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AN INVESTIGATION OF THE RELATIONSHIP BETWEEN THE COMPONENTS OF TUMOUR MICROENVIRONMENT, SIGNAL TRANSDUCTION PATHWAYS AND OUTCOME IN BREAST CANCER

A THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (PhD)

BY

DR. FADIA J. A. GUJAM

M.B.B.S., M.Sc.

Based on research conducted in the academic Unit of Surgery;

Royal Infirmary and Institute of Cancer Research,

College of Medical, Veterinary and Life of Sciences,

University of Glasgow-UK (May 2016)

Abstract

Breast cancer, the most commonly diagnosed type of cancer in women, is a major cause of morbidity and mortality in the western world. Well-established risk factors of breast cancer are mostly related to women's reproductive history, such as early menarche, late first pregnancy and late menopause. Survival rates have improved due to a combination of factors, including better health education, early detection with large-scale use of screening mammogram, improved surgical techniques, as well as widespread use of adjuvant therapy.

At initial presentation, clinicopathological features of breast cancer such as age, nodal status, tumour size, tumour grade, and hormonal receptor status are considered to be the standard prognostic and predictive markers of patient survival, and are used to guide appropriate treatment strategies. Lymphovascular invasion (LBVI), including lymphatic (LVI) and blood (BVI) vessel invasion, has been reported to be prognostic and merit accurate evaluation, particularly in patients with node negative tumours who might benefit from adjuvant chemotherapy. There is a lack of standard assessment and agreement on distinguishing LVI from BVI despite the major challenges in the field. A systematic review of the literatures, examining methods of detection and the prognostic significance of LBVI, LVI and BVI, was carried out. The majority of studies used haematoxylin and eosin (H&E) and classical histochemistry to identify LVI and BVI. Only few recent studies used immunohistochemistry (IHC) staining of the endothelium lining lymphatic and blood vessels, and were able to show clear differences between LVI and BVI. The prognostic significance of LBVI and LVI was well-documented and strongly associated with aggressive features of breast tumours, while the prognostic value and the optimal detection method of BVI were unclear.

Assessment and prognostic value of LBVI on H&E sections ($LBVI_{H\&E}$) was examined and compared to that of LVI and BVI detected using IHC with D2-40 for LVI ($LVI_{D2–40}$) and

Factor VIII for BVI (BVI_{FVIII}) in patients with breast cancer including node negative and triple negative patients (n=360). $LBVI_{H\&E}$, $LVI_{D2–40}$ and BVI_{FVIII} were present in 102 (28%), 127 (35%) and 59 (16%) patients respectively. In node negative patients (206), $LBVI_{H\&E}$, $LVI_{D2–40}$ and BVI_{FVIII} were present in 41 (20%), 53 (26%) and 21 (10%) respectively. In triple negative patients (102), $LBVI_{H\&E}$, $LVI_{D2–40}$ and BVI_{FVIII} were present in 35 (29%), 36 (35%) and 14 (14%) respectively. $LBVI_{H\&E}$, $LVI_{D2–40}$ and BVI_{FVIII} were all significantly associated with tumour recurrence in all cohorts. On multivariate survival analysis, only $LVI_{D2–40}$ and BVI_{FVIII} were independent predictors of cancer specific survival (CSS) in the whole cohort (P=0.022 and P<0.001 respectively), node negative (P=0.008 and P=0.001 respectively) and triple negative patients (P=0.014 and P<0.001 respectively). Assessment of LVI and BVI by IHC, using D2-40 and Factor VIII, improves prediction of outcome in patients with node negative and triple negative breast cancer and was superior to the conventional detection method.

Breast cancer is recognised as a complex molecular disease and histologically identical tumours may have highly variable outcomes, including different responses to therapy. Therefore, there is a compelling need for new prognostic and predictive markers helpful of selecting patients at risk and patients with aggressive diseases who might benefit from adjuvant and targeted therapy. It is increasingly recognised that the development and progression of human breast cancer is not only determined by genetically abnormal cells, but also dependent on complex interactions between malignant cells and the surrounding microenvironment. This has led to reconsider the features of tumour microenvironment as potential predictive and prognostic markers.

Among these markers, tumour stroma percentage (TSP) and tumour budding, as well as local tumour inflammatory infiltrate have received recent attention. In particular, the local environment of cytokines, proteases, angiogenic and growth factors secreted by inflammatory cells and stromal fibroblasts has identified crucial roles in facilitating tumour

growth, and metastasis of cancer cells through lymphatic and/or blood vessel invasion. This might help understand the underlying process promoting tumour invasion into these vessels. An increase in the proportion of tumour stroma and an increase in the dissociation of tumour cells have been associated with poorer survival in a number of solid tumours, including breast cancer. However, the interrelationship between these variables and other features of the tumour microenvironment in different subgroups of breast cancer are not clear. Also, whether their prognostic values are independent of other components of the tumour microenvironment have yet to be identified.

