores. The distance between the cores was 0.7 mm.

On completion of each recipient TMA block, it was then placed on a glass slide face down and heated for 30 minutes at 40°C followed by cooling it for 10 minutes. These last steps promote homogenization of the sampled cores with the recipient block paraffin wax.

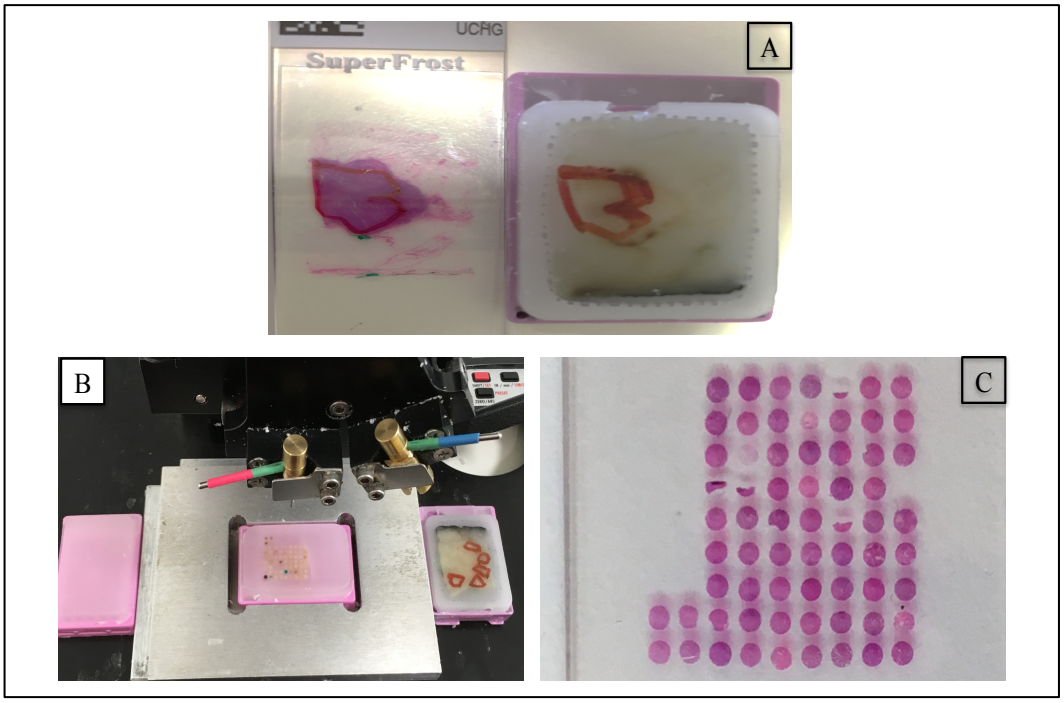


Figure 2.1. Summary of the steps of TMA construction (**A**) An H&E-stained whole section in which the tumour area is labeled and matched into the corresponding FFPE block (**B**) A donor block and a TMA block during the construction of the TMA (**C**) Picture of H&E stained TMA array with 4 controls at the left top corner as an identifier.

For the majority of the cases, tumour was cored in quadruplicate onto the TMA on different recipient blocks. In minority of the cases, due to small tumour size or difficult nature of the tumour hindering punching, only one or two cores were obtained.

A maximum of 72 tissues cores were placed on a single block (Table 2.1.). The TMA was constructed in the Discipline of Pathology, NUIG by research assistant Ms Nessa Keane and Mr. Mark Webber.

Table 2.1. Number of tumour cores on each TMA

Array	Number of TNBC cores	Number of control cores
TNBC TMA (1A, 1B, 1C, 1D)	58	5
TNBC TMA (2A, 2B, 2C, 2D)	58	5
TNBC TMA (3A, 3B, 3C, 3D)	61	2
TNBC TMA (4A, 4B, 4C, 4D)	60	3
TNBC TMA (5A, 5B, 5C, 5D)	71	1
TNBC TMA (6A)	51	4

The presence of tumour was confirmed by review of the Hematoxylin and Eosin (H&E) stained sections. Finally, the TMAs were scanned using Olympus (VS120) slide scanner in Discipline of Pathology, NUI Galway.

2.1.1.4. Validation of TNBC status on the TMA

The TNBC status was confirmed on the TMA. To this end, a TMA of TNBCs was constructed. The process for including patients in the final cohort is outlined in figure 2.1. From a total of 389 tumours from 365 patients with TNBC, the TNBC status was confirmed in 287 of these.

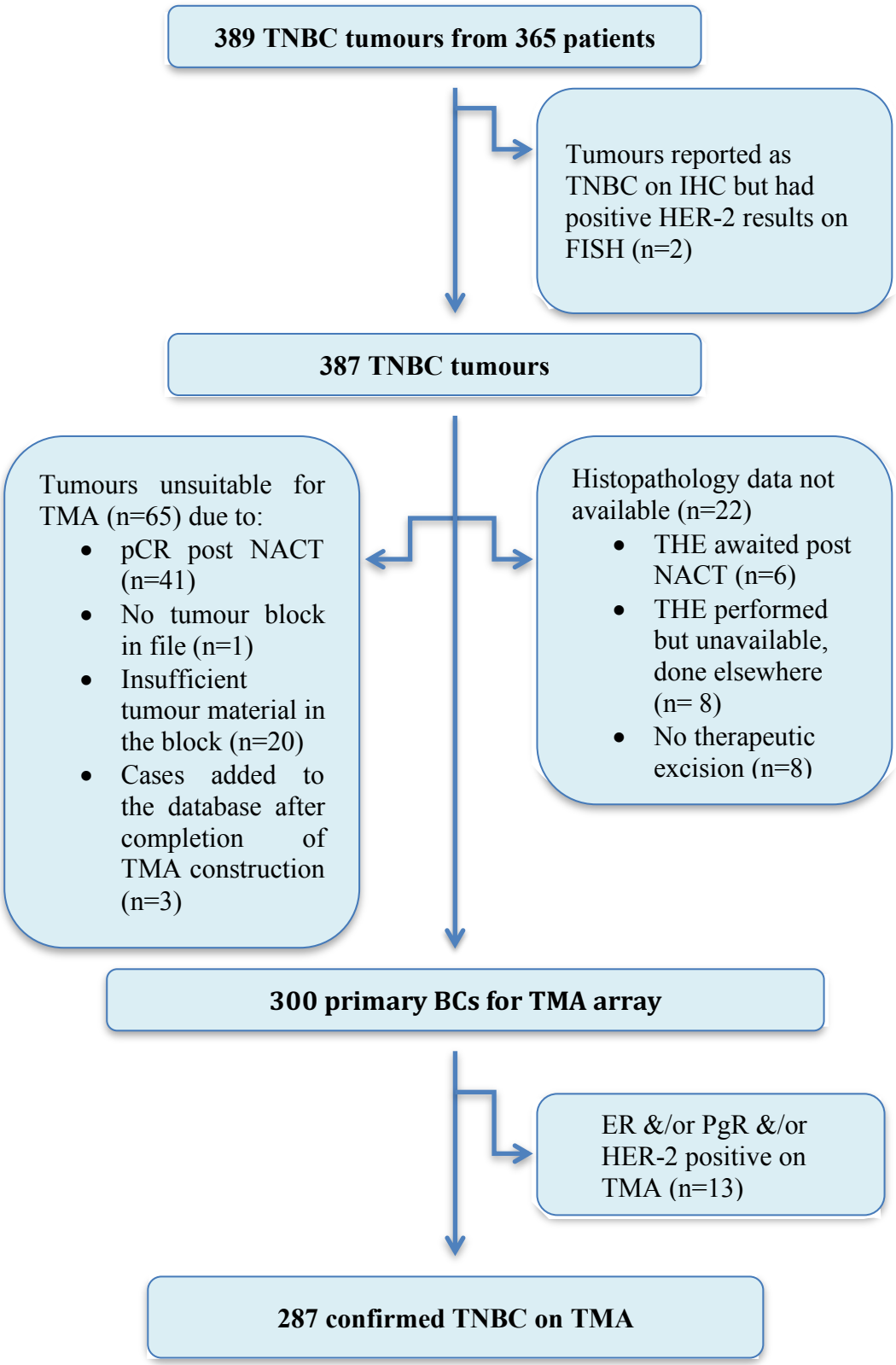


Figure 2.2. Flow chart of TNBC cohort, and strategy for tumour selection and exclusions

2.1.1.5. Histological assessment of the tumour type, grade and sTILs.

Assessment of tumour type, grade and sTILs was done on full-face sections and on needle core biopsy (NCB) from those who had pCR. The following parameters were assessed: by two pathologists (AS and GC).

- Tumour histological type.
    - In cases diagnosed with medullary features, we assessed the proportion of the tumour with the following properties:
        - Pushing or circumscribed margin
        - Syncytial growth pattern/ solid sheets
        - High nuclear grade
        - Abundant lymphoplasmacytic infiltrate
    - In cases of diagnosed metaplastic carcinoma, assessment of the neoplastic cell phenotype was done according to the WHO classification.
- Tumour grade (using Elston and Ellis' modified Bloom Richardson system).[228]
- sTILs. Previous studies defined it as the percentage of the tumour stroma occupied by mononuclear cells (lymphocytes and/or plasma cells).[110, 387, 426, 427] Using the recommendation of the TILs working group (2014), we examined TILs within the stroma of the invasive tumour, excluding the areas of necrosis, DCIS, cavity or previous biopsy sites.[427] As the density and distribution of the mononuclear cells was heterogeneous throughout the tumour stroma, an average percentage was given on a continuous scale (0-100) then categorized on a scale 0-4 as follows (0= No mononuclear infiltrate, 1=>10%, 2= 10-25%, 3=25-50%, 4= >50%).
- Presence or absence of necrosis within the invasive tumour.

2.1.2. WOI BC series

2.1.2.1. WOI BC series

Both COX-2 and iNOS are novel markers and their expression across all BC subtypes is unknown, therefore expression of these markers was examined in a series of BCs that included all subtypes that had been previously constructed. For this purpose, a database comprising all BCs diagnosed at GUH from 1999 – 2005 was used. This database and TMA had been previously created in the Discipline of Pathology, NUI Galway.[428] Tumour material comprised 666 invasive BCs, including 8 % TNBCs. The clinicopathological characteristics of the WOI series are detailed in Table 2.2. Routine biomarkers were previously done and scored. The details of the antibodies used and the biomarker profile of the WOI BC series are shown in Appendix II.

Table 2.2. Clinicopathological features of the WOI BC series

Parameter (n[a])		**Number (%)**
Invasive Tumour type (661)	Ductal	509 (77)
	Lobular	102 (15)
	Mucinous	14 (2)
	Tubular	5 (1)
	Microinvasive ductal	5 (1)
	Medullary	5 (1)
	Mixed[b]	8 (1.5)
	Others[c]	8 (1.5)
Invasive tumour grade (601)	1	82 (14)
	2	307 (51)
	3	212 (35)
Tubule formation (583)	1	31 (5)
	2	74 (13)
	3	478 (82)
Nuclear pleomorphism (583)	1	7 (1)
	2	287 (49)
	3	289 (50)

Table 2.2. (cont'd.)

Parameter (n[a]=)			Number (%)
Mitotic count (583)	1		367 (63)
	2		103 (18)
	3		113 (19)
Invasive tumour size (mm) (625)	≤20		239 (38)
	21-50		314 (50)
	>50		72 (12)
Lymphovascular invasion (386)	Present		203 (53)
	Absent		174 (45)
	Probable		9 (2)
Nodal status (596)	Positive [n=302 (51%)]	1-3 positive nodes	165 (28)
		4-9 positive nodes	88 (15)
		≥10 positive nodes	49 (8)
	Negative		294 (49)
Patient outcome (663)	Alive, no disease		301 (45)
	Alive, loco-regional disease		191 (29)
	Alive, distant metastases		58 (9)
	Dead with disease progression		87 (13)
	Dead with no disease progression		26 (4)
ER (522)	Positive		344 (66)
	Negative		178 (34)
PgR (534)	Positive		297 (56)
	Negative		237 (44)
HER-2 (602)	Positive		85 (14)
	Negative		517 (86)
CK5/6 (463)	Positive		42 (9)
	Negative		421 (91)
CK14 (444)	Positive		94 (21)
	Negative		350 (79)
EGFR (527)	Positive		77 (14)
	Negative		450 (85)
Bcl-2 (534)	Positive		290 (54)
	Negative		244 (46)
p53 (523)	Positive		105 (20)
	Negative		418 (80)

n[a] = no. of cases with data available; Mixed[b]: mixed ductal and lobular=10(2%) plus mixed Ductal and papillary or cribriform=3(0.5); Others[c]: Apocrine n= 3; Invasive papillary n=2; Lymphoma n = 1; cribriform carcinoma n = 1 and metaplastic carcinoma n = 1.

2.2. IHC

2.2.1. Staining

Staining of the TNBC TMA

Sections from the TNBC TMAs were cut at 4μm and transferred onto polysine (VWR International) slides for immunohistochemical staining. Immunohistochemical assessment was carried out on the TNBC TMA series for routinely used biomarkers. Ms Karen Scahill performed immunostaining for the routine diagnostic antibodies ER, PgR, HER-2, AR, CK5/6, p53 and Bcl-2 in the Department of Anatomic Pathology, GUH using the Ultra Ventana Benchmark automated slide staining systems according to the manufacturer's protocol. Immunostaining for EGFR and CK14 was performed by Mr. Tony O'Grady in the Department of Histopathology, Royal College of Surgeons of Ireland (RCSI), using an automated immunostainer (Bond™ III system – Leica MicroSystems™, Newcastle, U.K.) and according to the manufacturer's protocol. Standard indirect immunoperoxidase procedures were used. The list of primary antibodies, clones, source and the dilutions that were used in this work on the TNBC cohort are given in Table 2.3.

Table 2.3. Primary (routine) antibodies: sources and optimization protocol

Ab	Source	Clone	Dilution	Detection Kit	Antigen retrieval
ER	Thermo-scientific	SP1 Rabbit monoclonal Ab	1:100 for 32 minutes at 37C	Ultraview DAB	CC1[a] for 36 minutes
PgR	Leica	16/SAN27 Mouse monoclonal Ab	1:200 for 32 minutes	Ultraview DAB	CC1[a] for 64 minutes
HER-2	Roche	4B5 Rabbit monoclonal Ab	Pre-filled dilution for 12 minutes at 37C	Ultraview DAB	CC1[a] for 32 minutes
AR	Dako	AR441 Murine monoclonal Ab	1:50 for 32 minutes at 37C	Optiview DAB detection kit	CC1[a] for 34 minutes
CK5/6	Dako	D5/16 B4 Mouse monoclonal Ab	1:100 for 24 minutes at 37C	Optiview DAB detection kit	CC1[a] for 32 minutes
CK14	Leica	LL002 Mouse monoclonal Ab	1:400 for 30 minutes at 25C	Optiview DAB detection kit	ER1[c] for 20 minutes
EGFR	Leica	EGFR.25	1:100 for 60 minutes at 25C	Optiview DAB detection kit	ER2[b] for 20 minutes
p53	Roche	D0-7 Mouse monoclonal Ab	Pre-filled dilution for 32 minutes at 37°C	Optiview DAB detection kit	CC1[a] for 64 minutes
Bcl-2	Dako	124 Mouse monoclonal Ab	1:100 for 32 minutes	Optiview DAB detection kit	CC1[a] for 36 minutes

CC1[a]: Ventana Antigen Retrieval Cell Conditioning Buffer 1 (Tris/Borate/EDTA Buffer pH8.4), ER2[b]: EDTA based epitope retrieval ready to use solution (pH 9.0), ER1[c]: Citrate based epitope retrieval ready to use solution (pH 6.0)

COX-2 and iNOS staining

We selected our COX-2 and iNOS antibodies according to published data for available commercially validated antibodies.[191, 429] They were optimized for use on breast tissue, manually by Mr. M. Webber at the Discipline of Pathology, NUI Galway. Summary of antibodies, sources, clones, dilutions and optimization protocols are given in Table 2.4.

Sections of the TMA blocks of the WOI BC series were cut at 4μm and transferred onto prewashed polysine coated slides (VWR International) for immunohistochemical staining. Sections were dried overnight on a heated slide rack at 37°C, followed by incubation in an oven for 30 minutes at 60°C to liquify the wax. Then the slides were deparaffinised in xylene for 10 minutes, and subsequently hydrated by submersion in graded ethanol solutions and rinsed with distilled water. Antigen retrieval was performed as described in Table 2.4. using a Biocare NXGen Pressure Cooker, followed by administration of 2.5% horse serum for 20 minutes to block any non-specific binding sites of the antibodies. Afterwards, the appropriate concentration of the primary antibody (as mentioned in Table 2.4.) was added to the sections which were then incubated in a humidified chamber at room temperature overnight. ImmPRESS^{TM} universal reagent (Vector) secondary antibodies complex solution which is bound to peroxidise, was then put on to the sections for 40 minutes succeeded by another rinse in PBS. For the development of chromogen, ImmPACT^{TM} diaminobenzidine (DAB) (Vector) was utilised to visualise the colour reaction under the light microscope. The reaction was stopped by immersion of the slides in distilled water once the optimal staining is seen. The time needed for chromogen development was adjusted for each antibody on the first TMA slide then standardized on the rest of the TMA slides used for the same antibody. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

Table 2.4. Primary (novel) antibodies: sources and optimization protocols

Antibody	Pretreatment- antigen retrieval	Primary antibody: Manfacturer, clone, type, dilution	Incubation
COX-2	HIER Citrate buffer (pH: 6) for 15 minutes at 110C at PC	Thermo-scientific SP21 Rabbit monoclonal Ab 1:100	Overnight @ 4C
iNOS	HIER Citrate buffer (pH: 6) for 15 minutes at 110C at PC	BD transduction 6/iNOS/NOS Type II Mouse monoclonal Ab 1:100	Overnight @ 4C

PC: pressure cooker; RT: room temperature

2.2.2. Evaluation of IHC

Evaluation of staining for routine antibodies as well as additional research biomarkers on the TNBC TMA series was carried out manually by at least two observers (Dr. AS with Prof. GC and/or Dr. Helen Ingoldsby (HI)). Each was blinded to the other's results and to the clinicopathological data. A single observer (AS) scored all IHC on the series and a second observer (GC or HI) then independently reviewed ten to twenty percent of cases (randomly selected). To ensure reproducibility, scores given by both observers were compared and the Kappa coefficient calculated. Both observers (AS and GC or HI) reviewed any cases with discrepant scores together and agreed on a final score. For the tumours that had results from two or more cores, those cores were reviewed by AS again and a final score for each tumour on the array was determined by combining and averaging the results of all cores for that tumour.

For most markers, IHC was scored semi-quantitatively in which the unequivocal percentage of positive cells and the intensity of staining were recorded. The cut-off values and the categorizing scoring systems that were used to indicate positive versus negative staining were chosen in a different way for each of the markers according to the literature.

For Validation of the TNBC status, ER, PgR and HER-2 were evaluated on cases on the TMA. For ER and PgR, the percentage of tumour cells with nuclear

staining and the intensity were recorded then categorized according to the Allred scoring method.[430] The percentage of positive cells with nuclear staining was recorded then assigned a proportion score (score 0= no staining, score 1= <1%, score 2= 1-10%; score 3= 11-33%, score 4= 34-66% and score 5= > 66%) while the intensity of staining is scored on a tier of 0-3 as follows (score 0= no staining, score 1= weak, score 2= moderate; score 3= strong).[431]

ER and PgR positivity was only considered when >1% of the tumour cells show nuclear expression for those markers, regardless of the intensity result.[432]

HER-2 expression was graded as recommended by the guidelines in 2013,[433] as follows: Score 0: no staining or faint incomplete membranous staining in ≤10% of the tumour cells; 1+: faint partial membrane staining in >10% of the tumour cells; 2+: weak-or-moderate incomplete membrane staining in >10% of the tumour cells or complete circumferential intense membrane staining in ≤10% of the tumour cells; 3+: strong complete membrane staining in >10% of the tumour cells.

For the remaining markers, the percentage of positive cells (either nuclear or cytoplasmic, depending on the antibody) and the staining intensity were recorded then categorized. The cut-off values were based on those reported in the literature and/or on statistical analysis. A cut-off for positivity of 10% cytoplasmic and/or membranous staining was used for CK5/6 and CK14, similar to the threshold implemented by other basal BC studies using IHC.[286, 359, 434] Membranous stain was evaluated for EGFR immunohistochemical test and was scored in a scale (0-3) according to the scoring guidelines for HER-2 protein. However, score 2+ in addition to 3+ are regarded as positive.[286]

Nuclear expression was evaluated for AR and p53, while cytoplasmic expression for Bcl-2 was evaluated. For each of the above, the percentage of unequivocal positive tumour cells was estimated on a continuous scale (0-100%). The intensity was designated on a scale (0-3) similar to that used with ER and PgR scoring. The threshold for AR has varied in the literature but a value between 1% and 10% is most commonly reported.[435-438] We segregated the cohort into 2 subgroups: AR negative (<10% expression), and AR positive tumours (≥10%

expression). p53 was dichotomized at 10% similar to other studies done recently on BC.[439-442]

Cytoplasmic staining was evaluated for each of the COX-2 and iNOS on the WOI BC TMAs. The staining was scored as an absolute percentage from 0-100% and the intensity from 0-3. The result was then categorized according to the method used by Glynn *et al.*, 2010.[191, 429] The scoring method was as follows: intensity (0 to 3): negative, weak, moderate, strong; distribution (0 to 4): <10%, 10% to 30%, >30% to 50%, >50% to 80%, >80%. Then, the total score was divided into four subgroups: negative (0 or 1), weak (2 or 3), moderate (4 or 5), and strong (6 or 7). Of note, where there was heterogeneity of the staining intensity, the intensity present in the greatest percentage is the one recorded.

2.3. Fluorescence in situ hybridization (FISH) for HER-2

FISH was performed for the cases with equivocal results (2+) for HER-2 immunohistochemical analysis on the TMA, by Dr. Allan O'Keeffe in the Anatomical Pathology Department, GUH. The test was performed on 4 μm thick whole tissue sections using the HER-2 FISH-kit (PathVysion HER-2 06N46). FISH was scored according to ASCO/CAP guidelines (2013),[433] which reports the average HER-2/neu count to average CEP17 count and calculated ratio.[433]

2.4. Statistical analysis

The majority of statistical analyses was done by Dr. Sharon Glynn, Lecturer of Pathology, School of Medicine, NUI Galway. Descriptional statistics were done by Dr. AS.

- Comparisons between the expression of each immunohistochemical antibody, clinicopathological features and sTILs were analyzed using either by Fisher's exact test or chi-squared test.
- A *t* test was used to analyze differences between between the means in two unrelated groups.
- Kaplan-Meier estimates were plotted for survival (DFS, MFS, BCSS, OS), and the log rank test was used to assess significance. Patients who died of causes not related to BC were excluded from BCSS.
- Cox regression univariate analysis (UVA) was used to calculate an adjusted hazard ratio (HR) and to evaluate any independent prognostic effect of the variables on patients' survival with 95% confidence interval (95% CI).
- Cox regression multivariate analysis (MVA) model was used to compute HR and 95% CI, adjusting for known prognostic variables (age, menopausal status, grade, tumour size, nodal status).
- To evaluate interobserver agreement and concordance of duplicated TMA cores for each of the markers, the κ agreement statistic was calculated.
- All statistical tests were two sided and p-values less than 0.05 were considered statistically significant.
- Data analysis was performed using the Stata/SE 14 (Stata Corp, College Station, TX), GraphPad Prism 5 (GraphPad Software, San Diego, CA) and SPSS (23) statistical software packages.

3. CHARACTERISATION OF A TNBC SERIES AND EVALUATION OF PROGNOSTIC ROLE FOR HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL VARIABLES

3.1. Introduction

The aims of this work are to generate a detailed pathological and clinical characterization of TNBCs diagnosed at GUH from January 2000 to December 2015 and to identify pathological features of prognostic significance. This analysis particularly focused on the evaluation of the histological types in TNBC and their assessment against clinical endpoints.

3.2. Characteristics of the TNBC series

There were 374 TNBC tumours included in the TNBC series, from 355 patients who were managed partially or fully in GUH between January 2000 and December 2015. This included four patients who had bilateral synchronous TNBC tumours; five patients with bilateral asynchronous TNBC tumours; eight patients with multiple synchronous ipsilateral TNBCs; and one patient possessed asynchronous ipsilateral TNBC primary tumours.

Clinical details, histopathological information, treatment, follow-up and outcome status were collected. Clinical features, TNM stage and outcome of the TNBC patients are summarised in Table 3.1. Pathological characteristics for the TNBC tumours are summarised later in Table 3.2.

Table 3.1. Demographics, TNM stage and outcome of the TNBC cohort

Parameter		**TNBC All patients (n=355) n (%)**	**NACT treated patients (n=97) n (%)**	**Patients not treated with NACT (n= 258) n (%)**
Age at diagnosis	<45 years	82 (23)	39 (40)	43 (17)
	≥45 years	273 (77)	58 (60)	215 (84)
Menopausal status	Pre-menopausal	124 (35)	57 (60)	67 (26)
	Post-menopausal	215 (61)	33 (34)	182 (71)
	Unknown	16 (4)	7 (7)	9 (3)
BRCA status	BRCA-1 Mutated	12 (3)	4 (4)	8 (3)
	BRCA-2 Mutated	7 (2)	2 (2)	5 (2)
	Unspecified mutation	1 (<1)	0 (0)	1 (<1%)
	No mutation	248 (70)	74 (76)	174 (67)
	Unknown status	87 (25)	17 (18)	70 (27)
Tumour stage	0		35 (36)	0 (0)
	Is		8 (8)	0 (0)
	1		27 (28)	86 (33)
	2		12 (12)	129 (50)
	3		5 (5)	14 (5)
	4		10 (10)	15 (6)
	X		0 (0)	14 (5)
Nodal stage	0		63 (65)	151 (59)
	1		12 (12)	48 (19)
	2		13 (13)	19 (7)
	3		8 (8)	16 (6)
	X		1 (1)	24 (9)
`Nodal status	Negative	214 (60)	63 (65)	151 (59)
	Positive	116 (33)	33 (34)	83 (32)
	Unknown	25 (7)	1 (1)	24 (9)
M1 at diagnosis	No	338 (95)	93 (96)	245 (95)
	Yes	17 (5)	4 (4)	13 (5)

Table 3.1. (Cont'd)

Parameter		TNBC All patients (n=355) n (%)	NACT treated patients (n=97) n (%)	Patients not treated with NACT (n= 258) n (%)
Survival	Alive, with disease progression	15 (4)	3 (3)	12 (5)
	Alive, no disease progression	250 (7)	76 (78)	174 (67)
	Dead, disease progression	74 (20)	17 (17)	57 (22)
	Dead, unknown cause	9 (3)	1 (1)	8 (3)
	Dead, other type of cancer	7 (2)	0 (0)	7 (3)
New events*	Local recurrence	17 (5)	1 (1)	16 (6)
	Distant recurrence	46 (13)	10 (10)	36 (14)
	Local and distant recurrence	14 (4)	6 (6)	8 (3)
	Non-TNBC breast cancer	3 (1)	1 (1)	2 (<1)
	Other cancer	9 (3)	0 (0)	9 (3)
Number of recurrence events/time	At 1 year	31 (9)	7 (7)	24 (9)
	At 3 years	65 (18)	15 (15)	40 (16)
Number of distant metastases events/time	At 1 year	18 (5)	6 (6)	12 (5)
	At 3 years	53 (15)	15 (15)	38 (15)
Number of deaths/time	At 1 year	23 (6)	5 (5)	15 (6)
	At 3 years	60 (17)	14 (14)	38 (15)
Number of BC-specific deaths/time	At 1 year	20 (6)	15 (15)	5 (2)
	At 3 years	52 (15)	38 (39)	14 (5)
Site of distant metastases	Bone	37 (10)	7 (7)	30 (12)
	CNS	36 (10)	7 (7)	29 (11)
	Liver	33 (9)	5 (5)	28 (11)
	Lung	33 (9)	7 (7)	26 (10)
	Pleura/peritoneum	18 (5)	3 (3)	15 (6)
	Soft tissue	18 (5)	4 (4)	14 (5)
	Distant LN	37 (10)	11 (11)	26 (10)
	Unknown	9 (3)	1 (1)	8 (3)

* Data for 10 cases were unavailable

3.2.1. Age and menopausal status

The median age of diagnosis was 55 years (range: 24-92 years), which is consistent with other studies (51-58 years).[443-445] TNBC was diagnosed in young females 'before the age of 35 years' only in 6.5% (n=23), which is lower than the ranges reported in other series (22.2%[446] and 13.98%[447]).

Sixty percent (215/355) of our cohort were diagnosed at post-menopausal status. This is consistent with other studies[359, 448, 449] that reported that TNBC is slightly less common in pre-menopausal women. It was noted that NACT was given to younger patients. In the patients >45 years only a fifth (21.2%; n=58/273) underwent NACT while the rest were NACT naïve (78.7%; n=215/273).

3.2.2. BRCA mutation status

The Irish NCMG (National Centre for Medical Genetics) recommend testing for BRCA mutation if a BC diagnosis is made in a female before the age of 40 years old, bilateral disease or coexistence with ovarian cancer.[450]

BRCA mutations occur in different proportions in different ethnicities; these were detected only in 7.5% of the investigated cases in our cohort (n=20/268). This is nearly less than half of the proportion studied in Australian and Polish populations,[443] and less than a quarter of that reported in American females.[455,451] In this work, BRCA mutation carriers were diagnosed at a significantly younger age (median= 39.5 years; mean±SD= 40.72±8.449) than BRCA non-carriers (median= 55 years; mean±SD= 55.88±13.69) ($p=<0.001$; independent T-test). This corresponds with other studies.[451, 452] BRCA1 mutation was detected more often than BRCA2 in this cohort (n=12/355; 3.4% versus 7/355; 1.97%). This prevalence pattern, but in different proportions, was noted by other studies.[445, 452-455] Of the twelve BRCA1 carriers, five patients had multiple tumours (four had bilateral TNBC, while one had ipsilateral asynchronous TNBCs).

Table 3.2. Pathological characteristics for the TNBC tumours

Parameter		TNBC Clinical cohort (n=374) n (%)	Post-NACT TNBC (n=104) n (%)	NACT naïve TNBC (n= 270) n (%)
Tumour size	0 (pCR)	42 (11)	42 (40)	0 (0)
	<20	133 (36)	36 (35)	97 (36)
	21-50	152 (41)	18 (17)	134 (50)
	>50	29 (8)	8 (8)	21 (8)
	Unknown	18 (5)	0 (0)	18 (7)
Tumour grade	1	2 (<1)	0 (0)	2 (<1)
	2	68 (18)	28 (27)	40 (15)
	3	299 (80)	76 (73)	223 (83)
	Unknown	5 (1)	0 (0)	5 (2)
Tubule formation	1	3 (<1)	0 (0)	3 (1)
	2	27 (7)	8 (8)	19 (7)
	3	338 (90)	95 (91)	249 (90)
	Unknown	6 (2)	1 (1)	5 (2)
Nuclear Pleomorphism	1	1 (<1)	0 (0)	1 (<1)
	2	23 (6)	9 (9)	14 (5)
	3	344 (92)	94 (90)	250 (3)
	Unknown	6 (2)	1 (1)	5 (2)
Mitotic count	1	63 (167)	26 (25)	37 (14)
	2	92 (24)	33 (32)	59 (22)
	3	212 (57)	43 (41)	169 (63)
	Unknown	7 (2)	2 (2)	5 (2)
Tumour type	Ductal	276 (74)	86 (83)	190 (70)
	Lobular	15 (4)	1 (<1)	14 (5)
	Metaplastic	30 (8)	9 (9)	21 (8)
	Medullary	7 (2)	0 (0)	7 (3)
	Atypical medullary	24 (6)	6 (6)	18 (7)
	Apocrine	14 (4)	2 (2)	12 (4)
	Others	4 (1)	0 (0)	4 (1)
	Unknown	4 (1)	0 (0)	4 (1)
LVI	Yes	114 (31)	29 (28)	85 (31)
	No	253 (68)	74 (71)	179 (66)
	Unknown	7 (2)	1 (1)	6 (2)

pCR: pathological complete response; LVI: lymphovascular invasion

3.2.3. Tumour size and nodal status

3.2.3.1. Tumour size and nodal status in chemotherapy naïve cases

For those who did not receive NACT, and for whom details of the pre-treatment tumour size and nodal status were available, the median invasive tumour size was 25mm (range: 5-110mm). This is similar to the tumour size of between 20 to 31mm reported by others.[448, 449, 456] TNBC is known to be a fast-growing tumour that acquires a larger size at presentation.[444] Only a third of the series were <20mm (n=133/374) (Table 3.2.). This is slightly more than other reports. Rakha *et al.*[359] reported that only a fifth of 281 TNBCs were <15mm and Suresh *et al.* reported that a fifth of 171 TNBCs were <20mm.[457]

UVA of pathological variables against DFS were tested using Cox proportional hazards test, in which the baseline that was used for each analysis was the lower value for the parameter being tested, i.e. pT1 for NACT-naïve patients. As expected, tumour size pT was associated with adverse outcome (DFS) (Table 3.3.). Increasing tumour size was associated with reduced DFS and with a gradational increase in the hazard ratio. T4 tumours were associated with increased likelihood of disease recurrence by a factor of 4.27 (HR= 4.27, 95% CI: 1.73-10.55, p= 0.002) (Table 3.3.). There was no significant difference in hazard of adverse outcome for T1 tumours compared to T2 tumours.

Lymph node involvement is one of the most important prognostic factors in breast cancer patients.[235, 458, 459] Lymph node status was classified according to the TNM 7th edition. Overall, the axillary status was assessed in 330/355 patients (93%), while it was unknown in 25/355 cases (7%) (Table 3.1.). The median number of the retrieved axillary nodes was 7 (range: 1-45). Axillary lymph node positivity was present in 35% (116/330) including 35% (83/234) of the chemotherapy naïve patients and 34% (33/96) of the post-NACT patients (Table 3.1). Hunag *et al.*[460] reported that 38% of 504 TNBCs had axillary metastases while Sun *et al.* reported it in 27.9% of 86 chemotherapy naïve TNBCs.[461] The median number of positive axillary nodes was 3 (range: 1-37).

UVA of nodal stage against DFS were tested using Cox proportional hazards test. The baseline that was used for each analysis was nodal stage 0 (node negative disease). As expected, the higher pathological nodal stage was a poor prognostic factor in NACT-naïve TNBC (Table 3.3). Compared to N0 tumours, N1 tumours had 0.69 increased risk of recurrence (95% CI: 0.332-1.46, p= 0.34), and N2 tumours significantly doubled the HR for DFS (HR= 2.18, 95% CI: 1.28-5.59, p= 0.009) while N3 tumours were associated with a significant five-fold increase in the risk of recurrence (HR= 45.73, 95% CI: 2.87-11.4, p= <0.001) (Table 3.3.). This reflects that the increase in the number of positive lymph nodes was associated with reduction in DFS in a step-wise fashion, with substantial risk when nodal disease affect more than three lymph nodes.

Table 3.3. Univariate analysis of the association between tumour size and nodal status with DFS in TNBC patients not treated with NACT

Variable	HR	95% CI	p value	n
DFS				226
pN1	0.69	0.332-1.46	0.34	
pN2	2.68	1.28-5.59	**0.009***	
pN3	5.73	2.87-11.4	**<0.001***	
				234
pT2	0.889	0.516 - 1.532	0.67	
pT3	2.18	0.97 - 4.9	0.059	
pT4	4.27	1.73 - 10.55	**0.002***	

p values in bold indicate statistically significant associations

MVA showed the tumour size (pT) as a significant independent predictor for MFS (HR= 1.67, 95% CI: 1.09-2.57, p= 0.018) and BCSS (HR= 2.07, 95% CI: 1.38-3.11, p= <0.001) but not for DFS (HR= 1.07, 95% CI=0.72-1.58, p=0.723) (Table 3.3.). Nodal stage also remained an independent prognostic factor for the DFS and MFS (HR= 1.77, 95% CI: 1.29-2.34, p= <0.001 and HR= 1.58, 95% CI: 1.15-2.22, p= 0.08 respectively) but not for BCSS (HR= 1.34, 95% CI: 0.94 - 1.90, p= 0.102) (Table 3.4.).

Table 3.4. Multivariate survival analysis for TNBC patients not treated with NACT

Variable	HR	95% CI	p value	n
DFS				206
Age at diagnosis	1.01	0.99-1.03	0.128	
Tumour grade	0.77	0.39-1.51	0.455	
pT stage	1.07	0.72 - 1.58	0.723	
pN	1.74	1.29-2.34	**<0.0001***	
MFS				206
Age at diagnosis	1.01	0.98 - 1.03	0.298	
pT	1.67	1.09 - 2.57	**0.018***	
pN	1.58	1.12 - 2.22	**0.008***	
BCSS				203
Age at diagnosis	1.01	0.99 - 1.04	0.154	
pT	2.07	1.38 - 3.11	**<0.0001***	
pN	1.34	0.94 - 1.90	0.102	

p values in bold indicate statistically significant associations

3.2.3.2. Tumour size and nodal status in post NACT cases (Response to NACT)

NACT is increasingly used in TNBC patients, giving outcomes equivalent to adjuvant therapy. In addition, it has many benefits with reduction in the tumour size allowing more conservative surgical excision and permission of assessment of the tumour response to treatment histologically. However, the precise sub-categorization of the different grades of pathological partial response (pPR) to NACT in this study was hindered because the reporting of response to therapy was not consistent throughout the years of the study. More recently, the post-NACT reporting became standardized with documentation of the presence of the tumour bed (including its size) and inclusion of a classification system for the grade.

For those who received NACT (n=97), a pCR was achieved in 42 cases (43%); in eight tumours, there was no residual invasive carcinoma but residual DCIS remained. Residual invasive disease was seen in 55 cases (57%). The median residual invasive tumour size was 17.5 mm (range: <1-100mm).

The survival data for those patients who received NACT was updated in March 2017 and this survival time was used for all survival analyses on these patients. UVA of ypT against survival was tested using Cox proportional hazards test, using ypT0 as the baseline for analysis. Increasing ypT size is associated with adverse DFS as highlighted by the increasing HR from 2.54 to 7.84, however significance was not attained for all ypT categories. Nonetheless, ypT4 was associated with an almost eight-fold increase in the hazard of an adverse DFS, MFS and BCSS (HR= 7.84, 95% CI=1.87 - 32.85, p=0.005; HR= 7.80, 95% CI= 1.86 - 32.67, p=0.005 and HR= 6.89, 95% CI= 1.72 - 27.60, p=0.006 respectively) (Table 3.5.).

Table 3.5. Univariate analysis of the association between tumour size post-NACT and outcome

Variable	HR	95% CI	p value	n
DFS				93
ypTis	8.66	0	1.00	
ypT1	2.54	0.63 - 10.15	0.188	
ypT2	4.44	1.06 - 18.6	**0.041***	
ypT3	5.40	0.9 - 32.37	0.065	
ypT4	7.84	1.87 - 32.85	**0.005***	
MFS				93
ypTis	1.76	0	1.00	
ypT1	2.09	0.50 - 8.77	0.312	
ypT2	3.48	0.78 - 15.57	0.103	
ypT3	5.47	0.91 - 32.78	0.063	
ypT4	7.80	1.86 - 32.67	**0.005***	
BCSS				96
ypTis	4.20	0	1.00	
ypT1	2.13	0.51 - 8.91	0.302	
ypT2	1.61	0.27 - 9.63	0.603	
ypT3	5.20	0.87 - 31.18	0.071	
ypT4	6.89	1.72 - 27.60	**0.006***	

p values in bold indicate statistically significant associations

For those who received NACT, UVA showed that post-NACT nodal status was also significantly associated with a marked reduction in survival (DFS, MFS and BCSS) (Table 3.6). Compared to ypN0 tumours, ypN3 tumours were associated with a significant increase in the risk of recurrence (HR= 17.27, 95% CI: 5.48 - 54.46, p= <0.001), distant metastases (HR= 18.75, 95% CI: 5.82 - 60.43, p= <0.001) and BC related deaths (HR= 41.99, 95% CI: 10.99 – 160.30, p= <0.001) (Table 3.6.).

Table 3.6. Univariate analysis of the association between nodal status post-NACT and outcome

Variable	HR	95% CI	p value	n
DFS				92
ypN1	3.44	1.05 - 11.21	**0.041***	
ypN2	1.99	0.54 - 7.37	0.304	
ypN3	17.27	5.48 - 54.46	**<0.001***	
MFS				92
ypN1	3.85	1.16 - 12.86	**0.028***	
ypN2	1.49	0.32 - 7.05	0.614	
ypN3	18.75	5.82 - 60.43	**<0.001***	
BCSS				95
ypN1	6.31	1.57 - 25.31	**0.009***	
ypN2	3.58	0.80 - 16.06	0.095	
ypN3	41.99	10.99 – 160.30	**<0.001***	

p values in bold indicate statistically significant associations

For UVA and MVA analysis of tumour size post NACT, the ypT categories were collapsed such that ypT0/is (i.e. cases with no residual invasive carcinoma but with or without residual in situ disease) were classified as a pCR. Any case where there was any residual disease in the breast (ypT1-4) was regarded as a non-pCR. A pCR (ypT0/pTisN0) was reported in 36 cases (37%).

On UVA, pCR was a strong prognostic factor for DFS (HR= 6.66, 95% CI: 1.54 - 28.58, p=0.011), MFS (HR= 5.90, 95% CI: 1.36 - 25.56, p= 0.018) and BCSS (HR= 10.30, 95% CI: 1.37 - 77.38, p= 0.023) (Table 3.7.).

On MVA, the strength of pCR as an important independent predictor was maintained against DFS (HR= 6.23, 95% CI: 1.54 - 28.58, p=0.018), MFS (HR=

5.08, 95% CI: 1.09 - 23.65, p= 0.038) and BCSS (HR= 8.52, 95% CI: 1.09 - 66.64, p= 0.041) (Table 3.7.).

Table 3.7. Univariate and multivariate survival analysis in NACT patients

Variable	HR	95% CI	p value	n
DFS				
Univariate Analysis				93
pCR	6.66	1.54 - 28.58	**0.011***	
Multivariate Analysis				85
pCR	6.23	1.36 - 28.50	**0.018***	
Age at diagnosis	0.99	0.95 - 1.04	0.776	
Grade	1.05	0.39 - 2.81	0.926	
Tumour type	0.64	0.27 - 1.49	0.298	
Basal phenotype	1.61	0.36 - 7.20	0.530	
MFS				
Univariate Analysis				93
pCR	5.90	1.36 - 25.56	**0.018***	
Multivariate Analysis				85
pCR	5.08	1.09 - 23.65	**0.038***	
Age at diagnosis	0.99	0.95 - 1.04	0.841	
Grade	0.85	0.31 - 2.37	0.759	
Tumour type	0.67	0.30 - 1.52	0.343	
Basal phenotype	1.47	0.32 - 6.63	0.619	
BCSS				
Univariate Analysis				96
pCR	10.30	1.37 - 77.38	**0.023***	
Multivariate Analysis				88
pCR	8.52	1.09 - 66.64	**0.041***	
Age at diagnosis	1.01	0.96 - 1.06	0.714	
Grade	1.14	0.40 – 3.26	0.813	
Tumour type	0.77	0.40 - 1.49	0.441	
Basal phenotype	1.32	0.29 - 6.07	0.719	

* Treatment with platinum was included in the MVA model against DFS (HR= 0.68, 95% CI: 0.18 - 2.56, p= 0.566), MFS (HR= 0.69, 95% CI: 0.18 - 2.65, p= 0.590) and BCSS (HR= 0.310, 95% CI: 0.04 - 2.50, p= 0.272). p values in bold indicate statistically significant associations

3.2.4. Tumour grade

As expected, the majority of the TNBC tumours (n=299/374; 80%) were grade 3. Grade 1 was occasionally seen (n=2/374; <1%) (Table 3.2.). This is consistent with other reports.[359, 462, 463]

In the TNBC cohort, there were 104 tumours from 97 patients who had an excision after NACT. In 40 of these, it was possible to assess tumour grade before and after NACT. In the remainder, there had either been a pCR, or the residual tumour after treatment was too small to grade, or the biopsy had been done in another centre. The overall grade did not change in 24 tumours (60%). The grade was lowered in 13 cases (32.5%), while raised in three cases (7.5%). These changes were due to a change in mitotic count score in all cases, which might be related to either the anti-proliferative chemotherapeutic effect or the unrepresentative biopsy for the mitotic count within the whole tumour. For statistical analysis, the final grade used was taken from the pre-treatment biopsy assessment. As the numbers of grade 1 tumours were very few, they were grouped with grade 2 for statistical analysis.

On Cox regression analysis, there was no association between tumour grade and outcome (Appendix III), which was also observed by others.[358, 359] The association between the components of tumour grade and outcome were examined independently (Appendix III). Nuclear pleomorphism was marginally associated with adverse DFS (Grade 3 vs. Grade 1 and 2, HR= 0.402, 95% CI 0.055-2.89, p = 0.055) but there was no association between either tubule formation and mitotic score and DFS (Appendix III).

3.2.5. Histopathological tumour type

Previous studies highlighted that the morphological subtype of the TNBC tumour has an important prognostic role.[324, 319, 464] Medullary carcinomas are recognized by their good outcome,[211] while metaplastic carcinomas are known to have poor response to chemotherapy and worse outcome.[328] Salivary gland-type tumours which arise in the breast are TN and have excellent prognosis.[346] bACC had been reported to have 100% 5-year survival.[346]

The histological subtypes were reviewed. There was no case of adenoid cystic carcinoma (ACC) or secretory carcinoma in our series. As reported, the most common histological type was IDC-NST (81.5%), followed by metaplastic carcinoma (5%) and lobular carcinoma (5%). While the medullary (3%), apocrine (3%) and atypical medullary carcinoma (1%) were less common. Four cases were categorised as 'other type' for statistical purposes, as only one case belonged to each of the following categories: mixed ductal and lobular, papillary, micropapillary and mixed ductal and micropapillary.

Upon review, tumour type was changed in 40 cases (Figure 3.1.). Twenty nine cases that were typed as IDC-NST were re-typed to apocrine (n=4); medullary (n=3); atypical medullary (n=13) and metaplastic (n=11). Three ILCs were re-typed as ductal (n=2) and metaplastic (n=1). Seven medullary carcinomas were re-typed into atypical medullary. None of the metaplastic carcinomas or the 'other types' was changed.

After review, the most common histological subtype in the TNBC cohort remained the IDC-NST (73.79%) and metaplastic carcinoma (8%). The atypical medullary constituted 6.4% (n=24) while the lobular and apocrine subtypes showed nearly similar incidence (4%, n=15 and 3.7%, n=14 respectively). The typical medullary carcinoma comprised only 1.8% (n=7) (Figure 3.2.).

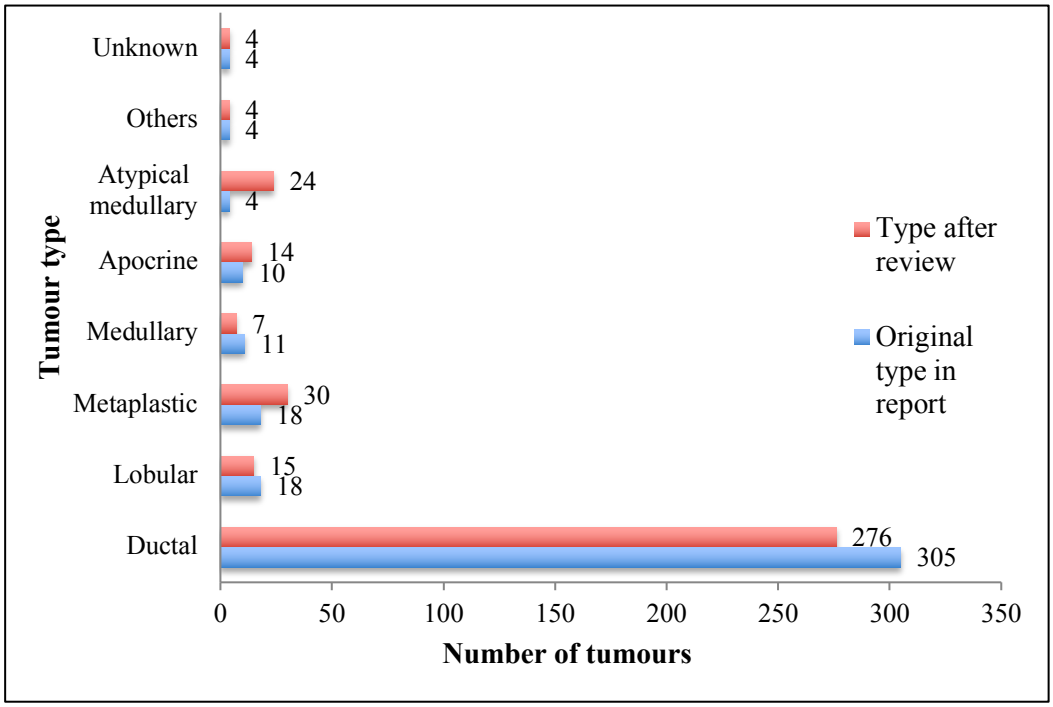


Figure 3.1. Re-classification of tumour type

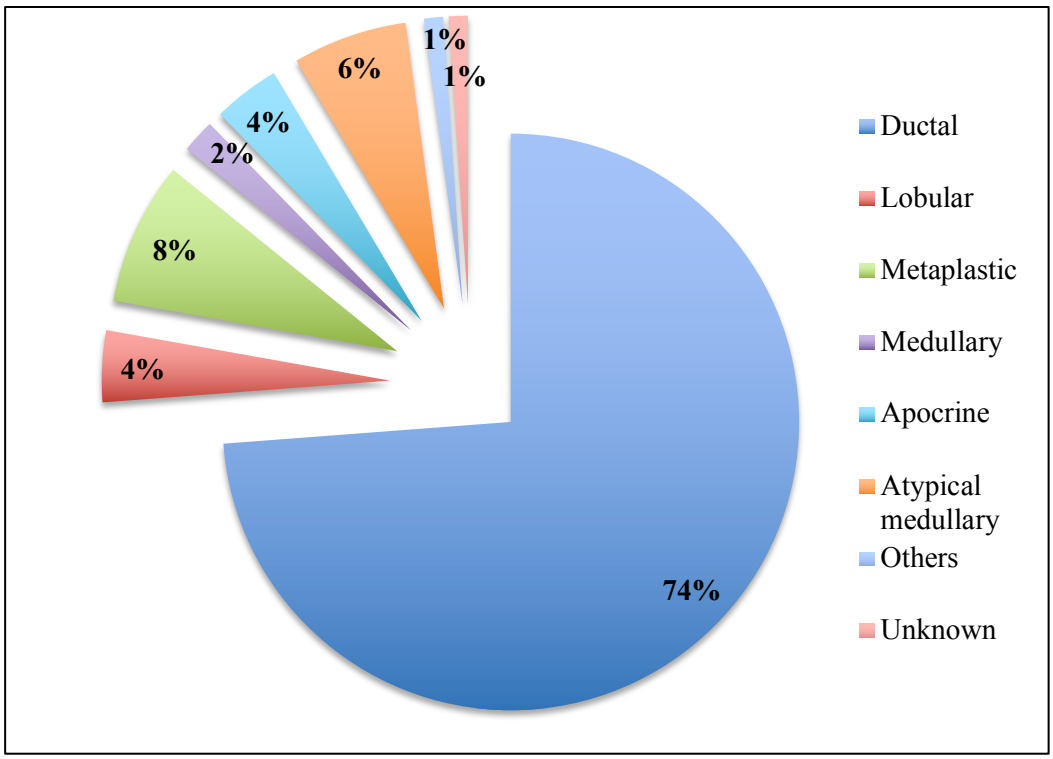


Figure 3.2. Distribution of histologic tumour types in the TNBC cohort

3.2.5.1. Prognostic value of the histological subtype

Statistical analysis was done on the final reviewed histological subtype. UVA using the log rank test and Kaplan-Meier survival curves was done to estimate the cumulative survival rates according to the histotypes. The tumour type showed a significant influence on the DFS and a borderline effect on OS (p= <0.001 and p=0.04 respectively). Invasive lobular carcinomas showed the maximum risk of recurrence while medullary carcinomas had the minimum risk of recurrence (Figures 3.3. and 3.4).

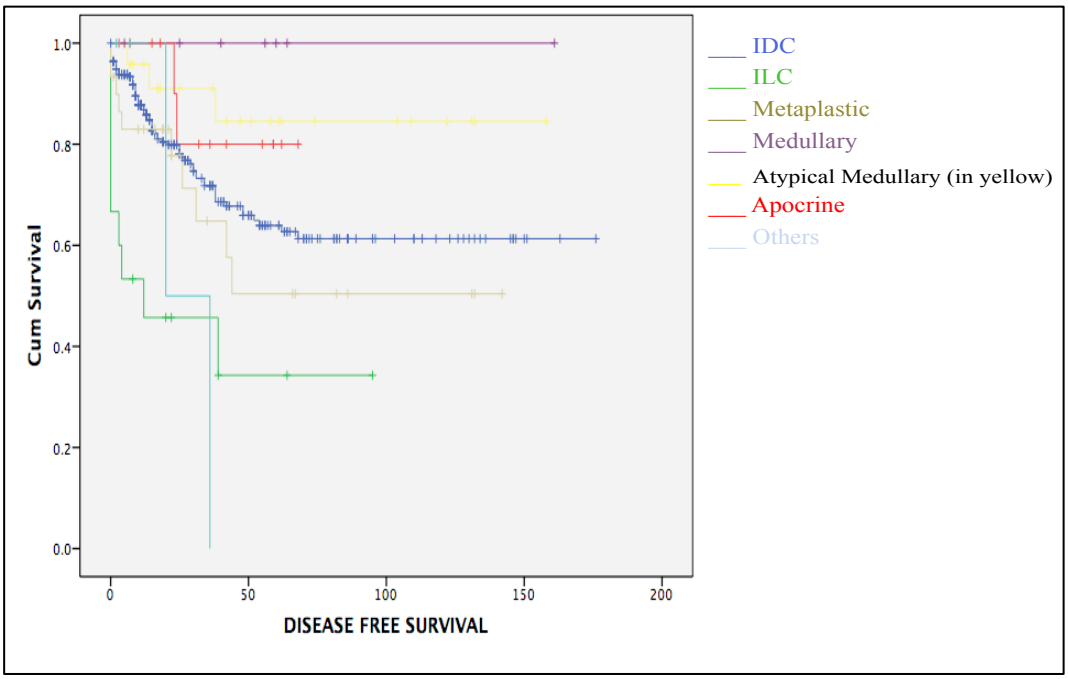


Figure 3.3. Kaplan Meier curve for DFS in TNBC according to the final histological subtypes

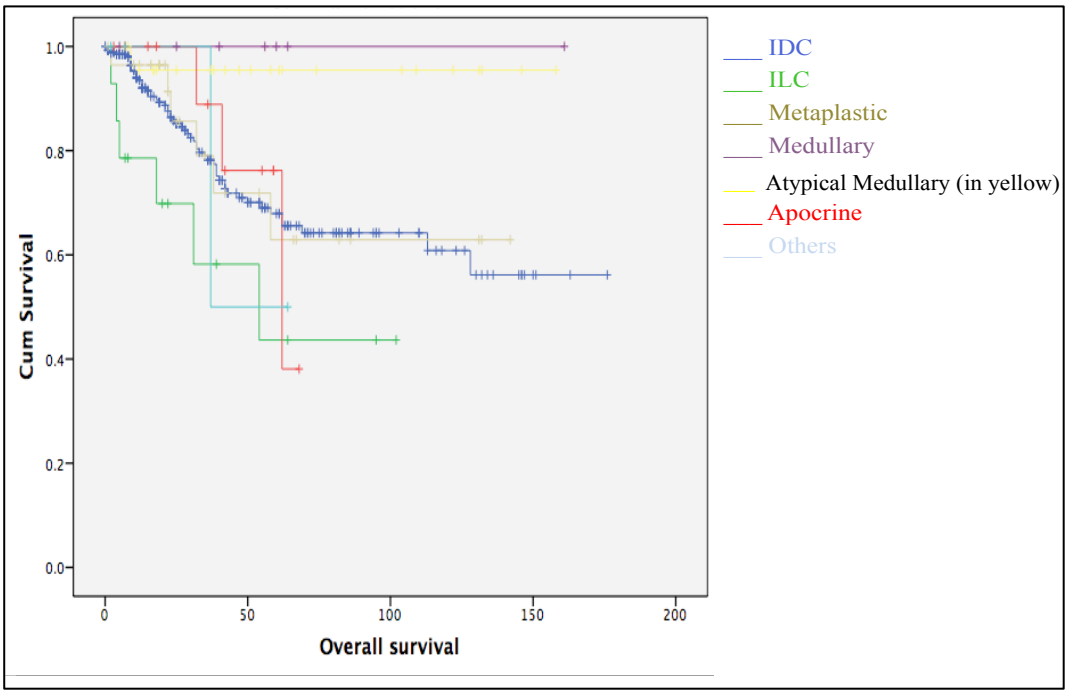


Figure 3.4. Kaplan Meier curve for OS in TNBC according to the final histological subtypes

3.2.5.2. Special histologic subtypes of TNBC

3.2.5.2.1. Medullary-like carcinoma

Medullary carcinomas represent less than 5% of all invasive BC.[211, 330] The diagnosis requires the presence of a lymphoplasmacytic infiltration, microscopic circumscription, syncytial growth pattern >75%, and grade 2 or 3 nuclei,[332, 333] and has poor interobserver reproducibility.[465, 466] Recently, the NHS guidelines recommend using a single term 'Invasive carcinoma with medullary features' for reporting classical medullary, atypical medullary and IDC with medullary characteristics as they mostly represent a continuum of a spectrum.[331] Although most of the studies showed that medullary carcinoma has a good prognosis,[332, 333] some authors reported a negative predictive effect.[467, 468] A significant proportion of the diagnosed medullary carcinomas are TNBC, while approximately 30% show hormone-receptor positivity and nearly 10% show HER-2 overexpression.[211]

As the diagnosis of medullary carcinoma and its subtypes depends on the assessment of the entire tumour, all the available tumour slides were reviewed. The median number of slides examined per case was 3 (range, 1-8).

The following morphological features were assessed:

- The intensity of tumoural lymphocytic inflammation was assessed on a semi-quantitative scale as absent, mild, moderate or marked.
- The growth pattern whether syncytial or not, and if any tubular differentiation present.
- The nature of the margin whether circumscribed, pushing or infiltrative.
- Presence of a fine fibrotic band surrounding the tumour.

This result of the review of the 32 cases resulted in a re-classification of tumour type as follows;

- The diagnosis changed from IDC-NST to atypical medullary in 13 cases.
- The diagnosis changed from IDC-NST to medullary carcinoma in 3 cases.
- Four cases originally reported as medullary were reclassified as atypical medullary.
- A diagnosis an atypical medullary carcinoma in four cases that were treated with NACT and had a pCR was not changed.

The median age of the patients with medullary carcinoma was 56 years (range: 51-76 years), six were postmenopausal and none of them was a BRCA mutation carrier. The median size of the invasive tumour was 27mm (range 8 to 43 mm). Only one tumour was associated with DCIS. None of the cases had lymphovascular invasion or regional lymph node involvement. With a median follow-up period of 56 months (range: 5-161 months), there were no recorded distant metastases or deaths.

Twenty-four tumours were categorized as atypical medullary carcinoma. The feature that commonly precluded a diagnosis of medullary carcinoma was the presence of an infiltrative border. The median age was 49.5 years (range: 24-77 years), and half of them were postmenopausal (n=12/24). Five (20.8%) patients were carriers of BRCA mutation, of which four patients had BRCA1 mutation and one patient had BRCA2 mutation. The BRCA mutation carriers acquired the neoplasm at a younger age than BRCA non-carriers (median 44 years versus 52 years). The median size of the atypical medullary tumours was 24 mm (range 0 to 60 mm). One third of all cases (n=8/24) had associated DCIS. Lympho-vascular invasion was seen in four tumours and regional lymph node involvement was recorded in five patients. A quarter of the patients (n=6/24) received NACT and three had a pCR. The median follow-up period was 49 months (range: 7-158 months). Distant metastases occurred only in one patient (BRCA non-carrier), six months after the initial diagnosis.

3.2.5.2.2. Basaloid carcinomas

Basaloid morphology is described in several neoplasms, in which the tumour cells portray dark enlarged nuclei and scant cytoplasm.[469, 470] The differential diagnosis for the cytohistological basaloid features in invasive carcinoma in the breast includes adenoid cystic carcinoma (solid type), IDC with basaloid features, small cell carcinoma, endocrine tumour and solid variant of papillary carcinoma. Tumours originating from salivary glands commonly incorporate ductular structures with biphasic cell lining, however they are less frequently seen in the solid variant of ACC,[471] which also lacks the characteristic cribriform growth pattern. Whether these tumours should be categorised as a separate entity is still debatable, however reports suggest that the mammary solid ACC displays better survival rates than IDC-NST and worse than typical ACC.[471]

TNBCs with this morphology were characterised in order to ascertain if this type of carcinoma had clinical relevance. On review of the six cases, all exhibited a basaloid cytomorphology with solid or trabecular growth pattern. All of the six tumours were devoid of ductular structures lined with dual cell population. Necrosis was only seen in one tumour. Three of the cases were associated with high grade DCIS. These tumours ranged in size from 21 to 44 mm (Median: 28.5

mm). The median age was 54 years (range: 33-69 years); none was in BRCA-mutation carriers. The median follow-up period was 26 months (range: 9-48 months) and lymph node metastases were detected in two cases, which subsequently attained distant disease progression, with a median DFS of 34 months. One of those patients died 39 months after diagnosis. The other five are alive, four with no evidence of disease progression at a median follow-up of 13 months.

3.3. Patient management

Although it is now accepted that TNBC should not be treated in a uniform fashion, yet the cornerstones of treatment are a combination of surgery, chemotherapy and radiotherapy. The treatment plans for the TNBC series are shown in Table 3.8.

Table 3.8. Treatment plans for the TNBC series

Parameter		**TNBC All patients (n=355) n (%)**	**NACT treated patients (n=97) n (%)**	**Patients not treated with NACT (n= 258) n (%)**
Surgery	Yes	340 (96)	97 (100)	243 (95)
	No	12 (3)	0 (0)	12 (4)
	Unknown	3 (<1)	0 (0)	3 (1)
Chemotherapy	NACT	97 (27)	97 (100)	0 (0)
	Adjuvant	174 (49)	6 (6)	168 (65)
	None	68 (19)	0 (0)	68 (26)
	Unknown	23 (6)	1 (1)	22 (9)
Adjuvant radiotherapy	Yes	252 (71)	81 (84)	171 (66)
	No	68 (19)	13 (13)	55 (21)
	Unknown	35 (10)	3 (3)	32 (13)

3.3.1. Surgical treatment

The majority of patients (96%, n= 340/355) underwent surgical treatment (Table 3.8.), that was either breast conserving therapy (BCT), BCT followed by mastectomy or an initial mastectomy. The final therapeutic surgical procedure was documented, i.e. if a patient underwent a wide local excision followed by mastectomy then it is recorded as a mastectomy. Fifty-eight percent (197/340) of the patients had BCT, forty-one percent (141/340) had mastectomy and the data for the surgery type for two patients was unavailable.