Therefore, the relationship between TSP, clinicopathological characteristics and outcome in patients with invasive ductal breast cancer, in particular node negative and triple negative disease was examined in patients with invasive ductal breast cancer (n=361). The TSP was assessed on the haematoxylin and eosin-stained tissue sections. With a cut-off value of 50% TSP, patients with ≤50% stroma were classified as the low-TSP group and those with >50% stroma were classified as the high-TSP group. A total of 109 (30%) patients had high TSP. Patients with high TSP were old age (*P*=0.035), had involved lymph node (*P*=0.049), Her-2 positive tumours (*P*=0.029), low-grade peri-tumoural inflammatory infiltrate (*P*=0.034), low CD68+ macrophage infiltrate (P<0.001), low CD4+ (P=0.023) and low CD8+ T-lymphocytes infiltrate (*P*=0.017), tumour recurrence (*P*=0.015) and shorter CSS (*P*<0.001). In node negative patients (n=207), high TSP was associated with low CD68+ macrophage infiltrate (*P*=0.001), low CD4+ (*P*=0.040) and low CD8+ T-lymphocytes infiltrate (*P*=0.016) and shorter CSS (*P*=0.005). In triple negative patients (n=103), high TSP was associated with increased tumour size (*P*=0.017) high tumour grade (*P*=0.014), low CD8+ T-lymphocytes infiltrate (*P*=0.048) and shorter CSS (*P*=0.041). The 15-year cancer specific survival rate was 79% vs 21% in the low-TSP group *vs* high-TSP group. On multivariate survival analysis, a high TSP was associated with reduced CSS in the whole cohort (*P*=0.007), node negative patients (*P*=0.005) and

those who received systemic adjuvant therapy (*P*=0.016), independent of other pathological characteristics including local host inflammatory responses. Therefore, a high TSP in invasive ductal breast cancer was associated with recurrence and poorer long-term survival. The inverse relation with the tumour inflammatory infiltrate highlights the importance of the amount of tumour stroma on immunological response in patients with invasive ductal breast cancer. Implementing this simple and reproducible parameter in routine pathological examination may help optimise risk stratification in patients with breast cancer.

Similarly, the relationship between tumour budding, clinicopathological characteristics and outcome was examined in patients with invasive ductal breast cancer (n=474), using routine pathological sections. Tumour budding was associated with several adverse pathological characteristics, including positive lymph node (*P*=0.009), presence of LVI (*P*<0.001), and high TSP (*P*=0.001) and low-grade general peri-tumural inflammatory infiltrative (*P*=0.002). In node negative patients, a high tumour budding was associated with presence of LVI (*P*<0.001) and low-grade general peri-tumural inflammatory infiltrative (*P*=0.038). On multivariate survival analysis, tumour budding was associated with reduced CSS (*P*=0.001), independent of nodal status, tumour necrosis, CD8+ and CD138+ inflammatory cells infiltrate, LVI, BVI and TSP. Furthermore, tumour budding was independently associated with reduced CSS in node negative patients (*P*=0.004) and in those who have low TSP (*P*=0.003) and high-grade peri-tumoural inflammatory infiltrative (*P*=0.012). A high tumour budding was significantly associated with shorter CSS in luminal B and triple negative breast cancer subtypes (all *P*<0.001). Therefore, tumour budding was a significant predictor of poor survival in patients with invasive ductal breast cancer, independent of adverse pathological characteristics and components of tumour microenvironment. These results suggest that tumour budding may promote disease progression through a direct effect on local and distant invasion into lymph nodes and

lymphatic vessels. Therefore, detection of tumour buds at the stroma invasive front might therefore represent a morphologic link between tumour progression, lymphatic invasion, spread of tumour cells to regional lymph nodes, and the establishment of metastatic dissemination.

Given the potential importance of the tumour microenvironment, the characterisation of intracellular signalling pathways is important in the tumour microenvironment and is of considerable interest. One plausible signalling molecule that links tumour stroma, inflammatory cell infiltrate and tumour budding is the signal transducer and activator of transcription (STAT).

The relationship between total and phosphorylated STAT1 (ph-STAT1), and total and ph-STAT3 tumour cell expression, components of tumour microenvironment and survival in patients with invasive ductal breast cancer was examined. IHC of total and ph-STAT1/STAT3 was performed on tissue microarray of 384 breast cancer specimens. Cellular STAT1 and cellular STAT3 expression at both cytoplasmic and nuclear locations were combined and identified as STAT1/STAT3 tumour cell expression. These results were then related to CSS and phenotypic features of the tumour and host. A high ph-STAT1 and a high ph-STAT3 tumour cell expression was associated with increased ER (P=0.001 and P<0.001 respectively) and PR (all P<0.05), reduced tumour grade (P=0.015 and P<0.001 respectively) and necrosis (all P=0.001). Ph-STAT1 was associated with increased general peri-tumoural inflammatory infiltrate (P=0.007) and ph-STAT3 was associated with lower CD4+ T-lymphocyte infiltrate (P=0.024). On multivariate survival analysis, including both ph-STAT1 and ph-STAT3 tumour cell expression, only high ph-STAT3 tumour cell expression was significantly associated with improved CSS (P=0.010) independent of other tumour and host-based factors. In patients with high necrosis grade, high ph-STAT3 tumour cell expression was independent predictor of improved CSS

(*P*=0.021). Ph-STAT1 and ph-STAT3 were also significantly associated with improved cancer specific survival in luminal A and B subtypes. STAT1 and STAT3 tumour cell expression appeared to be an important determinant of favourable outcome in patients with invasive ductal breast cancer. The present results suggest that STATs may affect disease outcome through direct impact on tumour cells, and the surrounding microenvironment.

The above observations of the present thesis point to the importance of the tumour microenvironment in promoting tumour budding, LVI and BVI. The observations from STATs work may suggest that an important driving mechanism for the above associations is the presence of tumour necrosis, probably secondary to hypoxia. Further work is needed to examine the interaction of other molecular pathways involved in the tumour microenvironment, such as HIF and NFkB in patients with invasive ductal breast cancer.