Surgery was not performed in twelve patients (3.38%), including seven patients (1.97%) who presented with metastatic TNBC; one patient (0.28%) with an associated lung cancer; one patient (0.28%) who opted for no treatment; and four patients (1.12%) where documentation for the reason of no surgery was unavailable.

In total, 348 surgical procedures were done. Two-hundred breast conservation surgeries were performed and one hundred forty eight mastectomies were performed. Of note, nine patients had synchronous or asynchronous bilateral or multifocal tumours and had multiple surgeries. From 2004 onwards, there was a shift from mastectomies in favour of the more conservative wide local excision as shown in Figure 3.5.

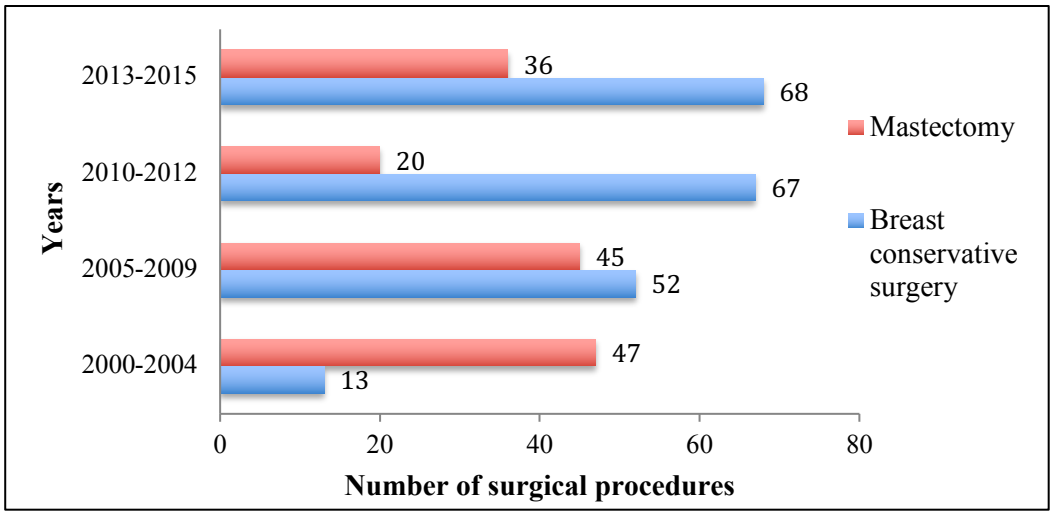


Figure 3.5. The final therapeutic surgical procedures rates for the TNBC cohort

3.3.2. Management of the axilla

Axillary lymph nodes were conventionally assessed by axillary lymph node dissection.[472] However many complications have been documented following this type of intervention such as lymphoedema and neuropraxia.[472] The advancements of the lymphoscintigraphic techniques supported the hypothesis of the sentinel lymph node concept.[473] Sentinel lymph nodes (SLNs) are the initial lymph nodes that receive lymphatic drainage from the primary tumour. Thus, in the presence of tumour nodal spread, they are the most likely to be involved by metastases.[473] The choice of the axillary surgery depends on the clinical and radiological presentation of the patient's axilla. In recent years, the minimally invasive axillary sentinel lymph node excisional biopsy (SLNB) has been favoured. This shift was particularly augmented after the validation of SLNB as a reliable axillary mapping technique and non-inferior predictor of the disease prognosis. Z11 randomized study was done on early stage BC (T1-2) and showed that axillary lymph node dissection does not add beneficial effect in patients with low axillary burden (fewer than three affected nodes) detected by SLN excision.[474] Therefore, we aimed to analyse the changing practice of management of the axilla in patients with TNBC over the time period of this study documented.

3.3.2.1. Diagnostic nodal assessment procedures

Upfront nodal assessment was performed in 26% (94/355) of patients. A summary of the diagnostic nodal assessment procedures and their results is presented in Table 3.9. The most common technique used was axillary lymph node needle core biopsy (ALN NCB) (55%; n=52/94) either alone or combined with another technique (Table 3.9.). A diagnostic axillary procedure was performed in 26% (93/355) of patients. Two-thirds of these patients (62/93) had axillary metastases (Table 3.9.). Follow-up of the cases who had a positive result on diagnostic lymph node procedure is summarized in the Appendix IV. Follow-up axillary procedure was done for 59 out of 62 patients (95%) with a positive diagnostic axillary procedure. Thirty-seven cases (63%) where there was positive nodal disease on diagnostic procedure were positive, while twenty-two cases (37%) were negative and 15 (68%) of these had NACT (Appendix IV).

Table 3.9. Diagnostic nodal assessment procedures

Lymph node assessment	Number (%)	Result	
		Positive	Negative
None	261 (73)	-	-
ALN NCB	49 (14)	40	9
ALN FNAC	26 (8)	14	12
ALN NCB & FNAC	2 (<1)	1	1
ALN NCB & SLN excision	1 (<1)	1	0
SLN excision	13 (4)	5	8
SLN excision & ALN excision	2 (<1)	1	1
SCLN FNAC	1 (<1)	1	0
Total	355 (100)	63	31

ALN NCB: axillary lymph node needle core biopsy; ALN FNAC: axillary lymph node fine needle aspiration cytology; ALN excision: axillary lymph node excisional biopsy; SLN excision: sentinel lymph node excision; SC FNAC: supraclavicular fine needle aspiration cytology.

Mapping of the major axillary diagnostic procedures used across the course of the study is shown in Figure 3.6. Upfront nodal assessment was rarely done before the year 2004. While from 2004 onwards, there was a gradual increase in favour of performing axillary nodal NCB after 2004 and axillary lymph node fine needle aspiration cytology (ALN FNAC) from circa 2009.

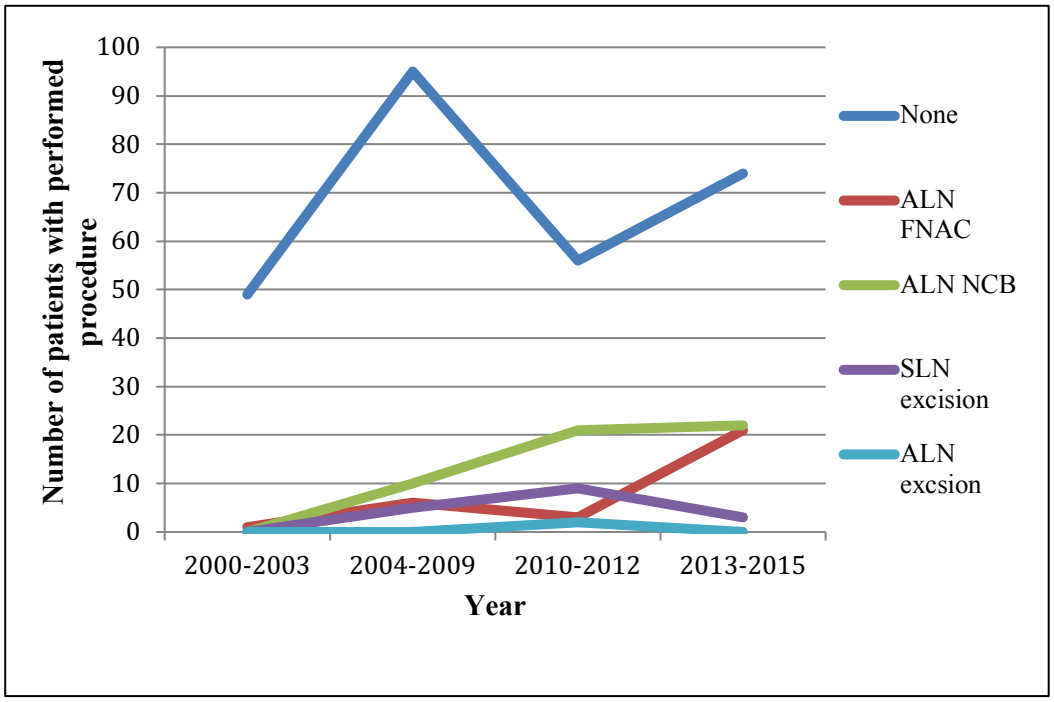


Figure 3.6. Diagnostic lymph node procedures across the years of the study

ALN FNAC: axillary lymph node fine needle aspiration cytology; ALN NCB: axillary lymph node needle core biopsy; SLN excision: sentinel lymph node excision; ALN excision: axillary lymph node excisional biopsy**.**

The positive predictive value for ALN NCB was slightly higher than that for ALN FNAC (97.5% versus 92.85%). In summary, our data highlights the beneficial value of the upfront diagnostic LN sampling and confirms that the incidence of inaccuracies, using NCB and FNAC, is very low (3.5%; n= 2/57) (Appendix IV).

3.3.2.2. Sentinel node biopsy practice

SLN excision was performed in 179/355 patients (50.4 %). In 17 cases (9.45%), it was done at the same time as the diagnostic procedure but the majority (90%, 162/180) were performed with the therapeutic procedure for the primary tumour. For those who had a SLN excision, the median number of SLNs sampled was 3 (range: 0-12). SLN metastases were reported in 24 of 179 patients (13.4%). The SLN nodal burden was predominantly low with <3 LN affected (n=22/24; 91.6%); and the median number of positive SLN was 1 (range: 1-4). Five patients (2.79%) had isolated tumour cells and were classified as negative (TNM 7th edition).

The subsequent management and results for the cases with positive SLN are illustrated in Figure 3.7. Out of the twenty-four cases with metastatic SLN, axillary clearance was done for nineteen patients (79.2%), while no further management was done for the remaining five cases (20.8%). Half of the cases who underwent axillary clearance (9/18) had residual axillary disease (Figure 3.7). With a median follow up of 43 months, five of those nine cases acquired recurrence (median DFS= 36 months) and three of them died (median OS = 43 months). On the other hand, no events (recurrence or death) occurred in the nine cases with negative axillary clearance or in the four cases with minimal nodal burden who had no further management.

Although our numbers are small and statistical analysis was not done, our retrospective results suggest that the SLN is beneficial in the management of TNBC, and that axillary dissection can be avoided in cases where the SLN disease burden is low.

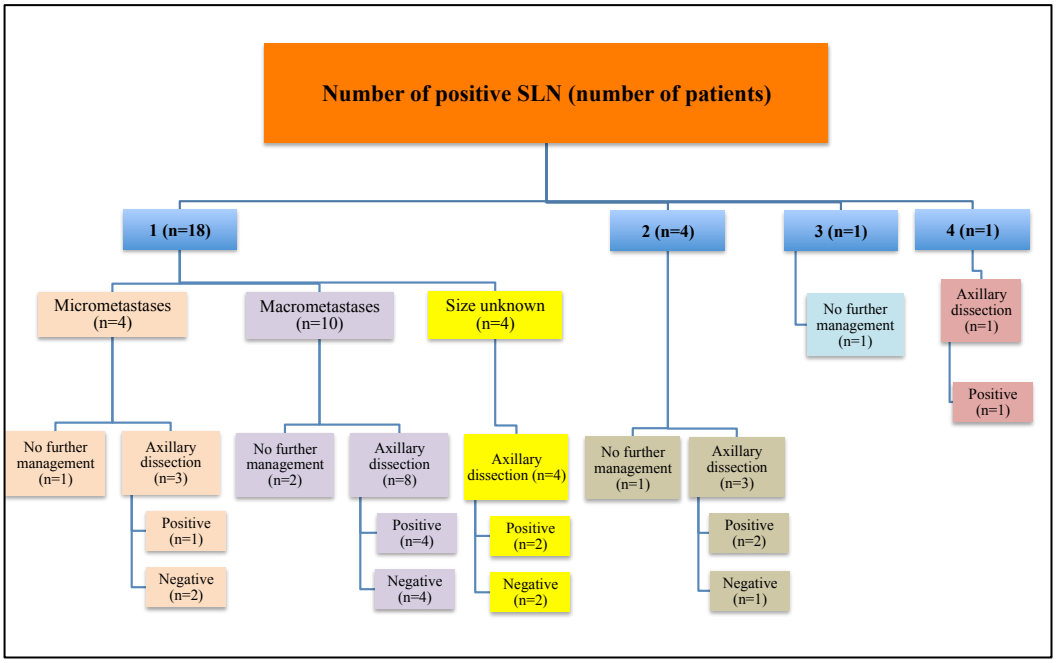


Figure 3.7. Management of cases with positive sentinel lymph nodes

n= number of the patients who had the specified amount of positive lymph nodes.

3.3.3. Adjuvant Radiotherapy

Adjuvant radiotherapy is usually given post-mastectomy in high-risk patients such as with tumours >5cm (pT3), metastatic axillary disease and/or presence of lymphovascular invasion.[421, 422] Ninety-one percent (181/197) of cases received adjuvant radiotherapy following BCT. Post-mastectomy radiotherapy was given in 69/141 (49%) cases. In keeping with the ESMO guidelines[422], it was given on the basis of high stage in 33.33% (23/69), positive axillary nodes in 37.68% (26/69) and the presence of lymphovascular invasion in 1.45% (1/69); while, the reason was unclear in the remaining 19 cases (27.5%).

3.3.4. Chemotherapy

In the TNBC series, nearly half of the patients received adjuvant chemotherapy (168/355, 47.32%) and a quarter of the patients received NACT (97/355, 27.32%). A minority (6/355, 1.69%) received both neoadjuvant and adjuvant therapy. One fifth of the patients (68/355) did not receive chemotherapeutic treatment, and details of the chemotherapy regimen was not available for the remaining 22 cases (6.2%).

Where adjuvant therapy was given, taxane was the most common drug used, either alone (64/168; 38.1%) or in conjunction with anthracycline (60/168; 35.71%). Anthracycline alone was used in 17.85% (30/168).

From 2004 onwards, there has been a gradual increase in the use of the NACT, with a noticeable shift from adjuvant chemotherapy in favour of the NACT in 2010 as shown in Figure 3.8.

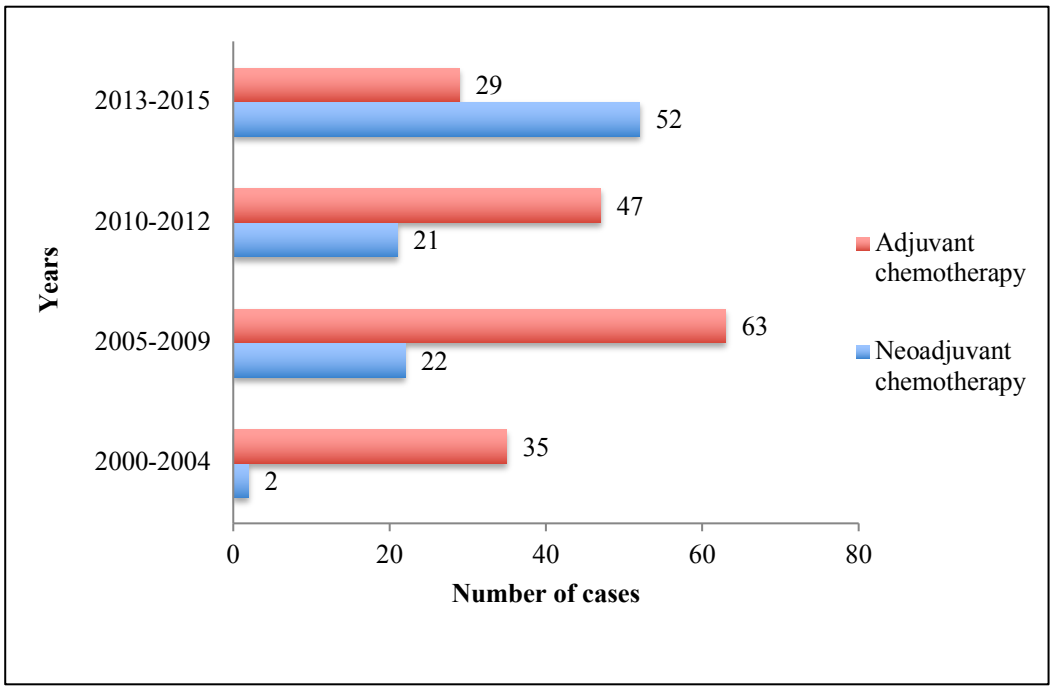


Figure 3.8. Pattern of chemotherapy administration for the TNBC

3.3.4.1. NACT

The clinical features of the TNBC patients who received NACT are summarised in Table 3.1. The median age was 48 years (range: 24 -73 years) and was lower than the age of those who did not received chemotherapy (median: 58.5; range: 29 - 92). The pathological features of the TNBC tumours (n=104) for the patients who underwent NACT are summarised in Table 3.2. In cases with residual invasive tumour, the median tumour size at resection after NACT was 17.5 mm (range, <1 – 100 mm). The most common type was ductal (n=93/104, 89.42%), followed by metaplastic subtype (n=5/104, 4.8%). The majority of the tumours were grade 3 (n=76/104, 73%), and the remainder of the tumours (n=28/104, 27%) were grade 2.

Most patients who received NACT (n=80/97, 82.4%) were given combination anthracycline and taxane therapy, and carboplatin was given in addition to 24 (24%) patients who were diagnosed from 2014 onwards. Data for the regimens used for the remaining 17 (17.6%) patients was not available at the time of this analysis. The median follow-up was 30 months (range, 3 - 126 months). Three patients who received NACT were metastatic at diagnosis. A further twenty (20.6%) patients experienced disease recurrence and one patient developed a new non-TNBC primary. Overall, nineteen (19.6%) patients died over the course of the follow-up.

3.3.4.2. Nodal status in NACT treated cases

The axillary status was assessed in 96/97 patients (99%). Two-thirds of the cases (63/79) had negative lymph nodes, while one third of the cases (33/97) had node positive disease either at pre-treatment or post-treatment assessment (Table 3.1).

Fifty-three out of the ninety-seven patients (55%) had pre-treatment axillary evaluation. Two-thirds of these cases (37/53) were positive for metastatic carcinoma. Post NACT axillary sampling confirmed metastatic disease in half of them (17/37) with treatment response seen in seven of these positive cases. For those with positive pre-treatment axillary evaluation and negative post NACT axillary sampling (20/37), chemotherapy effect was seen in 13 cases (13/20; 65%).

3.4. The outcome in the TNBC cohort.

At a median follow-up of 42 months, 74 patients (21%) died from their disease, including the 13 patients who were metastatic at diagnosis (Table 3.1.). Two-thirds of the BC-specific fatalities (52/74) occurred in the first three years after TNBC diagnosis, with a quarter of the deaths (20/74) happening in the first year (Table 3.1.). The 3-year BCSS was 85% (303/355). This is not far from other studies, which showed that up to 70% of the deaths in TNBC occur in the first five years after diagnosis.[295, 322]

A quarter of the patients, excluding those who presented with stage IV disease, experienced local and/or distant metastases (77/338) (Table 3.1.). The majority of these events (65/77; 84%) occurred in the initial three years after diagnosis. More than half of the cases that acquired distant disease progression developed it at multiple sites. Bone, distant lymph nodes and CNS were the most affected sites (Table 3.1.). Other studies have shown a propensity of TNBC metastases to the viscera, brain and lungs.[267, 270, 274] Excluding the patients who presented with stage IV disease, the 3-year DFS was 80% (273/338).

The outcome for patients with TNBC is similar to that reported for other series. The pattern of relapse is similar to the pattern reported for TNBCs with relapse occurring as metastatic disease as opposed to local recurrences, followed by a short time from first recurrence to death.[295]

3.5. Expression of the biomarkers in TNBC

One of the goals of this work is to evaluate different markers that might be of prognostic importance and/or involved in the biological pathways of TNBCs. We examined six markers: EGFR, CK5/6, CK14, AR, p53 and Bcl-2. The scoring method and the cut-off values are detailed previously (Materials and methods, section 2.2.2.). The overall number of positive and negative cases for each marker is shown in Table 3.10.

Table 3.10. Expression of biomarkers in TNBC series

Biomarker (n[a])		**n (%)**
AR	Positive	31 (12)
	Negative	227 (88)
EGFR	Positive	108 (45)
	Negative	134 (55)
CK5/6	Positive	85 (34)
	Negative	163 (66)
CK14	Positive	52 (22)
	Negative	188 (78)
p53	Positive	146 (60)
	Negative	94 (40)
Bcl-2	Positive	78 (32)
	Negative	165 (68)

3.5.1. AR in TNBC

Previous work by Burstein *et al.*[351] and Lehmann *et al.*[326, 352] identified the luminal androgen receptor subtype by gene expression analysis during the attempts to sub-classify TNBC. It represents a small proportion of the TNBC. However given the fact that TNBC lacks targeted therapy and that anti-androgen receptor therapies had been used successfully in prostatic cancer, this may have potential as an effective treatment for this small subgroup.

In our study, AR was assessed in 270 TMA tumour cores from 258 patients. With a cut-off of 10%, AR was positive in 31 (12.02%) and negative in 227 (87.98%) cases. This is in agreement with other studies. The published data has variable frequencies of expression of AR in TNBC ranging from 12.6% to 51.7%[359, 475-477] (Table 3.12.), however this may be partially influenced by the inconsistent cut-off thresholds for positivity (1% versus 10%), the variability of the applied anti-androgen antibodies as well as the variations in studied populations. Association between the clinicopathological features and AR was tested by the Chi squared test (Table 3.11.). AR effect on survival was tested using the COX regression test.

Table 3.11. Association between AR expression and clinicopathological features in TNBC

Parameter		AR positive (n=31) n (%)	AR negative (n= 228) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	3 (4)	79 (96)	82	8.20	**0.004***
	Post-menopausal	27 (16)	143 (84)	166		
	Unknown	1 (14)	6 (86)	7		
Tumour grade	1	0 (0)	1 (100)	1	15.84	**<0.0001***
	2	12 (32)	26 (68)	38		
	3	19 (9)	197 (91)	216		
Tubule formation	1	0 (0)	2 (100)	2	2.75	0.252
	2	5 (23)	17 (77)	22		
	3	26 (11)	205 (89)	231		
Nuclear Pleomorphism	1	0 (0)	1 (100)	1	4.56	0.102
	2	4 (31)	9 (69)	13		
	3	27 (11)	214 (89)	241		
Mitotic count	1	12 (35)	22 (65)	34	24.25	**<0.0001***
	2	9 (17)	44 (83)	53		
	3	10 (6)	158 (94)	168		
	x	1 (20)	4 (80)	5		
Tumour type	Ductal	19 (9)	188 (90)	207	35.54	**<0.0001***
	Lobular	5 (29)	12 (71)	17		
	Metaplastic	1 (8)	11 (92)	12		
	Medullary#	1 (10)	9 (90)	10		
	Apocrine	5 (83)	1 (17)	6		
	Others	0 (0)	2 (100)	2		
Tumour stage (pT)	1	7 (9)	70 (91)	77	2.19	0.533
	2	15 (12)	107 (88)	122		
	3	3 (21)	11 (79)	14		
	4	2 (18)	9 (82)	11		
Nodal stage (pN)	0	13 (9)	127 (91)	140	6.35	0.095
	1	5 (11)	38 (89)	43		
	2	4 (22)	14 (78)	18		
	3	4 (29)	10 (71)	14		
	x	1 (11)	8 (98)	9		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

AR positivity was associated with post-menopausal status (p= 0.004), lower-grade (p= 0.004) and slowly proliferative tumours (<0.0001) (Table 3.11.). Previous studies had also depicted the AR unregulated BC, across all subtypes, is more likely to be associated with older age at diagnosis[478, 479] and lower nuclear grade.[480, 481] Similarly, in TNBC, a high AR level is mostly seen in postmenopausal[438, 477] or older age women.[435, 476, 482] Our results support the concept that quadruple negative BC (AR negative TNBC) have more aggressive features. Likewise, there is an agreement in most of the published literature that, in TNBC, the AR expression is inversely correlated to the grade[359, 435, 438, 475-477, 481] and proliferation index.[476, 483] Confirmatory to our data, a meta-analysis by Wang *et al.* for 2826 TNBCs showed that AR presence paralleled the postmenopausal status and lower tumour grade.[484] On the contrary, Hu *et al.* recently published an odd observation, that AR positive TNBC was associated with high grade and low proliferation index.[437]

As expected, AR expression was significantly associated with the histological morphology of apocrine carcinoma (<0.0001) (Table 3.11.). Our results confirmed those by Choi *et al.* who observed that 77.8% of the tumours with apocrine morphology express AR positivity.[435] Similarly, Safarpour *et al.* reported that 55% of AR positive TNBC displayed classic apocrine morphology.[485]

COX regression analysis showed that AR expression in TNBC is not associated with DFS (HR= 1.263, 95% CI: 0.665 - 2.405, p=0.475). There is controversy in the literature regarding the AR impact on outcome. Some studies[359, 437, 438, 477, 486], but not all[435, 482, 483], recognized AR as an independent prognostic indicator of better survival. McGhan *et al.*[482] and Pistelli *et al.*[483], analogous to our research, did not find an association between AR positivity and survival rates. On the contrary, Choi *et al.* reported that AR expression was associated with increased probability of death, yet not associated with disease recurrence.[57] Wang *et al.* meta-analytical study of 2826 TNBCs showed significant association with DFS, but not with OS, on UVA and MVA.[484] The discrepancies in the AR relation with survival might be due to the small numbers of TNBC included in most studies, variability of the used antibodies and positivity threshold with different

proportions of the AR positive cases in the cohort, dissimilarities in the adjuvant and neo-adjuvant treatment with different sensitivities to drugs. The length of follow-up may be also important. The median follow-up period was 28.8 and 52.4 months in the studies by McGhan *et al.*[482] and Pistelli *et al.*[483] respectively. The median follow-up in our study is 40 months, which is within this range. However some of the studies, which showed survival significance for AR, had longer follow-up e.g. the median follow-up for the studies done by He *et al.*[436] and Hu *et al.*[437] were 72 and 64 months respectively.

A limitation of this work was that the analysis of AR was not possible in patients who had a pCR post NACT. Sakata *et al.* investigated 145 TNBCs and highlighted that AR positivity predicted lower pCR.[476] The small numbers of patients with AR status who were treated with NACT precluded assessment of the association between AR and response to NACT.

In summary, our results suggest that AR can be used to classify a subset of TNBC patients that might benefit from anti-androgen targeted therapy. However, it did not show a prognostic significance.

Table 3.12. Reports of associations between AR expression and clinicopathological parameters and outcome in TNBC

Study year	n	AR antibody	Cut-off for AR positivity	Frequency of AR positivity	Parameters examined	Significant association with parameters for AR positivity	Follow up period (months)	Impact of AR expression on outcome
Rakha *et al.*, 2007[359]	282	BioGenex F39.4.1 1:30	≥0%	36 (12.8%)	Grade, LN status, tumour size, LVI	Lower histologic grade	Median OS = 73 (1-206)	Improved RFS (p = 0.038) and MFS (p = 0.049)
Luo *et al.*, 2010[438]	137	Zymed PV6000G 1:100	H score	38 (28%)	Menopausal status, tumour grade, LN status	Postmenopausal status, lower grade, lack of LN metastasis	NR	Improved DFS and OS
Park *et al.*, 2010[481]	63	Dako AR441	≥10%	22 (35%)	Age, menopause, BMI, tumour grade, size, TNM, LN status	Smaller tumours, lower grade	NR	NR
Tang *et al.*, 2012[477]	127	Dako AR441 1:100	≥10%	16 (12.6%)	Age, Menopause, tumour grade, stage, size, LVI, LN status, ALDH1, e-cadherin	Lower grade, postmenopausal status	Median DFS = 35 Median OS = 44	Improved DFI on UVA but not on MVA. Improved OS on UVA and MVA
He *et al.*, 2012[436]	287	Dako AR441 1:500	≥5%	74 (25.8 %)	Age, tumour grade, size, LN metastases, number of positive nodes, type of surgery	LN metastases	Median = 72	Improved DFS on UVA and MVA
McGhan *et al.*, 2014[482]	94	Dako AR441 1:50	≥10%	22 (23 %)	Age, menopause, BMI, tumour grade, LVI, LN status	Older age, higher tumour stage, LN metastases	Median= 28.8 (0–118.8)	No association with RFS or OS

Table 3.12. (Cont'd.)

Study year	n	AR antibody	Cut-off for AR positivity	Frequency of AR positivity	Parameters examined	Significant association with parameters for AR positivity	Follow up period (months)	Impact of AR expression on outcome
Pistelli *et al.*, 2014[483]	81	BioGenex F39.4.1 1:60	≥10%	15 (18.8%)	Age, menopause, tumour grade, stage, size, LVI, LN status, Ki67, necrosis, lymphocytic infiltrate	Lower Ki67, presence of LVI	52.4 (2.5-95)	No association with DFS or OS on UVA
Abd-Elazeem *et al.*, 2014[475]	56	Santa Cruz sc-816 1:15	≥10%	29 (51.78%)	Age, tumour size,grade, LN status , Ki67, Claudin 4	Younger age, smaller tumour size, lower grade, lack of metastases	NR	NR
Choi *et al.*, 2015[435]	492	Epitomics ER179 (2) 1:200	≥1%	87 (17.7%)	Age, tumour size, LN status, grade, LVI	Older age, apocrine subtypt, lower grade	Median= 72 (1–202)	No association with DFS. Worse OS
Collina *et al.*, 2016[486]	207	Dako AR441 1:75	≥1%	49 (23%)	Age, menopause, tumour grade, size, LN status, Ki67, BMI, Diabetes	Diabetes	NR	Improved OS on UVA
Hu *et al.*, 2017[437]	360	NR	≥10%	113 (31.4%)	Age, menopause, tumour grade, size, LN status , LVI, Ki67, p53, ck5/6	Postmenopausal status, high grade, p53 positivity, ck5/6 negativity, low ki-67	64	Improved DFS and OS
Sakata *et al.*, 2017[476]	145	NR	≥10%	49 (33.8%)	Age, nuclear grade, Ki-67 index, pCR	Older age, low nuclear grade, low Ki-67 index, lower pCR	Median = 56	NR

AR: androgen receptor; LVI: Lymphovascular invasion; LN: lymph node; pCR: pathological complete response; NR: not reported; RFS: recurrence free survival; MFS: metastases free survival; DFS: disease free survival; DFI: disease free interval; OS: overall survival; UVA: univariate analysis; MVA: multivariate analysis

3.5.2. Basal status

Previous work by Burstein *et al.*[351] and Lehmann *et al.*[326, 352] identified two subsets within the basal subtype of the TNBC that behave differently. In addition to the expression of one of the basal markers, BL1 tumours also express genes associated with proliferation, represented by high Ki67 indices on mRNA and immunohistochemical levels.[326, 352] The rapid proliferation within this subtype suggests that this subset within the basal TNBCs would respond well to antimitotic agents.[326, 352]

The basal markers were positive in less than half of the TNBC series. The immunohistochemical expression for EGFR, CK5/6 and CK14 were assessable in 242, 248 and 240 cases on the TMA. EGFR, the most frequently expressed basal marker, was expressed in 108 cases (44.63%). CK5/6 was positive in 85 cases (34.27%), while CK14, the least expressed basal marker, was expressed in 52 cases (21.67%). The association between these markers and clinicopathological data and outcome is presented in Appendices V-VIII. None of these markers showed an association with any of the clinicopathological features or with outcome. Sobande *et al.* demonstrated that EGFR was not linked with pathological features or with survival.[487]

In total, two-thirds (171/262) of the TNBC tumours were classified as basal, while a third (91/262) were non-basal. There was no association between basal status and any of the endpoints, although some studies pointed towards an inferior outcome associated with basal markers,[206, 282] others did not reproduce the same findings. Rakha *et al.* identified a prognostic influence for the basal phenotype only if the nodes are clear, but it did not affect outcome in TNBC with nodal metastases.[359]

This supports the hypothesis by Burstein *et al.* that the basal phenotype alone might not have prognostic significance.[351] They identified within the basal tumours a subgroup that has intense immune response responsible for a better outcome while the subset that has weak immune response was associated with the worst outcome.[351] Recently, Milioli *et al.* detected different survival

outcomes for the two basal-like subtypes that they segregated through genomic profiling.[487]

Further subtyping of the TNBC according to AR status and sTILs, in addition to the basal status, did not reveal a prognostic value (Appendix IX).