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Author's Declaration

I hereby declare that all the work in this thesis, unless otherwise indicated below, is entirely my own contribution. This work was performed by me in the Academic Unit of Surgery, Glasgow Western infirmary, Glasgow Royal Infirmary and Wolfson Wohl Cancer Research Centre-Institute of Cancer Sciences, University of Glasgow between April 2012 to May 2016 under supervision of Professor Donald C McMillan and Dr Joanne Edwards.

The clinical cohort was provided by Dr Julie C Doughty, Department of Surgery, Western Infirmary, Glasgow. The clinicopathological data included age, histological tumour type, grade, tumour size, lymph node status and treatment were retrieved from the pathology reports and put on database by Dr E Jenniofer Campbell Department of Pathology, Western Infirmary, Glasgow.

Dr Zahra MA Mohammed performed immunohistochemical staining for oestrogen receptor (ER), progesterone receptor (PR) status, Her-2 status and Ki67 proliferative index, cellular inflammatory infiltrates markers including CD68, CD4, CD8, and CD138, as well as part of the general peri-tumoural inflammatory infiltrate and tumour necrosis assessment at Glasgow Royal Infirmary and Glasgow Western Infirmary.

Tissue micro arrays and slide scanning were made by Clare Orange in the Department of Pathology, Southern General Hospital, Glasgow.

Inter-observer reproducibility assessment was performed with the assistance of Dr James J Going, Dr Joanne Edwards, Dr Zahra MA Mohammed at Departments of Pathology Southern General Hospital, Royal infirmary and Wolfson Wohl Cancer Research Centre, Glasgow.

List of publications

The work presented in this thesis has resulted in the following full paper publications and poster presentations.

1-The role of lymphatic and blood vessel invasion in predicting survival and methods of detection in patients with primary operable breast cancer.

Fadia JA Gujam, James J Going, Joanne Edwards, Zahra MA Mohammed, Donald C McMillan. Crit Rev Oncol Hematol 2014;89(2):231-41.

2-Immunohistochemical detection improves the prognostic value of lymphatic and blood vessel invasion in primary ductal breast cancer.

Fadia JA Gujam, James J Going, Zahra MA Mohammed, Clare Orange, Joanne Edwards, Donald C McMillan. BMC Cancer 2014; 676.

3-Immunohistochemical ascertment improves the prognostic value of lymphatic and blood vessel invasion in primary ductal breast cancer. Annals of Oncology 25 (Supplement 4): iv85–iv109, 2014,doi:10.1093/annonc/mdu327.21. (Abstract).

4-The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with operable ductal breast cancer.

Fadia JA Gujam, Joanne Edwards, Zahra MA Mohammed, James J Going, Donald C McMillan. Br J Cancer 2014;111, 157–165.

5-The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer.

Fadia JA Gujam, Joanne Edwards, Zahra MA Mohammed, Donald C McMillan, James J Going. B J Cancer 2015;113(7):1066-74.

6-The relationship between total and phosphorylated STAT1 and STAT3 tumour cell expression, components of tumour microenvironment and survival in patients with invasive ductal breast cancer.

Fadia J.A. Gujam, Donald C. McMillan, Joanne Edwards. Oncotarget DOI: 10.18632/oncotarget.12730

Poster presentation:

1-The relationship between the tumour-stroma percentage, clinicopathological characteristics and outcome in patients with invasive ductal breast cancer presented at 9th EBCC (European Breast Cancer Conference), March-2014, Glasgow UK.

2-The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer presented at World Congress on Breast Cancer August -2015, Birmingham UK.

List of abbreviation

BCL	B-cell lymphoma
BVI	Blood vessel invasion
CAF	Cancer associated fibroblast
CCND1	Cyclin D1
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CSS	Cancer specific survival
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Endothelial mesenchymal transition
ER	Oestrogen receptor
FISH	Fluorescent in situ hybridisation
H&E	Hematoxylin and eosin
Her-2	Human epidermal growth factor receptor-2
HIF-1	hypoxia-inducible factor-1
ICCC	Interobserver intraclass correlation coefficient
IFNs	Interferons
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-17	Interleukin-17
JAK	Janus kinase
Ki67	Ki67 proliferative index
K-M score	Klintrup-Mäkinen score
LBVI	Lymphovascular invasion
LIF	Leukaemia inhibitory factor
LVI	Lymphatic vessel invasion
MCL-1	Myeloid cell leukemia 1
NF-κB	Nuclear factor-Kappa B
OS	Overall survival
OSM	Oncostatin M
PDGF	Platelet derived growth factor
Ph-STAT1	Tyrosin (701) phosphorylated STAT1
Ph-STAT3	Tyrosin (705) phosphorylated STAT3

PR	Progesterone receptor
RFS	Relapse free survival
STAT	Signal transducer and activator of transcription
TAM	Tumour associated macrophage
TBX21	T-box 21
T_H	T helper
TMA	Tissue microarray
VEGF	Vascular endothelial growth factor

Dedication

I dedicate this work to my lovely parents Joumaa and Fatma Gujam, who supported me throughout my life and my studies. This work is also dedicated to my husband Anwar Aboukdir for the continuous encouragement and to my lovely daughter Maryam, who dislodged the cloud and brightened my life again. I also dedicate this work to my sisters and brothers in particular my little brother Abdulhafid for his endless love.

Chapter 1 Introduction

1.1 Epidemiology of breast cancer

Breast cancer is a complex and heterogeneous disease, comprising distinctive histological patterns. It is the second most common cancer in the world and a major cause of morbidity and mortality. Breast cancer is the most frequent malignancy in women, accounting for 23% of all female cancers and ranks second worldwide (>10% of all new cancer), with more than a million women are diagnosed with breast cancer every year. It is also the most common cause of cancer death among women worldwide (Ferlay et al., 2010).

1.1.1 Incidence and mortality

Although breast cancer is now the most frequent cancer both in developed and developing regions, the incidence rates of breast cancer vary considerably across the world. The highest incidence rates are in Western Europe, North America and Australia, whereas Eastern Africa and Eastern Asia have the lowest rates (Key et al., 2001; Ferlay et al., 2010). However, the range of mortality rates is lower than that for incidence, due to the improved survival rates for breast cancer in (high-incidence) developed countries. In 2008 breast cancer accounted for >450,000 deaths globally, with the highest mortality rates occurring in developing areas (269,000 deaths) compared to that of developed regions (189,000 deaths) (Ferlay et al., 2010).

In the UK, breast cancer was the most common cancer in 2014, with more than 55,000 new cases diagnosed, and with an estimated lifetime risk affecting one in eight people. Despite the increased incidence rates, probably due to the introduction of screening program, improved surgical techniques and the widespread use of adjuvant chemotherapy, mortality rates have fallen by 40% since mid-1980s. However, breast cancer remains the third most common cause of female cancer death, and accounts for >11% of all female cancer

mortality. In the year 2014, there were 11,600 breast cancer deaths compared to around 13,705 breast cancer deaths in 1992 (Cancerstats, 2014).

In Scotland, breast cancer is the most frequently diagnosed cancer in women, with a frequency of 28% of all female cancers and an estimated lifetime risk of 1 in 10. In west of Scotland approximately 2300 new cases of breast cancer are diagnosed each year (West of Scotland Cancer Network, 2015). Death rates from breast cancer in Scotland have decreased by almost 11% over the last decade, despite the increase in incidence (isdscotland, 2012).

Breast cancer survival has improved over the last decade. In the UK almost 90% of women with breast cancer will survive their cancer for 5 years or more after diagnosis (Cancerstats, 2014). In England and Wales, 5 years survival rates for women diagnosed between 2010 and 2011 is 87% (Cancerstats, 2014). In west of Scotland, 5 years relative survival for patients diagnosed between 2007 and 2011 is 88%. The highest survival rate (90.6%) was reported for women aged 45-64 years at diagnosis (isdscotland, 2012).

1.1.2 Aetiology and risk factors

It is well established that breast cancer is a hormone-related disease, in which oestrogen induces some breast epithelial cell growth and proliferation (Pike et al 1993). However, the causes of breast cancer are complex and cannot be explained by a single etiological aspect. Several well-established risk factors play a significant role in the development of such tumours (Table 1.1).

1.1.2.1 Geographical variation

Breast cancer age-standardised incidence and mortality shows a strong geographic pattern between Western and Eastern countries and within one country. In the UK, England and

Scotland have significantly higher incidence rates compared with Wales. For almost 20 years, the incidence of breast cancer had been lowest in Northern Ireland compared with the rest of the UK; however, there was no significant variation in mortality across the United Kingdom (Westlake and Cooper, 2008).

In the United States, a substantial regional variation in breast cancer incidence and mortality rates has been observed for decades. Most notably, an increase in the rates of breast cancer among women in California has been historically higher than those in many other areas of the USA and the world (Parkin et al., 1999). Mortality rates in the Northeast United States have also been notably higher than rates in other areas of the USA (Canto et al., 2001). Studies of cancer incidence among immigrants from eastern Asia (a low-risk area) to United States (a higher-risk area) show an increase in incidence of breast cancer with figures doubling within 10 years of arrival, highlighting the important role of the environment and lifestyle in breast cancer development (McPherson et al., 2000; Key et al., 2001).

1.1.2.2 Age

The single most important risk factor strongly related to breast cancer incidence is age. The risk of breast cancer increases throughout a woman's lifetime, with the highest incidence rates in older women (Ferrer et al., 2005), supporting a link with reproductive hormones. In the UK, almost half (48%) of breast cancers affect people aged 65 and over, while the highest incidence rates are in women aged 85 and over (Cancerstats, 2014). The annual incidence rate of breast cancer in women 80-85 years old in the United States is 15 times higher than that in women aged 30-35 years old (American Cancer Society, 2012).

Age at menarche and menopause

Early age at menarche has been consistently associated with an increased risk of breast cancer and the estimated decrease in risk per five-year delay in menarche is 22% (Vogel, 2008). Late menopause (more than 55 years) doubles the risk of breast cancer than those whose menopause occurs before the age of 45. Risk of breast cancer development increases by almost 3% for each year of delay in the menopause, natural or induced by surgery (Hulka and Moorman, 2008, Collaborative group, 2012).