3.5.3. Bcl-2 and p53

Bcl-2 is an established positive prognostic factor in BC[488], across all subtypes.[489] Bcl-2 expression was scorable in 243 TMA cores. Expression of Bcl-2 in 10% or more of the tumour cells was considered positive. Nearly one third of the assessed cases showed Bcl-2 positivity (n=78; 32.1%), while the rest were negative (n=165, 67.9%). The association between the clinicopathological features and Bcl-2 was tested by the Chi squared test (Appendix X). Bcl-2 positivity was significantly associated with postmenopausal status (p= 0.016) (Appendix X).

With regards to outcome, univariate COX regression revealed that Bcl-2 expression was associated with reduced likelihood of recurrence (HR 0.54, 95%CI: 0.32-0.93, p = 0.026), metastases (HR 0.52, 95%CI: 0.27-0.98, p = 0.045), and BC specific death (HR 0.43, 95%CI: 0.23-0.80, p = 0.008) (Table 3.13). This effect was maintained when adjusted for age at diagnosis, tumour grade, and menopausal status on multivariate analysis for MFS and BCSS (HR 0.51, 95% CI 0.26-0.99, p = 0.049 and HR 0.44, 95% CI 0.23-0.85, p = 0.014 respectively) (Table 3.13), but not for nodal status or tumour size.

Using the Kaplan-Meier curves with log rank test, Bcl-2 expression was associated with longer DFS (p=0.022), MFS (p=0.040) and BCSS (p=0.006) compared with Bcl-2 negativity (Figures 3.9A, 3.9B and 3.9C respectively). Our results are in line with published data regarding Bcl-2 in TNBC.[490, 491]

p53 mutations had been shown in the literature to be expressed in higher rates in TNBC than non-TNBC.[396] In our study, p53 expression was scorable in 240 TMA cores, and was considered positive if expressed in 10% or more of the tumour cells. Similar to the literature, p53 was expressed in a high proportion of the TNBC cohort (n=146, 60%). There was no association between p53 and clinicopathological parameters (Appendix XI) or outcome (Appendix VIII).

Table 3.13. Univariate and multivariate analysis of association of Bcl-2 with survival

Variable	HR	95% CI	p value	n
DFS				
Univariate Analysis				233
Bcl-2	0.549	0.32-0.93	**0.026***	
Multivariate Analysis				225
Bcl-2	0.58	0.33-1.00	0.052	
Age at diagnosis	1.01	0.99-1.04	0.222	
Tumour grade	0.96	0.52-1.76	0.900	
Menopausal status	0.76	0.35-1.63	0.485	
MFS				
Univariate Analysis				233
Bcl-2	0.52	0.27-0.98	**0.045***	
Multivariate Analysis				225
Bcl-2	0.51	0.26-0.99	**0.049***	
Age at diagnosis	1.01	0.98-1.04	0.376	
Tumour grade	1.05	0.49-2.25	0.895	
Menopausal status	0.75	0.3-1.87	0.542	
BCSS				
Univariate Analysis				230
Bcl-2	0.43	0.23-0.80	**0.008***	
Multivariate Analysis				222
Bcl-2	0.44	0.23-0.85	**0.014***	
Age at diagnosis	1.03	1.00-1.07	**0.015***	
Tumour grade	1.35	0.62-2.89	0.441	
Menopausal status	0.51	0.20-1.27	0.150	

p values in bold indicate statistically significant associations

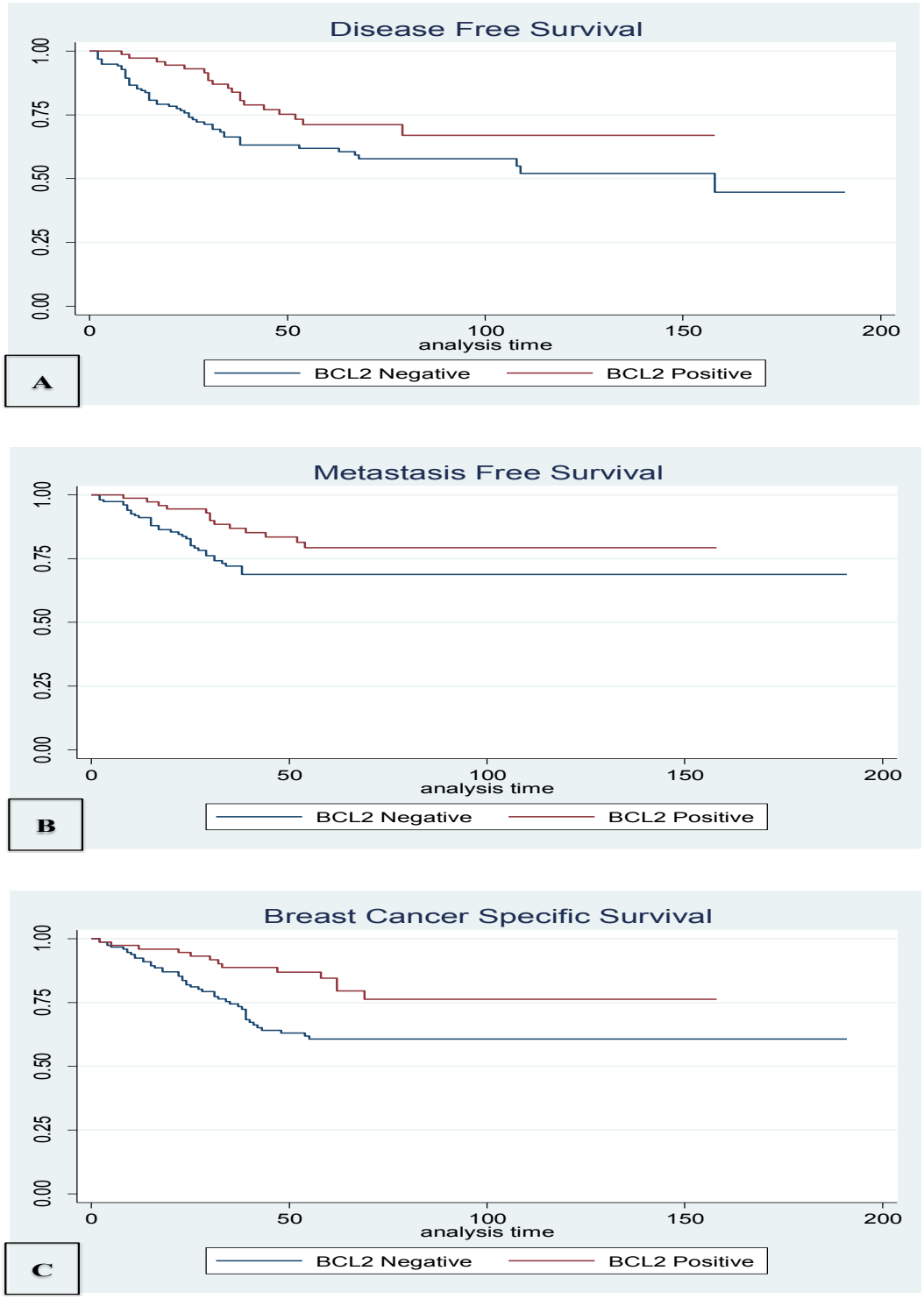


Figure 3.9. Association between Bcl-2 expression and DFS (A), MFS (B) and BCSS (C)

3.6. Summary

In this work, a retrospective review of clinical, pathological and outcome data for 355 TNBC patients, managed mainly in GUH between January 2000 and January 2016, is presented. This is one of the largest reviews of outcome for TNBCs outside of the clinical trial settings.

The overall outcome for TNCB was similar to that reported by others with 22% relapse and 21% death from disease, after a median follow-up of 42 months.

In patients who had no NACT treatment, nodal status was an independent predictor of DFS and MFS but not of BCSS; and tumour size was an independent predictor of MFS and BCSS.

In patients who received NACT, a pCR was the only independent predictor of all outcome endpoints. This highlights the importance of accurate assessment of the post-NACT specimens. This includes the proper grossing and adequate sampling, which can be challenging particularly when no remaining mass can be identified macroscopically. Therefore, the insertion of a clip and accessibility of radiological information, about the site and size of the pre- and post-treatment tumour, to the pathologist are essential. Guidelines are published, but characterization and quantification of the degrees of non pCR remain difficult and subject to variation between pathologists.[492] There are many grading systems for NACT response described in the literature, yet there is no consensus between breast pathologists and oncologists regarding a system that proved superior in terms of clinical relevance. The amount of residual tumour can vary from the presence of few viable tumour cells up to entirely resistant tumour with no response or change in its original size or cellularity. Including the residual tumour burden provides a measurable parameter that would be useful for testing the differences in outcome for various degrees of NACT response.[493] In summary, there is an essential need for a universally accepted standardized reporting protocol for the macroscopic and microscopic assessment of the post-NACT BC, to ensure the validity of the survival analyses in such cohorts.

Other pathological features of grade and tumour type were not independent predictors of outcome.

None of the other biomarkers examined by immunohistochemistry i.e. basal markers, AR and p53 was independent predictors of outcome. The basal phenotype as well as the four subtypes of TNBC, based on the stated markers in addition to the sTILs amount, did not show a clinical importance in this cohort. Only Bcl-2 showed a potential independent prognostic role for MFS and BCSS. TNBC tumours with high expression of Bcl-2 were associated with a longer survival rate on univariate and multivariate analyses, but not with nodal status or tumour size which were included in the model.

Due to the relatively low incidence of TNBC, the majority of single institutional studies include small number of patients. Our series is larger than other cohorts, yet the number of patients is still limited (n=355). To increase the statistical power, pooling of studies and multi-centric studies are warranted. Our group aims to prospectively increase the TNBC series across the coming years.

A longer follow-up time might be beneficial as a minority of the cases in this series had a short follow-up. Yet, the median follow-up at the time of this analysis was 42 months, and for TNBC the incidence of distant recurrence peaks between the first and third years after its diagnosis.

4. THE ROLE OF sTILs IN TNBC

4.1. Introduction

The development of normal tissue growth patterns in malignancies changes the inherent structure of the tissue, thereby prompting the body's immune response in an attempt to eradicate the cells that have transformed. In some instances, not all of these cells are eradicated and can become resistant to the immune system. This is known as immunoediting. If the body can effectively generate tumour-specific immune responses, the cancer can be prevented or controlled.[494]

Immunoediting is the process of immunosurveillance and tumour progression based on three phases: (1) elimination, also known as immunosurveillance, where tumour cells are eliminated by the immune system; (2) equilibrium, where tumour cells that escaped during phase one are selected for growth because they have acquired immune resistance; and (3) escape, where tumour cell escape the immune system entirely, further demonstrating their immune resistance. BC produces an immune response by promoting tumourigenesis as well as suppression via immune surveillance.[495]

Tumour cells can suppress TILs by directly suppressing antitumour immune cells or by recruiting immunosuppressive subgroups. Previous studies show that T cells, $CD4^+$ Th1 cells, $CD8^+$ cytotoxic T cells, NK cells, M1 macrophages, and dendritic cells suppress tumour growth, however $CD4^+$ forkhead box P3 ($FOXP3^+$), $CD4^+$ Th2 cells, M2 macrophages, and myeloid-derived suppressor cells promote tumour growth.[494-497]

Studies show that T-lymphocytes make up 75% of TILs, B-lymphocytes make up less than 20%, monocytes make up less than 10%, and natural killer/natural killer T cells make up less than 5% of all TILs.[104-106] T cells have the ability to move from the tumour to the peritumoural sites and can in turn control differentiation of M2 macrophages, which promote tumour growth or antitumour M1 macrophages, which suppress tumour growth.[498]

Many studies in the literature have indicated that TILs are important antitumour biomarkers in different types of cancer including BC.[107, 376, 377, 499-501] TILs can be

found within the tumour itself but their relative amount is reliant on the type and location of the cancer.[99] Furthermore, innovative targeted immunocell therapies such as CTLA-4, PD-1, and PD-L1, with/without chemotherapy, have shown an increased immune response in patients with melanoma and non–small cell lung cancer.[502] Quantitative analysis of TIL subpopulations will aid prediction of treatment response and ultimately prognosis.[102] It also generates hope for the use of new individualized immunotherapy treatment in TNBC patients.

TILs can be classified into two types, which is determined by the site they ultimately infiltrate e.g. in the tumour stroma (i.e. sTILs) or tumour cell islets (i.e. iTILs). iTILs, as their name suggests, are found within the tumour itself, and are in direct contact with the tumour cells.[103] While sTILs and iTILs are closely correlated, sTILs show a more consistent association with outcome than iTILs which is likely due to their greater number when compared to intratumoural TILs.[427]

Published data points to the variability of the quantities and populations of TILs according to the histo-morphologic and immunohistochemical types of BC molecular and histological subtypes. [107-109, 111] Marked lymphocytic infiltration defines one of characteristics of medullary carcinoma; while noticeable infiltrates are reported also in significant proportions of invasive ductal carcinomas. Sparseness of TILs was observed with metaplastic and lobular carcinoma.[391, 503] Surveying the published studies confirmed that intense sTILs are seen more frequently in TNBC and HER-2-positive versus hormone dependent tumours.[394]

The main aims of this work are:

1. To investigate the association between sTILs and clinicopathological factors and outcome in TNBC
2. To examine the effect of NACT on sTILs in TNBC

4.2. Study of sTILs in TNBC

4.2.1. Characteristics of TNBC series for sTILs assessment

sTILs were assessable in 303 cases of TNBC from 290 patients in our series. Cases where there had been a pCR in the breast or where the THE was not available were not assessed. Therefore, the characteristics of those patients suitable for sTIL assessment are summarised in Table 4.1.

Demographics, treatment and follow-up outcome status for the 290 patients are summarized in Table 4.1. The median age at diagnosis was 56 years (range 24-92 years), with the majority of the patients being over 45 years (n=232/290, 80%) and postmenopausal (n=182/290, 63%). Although a quarter of the cohort had a family history of BC (n=75/290; 26%), only four percent of the patients were BRCA carriers (n=12/290, 4%). Two thirds of the patients had breast conservation while a third of the patients underwent mastectomies. Most of the patients received adjuvant radiotherapy (n=208/290; 72%). Adjuvant chemotherapy was given in 58% (n=169/290) while NACT was given in 17% (n=50/290) of the cases. This cohort included 50 patients who had received NACT and had residual disease post- NACT.

The histopathological details for the 303 tumours are given in Table 4.2. As expected, the most common tumour type was invasive ductal carcinoma (n= 219/303; 72%). The majority of the tumours were high grade (n= 249/303; 82%), and showed a high rate of nodal involvement (178/303; 59%). Overall, the median invasive tumour size was 24mm (range: 1-120 mm, mean: 27.5 mm). In NACT naiive tumours the median tumour size was 25 (range 1-120 mm), while in post-NACT tumours the median tumour size was 19.5 (range 1-100 mm).

At the time of this analysis, the median length of follow-up was 29.5 months (range <1 month – 176 months). Both the DFS and the OS ranged from <1 to 176 months with a median of 27 and 32 months, respectively. Among the 290 cases, nine patients had metastatic disease at presentation. Sixty-seven deaths (23%) were reported. There were 64 events of local and/or distant recurrence. Of these, 59 patients acquired distant metastases with or without local recurrence.

Table 4.1. Characteristics of TNBC series for sTILs assessment

Parameter (n= 290)		**TNBC cohort n (%)**
Age at diagnosis Range: 24-92 Median: 56 Mean±SD: 57.6±14.31	<45 years	58 (20)
	>45 years	232 (80)
Menopausal status	Pre-menopausal	94 (32)
	Post-menopausal	182 (63)
	Unknown	14 (5)
BRCA status	BRCA-1 Mutated	8 (3)
	BRCA-2 Mutated	3 (1)
	BRCA unspecified mutated	1 (<1)
	No BRCA mutation	208 (72)
	Unknown BRCA status	70 (24)
Family history of BC	Yes	75 (26)
	No	105 (36)
	Unknown	110 (38)
Surgery	Breast conservative surgery	197 (68)
	Mastectomy	93 (32)
Chemotherapy	NACT only	44 (15)
	NACT and adjuvant chemotherapy	6 (2)
	Adjuvant chemotherapy only	163 (56)
	None	57 (20)
	Unknown	20 (7)
Adjuvant Radiotherapy	Yes	208 (72)
	No	59 (20)
	Unknown	23 (8)
Survival	Alive, no disease	207 (71)
	Alive, disease progression	16 (5)
	Dead, with disease progression	55 (19)
	Dead, with no disease progression	12 (4)
Distant metastases	Yes	59 (20)
	No	231 (80)

Table 4.2. Pathological characteristics of the TNBC series for sTILs assessment

Parameter (n= 303)		**TNBC cohort n (%)**
Tumour type	Ductal (NST)	219 (72)
	Metaplastic	27 (9)
	Typical medullary	7 (2)
	Atypical medullary	20 (7)
	Lobular	14 (5)
	Apocrine	12 (4)
	Others	4 (1)
Invasive tumour size (mm)	<20	125 (41)
	21-50	149 (49)
	>50	28 (9)
	Unknown	1 (<1)
Tumour grade	1	2 (1)
	2	52 (17)
	3	249 (82)
Tubule formation	1	3 (1)
	2	25 (8)
	3	275 (91)
Nuclear pleomorphism	1	1 (<1)
	2	19 (6)
	3	283 (93)
Mitotic count	1	47 (16)
	2	67 (22)
	3	189 (62)
Vascular invasion	Yes	106 (35)
	No	196 (65)
	Unknown	1 (<1)
Nodal status	Positive	178 (58.7%)
	Negative	111 (36.6%)
	Unknown	14 (4.6%)
Skin involvement	Yes	7 (2.3%)
	Paget's disease	2 (0.7%)
	No	294 (97%)

4.2.2. Evaluation of sTILs in the therapeutic resections of TNBC

sTILs were scored according to the recommendation of the International TIL working Group (TILs IWG).[427] This was carried out by two pathologists (GC and AS) on a multihead microscope on a single representative H&E slide from each resected tumour. An average percentage of the tumour stromal area occupied by lymphocytic inflammatory cells was graded on a continuous scale (0-100), then categorised on a scale 0-4 as follows: (0= no mononuclear infiltrate, 1= <10%, 2= 10-24%, 3= 25-49%, and 4= ≥50%). The assessment excluded areas of necrosis, DCIS, cavity or previous biopsy sites.

Scores were recorded for 291 tumours. sTILs could not be scored in 12 tumours due to the nature of the tumour with minimal stroma i.e. metaplastic carcinoma, or if the tumour was so small or extremely hypocellular after neoadjuvant therapy. sTIL scores in the resection specimens ranged from 0 to 90% with a median and mean of 15% and 21.58%, respectively.

sTIL counts in TNBCs were categorised in four different ways. First, as increments of 10% because many reports have reported an association between an incremental increase of sTILs and outcome in TNBC.[389, 392, 504] Second, sTILs were represented as categorical variables as four sTIL categories (C1: sTILs 0-9%; C2: sTILs 10 - 24%; C3: sTILs 25 - 49%; C4: ≥50%). The distribution and frequency of cases according to the four sTIL categories is illustrated in Figure 4.1. Third, cases with sTILs <25% (category 1 and 2) were also considered sTILs low, while cases with ≥25% (category 3 and 4) were considered sTILs high (Figure 4.2.). The distribution and frequency of cases according to sTIL-low and high categories is illustrated in Figure 4.3. Finally, a threshold of 50% sTILs was used to define lymphocyte predominate cancer (LPBC).[389, 504, 505] Therefore, cases with sTILs <50% were also categorised as "non-LPBC" while cases with ≥50% was named 'LPBC'. The distribution and frequency of LPBC and non-LPBC is illustrated in Table 4.3.

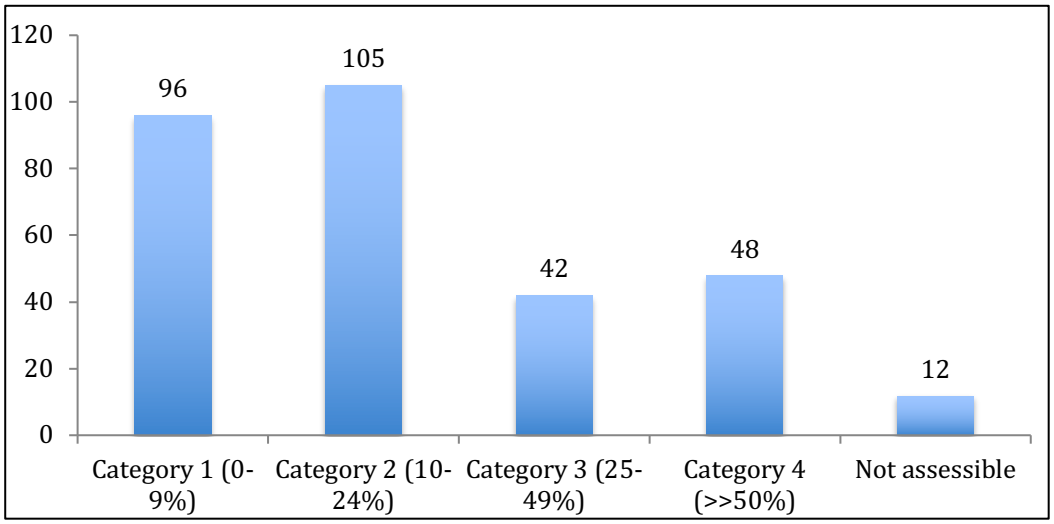


Figure 4.1. Distribution of cases according to four sTILs categories

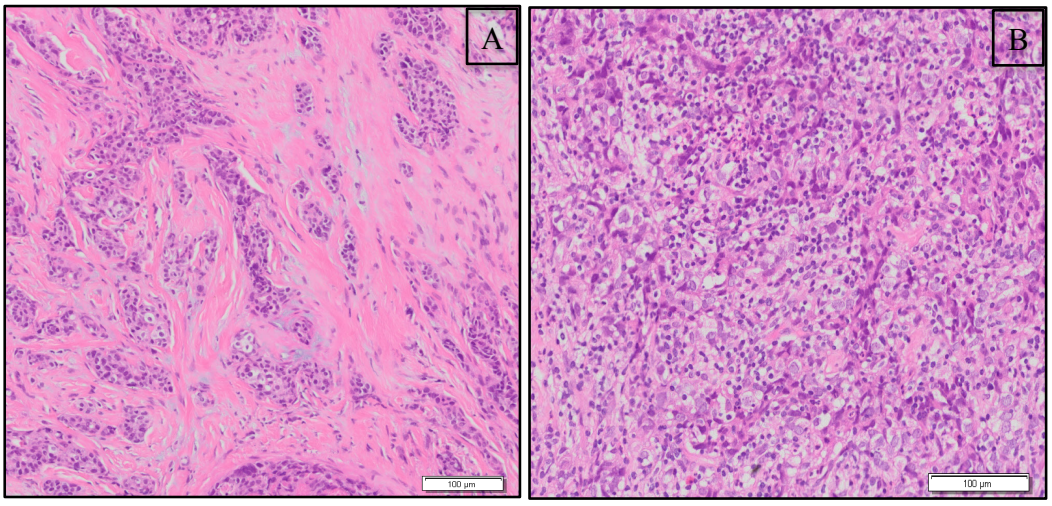


Figure 4.2. Representative areas for lymphocyte-low TNBC (A) and lymphocyte-high TNBC (B) (Olympus scanner)

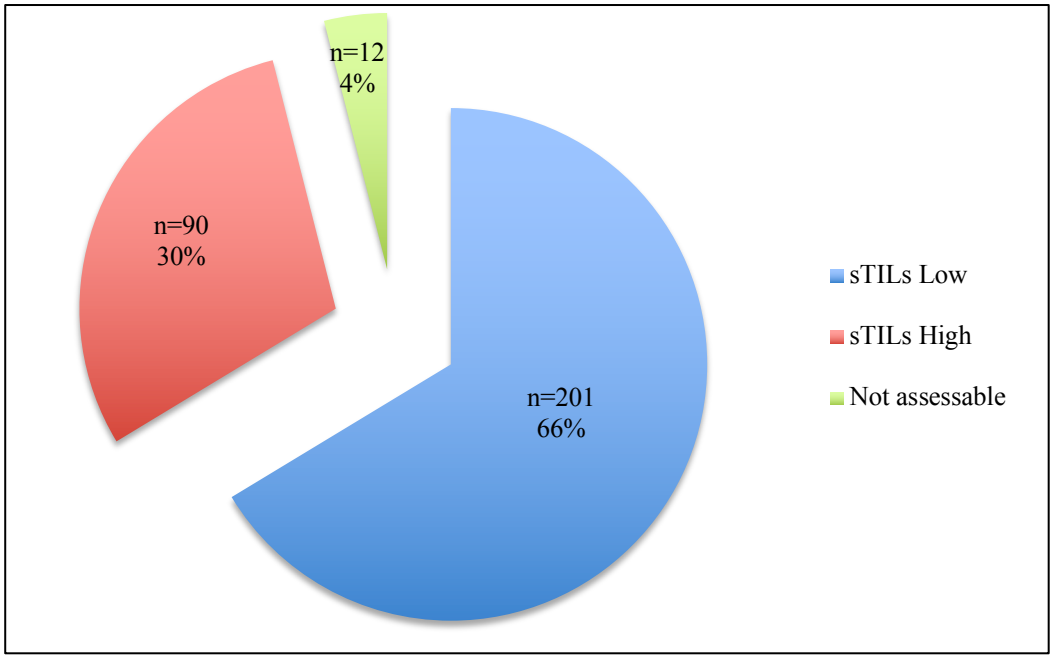


Figure 4.3. Distribution and frequency of sTILs low (sTILs<25%) and sTILs high (sTILs ≥25%) n = number of tumours within each sTILs category

Table 4.3. Distribution and frequency of LPBC

sTIL Category	**n (%) (n=303)**
sTILs non-predominant (<50%)	243 (80)
sTILs predominant (≥50%)	48 (16)
Not assessable	12 (4)
Total	303 (100)

4.2.3. Associations between sTILs and pathological variables

Associations between sTILs high (sTILs ≥25%) and pathological variables in TNBC are shown in Table 4.4. Associations between sTILs high and biomarkers in TNBC are shown in Appendix XII**.** Associations between LPBC (sTILs ≥50%) and pathological variables in TNBC are shown in Table 4.5. Associations between sTILs LPBC and biomarkers in TNBC are shown in Appendix XIII**.**

sTILs high status and LPBC showed an association with histological tumour type (p= <0.001), with frequent positivity in the typical and atypical medullary carcinomas and negativity in invasive lobular, metaplastic and apocrine carcinomas. The median sTILs within the typical and atypical medullary subtypes were 70% and 60% respectively. All the medullary carcinomas showed high sTILs (>25%), while 85.7% showed LPBC (sTILs >50%). Two thirds of the atypical medullary carcinomas showed high sTILs while LPBC was seen in half of this subtype. There was significant strong association between the tumour subtype and sTILs using the two cut-offs (p=<0.001, Chi square test). sTILs high status and LPBC were significantly associated with marked nuclear pleomorphism score 3 (p= 0.001 and p= 0.029, respectively). However, a significant association with the higher tumour grade and absent lymphovascular invasion was only observed with LPBC (p= 0.047 and p=0.003 respectively).

Table 4.4. Association between sTILs high and pathological variables in TNBC

Variable (n[a])		sTILs Low n (%)	sTILs High n (%)	Total	χ^2	p value
Tumour histological type (291)	Ductal	148 (70)	63 (30)	211	35.3	**<0.001***
	Metaplastic	20 (83)	4 (17)	24		
	T. Medullary	0 (0)	7 (100)	7		
	At. Medullary	7 (37)	12 (63)	19		
	Lobular	13 (93)	1 (7)	14		
	Apocrine	9 (75)	3 (25)	12		
	Others	4 (100)	0 (0)	4		
Tumour grade (291)	1	2 (100)	0 (0)	2	4.51	0.152
	2	38 (80)	10 (20)	48		
	3	161 (67)	80 (33)	241		
Tubule formation (291)	1	3 (100)	0 (0)	3	5.11	0.078
	2	20 (83)	4 (17)	24		
	3	178 (67)	86 (33)	264		
Nuclear pleomorphism (291)	1	1 (100)	0 (0)	1	14.6	**0.001***
	2	18 (100)	0 (0)	18		
	3	182 (67)	90 (33)	272		
Mitosis (291)	1	34 (77)	10 (22)	44	1.64	0.439
	2	43 (68)	20 (32)	63		
	3	124 (67)	60 (33)	184		
Invasive tumour size (mm) (290)	≤20mm	77 (66)	39 (34)	116	0.72	0.694
	20-50mm	104 (71)	42 (29)	146		
	>50mm	19 (68)	9 (32)	28		
LVI (290)	Absent	123 (66)	64 (34)	187	3.09	0.079
	Present	78 (76)	25 (24)	103		
LN status (277)	Absent	120 (71)	49 (29)	169	1.54	0.215
	Present	69 (64)	39 (36)	108		

T. Medullary: typical medullary; At. Medullary: atypical medullary; LVI: Lymphovascular invasion; LN: lymph node. p values in bold indicate statistically significant associations,

Table 4.5. Association between LPBC and pathological variables in TNBC

Variable (n[a])		sTILs Non-LPBC n (%)	sTILs LPBC n (%)	Total	χ^2	p value
Tumour histological type (291)	Ductal	180 (85)	31 (15)	211	45.5	**<0.001***
	Metaplastic	24 (100)	0 (0)	24		
	T. medullary	1 (14)	6 (86)	7		
	At. medullary	9 (47)	10 (53)	19		
	Lobular	14 (100)	0 (0)	14		
	Apocrine	11 (92)	1 (8)	12		
	Others	4 (100)	0 (0)	4		
Tumour grade (291)	1	2 (100)	0 (0)	2	6.11	**0.047***
	2	45 (94)	3 (6)	48		
	3	196 (81)	45 (19)	241		
Tubule formation (291)	1	3 (100)	0 (0)	3	1.08	0.581
	2	20 (83)	4 (17)	24		
	3	220 (83)	44 (17)	264		
Nuclear pleomorphism (291)	1	1 (100)	0 (0)	1	7.10	**0.029***
	2	18 (100)	0 (0)	18		
	3	224 (82)	48 (18)	272		
Mitosis (291)	1	41 (93)	3 (7)	44	5.39	0.067
	2	55 (87)	8 (13)	63		
	3	147 (80)	37 (20)	184		
Invasive tumour size (mm) (290)	≤20mm	93 (80)	23 (20)	116	1.50	0.472
	20-50mm	125 (86)	21 (14)	146		
	>50mm	24 (86)	4 (14)	28		
LVI (290)	Absent	147 (78)	40 (22)	187	8.92	**0.003***
	Present	95 (92)	8 (8)	103		
LN status (277)	Absent	141 (83)	28 (17)	169	<0.1	0.983
	Present	90 (83)	18 (17)	108		

LVI: Lymphovascular invasion; LN: lymph node. p values in bold indicate statistically significant associations

4.2.4 Associations between sTILs and outcome

For analyses of the association between sTILs and outcome, the following four sTILS categories were examined:

1. sTILs increments of 10%.
2. Four sTILs categories (C1: sTILs 0-9%; C2: sTILs 10-24%; C3: sTILs 25-49%; C4: ≥50%).
3. sTILs high (sTILs ≥25%) and low (sTILs <25%).
4. LPBCs (TILs ≥50%) and non-LPBCs (sTILs <50%).