Age at first birth and parity

Childbearing at a younger age lowers the risk of breast cancer. The relative risk of developing breast cancer is estimated to increase by 3% for each year of delay (Collaborative Group, 2002). First full-term pregnancy at a younger age of <30 years, and multiple pregnancies reduces the risk of breast cancer over the long term. However, there is an increased risk of breast cancer in the immediate period after childbirth, perhaps due to oestrogen effects causing extensive terminal ducts differentiation during pregnancy and their subsequent involution after birth. This excess risk, however, gradually diminishes, and the effect of birth is rather to protect against the disease for the rest of the women's life (Key et al., 2001). Risk of breast cancer, in addition, is reduced by 7% with each full-term pregnancy, and overall women who have had children have a 30% lower risk than nulliparous women (Collaborative Group, 2002).

1.1.2.3 Breast feeding

Breast feeding has consistently been shown to reduce the risk of breast cancer, suggesting a protective role. The greater protection is associated with longer duration and the risk is reduced by 4% for every 12 months of breastfeeding. Furthermore, the risk of breast cancer in women who had breastfed for more than two years was 33% lower than those

who had never breastfed (Lodha et al., 2011). The possible reason for this is highly related to delayed ovulation during lactation and suppressed levels of oestrogen after breast feeding (Newcomb et al., 1994). On the other hand, a major contribution to the high incidence of breast cancer in developed countries has been attributed to the lack of breast feeding or short lifetime duration of breast feeding in these countries (Hulka and Moorman, 2008).

1.1.2.4 Family history and genetic predisposition

Inherited mutations in certain genes like BRCA1, BRCA2, p53, PTEN and ATM are consistently linked to risk of breast cancer development (Key et al., 2001; McPherson et al., 2000; Hulka and Moorman, 2008). The risk increases four-folds if two or more first-degree relatives were affected at an early age (Hulka and Moorman, 2008). The estimated lifetime chance of developing breast cancer for BRCA1 and BRCA2 mutated genes carriers is 50% to 85% by the age of 70 (Antoniou and Easton, 2006). Nevertheless, only 5% to 10% of breast cancers results from genetic factors, and 25% of cases diagnosed before 30 years of age are attributed to mutated genes (Thull and Vogel, 2004; Key et al., 2001; Thull and Vogel, 2004; Lodha et al., 2011).

1.1.2.5 Benign breast disease

History of benign breast diseases increases the risk of breast cancer development. Lower category risk lesions such as cyst, adenosis, mammary duct ectasia, metaplasia, and hyperplasia without atypia are associated with a slight increase in the risk of breast cancer among women older than 50 years of age (Santen et al., 2007). Biopsies performed for proliferative disease with cellular atypia account for a fivefold increase of breast cancer risk. Approximately 40% of women with a family history of breast cancer and atypical hyperplasia subsequently develop breast cancer (Hartmann et al., 2005).

1.1.2.6 Exogenous hormones

Oral Contraceptives

Current use of combined (oestrogen and progestin) oral contraceptives may slightly increase the risk of breast cancer, probably due to their oestrogen content (Key et al., 2001; Cuzick, 2003; Lodha et al., 2011). The risk is particularly higher for women who had used such pills in their reproductive age however, women who have stopped using oral contraceptives for 10 years or more have the same risk as women who never used the pills (Collaborative Group, 1996).

Hormonal replacement therapy

Current use of hormone replacement therapy (HRT), oestrogen-progestin combinations or oestrogen only formulation, has been shown to increase the risk of fatal breast cancer (1.6 to 2 fold), with a higher risk associated with longer use (Million Women Study Collaborators, 2003; Heiss et al., 2008). Long-term use of 5 years or more among current or recent users appears to be associated with a 30–50% increase in breast cancer risk. Reports from both the US Nurses Health Study and the Collaborative Group support these estimates (Colditz et al., 1995; Collaborative Group, 1997). Among current HRT users in the Nurses Health Study, older women (aged 60–64) who had used oestrogens for at least 5 years had double the risk of women who reported no hormone use. However, the increased risk appears to diminish within 5 years of discontinuation of hormone use. The effect is substantially greater for oestrogen-progestagen combinations than for other types of HRT (Million Women Study Collaborators, 2003)

1.1.2.7 Radiation exposure

Ionising radiation is an established risk factor for breast cancer and is strongly related to dose and age at exposure. Women who received diagnostic x-rays to the chest for

tuberculosis or pneumonia between the ages of 10 to 29 have a three-fold increase in the risk of breast cancer (John et al., 2007). Studies show a 12- to 25-fold increased risk of secondary breast cancer among women who received chest radiotherapy for Hodgkin's lymphoma before the age of 30 (Alm et al., 2008). Women with BRCA1 or BRCA2 mutations also have high risk, especially if exposed before 30 years of age (Pijpe et al., 2012).

1.1.2.8 Life style related factors

A significant proportion of breast cancers can be linked to lifestyle factors such as obesity, diet, alcohol consumption tobacco smoking and physical activity. Overweight and obesity are associated with increased incidence and mortality of breast cancer (Calle et al., 2003, Cuzick, 2003; Ahn et al., 2007; Reeves et al., 2007; Begum et al., 2009). Specifically, 7-15% of breast cancer cases are attributed to obesity in developed countries (Lahmann et al., 2004; Renehan et al., 2010; Parkin and Boyd, 2011). Obesity and breast cancer risk varies according to menopausal status. For younger women, being obese appears to protect against breast cancer risk (Peacock et al., 1999; Weiderpass et al., 2004) with an estimated 20-40% reduction in cancer risk (van den Brandt et al., 2000; Reeves et al. 2007; Nelson et al., 2012). In contrast, postmenopausal obesity has been reported to be associated with increased risk of breast cancer (Ahn et al., 2007; Begum et al., 2009; Rose and Vona-Davis, 2010). In addition, results from the EPIC study and the Million Women Study have reported that obese women have a 30% higher risk of postmenopausal breast cancer than women with a healthy weight (Lahmann et al., 2004; Reeves et al., 2007). In respect to breast cancer subtype and obesity, a meta-analysis of 11 studies suggested that adult weight gain is predictive of a two-fold increase in the risk of hormone receptors positive breast cancers (Vrieling et al., 2010).

This inverse relationship can be explained by the different impact of premenopausal and postmenopausal obesity on endogenous hormone levels. The normal balance of oestrogen and progesterone levels in obese premenopausal women is disrupted with a decrease in sex hormone binding globulin and a minimal increase in exposure to oestrogen, but a decrease in breast exposure to progesterone (Stephenson and Ross, 2003; Dowsett and Folkerd et al., 2014). In postmenopausal women, where adipose tissue becomes the main site of oestrogen biosynthesis, androgens from the adrenal gland are converted into oestrogens in fat cells via aromatisation, resulting in increased concentration of plasma oestradiol (Ahn et al., 2007; Rose and Vona-Davis, 2010).

Diet and nutrition -fat intake in particular- have been proposed to be one of the reasons for the observed geographical difference in breast cancer rates. Several prospective epidemiological studies have suggested an increase in breast cancer risk with increased consumption of dietary fat (Cho et al., 2003; Thiebaut et al., 2007; Sieri et al., 2008), with saturated fat doubling the risk of breast cancer in the higher consumption group (Sieri et al., 2008). For example, a study assessing risk related to tumour subtypes demonstrated that vegetable oil–based margarine was associated with a 31% greater risk of hormone receptor negative breast cancer (Wirfait et al., 2011). A greater risk of hormone receptor positive and Her-2 negative tumours was linked to diet high in saturated fat (Sieri et al., 2014). However, some previous studies have reported a weak or no relationship between fat intake and breast cancer (Willett, 2001; Prentice et al., 2009; Romieu, 2011; Martin et al., 2011).

Studies that evaluated the role of red and/or processed meat on breast cancer risk have been inconsistent. A meta-analysis provided no conclusive evidence that red meat or processed meat acts as an independent risk factor for breast cancer (Alexander et al., 2010). In addition, the evidence for the protective roles of fruits, vegetables and dietary

vitamins (such as vitamin C and E) are limited and have proved inconclusive (Key et al., 2001; Gandini et al. 2000; Kushi et al. 2006). However, some evidence suggests that soy food intake seems to be inversely associated with the disease (Zhu et al., 2011; Zhang et al., 2012).

Alcohol intake is consistently associated with increased risk of breast cancer. This risk is dose-dependent and exists regardless of the type of alcoholic beverage consumed (Key et al., 2001; Li et al., 2003; Cuzick, 2003; Allen et al., 2009; Seitz et al., 2012). Low to moderate alcohol consumption (3-14 alcoholic drinks/week) is associated with a slight increase in the risk of breast cancer (Allen et al., 2009). The Million Women Study showed an increase in risk of approximately 7-12% with every extra unit of alcohol per day (Allen et al., 2009). Recent published meta-analysis consistently indicated a 40-50% increased risk of breast cancer in women consuming ≥3 alcoholic drinks/day (Seitz et al., 2012). The basis of this association is unclear, but may be caused by the influence of alcohol on the liver and so on hormone profiles (Boyle et al., 2003). Alternately changes in DNA and the significant increase in DNA methylation of target promoter genes may account for the increase in risk (Tao et al., 2011).

Several epidemiologic studies have evaluated the relationship between cigarette smoking and breast cancer risk (Cui et al., 2006; Collishaw et al., 2009; Secretan et al., 2009; Luo et al., 2011; Xue et al., 2011; Gaudet et al., 2013). Despite the quantity of data, this relationship still controversial and lack scientific consensus, probably because of the potential confounding by alcohol consumption (Hamajima et al., 2002; Gaudet et al., 2013). One recent study has reported that the rate of new breast cancer cases was 24% higher in smokers than in nonsmokers and 13% higher in former smokers than in nonsmokers. The combined meta-analysis of this study has found that the risk is especially

increased in women who started smoking at younger age (before the age of 20) or before the birth of their first child (Gaudet et al., 2013).

Moderate physical activity has been documented to be inversely associated with risk of breast cancer (Vainio, 2002; Lynch et al., 2011), with a risk reduction up to 30% for women with high levels of physical activity compared to women who have none (Key et al., 2001; Winzer et al., 2011). Previous studies have also suggested that exercise is associated with longer breast cancer survival, in that 2–3 hours of brisk walking per week reduced breast cancer recurrence and all-cause mortality by 40-67% compared with inactivity (Ibrahim and Al-Homaidh, 2010; McTiernan et al., 2010). Data from the Women's Health Initiative, showed that high levels of physical activity improved survival in postmenopausal women with breast cancer, even among those reporting low physical activity before diagnosis (Irwin et al., 2011).

Socio-economic status has been reported by several studies to be related to the risk of breast cancer, with higher risk in women living in high socioeconomic status (Kelsey et al., 1992; Robert et al., 2004; Hulka and Moorman, 2008). In contrast, lower income was associated with increased risk of more aggressive tumour characteristics, late stage disease and poorer outcomes (Clegg et al., 2009; Dunn et al., 2010).