DFS and OS were estimated by univariate analysis by the Kaplan-Meier survival analysis method, and were compared by the log-rank test and test for trend to assess the significance.

On UVA, sTILs were associated with both DFS and OS when sTILs were evaluated in increments of 10% (Table 4.6). Each 10% incremental increase in sTILs count was associated with a reduction in DFS in the order of 18% (HR 0.82, 95% CI 0.72-0.94, p = 0.003). This effect was maintained on multivariate analysis when adjusted for age at diagnosis, nodal status and tumour grade (HR 0.82, 95% CI 0.71-0.94, p = 0.005). Similarly, each 10% incremental increase in sTILs was associated with an improved overall survival by UVA (HR 0.85, 95% CI 0.74-0.97, p = 0.017 and HR 0.84, 95% CI 0.73-0.96, p = 0.012 respectively) adjusted for the same variables (Table 4.6).

Decreasing numbers of TILs in 10% increments were associated with a significant trend toward poor DFS (p=0.199, Log rank test) and poor OS (p=0.394, Log rank test).

Table 4.6. Univariate and multivariate analysis of association of sTILs (per 10% increase) with DFS and OS

Variable	HR	95% CI	p value	n
DFS				
Univariate Analysis				282
sTILs	0.82	0.72-0.94	**0.003***	
Multivariate Analysis				268
sTILs	0.82	0.71-0.94	**0.005***	
Age at diagnosis	1.00	0.98-1.02	0.598	
Nodal Status	2.33	1.41-3.83	**0.001***	
Tumour grade	0.86	0.47-1.57	0.628	
OS				
Univariate Analysis				282
sTILs	0.85	0.74-0.97	**0.017***	
Multivariate Analysis				264
sTILs	0.84	0.73-0.96	**0.012***	
Age	1.02	1.00-1.04	0.023	
Nodal Status	2.73	1.61-4.61	**<0.001***	
Tumour grade	1.21	0.60-2.45	0.578	

p values in bold indicate statistically significant associations

Analysis of the association between sTILs and outcome, when sTILs were expressed as four categories (C1: sTILs 0-9%; C2: sTILs 10-14%; C3: sTILs 25-49%; C4: ≥50%), revealed that sTILs had an independent association with both DFS and OS (HR 0.71, 95% CI 0.55-0.91, p = 0.007 and HR 0.78, 95% CI 0.61-0.99, p = 0.043 respectively) (Table 4.7.). This effect was maintained when adjusted for age at diagnosis, nodal status and tumour grade on MVA (HR 0.70, 95% CI 0.54-0.90, p = 0.006 and HR 0.74, 95% CI 0.57-0.96, p = 0.022 respectively) (Table 4.7.).

Table 4.7. Univariate and multivariate analysis of association of sTILs (four categories) with DFS and OS

Variable	HR	95% CI	p value	n
DFS				
Univariate Analysis				282
sTILs	0.71	0.55-0.91	**0.007***	
Multivariate Analysis				268
sTILs categories	0.70	0.54-0.90	**0.006***	
Age at diagnosis	1.00	0.98-1.03	0.598	
Nodal Status	2.33	1.42-3.83	**0.001***	
Tumour grade	0.86	0.47-1.57	0.628	
OS				
Univariate Analysis				277
sTILs categories	0.78	0.61-0.99	**0.043***	
Multivariate Analysis				265
sTILs	0.74	0.57-0.96	**0.022***	
Age at diagnosis	1.02	1.00-1.04	0.028	
Nodal Status	2.75	1.62-4.65	**<0.0001***	
Tumour grade	1.18	0.58-2.37	0.642	

p values in bold indicate statistically significant associations

The Kaplan Meier graphs show an association between the four categories of TILs and DFS (p=0.042, Log rank test) but not with OS (p=0.190, Log rank test) (Figures 4.4A and 4.4B respectively).

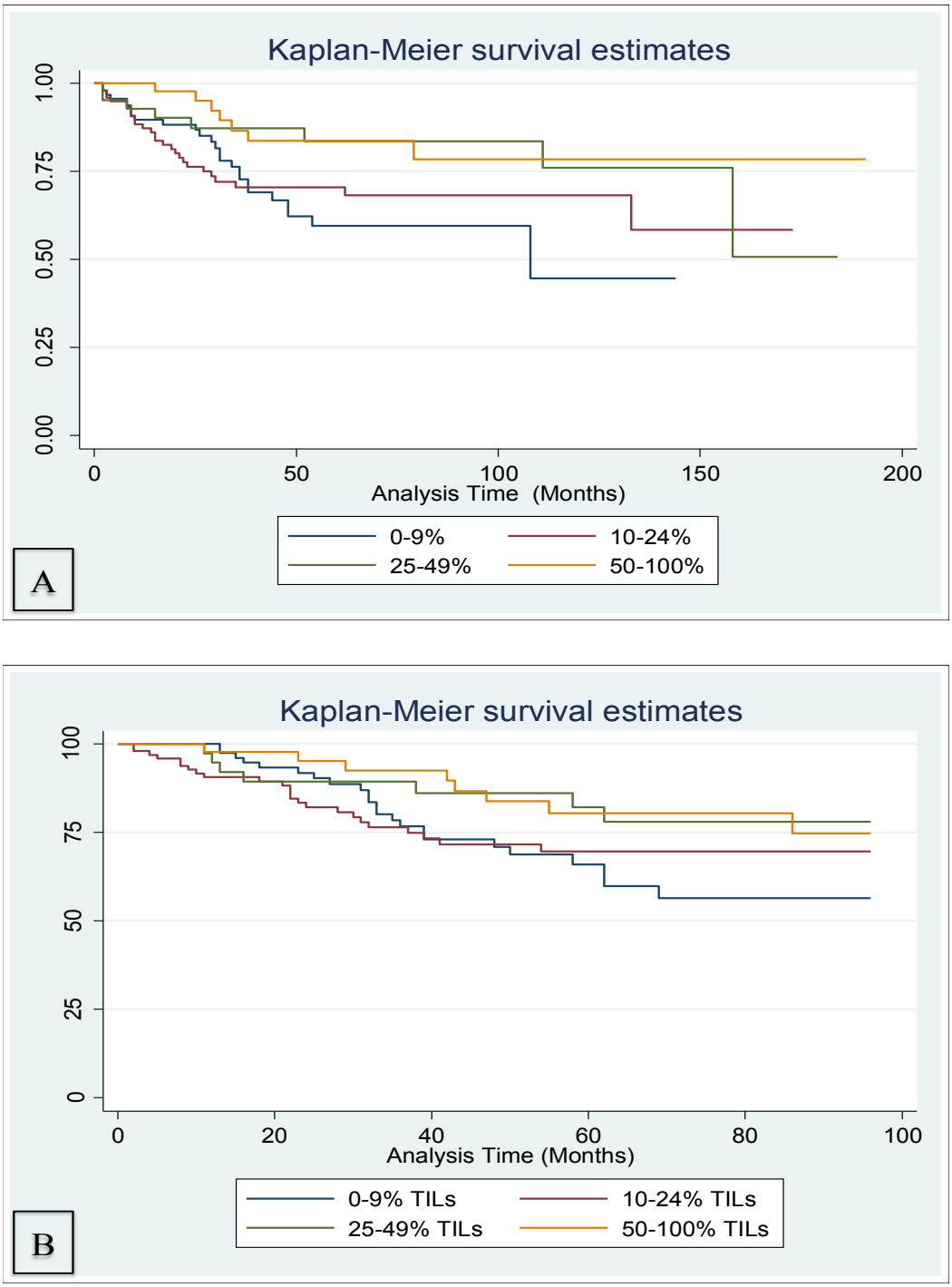


Figure 4.4. Association between sTILs four category groups and DFS (A) and OS (B) sTILs categories [Category 1 (C1): sTILs 0-9%; Category 2 (C1):: sTILs 10-14%; Category 3(C1):: sTILs 25-49%; Category 4 (C4)]

Multivariate Cox proportional hazard regression analysis, expressing sTILs as sTIL-low (< 25%) and those with sTIL> 25% as sTIL-high and adjusting for age, lymph node status and tumour grade, revealed that sTIL-high was an independent predictor of DFS (HR 0.44, 95% CI 0.25-0.79, p = 0.007) and OS (HR 0.56, 95% CI 0.32-0.99, p = 0.047) (Table 4.8.). The magnitude of the effect on DFS was greater for this categorization than when sTILs were treated in increments of 10% or divided into four categories. Using the log rank test, sTILs-high cases were associated with a significantly better DFS (p=0.004), but non-significant longer OS (p=0.057) (Figures 4.5A and 4.5B respectively).

Table 4.8. Multivariate analysis of association of sTILs high (sTILs <25%) with DFS and OS

Variable	HR	95% CI	p value	n
DFS				268
sTILs	0.44	0.25-0.79	**0.007***	
Age at diagnosis	1.00	0.98-1.02	0.685	
Nodal Status	2.29	1.40-3.76	**0.001***	
Tumour grade	0.81	0.44-1.47	0.491	
OS				264
sTILs	0.56	0.32-0.99	**0.047***	
Age at diagnosis	1.02	1.00-1.04	0.030	
Nodal Status	2.52	1.51-4.21	**<0.001***	
Tumour grade	1.15	0.57-2.29	0.698	

p values in bold indicate statistically significant associations

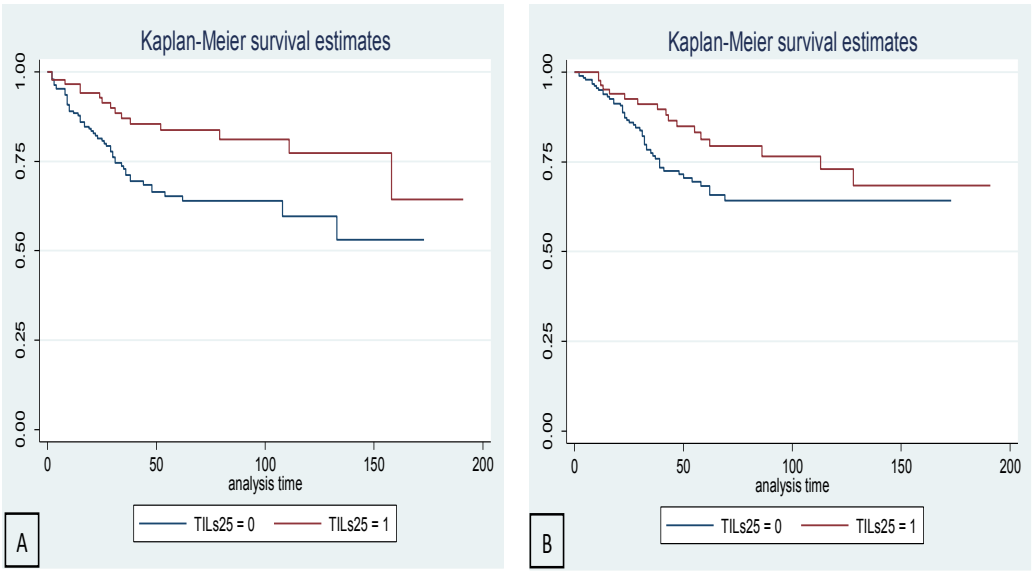


Figure 4.5. Kaplan-Meier curves for DFS (A) and OS (B) in TNBC labelled according to the sTILs high (sTILs >25%)

Finally, MVA of the association between LPBC (sTILs ≥50%) and both DFS and OS was examined adjusting for the same co-variables (age at diagnosis, node positivity, tumour grade). Nodal status was, as expected, the strongest independent predictor of both DFS and OS. Patients who had non-LPBC had a reduced DFS and OS reflected by a HR of 52% (95% CI 0.22-1.06, p = 0.07) and 41% (95% CI 0.28-1.23, p = 0.162) respectively. While this did not maintain significance, nonetheless LPBC remained in the final model (Table 4.9.). Using the log rank test, sTILs predominant TNBC showed significantly better DFS (p=0.036), yet with OS the difference was not significant (p=0.209) (Figures 4.6A and 4.6B respectively).

Table 4.9. Multivariate analysis of the association of LPBC (sTILs >50%) with DFS and OS

Variable	HR	95% CI	p value	n
DFS				268
sTILs (LPBC vs non-LPBC)	0.48	0.22-1.06	0.070	
Age at diagnosis	1.01	0.99-1.03	0.531	
Nodal Status	2.18	1.33-3.56	0.002	
Tumour grade	0.82	0.45-1.50	0.529	
OS				264
sTILs (LPBC vs non-LPBC)	0.59	0.28-1.23	0.162	
Age at diagnosis	1.02	1.00-1.04	0.021	
Nodal Status	2.44	1.46-4.06	0.001	
Tumour grade	1.15	0.57-2.31	0.690	

p values in bold indicate statistically significant associations

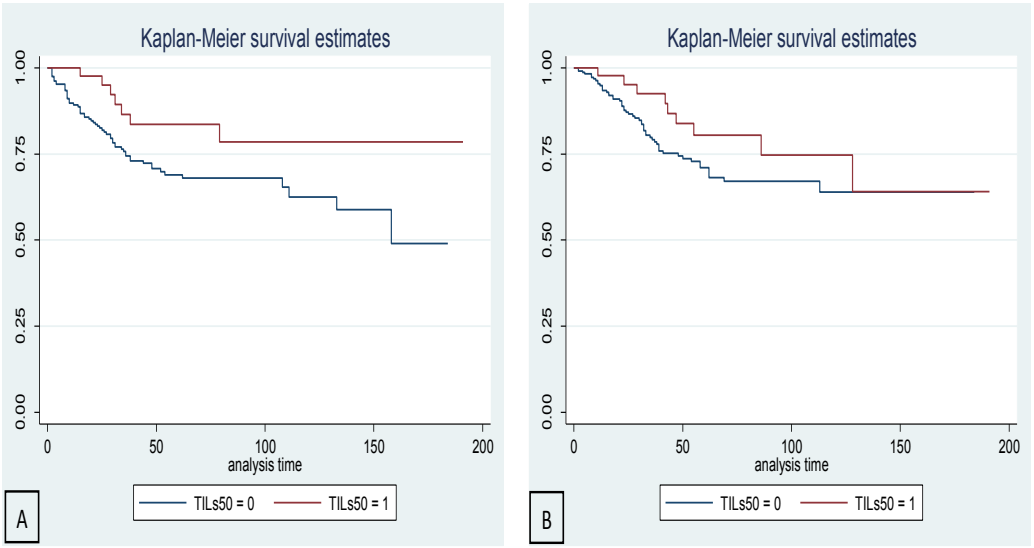


Figure 4.6. Kaplan-Meier curves for DFS (A) and OS (B) in TNBC labelled according to the sTILs predominance (sTILs >50%)

4.3. Comparison of sTILs in pre-NACT needle-core biopsies and surgical resection specimens

4.3.1. Correlation of sTILs proportions in pre-treatment needle-core biopsies and post-treatment surgical resection specimens

As we included in this study TNBC cases from patients who had partial response to NACT on THE with others who did not receive NACT, it was imperative to investigate whether the degree of the sTILs in the THE corresponds to pre-NACT NCB.

Twenty-six paired pre-treatment NCB and their post-treatment residual disease on surgical THE were examined for the degree of the sTILs infiltrate on H&E stained slides. The median number of biopsy cores taken was 3 and the range was between 1 to 7 cores/case.

The median residual post-neoadjuvant tumour size was 25mm (range: 0.5-100mm) and 15 tumours (57.7 %) were grade 3 while 11 (42.3%) were grade 2. The majority of the tumours (80.7%, n=21/26) were invasive ductal carcinoma (NST), two tumours (7.7%) were metaplastic carcinoma, two tumours (7.7%) were atypical medullary carcinoma and one tumour (3.9%) was classified as apocrine carcinoma. Seventeen cases (65.4%) were treated with anthracycline and taxane. Six cases (23.1%) were treated with platinum in addition to anthracycile and taxane regimen. The records of the neoadjuvant regimen could not be retrieved for 3 cases (11.5%).

There was no difference between sTILs counts in NCBs and the corresponding THE specimens. The median sTIL count was the same in both (10%) with a range of 0 % to 90% observed in NCBs and 0 % to 80% in THEs specimens. There was no difference in the mean sTILs in NCBs and THE specimens (mean = 15.25% and 18.77% respectively, $p = 0.317$, paired t-test). Figure 4.7. shows the spread of sTILs counts in biopsy and THE samples.

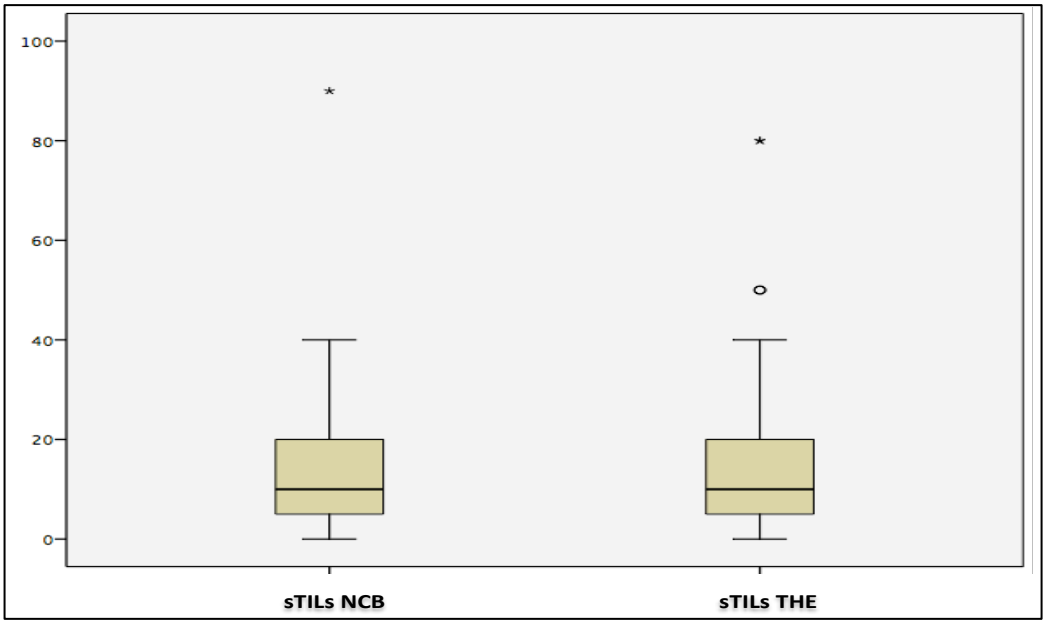


Figure 4.7. Parallel boxplots of sTILs counts in biopsy and resection samples

sTILs NCB: stromal tumour infiltrating lymphocytes in needle core biopsy; sTILs THE: stromal tumour infiltrating lymphocytes in therapeutic excision specimen.

For all cases, the difference in the absolute sTILs values between the paired NCB and THE for each case was low, and ranged from 0 to 45% with a median and mean of 5% and 11.31% respectively. There was a strong correlation between the sTILs values in NCB and subsequent THE specimen (Pearson's correlation coefficient (R) of 0.67, p=0.002). The explained variance (R2) showed that 45% of the excision results for sTILs can be predicted by the NCB results.

The sTILs in the NCB and the corresponding resection are shown in Figure 4.9. A change in the sTIL count ≥20% was observed in 4 cases (15.38%). the sTIL count was higher in the resection than it was in the corresponding NCB in three cases, and was lower in the other. There was no difference between the number of cores examined in these four cases (median 3) and the median number of cores taken for all 26 cases, suggesting that the difference was not related to the number of cores examined.

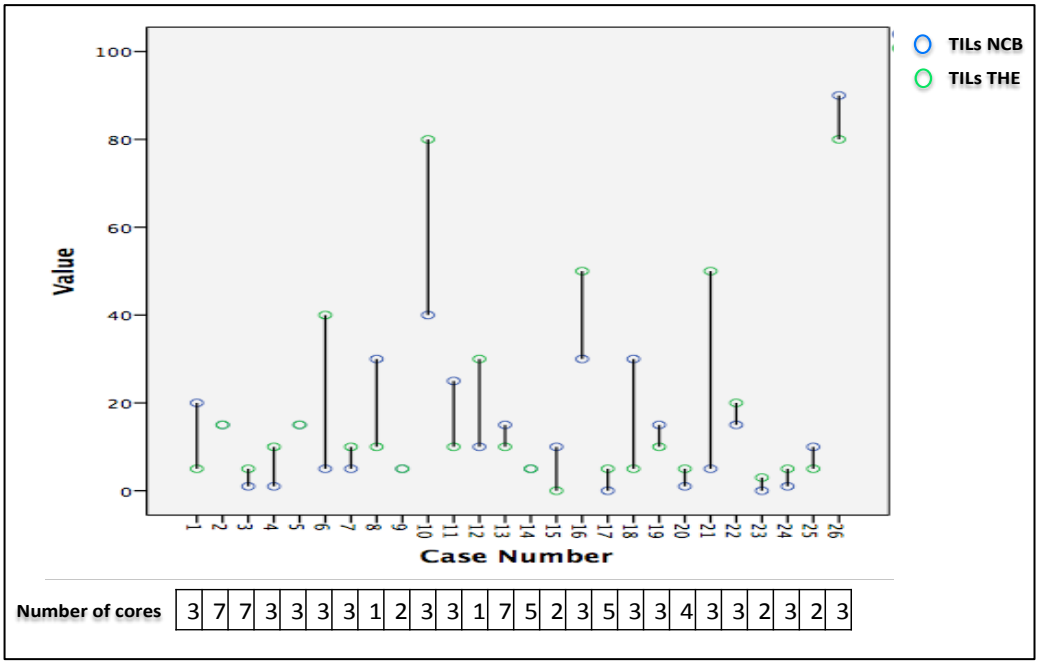


Figure 4.8. Differences in paired biopsy and resection sTILs percentages

sTILs NCB: stromal tumour infiltrating lymphocytes in needle core biopsy; sTILs THE: stromal tumour infiltrating lymphocytes in therapeutic excision specimen.

Therefore, in this pilot series there was no appreciable difference in sTIL counts in NCBs pre and post NACT treatment in those cases for whom a pCR was not achieved.

4.4. Discussion

sTILs have been proposed as a potential measure of the immune response to the presence of neoplastic cells. A number of studies support sTILs, assessed by light microscopy, as a potential predictor of outcome in women with TNBC. However, sTILs have been evaluated by a small number of groups. The assessment of sTILs is not routinely used in diagnostic practice and as with many markers is likely to be subject to variation in interpretation. Over the last few years, many groups have suggested different scoring systems for semi-quantitative assessment of sTILs. In 2014, the international TILs working group recommended assessment of sTILs on a continuous scale, upon examining the entire invasive tumour area on a single representative full-face H&E stained slide. The assessment includes but is not exclusive to the hotspots.[427]

In this work, sTILs were assessed in our series of TNBC with the aim of investigating its relationship with clinico-pathological features and outcome. In TNBC, sTILs high and LPBC was significantly associated with tumour type, being more common in medullary and atypical medullary cancers. sTILs LPBC was also associated with higher tumour grade, higher nuclear pleomorphism and absent lymphovascular invasion. An increasing level of sTILs, as 10% increments, was a significant predictor of outcome both DFS and OS on both uni- and multi-variate analysis. The magnitude of the effect on outcome was greater when sTILs was treated as a categorical variable and when a 25% threshold was used, sTIL high tumours had a significantly worse DFS and OS compared with those with sTILs <25% (reflected in a HR of 0.44 and 0.56 respectively) that was maintained on multivariate analysis. Those cases where the sTIL infiltrate reached the level of LPBC showed a similar association with DFS and OS however this did not attain statistical significance. For those who were treated with NACT, there was no significant difference in the sTIL counts in paired pre- and post-NACT samples.

4.4.1. Reported associations of sTILs in cancer

TILs are commonly observed in several tumour types. Increased TILs, particularly CD8+ lymphocytes, were associated with better prognostic features in cancers of the colon,[374, 375] ovary,[376] lung[377] and in malignant melanomas.[378, 379] Tumour regression can be accomplished just by the activation of the immune system (through administration of T-cell growth factors as interleukin-2). This was first described in melanomas.[380, 381] More recently, clinical studies revealed enhanced survival for melanoma patients on administration of the T-cell checkpoint inhibitor ipilimumab, alone or in combination with other drugs.[382, 383]

Over the last thirty years, several studies examined the presence of inflammation as a prognostic marker within BC. However, most of the earliest reports lacked the detailed documentation about the methodology used for the assessment of tumour-associated inflammation.[384-386, 506] An association between prominent inflammation and better prognosis was outlined by some studies[384-386] but not all.[506] Fifteen years ago, unveiling the molecular heterogeneity of BC was gained through gene expression profiling, which disclosed the fact that BC is not a single disease.[249] At the same time, the insights about the role of the tumour's immune microenvironment were expanded substantially and subsequent development of immune cell-therapy commenced an era for new therapeutic strategies.[382, 383] This led to revisiting the role of TILs and investigating it more comprehensively within different BC molecular subtypes. The recent research on BC emphasizes that some of its molecular subtypes are immunogenic. This is predominantly shown in the TN and HER-2-positive BCs, in which higher TILs prevalence was observed.[108, 110, 391, 394, 507] A recent systematic review which included 13,914 BCs confirmed that intense sTILs are seen more frequently in TNBC and HER-2-positive BC than in ER-positive tumours (median: 20% and 16% versus 6% respectively).[394] This had been attributed to the higher genomic instability of these two subtypes, particularly the TNBC. TNBC displays a complex mutational pattern with numerous genetic alterations.[508] Similarly, the HER-2-positive and luminal B subtypes are characterized by genetic instability centered on focal elevated DNA amplifications while luminal A showed low degree of genetic instability and did not portray a complex mutational pattern.[508]

Genetic mutations can lead to production of innovated abnormal amino acids that can be expressed and presented on the surface of the oncogenic cells (tumour neoantigens). Fortunately, the immune system can recognize it as non-self and initiate its fight to destroy it.[509] This is postulated to gather the TILs, which in turn modulate the prognosis for those tumours.

Large cohorts of BC patients were examined for iTILs and sTILs. As noted by the IWG, iTILS are usually associated with sTILs.[427] A prognostic value for the iTILs has not been demonstrated. In the BIG 02-98 trial, Loi and colleagues studied the TILs in both compartments within >2000 BC, including 256 TNBCs, enrolled in a randomized trial. They described that linear increase in iTILs was significantly paralleled with an improvement in the survival of TNBC but not in luminal and HER-2-positive BC.[110] This parallel association remained existent, although non-significant, within 481 TNBCs examined by Adams *et al.*[504] A contradictory result, with no association between iTILs and prognosis in 425 TNBCs, was published by Chinese investigators.[510] There may be several reasons for this. One element is that there are scant numbers of cases incorporating iTILs and so the dynamic range for evaluation of iTILs is low. Also, the investigators noted that more effort is exerted to identify the iTILs on H&E, which might be a contributory factor.[427] On the other hand, data about the prognostic value of sTILs in BC was more homogeneous, particularly within the TNBC. This will be discussed in section 4.4.3.

Recently, a Korean group conducted an observational study focusing on sTILs among 447 HER-2-positive BCs. Interestingly, their analysis revealed that high TILs was prognostic within hormone receptor negative HER-2-positive BC but not within hormone receptor positive HER-2-positive BC.[511] Other studies demonstrated that women with HER-2-positive BC, treated with trastuzumab and chemotherapy, in which their tumours harboured abundant TILs, were less likely to develop recurrence [389, 512] and more likely to have pCR.[512] On the contrary, the results from the N9831 trial confined the prognostic value of TILs to the patients treated with chemotherapy alone without trastuzumab.[513]

Lastly, a recent meta-analysis which investigated 13,100 BC patients confirmed that hormone independent and poorly differentiated tumours were coupled with

high sTILs. Moreover, superior survival rates and pCR were significantly linked to prominent TILs within the entire analyzed BC population.[107]

4.4.2. Reported frequencies of sTILs in TNBC

In our analysis, the median sTILs value was 15%, which is comparable with other studies published. Recorded sTILs median value levels varied in the literature between 10%[352, 504] and 25%[389] (Table 4.10.). Our result is similar to Dieci *et al.* (2014)[388] and Tian *et al.* (2016)[510] (median 15% and 14.2% respectively), and slighly lower than a recent Italian study done on 897 TNBCs [505] who outlined equal median (20%) to that reported in the BIG 02-98 trial.[110]

Different thresholds for the cut-off to distinguish LPBC have been used globally and there is no agreed threshold yet. As recommended by the IWG, a continuous scale should be measured.[427] Cut-offs used to designate lymphocyte-rich BC or LPBC differed widely between 10%[514, 515] and 60%,[373, 516] while most studies used a 50%[392, 504, 505] threshold. Similar to our study, two published reports examined the significance of two or more cut-off values in a trial, to pinpoint a meaningful cut-off percentage that can be used in a clinical setting.[391, 510] Kotoula *et al.* evaluated sTILs in 2618 BCs including 327 TNBCs. They tested the prognostic significance of sTILs at three different thresholds (>25%, >35% and >50%) and the three cut-offs were associated with significant statistical results. The association between sTILs and DFS with the > 25% and >50% cut-offs (HR= 0.15, log rank p=0.010 and HR= 0.12, log rank p=0.011 respectively) were slightly stronger than >35% cut-off (HR= 0.36, log rank p=0.02).[391] Tian *et al.* analyzed the impact of sTILs in 425 TNBCs on survival when using the cut-offs of >20% and >50%. They found that the >20% is a more useful cut-off. sTILs >20% were associated with better DFS and OS on univariate (HR 0.24; 95% CI: 0.10–0.60, p = 0.002 and HR 0.16; 95% CI 0.05–0.51, p = 0.002 respectively and multivariate models ((HR 0.17; 95% CI 0.05–0.53, p = 0.003, HR 0.26; 95% CI 0.08–0.84, p = 0.02 respectively). While there was no significant association with survival at sTILs >50% threshold.[510] LPBC (sTILs ≥50%) constituted 15.8% of our cohort, which is an intermediate value between the proportions described by Loi *et al.* and Kreike B *et al.* (11.6 and 12% respectively)[353, 389] and Pruneri *et al.* and Hida *et al.* (21.9% and 20.77%

respectively).[505, 517] The highest density of sTILs was conveyed in a Korean study in which 32.5% of 769 TNBCs displayed ≥70% sTILs[390] while within a German population 36.5% of 313 TNBCs presented with ≥50%[516]. On the contrary, much lower frequencies of LPBC (sTILs ≥50%) were documented by Beckers *et al.*, Adams *et al.* and Dieci *et al.* (3.7%, 4.4% and 5% respectively).[392, 504, 518]

The dissimilarities in the sTILs median values and the frequency of LPBC in TNBC might be influenced by several factors such as the ethnic differences of the studied populations with different immunological profiles, different nature and quantity of the assessed material (whether it is TMA, core biopsy or full-face of one or more H&E slide, H&E versus IHC). Different cut-offs and interobserver variability in the assessment of TILs may also play a role.