Table 1-1 Breast cancer risk factors

Risk factor	Relative risk	High risk group
Geographical area	5	America/ Northern Europe
Age	>10	Postmenopausal age
Early menarche	2	<12 years of age
Late menopause	2	>55 years of age
Late onset childbearing	1-2	First full-term>33years
Breastfeeding	1-2	Never breastfeed a child
Genetic mutation	4	Women < 40 + BRCA1 and/or BRCA2
Family history	2.1-4	≥2 first-degree relatives with breast cancer diagnosed at young age
Previous breast disease	2-5	Atypical hyperplasia
Exogenous hormones:		
Oral contraceptive	1-2	Resent use
HRT	1.5-2	≥5 years use
Radiation exposure	3	High dose radiation to chest in young females after age 10
Body weight:		
Postmenopausal	1-2	BMI>35 for both group
Premenopausal	0.8	
Alcohol consumption	1.3	Dose-dependent

HRT: hormone replacement therapy, BMI: body mass index. Adapted from McPherson et al., 2000, and Hulka and Moorman, 2008.

1.2 Breast Cancer Prognostic and Predictive Factors

Breast cancer is characterised by its molecular and clinical diversity. While some patients experience long cancer-specific survival, an aggressive disease with poor outcome rates might affect others. A number of tumour and patient-related factors can be identified in order to understand the clinical behaviour of the newly diagnosed tumour and determine prognosis and survival. Importantly, these factors help optimise individual treatment plan to provide efficient therapy and avoid unwanted side effects of overtreatment (Cianfrocca and Goldstein, 2004; Weigel and Dowsett, 2010).

In this era of high-throughput methods, several novel biomarkers have been reported for prognostic and predictive purposes. Only a few have made their way into clinical routine due to the lack of sufficient validation, however, the identification of new markers has led to a more definitive insight into tumour biology and substantiates the importance of the existing biomarkers (Weigel and Dowsett, 2010).

While prognostic factors supply information on the course of a disease (recurrence-free and total survival) and are independent of the adjuvant therapy, predictive factors provide prior information on the likelihood of the response of a tumour to a defined therapeutic intervention and are associated with tumour sensitivity or resistance to that therapy (Cianfrocca and Goldstein, 2004; Weigel and Dowsett, 2010). Several prognostic and predictive factors are well established and routinely used in clinical practice purely as a prognostic or predictive, or as both (Table 1.2).

Table 1-2 Summary of well-established breast cancer prognostic and predictive factors

Factor	Prognostic	Predictive
Patient age	Yes	
Nodal status	Yes	
Tumour size	Yes	
Histological grade	Yes	
Histological tumour type	Yes	
Lymphovascular invasion	Yes	
Steroid hormone receptors	Yes	Yes
Her-2 overexpression	Yes	Yes

1.2.1 Tumour characteristics

1.2.1.1 Nodal status

The axillary lymph node status is the most valuable factor for the cancer specific and overall survival in breast cancer (Weiss et al., 2003; Cianfrocca and Goldstein, 2004). Node positive patients have about a 4–8 times higher mortality rates than those without nodal involvement (Fisher et al., 2001; Arriagada et al., 2006). The presence of involved lymph node correlates directly with the outcome, and the more nodes involved the worse the prognosis. Prognosis for patients with ≥10 involved axillary nodes showed 70% reduced survival at 10 years than those with 1–3 involved nodes (Weiss et al., 2003; Cianfrocca and Goldstein, 2004). Although lymph node status has no predictive value for response to treatment, it provides a very reliable assessment of local tumour spread and is the most consistent prognostic factor used in making decisions for adjuvant therapy. Therefore, careful histological examination should be carried out for all excised axillary lymph nodes (Cianfrocca and Goldstein, 2004).

Recently, the use of sentinel lymph node (SLN) biopsy has replaced standard axillary dissection since standard axillary dissection is associated with increased morbidity after surgery and does not contribute to survival. SLN could accurately stage the axilla in 96% of patients and identifies lymph nodes at the highest risk of harbouring metastatic disease (Albertini et al., 1996; Giuliano et al., 2011).

1.2.1.2 Tumour size

Tumour size is one of the strongest independent prognostic indicators for breast cancer (Aebi et al., 2000) even after long term follow-up (Arregada et al., 2006). Increased tumour size correlates positively with reduced survival, presence of regional lymph nodes, and with increased distant recurrence (Weiss et al., 2003; Cianfrocca and Goldstein, 2004;

Chen et al., 2010). After nodal status, tumour size is the most powerful prognostic factor related to prognosis, and for patients with node negative tumours, tumour size is the best tumour factor used to identify prognosis and to make adjuvant treatment decisions (Cianfrocca and Goldstein, 2004). Node negative patients with a tumour of 2–5 cm have lower 10-year overall survival compared to those with a tumour smaller than 1 cm, 66% vs 79%, respectively (Chia et al., 2004). Patients with T1-T2 breast cancer with 1-3 positive lymph nodes have higher local and distant recurrence, and lower overall and cancer specific survival compared to patients with negative lymph nodes (Chen et al., 2010).