4.4.3. Reported clinicopathological and prognostic associations with sTILs in TNBC

Our investigation demonstrated that LPBC (sTILs ≥50%) was associated with poor differentiation (higher grade), increased nuclear pleomorphism, certain histological subtypes and absence of lymphovascular invasion. The available data regarding the relationship of sTILs, within the TNBC subtype, with other clinicopathological parameters are limited. Similar to our data, there is evidence highlighting the significant prevalence of elevated sTILs within the high grade TNBC tumours in many cohorts.[107, 110, 386-392] The numbers of the special histological subtypes within the TNBC cohort was small in other studies as well as in ours. Yet keeping with the limited literature, carcinomas with medullary features were associated with high sTILs counts, while ILC and metaplastic carcinomas had low to absent sTILs.[386, 390, 503]

The association with LVI was significant only in two studies,[390, 519] however it conflicts with our data in which LPBC is associated with absent LVI. Tian *et al.* described an inverse association between age and concentration of sTILs.[510] A similar correlation was noted by the Nottingham group when CD8+ lymphocytes were investigated in BC.[520] Loi and collaborators presented, of the 2015 San Antonio BC Symposium, the results of a pooled analysis of sTILs in 991 TNBC

patients. They found no association between sTILs with either age or lymph node status.[521] Further large-scale studies and meta-analysis are needed to explore the relationship between sTIL and different clinicopathological parameters with the aim of understanding their interactions and tailor the individualized proposed immunotherapy.

In our study, sTILs were associated with improved DFS and OS when sTILs were evaluated in increments of 10%, both on UVA and MVA. The four-scale categorization of sTILs showed a greater prognostic impact on both DFS and OS than that with an incremental increase of sTILs. The magnitude of the sTILs effect was even more significant when we used the > 25% cut-off to define high sTILs. Using the univariate log rank test, sTILs-high cases were associated with a significantly better DFS but non-significant longer OS. While on MVA, sTIL-high was an independent predictor of DFS and OS. Finally, the association between DFS and LPBC was significant on the univariate log rank test, but not on the MVA. There was no association between LPBC and OS neither on the logrank UVA nor on the MVA.

All[110, 353, 389-392, 504, 510, 517, 518, 522, 523] published studies except two[393, 524] have reported the presence of sTILs, assessed on H&E, is associated with an improved prognosis in operable TNBC. The characteristics and findings of these studies are summarized in Table 4.9. Park *et al.* studied the sTILs on FF H&E slides for 121 NACT naïve TNBC tumours in which the majority of the tumours were low stage (pT1/2 = 96%; pN0= 87%).[524] Interestingly, a detailed investigation of the relationship of sTILs with different BC stages was recently researched through a large-scaled Korean study. The enhanced outcome was significantly associated with the advanced stages but not with stage I.[390] This highlights the need for more studies and analysis of sTILs in TBNC according to the disease burden. While Polónia *et al.* evaluated sTILs on TMA for 536 BCs including 79 TNBCs and detected a better prognostic influence for elevated sTILs (>30%) in ER-negative and vimentin positive BCs.[393] They also noticed a non-significant trend between sTILs and survival in TNBC.[393] The limitations of this study include the small number of TNBCs and the nature of the evaluated material being a TMA core, instead of the FF sections as recommended by the IWG. Mori *et al.* studied

a moderately sized cohort of TNBC (n= 248) and observed that the high sTILs did not affect the recurrence free survival (RFS) but significantly improved the OS on univariate and multivariate analysis.[525] Krishnamurt *et al.*, proposed that the location of the sTILs indicates prognostic importance. They concluded that the risk of recurrence and death were influenced by the peripheral sTILs, but not the overall sTILs.[526]

This work presented in this thesis showed that dicotomization of sTILs into two categories had a stronger association with outcome that when sTILs was evaluated as 10% increments. On multivariate analysis, women with sTIL-high (sTILs ≥25%) tumours were significantly less likely to develop a disease relapse and had an improved OS than those with sTIL-low tumours. This was independent of age at diagnosis, tumour grade and nodal status. However, when the LPBC status (sTILs ≥ 50%) was analysed, as stratified in the BIG02-98 study, the associations between LPBC status and DFS and OS were observed and LPBC remained in the final multivariate model with age at diagnosis, nodal status and tumour grade but did not reach statistical significance (p=0.070 and p=0.162 respectively, multivariate cox regression test). Although this result contradicts those of Loi *et al.*[110], it concurs with those of Adams *et al.*[504] and Tian *et al.*[510]

Our findings, in line with previous published data, support the concept that some TNBC tumours are associated with a successful immune response that modulates their prognosis. It further supports the consensus that sTILs have prognostic significance in TNBC and that tumours with a high level of sTILs have a better outcome. The precise threshold for a prognostic role for sTILs in clinical practice is uncertain. This work suggests that the magnitude of the prognostic effect of sTILs is greater when a threshold of 25% is used. As with other series, the size of our series of TNBC patients is too small to have the power to detect the strength of the association between LPBC (i.e. a threshold of 50%) and outcome in TNBC.

Table 4.10. Reports of association between sTILs (on H&E) and outcome in TNBC

Study, year	n	Method of sTILs assessment	Median sTILs (Range)	Frequency of sTILs	Follow-up (months)	Significant association with outcome for sTILs			
						Univariate		Multivariate	
						DFS	OS	DFS	OS
Kreike *et al.*, 2007[353]	97	H&E (2-6 FF slides per tumour)	NR	None= 8.24% Minimal*= 41.23% Moderate**= 34% Extensive*** = 12.37% Non-scorable= 4.12%	61	Yes[a,b]		Yes[a,b]	
Loi *et al.*, 2013 (BIG 02-98 trial)[110]	256	H&E (FF section)	20% (2.5-75%)	LPBC sTILs (>50%) = 10.6%	96	Yes[c]	Yes[c]	Yes[c]	Yes[c]
Loi *et al.*, 2014 (FinHer trial)[389]	134	H&E (FF section)	25% (3-85%)	LPBC sTILs (>50%) = 11.6%	62	Yes		Yes	
Adams *et al.*, 2014 (ECOG 2197 ECOG 1199 trial)[504]	481	H&E (FF section)	10%	sTILs (0%) = 20% sTILs (10%) = 49% sTILs (20-40%) = 26.6% sTILs (>50%) = 4.4%	127	Yes[a,d,e]	Yes[a,d,e]	Yes[a,d,e] No[c]	Yes[a,d,e] No[c]
Dieci *et al.*, 2015[392]	199	H&E (FF section)	15% (0-90%)	Low sTILs (<50%) = 85% High sTILs (>50%) = 15%	152				Yes[d]
Kotoala *et al.*, 2015[391]	327	H&E (FF section)	NR	Three cut-offs tested sTILs (>25%) = 23.8% sTILs (>35%) = 13.6% sTILs (>50%) = 8%	NR	Yes[f]		Yes[f]	
Beckers *et al.*, 2016[518]	161	H&E (TMA)	NR	Mild (<30%) = 45% Moderate (30-60%) = 21.3% Marked (>60%) = 3.7%	55		Yes[g,h]		Yes[g,h]
Pruneri *et al.*, 2016[505]	897	H&E (FF section, 3 slides)	20%	LPBC sTILs (>50%) = 21.9%	98	Yes[c,d]	Yes[c,d]	Yes[c,d]	Yes[c,d]

Table 4.10. (Cont'd)

Study, year	n	Method of sTILs assessment	Median sTILs (Range)	Frequency of sTILs	Follow-up (months)	Significant association with outcome for sTILs			
						Univariate		Multivariate	
						DFS	OS	DFS	OS
Park *et al.*, 2016[522]	767	H&E (FF all tumour slides)	NR	Low sTILs (<10%) = 23% High sTILs (<10%) = 74%	NR	Yes[b]	Yes[b]	Yes[b]	Yes[b]
Lee *et al.*, 2016[390]	769	H&E (FF section)	NR	sTILs (<10%) = 24.4% sTILs (20-30%) = 22.2% sTILs (40-60%) = 20.8% sTILs (70-100%) =32.5%	NR	Yes[d]	Yes[d]	Yes[d]	Yes[d]
Park *et al.*, 2016[524]	121	H&E (FF section)	NR	Positive sTILs (>10%) = 62% LPBC (>60%) = 19%	NR	No[b,c]	No[b,c]	No[b,c]	No[b,c]
Tian *et al.*, 2016[510]	425	H&E (FF section)	14.2% (0-90%)	sTILs 0-10% = 65.3% sTILs 11- 30% = 23.2% sTILs 31-50% =7% LPBCs (50%)= 3.5%	48	Yes[d,i] No[c]	Yes[d,i] No[c]	Yes[d,i] No[c]	Yes[d,i] No[c]
Hida *et al.*, 2016[517]	154	H&E (FF section)	NR	Low sTILs (<10%) = 12.33% Intermediate (10-50%) = 66.89% High sTILs (>50%) = 20.77%	45	Yes[b,j]		Yes[b,j]	
Mori *et al.*, 2017[525]	248	H&E (FF section)	NR	LPBC (>50%) = 47.6%	68	No[c,k]	Yes[c]	No[c,k]	Yes[c]
Polónia *et al.*, 2017[393]	79	H&E (TMA)	NR	sTIL<30%: 81% sTIL<30: 19%	120	No[h]	No[h]		
Krishnamurt *et al.*, 2017[526]	157	H&E (FF section)	NR	sTILs <5% = 39% sTILs 5-10% = 30% sTILs 10-50% =25% LPBCs (>50%)= 6%	NR	Yes[d,l] No[d,m]	Yes[d,l] No[d,m]	Yes[d,l] No[d,m]	Yes[d,l] No[d,m]

*:<10 lymphocytes/HPF, ** :easily identified lymphocytes but no large aggregates, ***: Big aggregates in >50% of the tumour; a: Distant-disease free survival, b: analysis at10% cut-off, c: analysis at 50% cut-off, d: analysis as continuous variable with 10% increments, e: analysis at (0% vs >0%), f: cut-off at 25%,35% and 50%, g; Breast cancer specific survival, h; analysis at 30% cut-off, i: analysis at 20% cut-off, j: analysis at 3 grade system, k: Recurrence-free survival, l: Peripheral TILs, m: overall TILs

4.4.4. Limitations of our study and future directions

Our study had several limitations. At the time of this analysis, the follow up time was short for some cases because we undertook the analysis on THE specimens and some of the patients had a short follow-up as their therapeutic procedures were performed at the beginning of last year (2016). However, the median follow up was 36 months that was reached by 145 patients and for TNBC the risk of relapse is highest in the first 3 years.[527] The follow-up of 2548 women diagnosed with TNBC in a Dutch study showed that nearly 75% of the events occur in the first 3 years.[528]

We included cases from patients who had received NACT prior to their resection with those treated with adjuvant chemotherapy and so the study cohort comprised chemotherapy naïve tumours as well as tumours treated with NACT. However we noted that there was no difference in the sTILs scores in the pilot study of 26 paired pre-treatment NCBs and corresponding post-NACT excision specimens. Neither was stratification of the prognostic value of sTILs according to the treatment regimens or agents given tested in this work. However, sTILs was a strong prognostic factor within the entire cohort regardless of the treatment differences, and this corresponded to previous published data in the adjuvant[110, 389, 504, 505] and neoadjuvant setting.[392, 517]

The effect of NACT on sTILs has been studied sparsely with few published data including small numbers of cases and presenting conflicting results. Demaria *et al.* exerted a comparative study for TILs in pre-treatment and post-treatment tissue for a small cohort (n=25) of all types of BC who completed taxane-based NACT (paclitaxel). They concluded that anti-tumour lymphocytic response can result from the exposure to NACT, as there was a rise in the TILs within one third of the post-treatment residual tumours which did not show TILs on the pre-treatment tissue (7/21).[529] A further study by Dieci *et al.* focused on analysis of paired specimens for tumours with high-TIL on post-chemotherapy specimens (n=27). They demonstrated that an intensification of the immune reaction was observed in 96% of these tumours, which was additionally associated with better prognosis.[388] In our study, we analyzed a small number of unselected cases including those with both low and high sTILs counts, while Demaria *et al.*

exclusively studied the cases with low-TILs on pre-treatment cores and Dieci *et al.* limited their investigation to the post-treatment specimens with high-TILs scores. Nevertheless, we are in partial agreement that a proportion of the BC tumours can experience an increase in sTILs due to the NACT effect. Additional evidence was supplemented by a French study that explored the counts of different T-cell subtypes on 56 paired tumours using immunohistochemical staining. Their research concluded that the NACT had no effect on the amounts of the CD3 and CD8 lymphocytes but significantly reduced the Foxp3 immunosuppressive T cell counts.[530] Hida *et al.* reported that 11/26 TNBC cases showed higher sTILs in the excision, 7/26 had equivalent amounts while 8/26 revealed lower sTILs post-chemotherapy. However, they categorized the sTILs into three categories (Low: <10; Intermediate: 10-50%; High: >50%) and compared the cases that changed from a category to another.[517]

Treatment differences between studied groups could provide an explanation for differences in sTILs pre and post treatment, and it would be interesting to explore the sTILs counts in relation to specific therapeutic agents e.g. platinum.

Another limitation of this work was that a small number of co-variables were included in the multivariate analysis of sTILs: age at diagnosis, nodal status and tumour grade. We were unable to test tumour type as an independent variable because most cases were ductal (NST) type. There was a strong association between typical and atypical medullary types and sTILs. However, there were insufficient cases to examine if the prognostic effect of sTILs was independent of medullary type (or medullary-like) phenotype. As expected nodal status was the strongest predictor of outcome by multivariate analysis in our series and determination of the significance of sTILs in the different nodal groups should be determined in a larger series.

Interobserver variability was not adequately tested in this study as the two observers assessed sTILs together on a multiheaded microscope. A further study within our group is in progress to study the inter-observer variability of sTILs. Swisher *et al.*, assessed the interobserver variability for sTILs on H&E stained pre-treatment core biopsies from 75 TNBC tumours, by four breast pathologists. There was moderate agreement (kappa=0.57) when the sTILs were categorized

i.e. (<10%, 10-50% and >50%), however a continuous scale was not evaluated in this analysis.[531]

Counting of sTILs proportions is a relatively subjective method in which 'eyeball' estimates are used. Computer based image analysis may represent an objective method. It had been used successfully in evaluation of the biomarker expression.[532] A recent comparative study between computerized versus manual scoring for sTILs on BC revealed excellent correlation results on 377 samples.[533] The manual approach was used in most of the published sTILs studies to date and yielded promising results even on H&E alone. Moreover, it is a method that can be used by most pathologists during routine practice. On the other hand, it is challenging and time-consuming to outline the boundaries of the tumour stroma using the image analysis software. The pathologists' examination of sTILs can be done in a timely-manner during routine histological examination using the proposed four-scale grading system. An equivalent three-scale grading system for TILs is used in clinical practice and is integrated in the recommended datasets for reporting malignant melanoma.[534] A validation for the use of a comparable grading system for sTILs is supported by Swisher *et al.*.[531] This can be applied in the clinical setting with an acceptable degree of reproducibility, however the limited sample size and small number of participating pathologists limit its predictive power. We followed the recommendations of IWG, averaging the sTILs on a representative FF H&E slide, however we noted that the density of the sTILs varies in different fields of the tumour with higher concentration (hot spots) at the edges of the invasive tumour.

Tumour heterogeneity and the effect of sampling impacts on the evaluation of many prognostic and therapeutic factors in BC. The NCB is a small fraction of the overall tumour while the resection tissue full-face slide represents a larger proportion from the tumour. Notwithstanding, in most cases of TNBC tissue will only be available in the form of a NCB because NACT is now the standard of care for TNBC. The heterogeneity of the tumour stroma had been described recently[535-537] and the effect of sampling has been established in many studies, exposing the heterogeneity of the BC grade as well as the immunohistochemical expression.[538-541] Heterogeneity of sTILs scores was noted on the excision

specimens in other studies.[391, 526, 542] Although the IWG had no consensus regarding this issue, they favoured examination of whole tissue tissue sections over NCB.[427] A recent American study emphasized the heterogenous distribution of sTILs within BC. They examined the prognostic influence of the location of sTILs (peripheral versus overall) in TNBC and ER-positive tumours. They perceived that longer survival was associated with higher peripheral but not overall sTILs in TNBC. While sTILs, regardless to their location, did not impact survival in ER-positive tumours.[526]

Our pilot study compared sTILs in paired biopsy and surgical excision specimens in a 26 cases of TNBC who received NACT and while it examined tissue heterogeneity to a degree, it was confounded by the fact that the resection was post NACT. There were very few discordances between the paired samples in the pilot study. While it would seem that a better representation of the tumour might be expected when more cores were examined, the four cases where a >20% difference in sTILs was observed had the same number of cores as the 26 cases overall (median = 3). However, a statistical comparison was not applicable because of the small number of discrepancies and the limited number of cases in this study.

Cohort size was also a limitation. TNBCs comprise less than 20% of all cancers and so most single institution TNBC series are small. The size of the TNBC in this work was too small to have the power to accurately evaluate the most appropriate threshold for a cut-off for sTILs or to determine if the prognostic effect of sTILs was independent of the medullary type.

Lastly, we examined sTILs on only H&E without further immunohistochemical characterization of the functional subsets of the lymphocytes, however it was proved to be adequate for prediction of the outcome with consistent results across the published studies done on TNBC. Determining the subtyping of infiltrating lymphocytes may provide additional information that might help understanding the mechanisms behind the tumour's immune microenvironment in TBNC.

5. CHARACTERIZATION OF COX-2 AND iNOS EXPRESSION IN BC

5.1. Introduction

Inflammation associated carcinogenesis is incited through the inflammatory cells and the inflammatory cytokines secreted into the tumour microenvironment.[69, 70] The combined effect of these can direct the host inflammatory response to be either anti-tumourigenic or pro-tumourigenic.[69, 70] Two inflammation-associated enzymes that had been under the spotlight for researchers to explore their role in tumour initiation, progression and prognosis, are the COX-2 and the iNOS. These enzymes (COX-2 and iNOS) are subjected to cross-talk with NF-κB, [72, 543] which is also regulated by estradiol.[81] Abnormal production of COX-2 and iNOS in turn leads to overproduction of pro-inflammatory mediators, prostaglandins and NO, and are found in many precancerous and malignant lesions.[199, 544] Previous animal studies on both COX-2 and iNOS have revealed that targeted inhibition of COX-2 and iNOS can prevent inflammation-associated carcinogenesis.[200-202] The role of COX-2 in colonic tumourigenesis is established in earlier studies.[154545] However its role in BC and its subtypes remains questionable. Whereas iNOS potentiates the production of nitric oxide, which is described by some authors as a double-edged sword due to opposing functions in cancer biology influenced by its concentration.[187] The value of iNOS detection in BC is still unclear and controversial. In particular, studies by Glynn *et al.*[191, 429] showed that COX-2 and iNOS expression is associated with worse BC specific survival in ER-negative breast tumours. Therefore, we aimed in this work to investigate the association between COX-2 and iNOS immunohistochemical expression in BC cells and its pathological parameters and outcome.

5.2. Evaluation of COX-2 and iNOS IHC in BC

COX-2 and iNOS were evaluated on 450 and 422 of 666 BC using the WOI BC series. This cohort included all types of BCs and 8% were TNBC (52/666). This was used because the expression of these markers remains largely unknown in all subtypes of BC. After excluding the uninformative TMA cores from the study, tumours were scored for COX-2 within 450 cores by AS. Twenty-five percent of the informative TMA cores were additionally double-scored by GC. There was agreement on COX-2 staining intensity in 81.2% of the double-scored cores (kappa value = 0.74). Also, there was very good agreement on the COX-2 staining percentage categories (kappa value = 0.7) with agreement on 77.7% of the double-scored cores.

Tumours were scored for iNOS within 422 cores by AS and twenty-five percent of the informative TMA cores were additionally double-scored by GC. For these cases, there was good agreement for scoring intensity with concordant scores recorded in 75.5% of cases (kappa value = 0.65). Scoring the percentage of the positive was more heterogeneous than scoring the iNOS intensity with agreement between both scores reached for 70.5% of cases (kappa value = 0.55), which indicated a moderate level of agreement. Discordant cases were reviewed and a consensus score assigned.

5.2.1. Expression of COX-2 in BC

Within the WOI BC series, 450 informative cores were assessed for COX-2 immunohistochemical staining. Overall, 48/450 cores (10.7%) revealed no staining while diffuse cytoplasmic staining was seen in 402/450 (89.3%). Different staining intensities and their distribution within the cases are shown in Figures 5.1 and 5.2. The cases were dichotomized into COX-2 positive and COX-2 negative depending on the sum score for the intensity of the staining and the proportion of the tumour cells expressing the protein. Fifty one percent (230/450 cores) were COX-2 positive while approximately 49% (220/450 cores) were COX-2 negative.

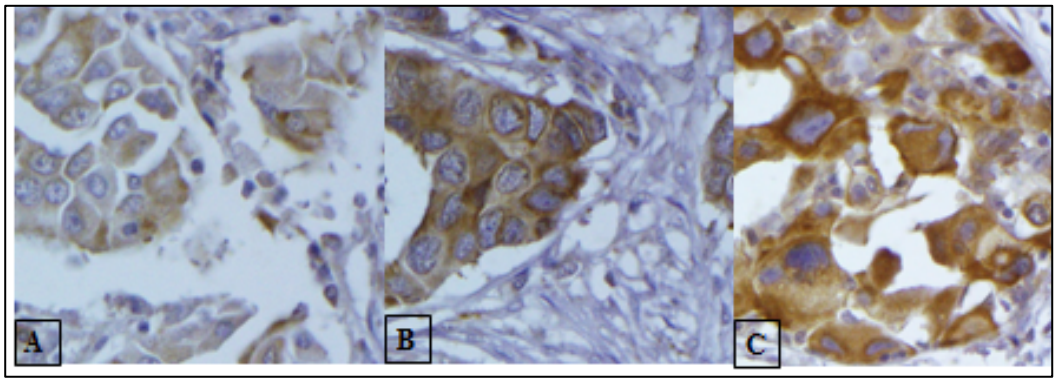


Figure 5.1. COX-2 cytoplasmic staining in BC Representative images of the spectrum of the cytoplasmic intensity observed for COX-2 in BC where A = weak, B= moderate and C= strong (x200)

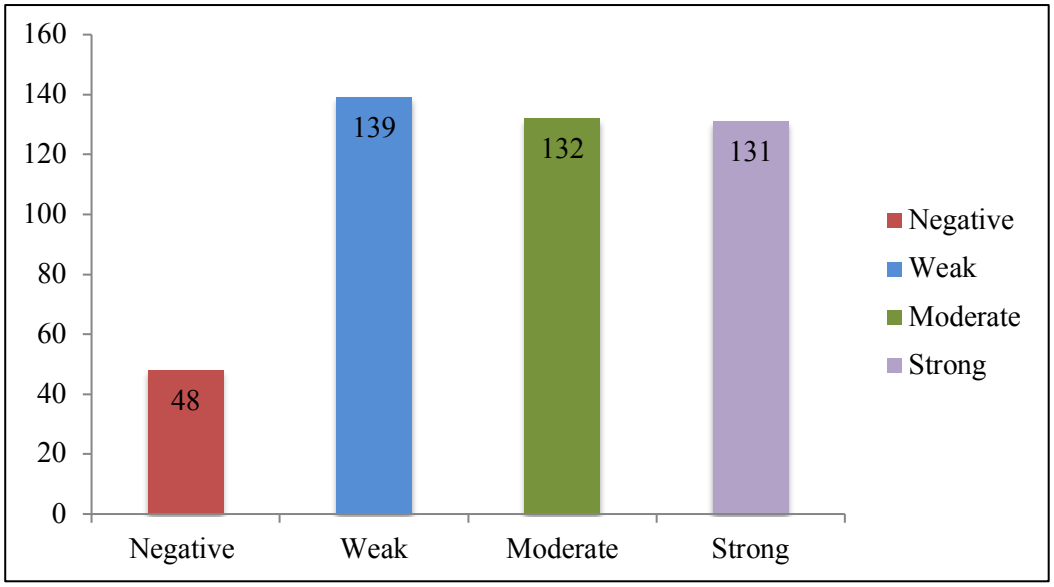


Figure 5.2. COX-2 expressions in BC

5.2.3. Associations between COX-2 expression and pathological parameters in BC

Associations between COX-2 expression and pathological variables in BC are shown in Table 5.1. In the entire cohort, a significant reverse association was observed between COX-2 positivity and tumour grade (p= <0.001) (Table 5.1). There was no association between COX-2 expression and tumour size, nodal status or stage.

Table 5.1. Association between COX-2 and tumour variables in BC

Variable		COX-2 negative n(%)	COX-2 positive n(%)	n	χ^2	p value
Tumour grade	1	17 (30)	39 (70)	56	15.41	**<0.001***
	2	85 (42)	114 (58)	198		
	3	92 (58)	67 (42)	159		
T stage	pT1	50 (41)	73 (59)	123	6.08	0.108
	pT2	117 (45)	141 (55)	258		
	pT3	34 (60)	23 (40)	57		
N stage	pN0	94 (45)	115 (55)	209	0.41	0.937
	pN1	57 (48)	61 (52)	118		
	pN2	28 (46)	33 (54)	61		
	pT3	17 (49)	18 (51)	35		
M stage	pM0	174 (46)	202 (54)	376	1.05	0.591
	pM1	21 (43)	28 (57)	426		

a, number of cases for which data was available; p values in bold indicate statistically significant associations.

COX-2 positivity was seen in a significantly higher proportion of ER and PgR-positive BC as compared to ER-negative and PgR-negative BC (p=<0.0001) (Table 5.2). There was a significant association between COX-2 positivity and higher Bcl-2 expression (p=0.04), lower EGFR, Ki67, p53 and E-cadherin expressions (p=0.026, p=0.035, p=<0.001, p=0.008 respectively) (Table 5.2).

The associations with the pathological variables were examined in ER-positive and ER-negative tumours. Within ER- BC, the significant relationship between COX-2 positivity and Bcl-2, p53 and E-cadherin were retained (p=0.04, p=0.015 and p=0.036 respectively) (Table 5.3) while in ER-positive tumours, no significant association was observed (Appendix XIV).

Table 5.2. Association between COX-2 and other biomarkers in BC

Variable		COX-2 negative n(%)	COX-2 positive n(%)	Total	χ^2	p value
ER	Negative	83 (63)	48 (37)	131	22.36	**<0.001***
	Positive	116 (39)	184 (61)	300		
PgR	Negative	87 (61)	55 (39)	142	19.86	**<0.001***
	Positive	110 (38)	176 (62)	286		
HER-2	Negative	192	178	370	3.89	0.143
	Positive	33	31	64		
Bcl-2	Negative	110 (56)	85 (44)	195	17.69	**<0.001***
	Positive	80 (36)	143 (64)	223		
CK5/6	Negative	137 (45)	166 (55)	303	1.61	0.204
	Positive	32 (54)	27 (46)	59		
EGFR	Negative	149 (43)	194 (57)	343	4.92	**0.026***
	Positive	39 (58)	28 (42)	67		
CK14	Negative	131 (49)	137 (51)	268	0.38	0.533
	Positive	35 (45)	43 (55)	78		
Ki67	Negative	123 (44)	160 (56)	283	4.43	**0.035***
	Positive	72 (55)	60 (45)	132		
p53	Negative	136 (42)	185 (58)	321	14.97	**<0.001***
	Positive	60 (65)	32 (35)	92		
E-cadherin	Negative	14 (29)	34 (71)	48	7.00	**0.008***
	Positive	181 (49)	185 (51)	366		

a, number of cases for which data was available. p values in bold indicate statistically significant associations

Table 5.3. Association between COX-2 and tumour variables in ER-negative BC

Variable		COX-2 negative n(%)	COX-2 positive n(%)	Total	χ^2	p value
Tumour grade	1	3 (43)	4 (57)	7	1.39	0.498
	2	18 (65)	10 (35)	28		
	3	56 (65)	30 (35)	86		
HER-2	Negative	62	35	97	0.64	0.422
	Positive	25	19	44		
Bcl-2	Negative	65 (68.)	30 (32)	95	4.23	**0.040***
	Positive	11 (46)	13 (54)	24		
CK5/6	Negative	48 (61)	30 (39)	78	<0.001	1.00
	Positive	16 (62)	10 (38)	26		
EGFR	Negative	44 (59)	30 (41)	74	1.24	0.265
	Positive	30 (70)	13 (30)	43		
CK14	Negative	48 (67)	24 (33)	72	0.58	0.445
	Positive	17 (59)	12 (41)	29		
Ki67	Negative	34 (60)	23 (40)	57	1.22	0.268
	Positive	43 (69)	19 (31)	62		
p53	Negative	39 (57)	30 (43)	69	5.92	**0.015***
	Positive	39 (78)	11 (22)	50		
E-cadherin	Negative	3 (33)	6 (67)	9	4.38	**0.036***
	Positive	76 (68)	36 (32)	112		

a, number of cases for which data was available. p values in bold indicate statistically significant associations

5.2.4. Association between COX-2 and patient outcome in BC

On UVA, COX-2 positivity did not indicate a difference in DFS or OS (p= 0.866 and p= 0.836, log rank test) neither in the entire BC cohort nor if grouped according to ER status (Appendices XV and XVI).

5.2.5. Expression of iNOS in BC

Within the WOI BC series, 417 informative cores were assessed for iNOS immunohistochemical staining. Overall within all the TMAs, only 12.7% (53/417) revealed no staining while 87.3% (364/417) revealed iNOS cytoplasmic immunohistochemical expression. The distribution of different staining intensities within the cases is shown in Figure 5.3. The cases were dichotomized into iNOS positive and iNOS negative depending on the sum score for the intensity of the staining and the proportion of the tumour cells expressing the protein. Nearly forty-five percent (189/417 cores) were iNOS positive while approximately 55% (228/417 cores) were iNOS negative.

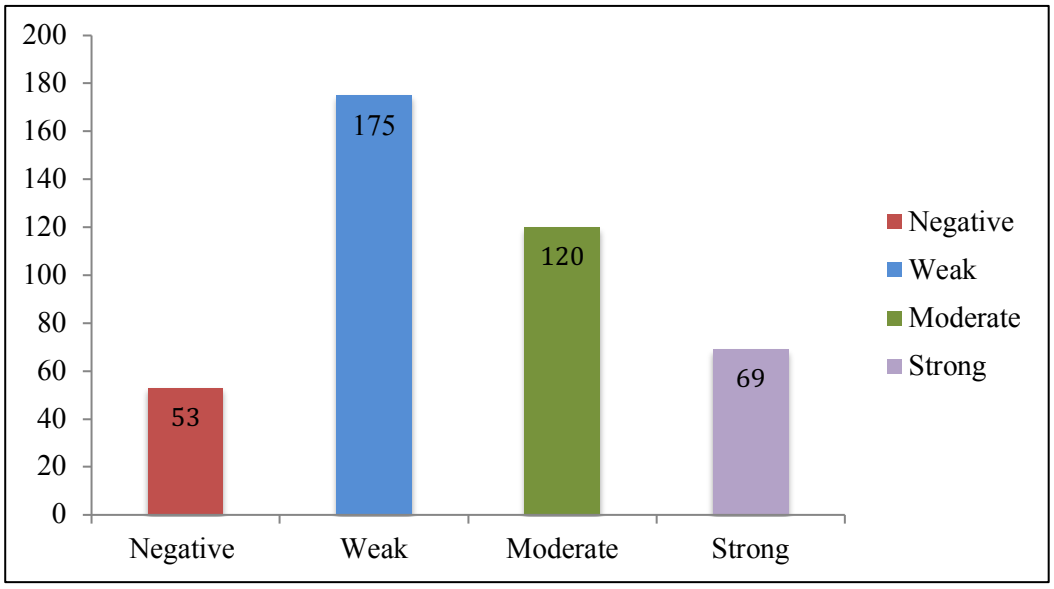


Figure 5.3. iNOS expression in BC

5.2.6. Association between iNOS and pathological parameters in BC

Associations between iNOS expression and pathological variables in BC are shown in Tables 5.4. to 5.7. In the entire cohort, a significant inverse association was observed between iNOS expression and tumour grade (p= 0.026) (Table 5.4.). There was a significant association between iNOS positivity and ER, PgR and CK14 expression (p=0.006, p=0.003 and p=0.016 respectively) (Table 5.5.). Within ER-negative BC, the significant relationship between iNOS expression and tumour grade was retained (p=0.019) (Table 5.6.) while in ER-positive tumours, significant association between iNOS expression and CK14 was still observed (Table 5.7.).

Table 5.4. Association between iNOS and tumour variables in BC.

Variable		**iNOS negative n(%)**	**iNOS positive n(%)**	**n**	χ^2	**p value**
Tumour grade	1	31 (58)	23 (43)	54	7.26	**0.026***
	2	98 (54)	84 (46)	182		
	3	103 (68)	48 (32)	151		
T stage	pT1	72 (63)	42 (37)	114	0.31	0.957
	pT2	150 (61)	96 (39)	246		
	pT3	30 (59)	21 (41)	51		
N stage	pN0	129 (64)	72 (36)	201	1.50	0.681
	pN1	66 (59)	46 (41)	112		
	pN2	31 (58)	23 (43)	54		
	pT3	19 (58)	14 (42)	33		
M stage	pM0	212 (60)	144 (40)	356	5.82	0.054
	pM1	35 (78)	10 (23)	45		

a, number of cases for which data was available; p values in bold indicate statistically significant associations.

Table 5.5. Association between iNOS and other biomarkers in BC

Variable		iNOS negative n(%)	iNOS positive n(%)	Total	χ^2	p value
ER	Negative	94 (71)	39 (29)	133	7.69	**0.006***
	Positive	141 (56)	110 (44)	251		
PgR	Negative	122 (70)	53 (30)	175	9.10	**0.003***
	Positive	116 (55)	96 (45)	212		
HER-2	Negative	104 (45)	129 (55)	233	2.19	0.334
	Positive	26 (57)	20 (43)	46		
Bcl-2	Negative	118 (65)	64 (35)	182	1.56	0.211
	Positive	125 (59)	88 (41)	213		
CK5/6	Negative	171 (61)	108 (39)	279	1.20	0.272
	Positive	40 (69)	18 (31)	58		
EGFR	Negative	195 (60)	128 (40)	323	0.91	0.338
	Positive	44 (67)	22 (33)	66		
CK14	Negative	153 (60)	102 (40)	255	5.75	**0.016***
	Positive	55 (75)	18 (25)	73		
Ki67	Negative	158 (59)	112 (41)	270	3.44	0.064
	Positive	86 (68)	40 (32)	126		
p53	Negative	188 (62)	116 (38)	304	0.29	0.587
	Positive	51 (59)	36 (41)	87		
E-cadherin	Negative	27 (64)	15 (36)	42	0.22	0.637
	Positive	210 (61)	137 (39)	347		

a, number of cases for which data was available; p values in bold indicate statistically significant associations

Table 5.6. Association between iNOS and tumour variables in ER-negative BC

Variable		iNOS negative n(%)	iNOS positive n(%)	Total	χ^2	p value
Tumour grade	1	1 (17)	5 (83)	6	7.96	**0.019***
	2	16 (59)	11 (41)	27		
	3	57 (71)	23 (29)	80		
HER-2	Negative	33 (49)	35 (51)	68	1.02	0.311
	Positive	19 (59)	13 (41)	32		
Bcl-2	Negative	65 (70)	28 (30)	93	2.91	0.088
	Positive	10 (50)	10 (50)	20		
CK5/6	Negative	49 (69)	22 (31)	71	0.11	0.734
	Positive	17 (65)	9 (35)	26		
EGFR	Negative	48 (70)	21 (30)	69	0.10	0.750
	Positive	28 (67)	14 (33)	42		
CK14	Negative	43 (64)	24 (36)	67	2.70	0.100
	Positive	22 (81)	5 (19)	27		
Ki67	Negative	33 (61)	21 (39)	54	2.35	0.125
	Positive	44 (75)	15 (25)	59		
p53	Negative	46 (71)	19 (29)	65	1.32	0.250
	Positive	29 (60)	19 (40)	38		
E-cadherin	Negative	6 (75)	2 (25)	8	0.40	0.523
	Positive	67 (64)	38 (36)	105		

a, number of cases for which data was available; p values in bold indicate statistically significant associations

Table 5.7. Association between iNOS expression and tumour variables in ER-positive BC.

Variable		iNOS negative n(%)	iNOS positive n(%)	Total	χ^2	p value
Tumour grade	1	29 (62)	18 (38)	47	3.78	0.151
	2	78 (53)	71 (47)	149		
	3	44 (66)	23 (34)	67		
HER-2	Negative	62 (44)	79 (56)	141	1.27	0.528
	Positive	7 (54)	6 (46)	13		
Bcl-2	Negative	49 (59)	34 (41)	83	0.01	0.895
	Positive	109 (60)	73 (40)	182		
CK5/6	Negative	114 (58)	83 (42)	197	2.99	0.083
	Positive	21 (75)	7 (25)	28		
EGFR	Negative	140 (58)	102 (42)	242	0.81	0.368
	Positive	13 (68)	6 (32)	19		
CK14	Negative	102 (57)	77 (43)	179	4.40	**0.036***
	Positive	32 (74)	11 (26)	43		
Ki67	Negative	119 (58)	86 (42)	205	0.35	0.554
	Positive	38 (62)	23 (38)	61		
p53	Negative	133 (59)	91 (41)	224	0.09	0.764
	Positive	21 (57)	16 (43)	37		
E-cadherin	Negative	19 (59)	13 (41)	32	<0.01	0.976
	Positive	136 (60)	92 (40)	228		

a, number of cases for which data was available; p values in bold indicate statistically significant associations

5.2.7. Association between iNOS and patient outcome in BC

On univariate analysis using Kaplan-Meier survival curves, iNOS positivity did not indicate a difference in DFS or OS (p= 0.799 and p=0.501, log rank test) neither in the entire BC cohort nor if grouped according to ER status. (Appendices XVII and XVIII).

5.3 Discussion

We examined the expression of COX-2 protein in BC. This enzyme is involved in inflammation as well as in the promotion of tumourgenesis and progression of some types of cancer. Cytoplasmic expression of COX-2 was present in 89.3% of cases, however using the detailed scoring system COX-2 was positive in 51.12% of the entire cohort. Within the entire BC cohort, COX-2 protein was associated with positive prognostic factors such as ER and PgR positivity and high Bcl-2 expression. COX-2 positivity was inversely associated with tumour grade and expression of EGFR, p53, Ki67 and E-Cadherin. These associations were not observed between COX-2 positivity and ER-positive tumours, while the association between COX-2 positivity and high Bcl-2, low p53 and reduced E-cadherin was retained in ER-negative tumours. In terms of outcome, COX-2 had no prognostic impact, neither in the entire cohort nor in the subgroups stratified according to ER status.

5.3.1. COX-2 expression in BC

This work demostrated that a high proportion of BC expresses the COX-2 protein (51.12%). This is consistent with previous reports, although there was a wide range (28-95%) within those studies.[155-157] This may reflect the non-uniformity in the staining protocols and antibodies used, differences in the examined cohorts, and scoring systems. Nevertheless these findings support a role for COX-2 deregulation in BC. A summary of the publications of COX-2 immunohistochemical expression and its associations with clinicopathological parameters and outcome are presented in Table 5.8.

Table 5.8. Reports of associations between COX-2 IHC expression and clinicopathological parameters and outcome in BC

Study year	n	COX-2 antibody	Scoring system	Cut-off for COX-2 positivity	Frequency of COX-2 positivity	Parameters examined	Significant association with parameters for AR positivity	Follow up period (months)	Impact of AR expression on outcome
Ristimäki *et al.*, 2002[160]	1576 TMA	Cayman mouse monoclonal (2.5 μg/ml)	Proportion and intensity[a]	Score ≥2	37%	Age, tumour size, grade, type, ER, PgR, HER-2, Ki67, p53	Larger tumour, high grade, ER and PgR negativity, HER-2 positivity, high Ki67, high p53, LN positivity, ductal phenotype	Median 6.8 years	Decreased DDFS in the entire cohort and in ER positive tumours on UVA, but not on MVA
Kelly *et al.*, 2003[546]	106 WTS	Cayman polyclonal 1:500	Product score of percentage and intensity[b]	Analysis for 4 categories[c]	85%	Tumour size, grade, type, ER, PgR, LN status	None	Mean 1.92 years	No correlation with survival
Denkert *et al.*, 2003[547]	221 WTS	Cayman mouse monoclonal 1:1000	Product score of percentage and intensity[b]	Score >7	36%	Age, tumour size, grade, type, ER, HER-2, Ki67, LVI, LN status	Larger tumour, high grade, ER negativity, LVI positivity, LN positivity	Median 7.25 years	Reduced DFS and OS on UVA. Borderline association with DFS. No association with OS on MVA
Wülfing *et al.*, 2003[548]	192 TMA	Cayman mouse monoclonal 1:200	Proportion and intensity[d]	Score ≥2	40.6%	Tumour stage, grade, ER, PgR, HER-2, Ki67, LVI, LN status	Advanced stage, high grade, ER and PgR negativity, high Ki67, LVI positivity, LN positivity	Median 5.92 years	No impact on DFS or OS
Nakopoulou *et al.*, 2005[549]	175 WTS	Santa Cruz polyclonal 1:120	Proportion/ intensity score	Moderate staining in >20%	68%	Menopause, tumour size, grade, type, stage, ER, PgR, HER-2, p53, topoIIα, PPARγ	Low grade, low nuclear grade, PgR positivity, low p53, low topoIIα, high PPARγ	Mean 9.25 years	No correlation with DFS or OS in UVA or MVA

Table 5.8. (Cont'd)

Study year	n	COX-2 antibody	COX-2 scoring system	Cut-off for COX-2 positivity	Frequency of COX-2 positivity	Parameters examined	Significant association with parameters for AR positivity	Follow up period (months)	Impact of AR expression on outcome
Holmes *et al.*, 2011[157]	2001 TMA	Labvision Rabbit monoclonal	Intensity/ Percentage (negative, 1-3)[e]	Score ≥1	28%	Tumour size, grade, stage, type, ER, PgR, HER-2, LN status	Larger size, advanced stage, lobular subtype, ER positivity, PR positivity, LN metatsases	NR	Worse prognosis
Park *et al.*, 2012[550]	861 TMA	SP21, 1:200; Biocare Medical,	Percentage score	>>10%	57.3%	Age, tumour size, grade, type, stage, ER, PgR, HER-2, Ki67	Older age, smaller tumour size, low grade, ER positivity, PgR positivity, low Ki67	Median 5.9 years	No association with survival in entire cohort or according to ER status. Worse survival is restricted to COX-2 positivity in proliferative tumours
Misron *et al.*, 2014[159]	144 TMA	LabVision-RM-9121-S Clone SP21 Rabbit monoclonal) at 1:50	Product score of percentage and intensity[b]	Score ≥5	75%	Age, tumour size, grade, ER, PgR, HER-2, LVI, LN invasion,	Low grade, ER positivity, PgR positivity	NR	Not examined
Simonsson *et al.*, 2017[551]	911 TMA	Abcam, polyclonal rabbit 1:750	Intensity score	Moderate staining intensity	39%	Tumour size, grade, ER. PgR, HER-2, Ki67, LN status	Older age, lower grade, ER positivity, PgR positivity, low Ki67	5 years	Association with lower risk for any BC event in the UVA but not in MVA. Not associated with DMFS or OS

a. 0: no staining, 1: weak diffuse staining or strong stain in <10%, 2: moderate/strong stain in 10–90%, 3: strong 90%; b. percentage (0–4) X intensity (0–3); c. Four categories: None (0) Low (1–4) Medium (5–8) High (9–12); d. 0: no staining, 1: weak, 2: moderate 10–90%, 3: strong 90%; e: Negative: no staining, 1: weak diffuse staining, 2: moderate/strong staining, 3: >90% strong intensity; LN: lymph node; LVI: lymphovascular; DDFS: distant disease free survival; UVA: univariate analysis; MVA: multivariate analysis; DFS: disease free survival; OS: overall survival; DMFS: distant metastases free survival

5.3.2. COX-2 and its relation to oestrogen in BC

In this work, COX-2 positivity was strongly associated with hormone positivity. This suggests that COX-2 deregulation may play a role in the pathogenesis of ER-positive BC. This is in agreement with the conclusions illustrated by several investigators.[157, 159, 552] Holmes and colleagues confirmed the existence of this correlation in a study that was done on 2001 BCs.[157] Moreover, this had been supported by transcriptional studies. Zhao *et al.* quantified COX-2 mRNA by reverse transcription-polymerase chain reaction in benign and malignant breast tissue.[164] The authors perceived noticeably higher COX-2 levels in ER-positive than in ER-negative breast tumours.[164] Nevertheless, in a British study, a direct association was present between COX-2 mRNA and PgR positivity; but not with ER status.[165] Previous research illustrated that COX-2 upregulation stimulates the conversion of androgen to oestrogen in breast tissue through prostaglandin production and aromatase activation[166-169, 553] (Figure 1.7). This can explain the bond between COX-2 and ER. Generally, most of the ER-positive breast tumours are associated with a better prognosis and less aggressive phenotype as well as differentiation and low proliferation rate,[554, 555] which agrees with our results that COX-2 was associated with ER-positive tumours, low histological grade and low Ki67. In addition, observations similar to ours were also reported by Park *et al.*[550] and Misron *et al.*[159] on analysis of 861 and 144 BCs respectively.

Moreover, inline with our results, a recently published Swedish study done on 911 patients assured that more favourable BC tumour characteristics such as small tumour size, hormone receptor positivity, low Ki67 in addition to HER-2 negativity were paralleled with COX-2 protein presence.[551] These results were not observed in the study by Glynn *et al.*,[429] who did not find any association between COX-2 expression and hormone receptor status or tumour grade.[429] Additional studies done on small number of BC tumours (maximum of 50 tumours) revealed no association between COX-2 expression and tumour grade or ER status.[556164,165]

In contrast to our result, Lui *et al.* stated that COX-2 protein level in oestrogen-independent MDA-MD-231 cells was higher than in oestrogen-dependent MCF-7 cells.[557] This was further described by investigations done on TMAs.[160, 548] The

latter studies and others also reported that COX-2 expression is associated with higher histological grade and larger tumour size.[155, 160, 548, 558]

Several factors can contribute to these contradictory conclusions. The absence of uniform criteria for positivity of COX-2 can result in divergent 'COX-2-positive' tumour samples across these studies. For example, van Nes *et al.* used a histogram (H-score) with a cut-off score of 148 for positivity.[558] The threshold used by Nakopoulou *et al.* was 20% moderate cytoplasmic expression while Ristimäki *et al.*[160] and Boland *et al.*[559] considered 10% moderate cytoplasmic expression as positive. A lower limit (5% cytoplasmic expression regardless of the intensity) was even used by other investigators.[560] In our study we used a scoring system based on summation of the intensity of the staining and proportion of the tumour cells expressing the marker, similar to that used in Glynn *et al.*,[429] with a cut-off score of ≥4. Adding to the diversity of the results are the differences in the sensitivity and specificity of several commercial COX-2 antibodies and the variability between the studied populations.

5.3.3. COX-2 and its association with other biomarkers and the possible role in BC carcinogenesis

Consistent with the key role of COX-2 in carcinogenesis and its putative role in the control of apoptosis, our work indicated a correlation between the presence of COX-2 and BCL-2 in BC. This is inline with previous published studies.[561, 562] A study by Singh *et al.* demonstrated that COX-2 markedly increased BCL-2 expression on MCF-7 cells[562] and additionally selective COX-2 blockers reversed this effect in an in vivo oncogenic mouse model of BC.[563] Similar findings of COX-2/BCL-2 co-expression were seen in TNBC and non-TNBC breast tumours,[561] suggesting that correlation is present independent of BC molecular subtype. Notably, many experiments revealed that upstreaming of BCL-2 mRNA and protein expression is also associated with the exposure to oestrogen,[564, 565] which is consistent with our finding that COX-2 was associated with both ER and BCL-2 positivity. Only one study reported a contradictory result, in which the researchers observed an enhancement of the BCL-2 levels, measured by western blot, after oral administraion of a selective COX-2 inhibitor in experimnetal rats.[566]

Moreover, a significant inverse association between COX-2 and E-Cadherin expression was apparent in our analysis. This raises the possibility that COX-2 may play a role in BC tumourigenesis. Bocca. *et al.* reported that COX-2 overexpression leads to E-cadherin reduction in MDA-MB-231 and MCF-7 BC cells.[567] In contrast COX-2 knockdown led to upregulation of E-cadherin,[568, 569] reduction of cell migration and invasion in different cancers in vitro.[570, 571] Moreover, this inverse association between COX-2 and E-cadherin was described in different types of cancers such as lung and bladder cancers.[572, 573] However, an association between COX-2 expression and nodal metastases was not observed in this work. A possible verification is that E-Cadherin downstreaming is an earlier event within the invasion/metastases sequence, which ensues before the establishment of nodal metastases.

5.3.4. COX-2 and outcome in BC

Many investigators have examined the clinical significance of COX-2 overexpression in BC. However, the results of these reports are still conflicting as shown in Table 5.8. Glynn *et al.*[429] previously noted a worse survival rates in ER-negative tumours. Our analysis did not show a significant association between survival and COX-2 expression. However, we noted a possible time effect for the COX-2 positivity in ER-negative disease (Figure 5.6 C and 5.7 C). Perhaps a longer follow up would detect evidence of such an association.

Overall, we hypothesize that the effects of COX-2 are context dependent and that the pathways involved in the COX-2 associated cancer development and progression may depend on BC subtypes. Further studies and meta-analysis are recommended.

5.3.5. iNOS in BC

In this cohort, iNOS expression in BC showed a significant inverse association with tumour grade. Furthermore, iNOS positivity was more common in ER-positive tumours compared with ER-negative ones. Similarly, there was high concurrence between iNOS expression and PgR and CK14 expression. Within ER-negative BC, the significant relationship between iNOS expression and

tumour grade was retained while in ER-positive tumours, significant association between iNOS expression and CK14 was still observed.

There is no consensus in the literature regarding the relationship between iNOS and oestrogen. Some studies in rat models suggest that oestrogen has a positive regulatory effect on the iNOS levels in different cells[574, 575] while other experiments revealed the opposite effect.[576, 577] In 2009, Ostad *et al.* investigated the effect of estradiol on the expression of iNOS in 2 BC cell lines.[578] They observed a direct effect of estradiol levels on iNOS titres.[578] This is consistent with our finding that iNOS expression was associated with ER-positive tumours. Furthermore, Karpuzoglu and Ahmed stated that COX-2 and other genes can be induced by iNOS-derived nitric oxide,[579] which can be by itself a link to its association with ER-positive BC. Vakkala *et al.* examined iNOS expression in both the tumour and stromal cells and concluded that their sum score is correlated with the PgR positivity.[580] On the contrary, other studies on human BC did not show similar association with hormone receptor status[191, 581] and a single study reported an association between iNOS and TN phenotype,[582] however this study investigated a small number of breast tumours *(27)*.

iNOS staining was directly associated with hormone receptor status in this study and it is not surprising that it also shows an inverse affiliation with tumour grade. A similar observation was reported in Austrian[583] and French[584] studies.

Contradictory to our data, Glynn *et al.*[191] and Dabbeche-Bouricha *et al.*[582] reported a proportional relationship between the tumour differentiation and iNOS expression. These differences might be related to the differences in the studied populations, interobserver variability and the sensitivity of the antibodies used. Moreover, the effects of NO on the cells are known to be contradictory according to its concentration. The NO within the tumour microenvironment can be sourced not only from iNOS enzyme present within the tumour cells, but also from the iNOS enzyme present in the tumour-associated inflammatory cells and stromal cells. Thus, iNOS expression was assessed in the concomitant inflammatory infiltrate, however most of the cores showed a deficient number of inflammatory cells or none. This is not surprising as our study was done on TMA, which typically aims to obtain the most cellular areas of the tumour. Therefore, it was difficult to get a real estimate of the NO amount in the tumour microenvironment.

In summary, this work revealed that COX-2 and iNOS were expressed more often in hormone receptor positive tumours and in low grade tumours, supporting the hypothesis that COX-2 may have a tumourigenic role in hormone dependent BC. Such a role could be through the PGE2 pathway, which can increase aromatase activity in breast cells with subsequent oestrogen production. An additional mechanism might be linked to the interactions between the anti-apoptotic protein Bcl-2 and COX-2. However, an association between either iNOS and COX-2 and outcome in all BCs, in hormone receptor positive or hormone receptor negative subsets was not observed.

5.3.6. Limitations of our study and future directions

IHC is a relatively simple technique that has been proven to be practically useful for decades. However, this method is liable to reaction bias and interpretation bias.[585] Therefore, the reliability and validity of the manual immunohistochemical technique for COX-2 and iNOS detection can represent potentially limiting factors in this study. We attempted to decrease run-to-run variation in expression by adhering to a standardized protocol that was initially optimized. Whole tissue sections of appropriate external controls were used during optimization and during immunohistochemical staining of the TMAs. These often showed a relatively homogeneous staining pattern for COX-2 and iNOS. Concordance of expression in duplicated cases, for COX-2 and iNOS, located on different TMAs showed a fair agreement. Tissue heterogeneity, intraobserver variability and technical factors might contribute to the disconcordance in scores.

Although the TMA allows the evaluation of large number of tumours in an identical standardized method, the cores constitute a small proportion of each tumour that might not be representative of the entire tumour. Therefore, it may represent a possible limiting factor in our work. However, several studies validated the use of the immunohistochemical methods on TMAs. These studies confirmed that the results of biomarker expression were highly concordant in TMA cores and whole tissue sections.[425, 586, 587]

6. SUMMARY AND FUTURE PRESPECTIVE

TNBCs constitute a subset of BC that lacks targeted treatment despite the recent advances in cancer therapy. Comprehensive genomic and expression array studies have revealed that TNBCs exhibit considerable molecular heterogeneity. This diversity has hindered the identification of predictive markers and targeted therapies.

This work analysed the clinical and detailed pathological characteristics of a TNBC series diagnosed at GUH from January 2000 to January 2016. This is one of the largest retrospective cohorts of TNBCs outside a clinical trial setting. The analysis particularly focused on the evaluation of the clinicopathological and immunohistochemical parameters in TNBC and their association with outcome.

The prognostic importance of the traditional parameters of tumour size and nodal status was confirmed. On MVA, nodal status was an independent predictor of DFS and MFS but not for BCSS in the chemo naïve group. Tumour size was an independent predictor of MFS and BCSS. In the post NACT setting, pCR was the only independent predictor of DFS, MFS and BCSS.

A range of parameters was examined as potential prognostic indicators. Only sTILs was an independent prognostic factor for both DFS and OS. The significance of incremental increases in sTILs in its association with an improved outcome shown by others was confirmed. The prognostic value of sTILs was maintained when it was expressed as a binary variable with a 25% cut-off.

None of the other biomarkers examined - AR, basal phenotype (EGFR, CK5/6, CK14), p53 - was independently predictive of outcome and neither were patient age, menopausal status or BRCA mutation status. Expression of Bcl-2 - a marker associated with good outcome in BC overall - was associated with a longer survival, MFS and BCSS, on multivariate analysis with age, grade and menopausal status, but not with nodal status or tumour size - as co-variables. The two inflammation associated enzymes COX-2 and iNOS, while associated with hormone receptor positive tumours, were not associated with outcome.

This work highlights the difficulty in identifying patients with subtypes of TNBC who will follow a different course and who may benefit from different treatment options. TNBC accounts for less than 20% of BCs and most published series are small. International consortia that pool the data from their respective TNBCs series would increase the power of studies to validate potential predictive markers. The follow up for patients who were treated with NACT was short. Most TNBC relapses occur within the first three to five years after diagnosis and an update of follow up on these patients within the coming years will be very informative.

In the absence of validated predictive markers in TNBC, a lot of research has been directed at modifying the chemotherapeutic regime. Incorporation of platinum agents into the standard anthracycline taxane backbone in the NACT setting has shown promising results with higher rates of pCR reported. Data on treatment regimes was not incorporated into this work and treatment will be crucial for future studies, and in particular the outcome for those patients who received carboplatin is eagerly awaited.

The validation of TILs as a potential prognostic marker for clinical use will require methodological studies designed to test different cut off points and carefully designed inter-observer and intra-observer studies. Also, it would be interesting to classify the functional types of the inflammatory infiltrate using immunohistochemical markers such as CD3, CD8 and FOXP3 and evaluate the roles of each subtype. This would also provide a better understanding of the mechanisms behind the tumour's immune microenvironment in TBNC. As well, with the emergence of immunotherapies such as a promising class of drugs to treat patients with different tumour types, markers such as PD1, PDL1 and CTLA4 should be investigated in TNBC.

Finally, the importance of a pCR as a consistent predictor of outcome has been confirmed in many studies as well as in this work. It is crucial that a consistent approach for the grossing and sampling of post NACT specimens is adopted. This will facilitate the comparison of pCR and of treatment response between studies

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Appendix I

Table A.1. Summary of the TNM staging system (7th edition)

Parameter	Stage		Description
T stage	Tx		Primary tumour cannot be assessed
	T0		No evidence of primary tumour
	T1 (Tumour ≤ 20 mm)	T(mi)	Tumour ≤1mm in largest extent
		T1a	Tumour greatest dimension (>1mm but ≤ 5 mm)
		T1b	Tumour greatest dimension (> 5mm but ≤ 10 mm)
		T1c	Tumour greatest dimension (>10mm but ≤ 20mm)
	T2		Tumour greatest dimension (>20mm but ≤ 50 mm)
	T3		Tumour > 50 mm in largest extent
	T4		Any size tumour with direct extension to chest wall (4a) or skin (4b) or both (4c) or inflammatory carcinoma (4d)
N stage	Nx		Regional LNs cannot be assessed
	N0		No regional LN metastasis identified histologically
	N(i+)		Isolated tumour cells (ITC): clusters of cells (≤ 0.2 mm or <200 cells)
	N1	N1(mi)	Micrometastasis (>0.2mm to <2mm) in 1-3 axillary LN
		N1a	Metastases in 1-3 axillary LNs with macrometastasis (>2mm) in at least 1 LN
		N1b	Metastasis in clinically occult internal mammary LN (detected by SLN)
		N1c	Metastases in 1-3 axillary LNs + Metastasis in clinically occult internal mammary LN (detected by SLN)
	N2	N2a	Metastases in 4-9 axillary LN
		N2b	Metastases in clinically apparent internal mammary LN in the absence of axillary LN metastases
	N3	N3a	Metastases in ≥10 axillary LNs OR infraclavicular LN
		N3b	Metastases in clinically apparent internal mammary LN + axillary LN metastases OR Metastases in >3 axillary LNs + Metastasis in clinically occult internal mammary LN (detected by SLN)
		N3c	Metastasis in ipsilateral supraclavicular LN
M stage	M0		No distant metastasis
	M1		Distant metastasis

Appendix II

Table A.2. Source and dilution of the antibodies used and the biomarker profile of the WOI BC series

Biomarker (manfacturer, clone, dilution)	n[a]	Status	n[a] |(%)	Cut-off for positivity
ER (Neomarker, clone SP, 1:100)*	522	Positive Negative	344 (66) 178 (34)	>10% nuclear positivity -
PgR (Leica, clone 16, 1:200)*	534	Positive Negative	297 (56) 237 (44)	>10% nuclear positivity
HER-2 (Dako)	602	Positive Negative	85 (14) 517 (86)	2+ with positive FISH result/3+ ***
EGFR (Dako, pharmDx kit)*	527	Positive Negative	77 (14) 450 (85)	Any membranous/cytoplasmic positivity
CK14 (Novo- castra, LL002, 1:20)**	444	Positive Negative	94 (21) 350 (79)	Any membranous/cytoplasmic positivity
CK5/6 (Dako, D5/16, 1:100)*	463	Positive Negative	42 (9) 421 (91)	Any membranous/cytoplasmic positivity
BCL-2 (Novocastra, bcl-2/100/D5, 1:200)**	534	Positive Negative	290 (54) 244 (46)	>10% cytoplasmic positivity
p53 (Dako, DO-7, 1:100)*	523	Positive Negative	105 (20) 418 (80)	>10% nuclear positivity
Ki67 (Dako, MIB-1, 1:200)*	540	Positive Negative	163 (30) 377 (70)	>10% nuclear positivity

n[a] : number of cases for which data is available

*Antigen retrieval was done using CC1 protocol for 30 minutes

** Antigen retrieval was done using ER2 protocol for 20 minutes

*** Scoring for HER-2 (ASCO/CAP recommendation, 2013) 0 = no staining; 1+ = weak, incomplete membrane staining in >10% of tumour cells; 2+= weak to moderately complete membrane staining in >10% of tumour cells; 3+= strong complete membrane staining in >30%

Appendix III

Table A.3.1 Univariate analysis of association between tumour grade and survival in TNBC

	HR	**95% CI**	**p value**
DFS (329)	0.918	0.525-1.6	0.765
MFS (330)	0.776	0.412-1.46	0.433
DFS (328)	0.918	0.525-1.6	0.765
p values in bold indicate statistically significant associations			

Table A.3.2. Univariate analysis of association between tumour tubule formation, nuclear pleomorphism and mitotic count with DFS in TNBC

		HR	**95% CI**	**p value**
Tubule formation (329)	2	0.451	0.05-4.04	0.477
	3	0.86	0.119-6.2	0.881
Nuclear Pleomorphism (329)	2	0.54	0.065-4.52	0.573
	3	0.402	0.055-2.89	0.055
Mitotic count (328)	2	0.908	0.468-1.764	0.78
	3	0.796	0.796-0.234	0.44
p values in bold indicate statistically significant associations				

Appendix IV

Table A.4. Follow-up of cases who had positive result on diagnostic lymph node procedures within the TNBC cohort

Diagnostic LN procedure	**Follow-up therapeutic LN procedure (type)**	**Follow-up therapeutic LN procedure (Result)**	**Comment**
ALN NCB (n=40)	ALND (n=35) SLN & ALND excisions (n=3) Awaited (n=1)* None (n=1)	Positive (n= 26) Negative (n=11) No lymph nodes retrieved (n=1) Unknown (n=2)	The 11 cases with negative ALN had NACT, and 10 of them showed effect to treatment in uninvolved nodes.
ALN FNA (n=14)	ALND (n=14)	Positive (n=8) Negative (n=5) No lymph nodes retrieved (n=1)	The 5 cases with negative ALN had NACT, and 4 of them showed effect to treatment in uninvolved nodes.
SLN excision (n=5)	ALND (n=5)	Positive (n= 3) Negative (n=2)	
ALN NCB ALN FNAC (n=1)	SLN excision (n=1)	Negative (n=1)	This case had NACT, and showed effect to treatment in uninvolved nodes.
ALN NCB SLN Excision (n=1)	None (n=1)		
SLN excision ALN excision (n=1)	ALND (n=1)	Negative (n=1)	

ALN NCB: axillary lymph node needle core biopsy; ALN FNAC: axillary lymph node fine needle aspiration cytology; ALN excision: axillary lymph node excisional biopsy; ALND: axillary lymph node dissection(clearance); SLN excision: sentinel lymph node excision; SC FNAC: supraclavicular fine needle aspiration cytology.

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Appendix V

Table A.5. Association between EGFR expression and clinicopathological features in TNBC

Parameter		EGFR positive (n=134) n (%)	EGFR negative (n= 108) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	42 (52)	38 (58)	80	0.339	0.560
	Post-menopausal	87 (47)	67 (43)	154		
	Unknown	14 (82)	3 (18)	17		
Tumour grade	1	1 (100)	0 (0)	1	0.939	0.625
	2	17 (59)	12 (41)	29		
	3	116 (55)	95 (45)	211		
	x	0 (0)	1 (100)	1		
Tubule formation	1	1 (100)	0 (0)	1	3.598	0.165
	2	8 (38)	13 (62)	21		
	3	125 (57)	94 (43)	219		
	x	0 (0)	1 (100)	1		
Nuclear Pleomorphism	1	1 (100)	0 (0)	1	2.201	0.333
	2	8 (73)	3 (28)	11		
	3	125 (55)	104 (45)	229		
	x	0 (0)	1 (100)	1		
Mitotic count	1	15 (58)	11 (42)	26	0.186	0.911
	2	26 (33)	23 (47)	49		
	3	93 (56)	73 (44)	166		
	x	0 (0)	1 (100)	1		
Tumour type	Ductal	109 (56)	87 (44)	196	10.672	0.221
	Lobular	10 (77)	3 (23)	13		
	Metaplastic	4 (36)	7 (64)	11		
	Medullary#	5 (50)	5 (50)	10		
	Apocrine	2 (29)	5 (71)	7		
	Others	4 (80)	1 (20)	5		
Tumour stage (pT)	1	32 (47)	36 (53)	68	4.426	0.219
	2	76 (63)	45 (37)	121		
	3	7 (58)	5 (42)	12		
	4	7 (58)	5 (42)	12		
	x	12 (41)	17 (59)	29		
Nodal stage (pN)	0	72 (55)	58 (45)	130	1.145	0.766
	1	27 (64)	15 (36)	42		
	2	10 (59)	7 (41)	17		
	3	8 (53)	7 (47)	15		
	x	17 (45)	21 (55)	38		
sTILs categories	0	40 (53)	36 (47)	67	0.616	0.893
	1	52 (58)	37 (42)	89		
	2	20 (56)	16 (44)	36		
	3	22 (54)	19 (46)	41		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

Appendix VI

Table A.6. Association between CK5/6 expression and clinicopathological features in TNBC

Parameter		CK5/6 positive (n=163) n (%)	CK5/6 negative (n= 85) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	52 (67)	26 (33)	78	0.0798	0.778
	Post-menopausal	105 (65)	57 (35)	162		
	Unknown	6 (75)	2 (25)	8		
Tumour grade	1	0 (0)	1 (100)	1	1.9145	0.384
	2	21 (66)	11 (34)	32		
	3	141 (66)	73 (34)	214		
	x	1 (100)	0 (0)	1		
Tubule formation	1	1 (50)	1 (50)	2	4.7833	0.091
	2	19 (86)	3 (14)	22		
	3	142 (64)	81 (36)	223		
	x	1 (100)	0 (0)	1		
Nuclear Pleomorphism	1	0 (0)	1 (100)	1	1.9173	0.383
	2	8 (67)	4 (33)	21		
	3	154 (66)	80 (34)	234		
	x	1 (100)	0 (0)	1		
Mitotic count	1	16 (57)	12 (43)	28	1.1937	0.551
	2	34 (64)	19 (36)	53		
	3	112 (67)	54 (33)	166		
	x	1 (100)	0 (0)	1		
Tumour type	Ductal	131 (65)	70 (35)	201	3.3342	0.912
	Lobular	10 (67)	5 (34)	15		
	Metaplastic	6 (55)	5 (45)	11		
	Medullary#	7 (70)	3 (30)	10		
	Apocrine	5 (83)	1 (17)	6		
	Others	3 (75)	1 (25)	4		
Tumour stage (pT)	1	44 (63)	26 (37)	70	6.0157	0.111
	2	79 (65)	43 (35)	122		
	3	12 (92)	1 (8)	13		
	4	10 (83)	2 (17)	12		
	x	83 (63)	48 (37)	131		
Nodal stage (pN)	0	33 (73)	12 (27)	45	3.6648	0.300
	1	14 (78)	4 (22)	18		
	2	8 (80)	7 (20)	15		
	3	1 (7)	14 (93)	15		
	x	44 (63)	26 (37)	70		
sTILs categories	0	48 (63)	28 (37)	76	0.4006	0.940
	1	60 (67)	29 (33)	89		
	2	26 (67)	13 (33)	39		
	3	29 (67)	14 (33)	43		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

Appendix VII

Table A.7. Association between CK14 expression and clinicopathological features in TNBC

Parameter		CK14 positive (n=188) n (%)	CK14 negative (n= 52) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	58 (77)	17 (23)	75	0.0815	0.775
	Post-menopausal	124 (79)	33 (21)	157		
	Unknown	6 (75)	2 (25)	8		
Tumour grade	1	1 (100)	0 (0)	1	0.3237	0.851
	2	23 (77)	7 (23)	30		
	3	163 (78)	45 (22)	208		
	x	1 (100)	0 (0)	1		
Tubule formation	1	1 (100)	0 (0)	1	0.7246	0.696
	2	16 (84)	3 (16)	19		
	3	170 (78)	49 (22)	219		
	x	1 (100)	0 (0)	1		
Nuclear Pleomorphism	1	1 (100)	0 (0)	1	0.3002	0.861
	2	8 (80)	2 (20)	10		
	3	178 (78)	50 (22)	228		
	x	1 (100)	0 (0)	1		
Mitotic count	1	21 (75)	7 (25)	28	0.2048	0.903
	2	38 (79)	10 (20)	48		
	3	128 (79)	35 (21)	163		
	x	1 (100)	0 (0)	1		
Tumour type	Ductal	149 (77)	44 (23)	193	5.1936	0.737
	Lobular	13 (81)	3 (19)	16		
	Metaplastic	7 (64)	4 (36)	11		
	Medullary#	9 (90)	1 (10)	10		
	Apocrine	6 (100)	0 (0)	6		
	Others	3 (100)	0 (0)	3		
Tumour stage (pT)	1	53 (77)	16 (23)	69	4.9514	0.175
	2	92 (78)	26 (22)	118		
	3	13 (100)	0 (0)	13		
	4	11 (92)	1 (8)	12		
	x	98 (75)	32 (25)	130		
Nodal stage (pN)	0	35 (85)	6 (15)	41	3.6283	0.305
	1	15 (88)	2 (12)	17		
	2	14 (88)	2 (12)	16		
	3	53 (77)	16 (23)	69		
	x					
sTILs categories	0	54 (74)	19 (26)	73	3.3447	0.341
	1	68 (26)	21 (24)	89		
	2	30 (81)	7 (19)	37		
	3	36 (88)	5 (12)	41		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

Appendix VIII

Table A.8.1. Univariate analysis of association between EGFR expression with DFS in TNBC

	HR	**95% CI**	**p value**
EGFR (233)	0.6991	0.428-1.1402	0.152
p values in bold indicate statistically significant associations			

Table A.8.2. Univariate analysis of association between biomarkers expression with BCSS in TNBC

	HR	**95% CI**	**p value**
CK5/6 (234)	0.9019	0.498-1.6318	0.733
CK14 (226)	0.9293	0.468-1.8455	0.834
P53 (226)	0.9556	0.5532-1.6508	0.871
p values in bold indicate statistically significant associations			

Appendix IX

Table A.9. Univariate analysis of the association between the four TNBC subtypes and survival

Variable	HR	95% CI	p value
DFS			
BLIS	1.25	0.74-2.10	0.398
BLIA	0.74	0.35-1.53	0.418
AR	1.10	0.51- 2.38	0.799
OS			
BLIS	1.52	0.88 - 2.65	0.135
BLIA	1.04	0.50 - 2.15	0.909
AR	1.54	0.73 - 3.26	0.262

p values in bold indicate statistically significant associations

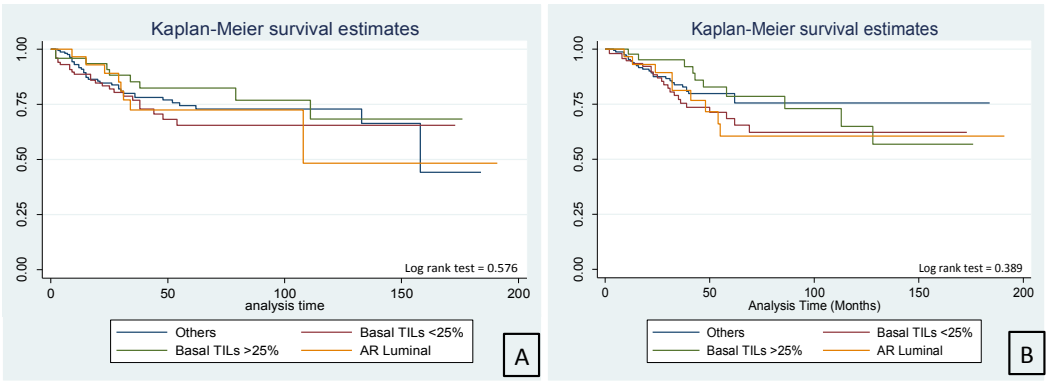

Figure A.9. Kaplan-Meier curves for DFS (A) and OS (B) of the TNBC subtypes

Appendix X

Table A.10. Association between Bcl-2 expression and clinicopathological features in TNBC

Parameter		Bcl-2 positive (n=165) n (%)	Bcl-2 negative (n= 78) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	45 (58)	33 (42)	78	5.8034	0.016
	Post-menopausal	115 (73)	42 (27)	75		
	Unknown	5 (63)	3 (37)	8		
Tumour grade	1	1 (100)	0 (0)	1	0.4910	0.782
	2	22 (69)	10 (31)	32		
	3	142 (68)	68 (32)	219		
	x	0 (0)	0 (0)	0		
Tubule formation	1	2 (100)	0 (0)	2	3.1878	0.203
	2	18 (82)	4 (18)	22		
	3	145 (66)	74 (34)	219		
	x	0 (0)	0 (0)	0		
Nuclear Pleomorphism	1	1 (100)	0 (0)	1	0.5650	0.754
	2	7 (64)	4 (36)	11		
	3	157 (68)	74 (32)	231		
	x	0 (0)	0 (0)	0		
Mitotic count	1	19 (68)	9 (32)	28	1.3374	0.512
	2	38 (74)	13 (26)	51		
	3	108 (66)	56 (34)	164		
	x	0 (0)	0 (0)	0		
Tumour type	Ductal	132 (67)	65 (33)	197	8.2702	0.309
	Lobular	10 (71)	4 (29)	14		
	Metaplastic	9 (75)	3 (25)	12		
	Medullary#	5 (50)	5 (50)	10		
	Apocrine	6 (100)	0 (0)	6		
	Others	3 (75)	1 (25)	4		
Tumour stage (pT)	1	48 (71)	20 (30)	68	3.1609	0.367
	2	77 (65)	42 (35)	119		
	3	11 (78)	3 (21)	14		
	4	11 (85)	2 (15)	13		
	x	18 (62)	11 (38)	29		
Nodal stage (pN)	0	83 (64)	47 (36)	130	7.2046	0.066
	1	28 (69)	13 (31)	41		
	2	17 (94)	1 (6)	18		
	3	12 (75)	4 (25)	16		
	x	25 (66)	13 (34)	38		
sTILs categories	0	50 (63)	29 (37)	79	1.9066	0.592
	1	61 (72)	24 (28)	85		
	2	26 (72)	10 (28)	36		
	3	27 (64)	15 (36)	42		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

Appendix XI

Table A.11. Association between p53 expression and clinicopathological features in TNBC

Parameter		p53 positive (n=94) n (%)	pa53 negative (n= 146) n (%)	n	χ^2	p value
Menopausal status	Pre-menopausal	29 (36)	52 (64)	81	0.7719	0.380
	Post-menopausal	63 (6)	88 (58)	151		
	Unknown	2 (25)	6 (75)	8		
Tumour grade	1	0 (0)	1 (100)	1	0.6770	0.713
	2	13 (40)	19 (60)	32		
	3	80 (39)	126 (61)	206		
	x	1 (100)	0 (0)	1		
Tubule formation	1	0 (0)	1 (100)	1	0.3960	0.820
	2	7 (33)	14 (67)	21		
	3	85 (39)	131 (61)	216		
	x	1 (100)	0 (0)	1		
Nuclear Pleomorphism	1	0 (0)	1 (100)	1	1.8008	0.406
	2	6 (55)	5 (45)	11		
	3	87 (38)	140 (62)	227		
	x	1 (100)	0 (0)	1		
Mitotic count	1	9 (32)	19 (68)	28	1.0695	0.586
	2	24 (44)	31 (56)	55		
	3	60 (38)	96 (62)	156		
	x	1 (100)	0 (0)	1		
Tumour type	Ductal	78 (40)	115 (60)	193	5.1936	0.737
	Lobular	4 (29)	10 (71)	14		
	Metaplastic	5 (38)	8 (62)	13		
	Medullary#	3 (33)	6 (67)	9		
	Apocrine	2 (33)	4 (67)	6		
	Others	2 (50)	2 (50)	4		
Tumour stage (pT)	1	26 (38)	43 (62)	69	0.5284	0.913
	2	44 (38)	70 (62)	114		
	3	6 (46)	7 (54)	13		
	4	5 (45)	6 (55)	11		
	x	51 (40)	75 (60)	126		
Nodal stage (pN)	0	15 (37)	26 (63)	41	0.3769	0.945
	1	6 (35)	11 (65)	17		
	2	5 (36)	9 (64)	14		
	3	26 (38)	43 (62)	69		
	x	44 (38)	70 (62)	114		
sTILs categories	0	31 (40)	47 (60)	78	1.5795	0.664
	1	30 (36)	54 (64)	84		
	2	17 (47)	19 (53)	36		
	3	14 (36)	25 (64)	39		

#Medullary types including the classical and atypical medullary. p values in bold indicate statistically significant associations

Appendix XII

Table A.12. Associations between sTILs high and biomarkers in TNBC

Variable		sTILs low n(%)	sTILs high n(%)	Total	χ^2	p value
AR	Negative	156 (68)	72 (32)	228	0.426	0.514
	Positive	23 (74)	8 (26)	31		
EGFR	Negative	95 (68)	44 (32)	139	0.30	0.862
	Positive	77 (70)	34 (30)	111		
CK5/6	Negative	115 (68)	54 (32)	169	0.025	0.874
	Positive	57 (67)	28 (33)	85		
CK14	Negative	128 (66)	65 (34)	193	2.356	0.125
	Positive	41 (77)	12 (16)	53		
Bcl-2	Negative	114 (69)	52 (31)	166	0.004	0.951
	Positive	56 (68)	26 (32)	82		
COX-2	Negative	99 (71)	41 (29)	140	0.006	0.937
	Positive	52 (71)	21 (29)	73		
iNOS	Negative	106 (69)	47 (31)	153	0.264	0.607
	Positive	43 (73)	16 (17)	59		
p53	Negative	66 (68)	31 (32)	97	0.113	0.737
	Positive	103 (70)	44 (30)	147		

a, number of cases for which data was available. p values in bold indicate statistically significant associations

Appendix XIII

Table A.13. Associations between sTILs high and biomarkers in TNBC

Variable		sTILs non-LPBC n(%)	sTILs LPBC n(%)	Total	χ^2	p value
AR	Negative	189 (83)	39 (17)	228	0.018	0.892
	Positive	26 (84)	5 (16)	31		
EGFR	Negative	115 (83)	24 (17)	139	0.049	0.825
	Positive	93 (84)	18 (16)	111		
CK5/6	Negative	140 (83)	29 (17)	169	0.009	0.923
	Positive	70 (82)	15 (18)	85		
CK14	Negative	157 (81)	36 (18)	193	2.544	0.111
	Positive	48 (91)	5 (9)	53		
BCL-2	Negative	139 (84)	27 (16)	166	0.404	0.525
	Positive	66 (80)	16 (20)	82		
COX-2	Negative	119 (85)	21 (15)	140	0.610	0.435
	Positive	59 (81)	14 (19)	73		
iNOS	Negative	127 (83)	26 (17)	153	0.852	0.356
	Positive	52 (88)	7 (12)	59		
p53	Negative	83 (86)	14 (14)	97	0.451	0.502
	Positive	121 (82)	26 (18)	147		

a, number of cases for which data was available. p values in bold indicate statistically significant associations

Appendix XIV

Table A.14. Association between COX-2 and tumour variables in ER-positive BC

Variable		COX-2 negative n(%)	COX-2 positive n(%)	Total	χ^2	p value
Tumour grade	1	13 (27)	35 (73)	48	5.1358	0.077
	2	63 (39)	100 (61)	163		
	3	33 (48)	36 (52)	69		
HER-2	Negative	113 (48)	121 (52)	234	1.933	0.380
	Positive	8 (44)	10 (66)	18		
BCL-2	Negative	42 (48)	46 (52)	88	3.1781	0.075
	Positive	70 (85)	12 (15)	82		
CK5/6	Negative	85 (40)	129 (60)	214	0.2769	0.599
	Positive	13 (45)	16 (55)	29		
EGFR	Negative	100 (39)	157 (61)	257	0.4020	0.526
	Positive	6 (32)	13 (68)	19		
CK14	Negative	78 (42)	109 (58)	187	0.7364	0.391
	Positive	16 (35)	30 (65)	46		
KI67	Negative	84 (39)	131 (61)	215	0.0499	0.823
	Positive	26 (41)	38 (59)	64		
p53	Negative	91 (38)	146 (62)	237	1.1846	0.276
	Positive	19 (47)	21 (53)	40		
E-cadherin	Negative	9 (26)	26 (74)	35	3.0290	0.082
	Positive	99 (41)	142 (59)	241		

a, number of cases for which data was available. p values in bold indicate statistically significant associations

Appendix XV

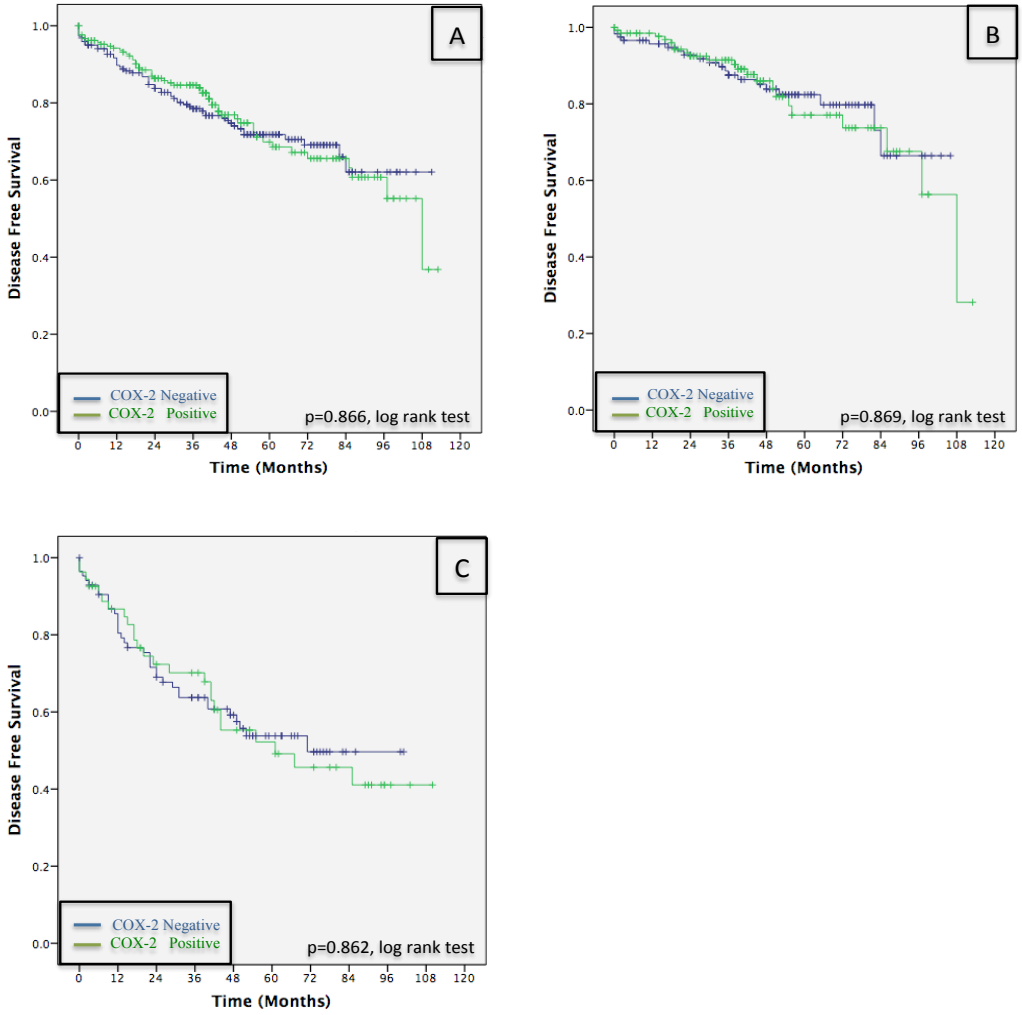

Figure A.15. Kaplan-Meier curves for DFS in BC according to COX-2 expression

(A) the entire cohort, (B) ER-positive and (C) ER-negative

Appendix XVI

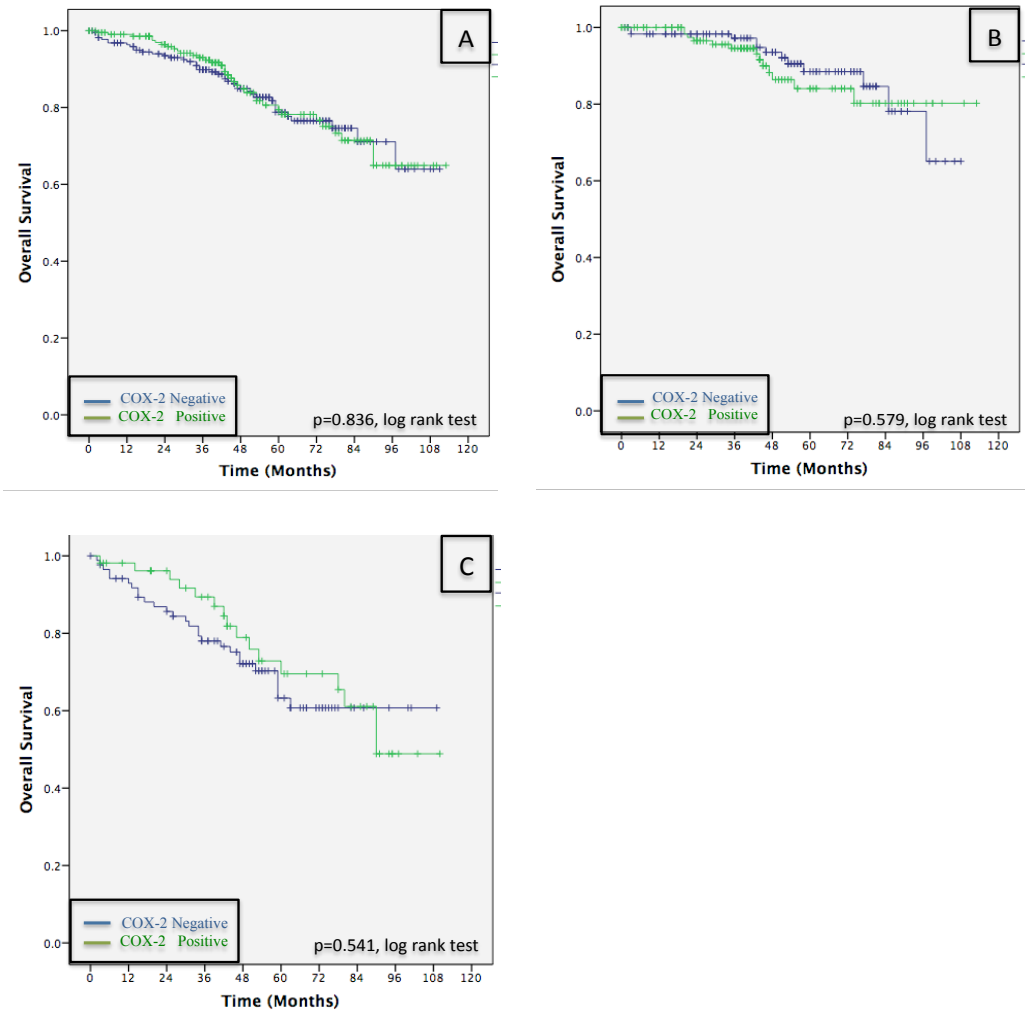

Figure A.16. Kaplan-Meier curves for OS in BC according to COX-2 expression

(A) the entire cohort, (B) ER-positive and (C) ER-negative

Appendix XVII

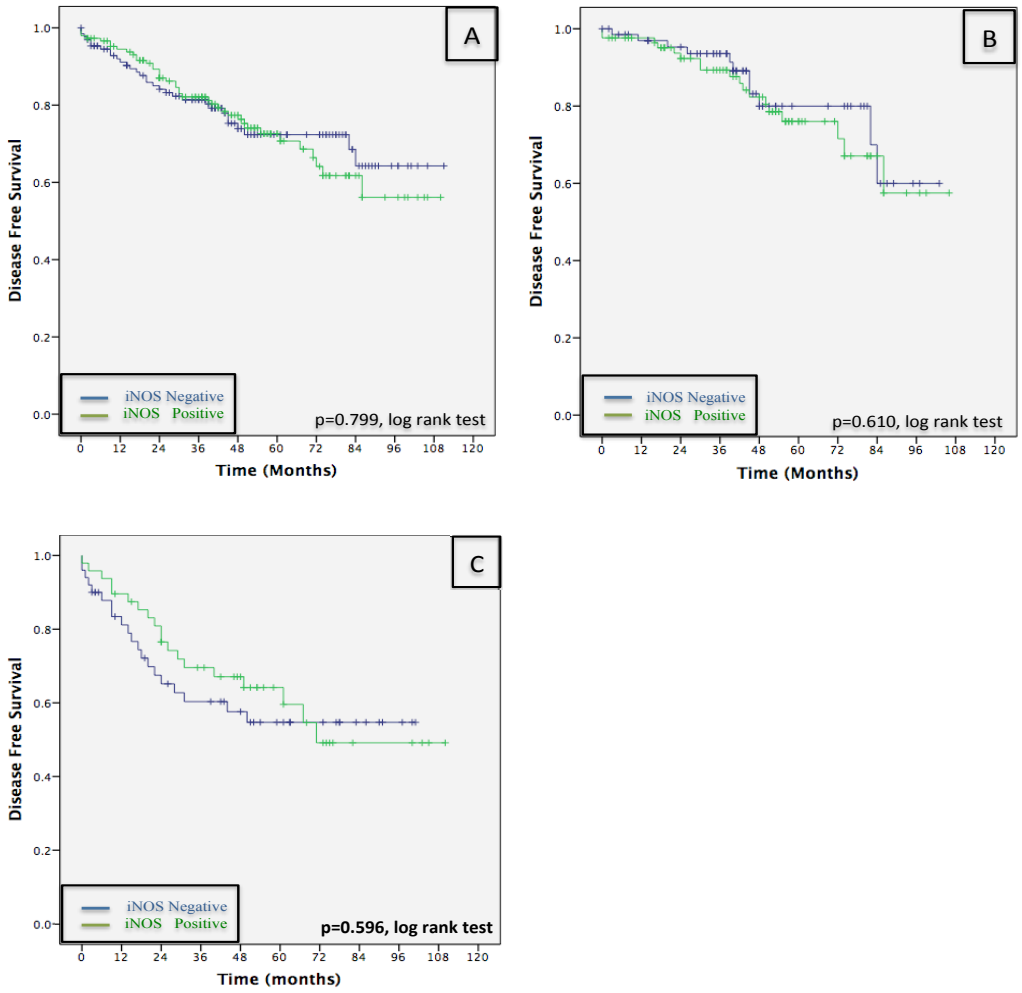

Figure A.17. Kaplan-Meier curves for DFS in BC according to iNOS expression (A) the entire cohort, (B) ER-positive and (C) ER-negative

Appendix XVIII

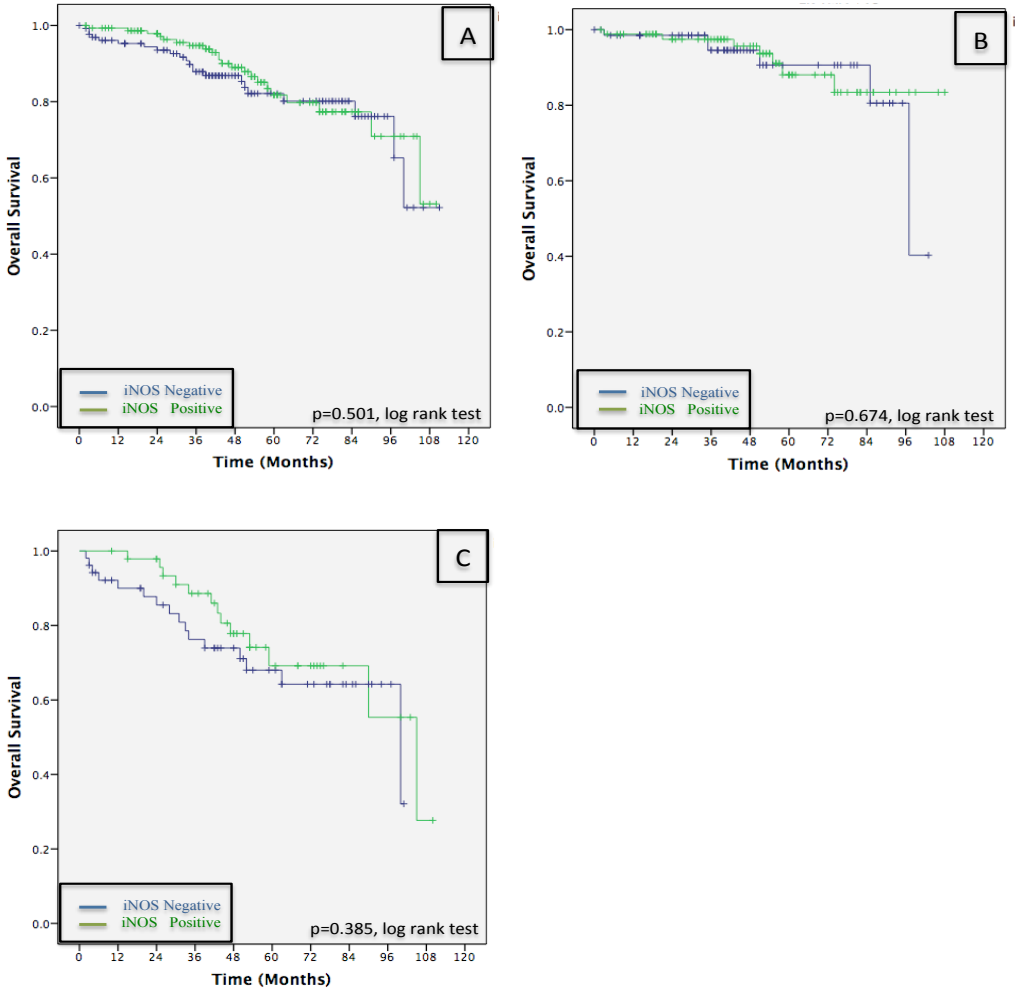

Figure A.18. Kaplan-Meier curves for OS in BC according to iNOS expression (A) The entire cohort, (B) ER-positive and (C) ER-negative