1.2.1.3 Histologic grade

Histologic grade, or the degree of tumour differentiation, (Figure 1.1) is widely recognized as an important tool in the histopathological assessment of breast cancer. The first modern description of the current grading system by Bloom and Richardson (1957) (Bloom and Richardson, 1957) was modified and validated by Elston and Ellis (1991) (Elston and Ellis, 1991). Three morphologic characteristics, including tubule formation, nuclear pleomorphism and mitotic count are scored from 1 to 3 and the three scores from each category are grouped to produce a score between 3 and 9 (Table 1.3). A score of 3–5 points is assigned grade 1 (well differentiated tumour), a score of 6–7 points is assigned grade 2 (moderately differentiated tumour), and a score of 8–9 points is assigned grade 3 (poorly differentiated tumour) (Elston and Ellis, 1991).

Table 1-3 Invasive breast cancer grade scoring (Modified Bloom and Richardson)

	Score 1	Score 2	Score 3
Tubule formation	>75% of the tumour	10-75% of the tumour	<10% of the tumour
Nuclear pleomorphism	nuclei with slight increase in size	nuclei with moderate increase in size and shape	Marked variation in size and shape
Mitotic count	≤4 mitotic counts / 10 high power fields	5–9 mitotic counts / 10 high power fields	≥10 mitotic counts / 10 high power fields
Overall grade	Grade 1 = 3–5 points Well–differentiated	Grade 2 = 6–7 points Moderately–differentiated	Grade 3 = 8–9 points Poorly–differentiated

Information taken from (Elston and Ellis, 1991)

Higher tumour grade has been consistently associated with lower survival rates (Aebi et al., 2000; Arriagada et al., 2006). Depending on other prognostic factors, such as nodal status or tumour size, cumulative survival among patients with the lowest score was 90–94% 10 years after diagnosis, and 30–78% among those with the highest score (Reed et al., 2000).

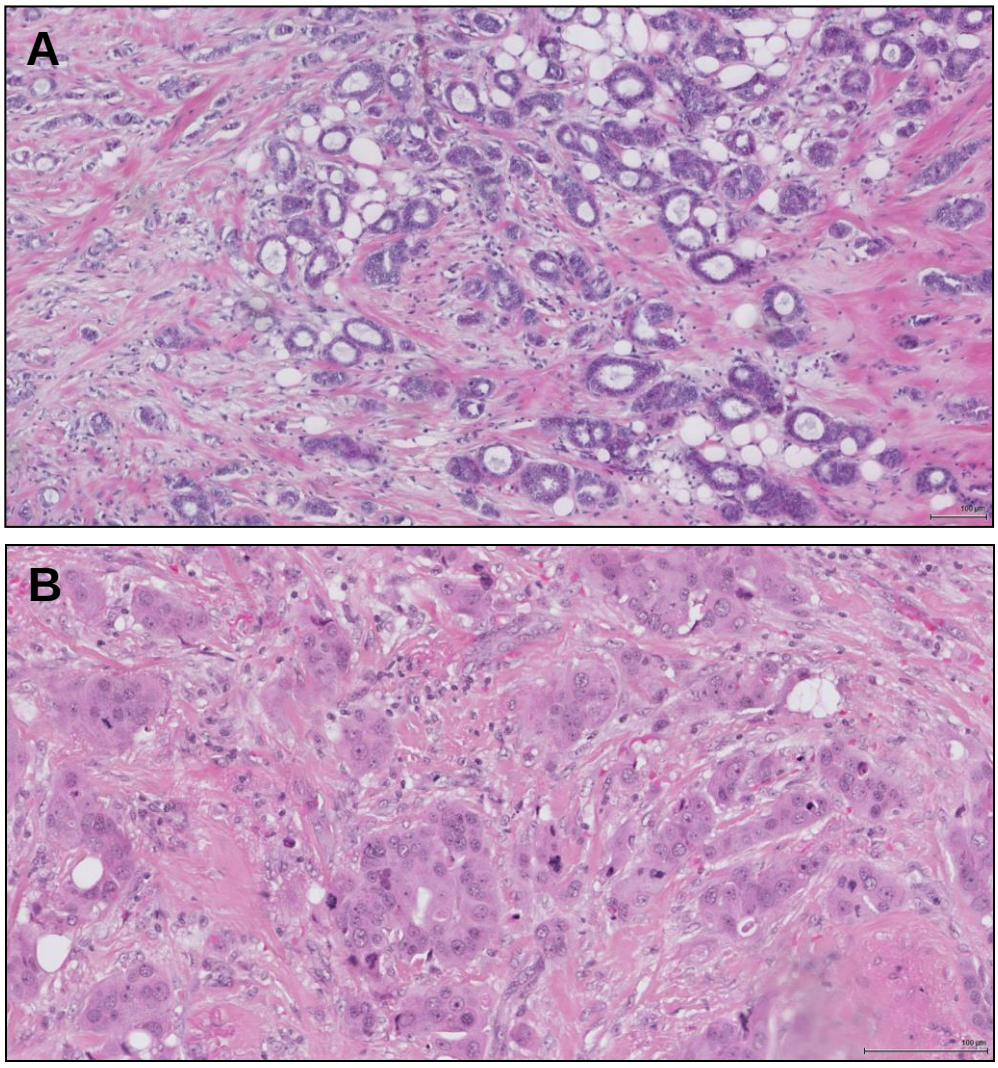


Figure 1-1 Tumour Grade

(A) Low-grade invasive ductal carcinoma. The majority of tumour cells form tubules that are lined by uniform tumour cells. (B) High-grade invasive ductal carcinoma characterised by solid growth of pleomorphic tumour cells with the numerous mitotic figures. Original magnification x20, scale 100µm.

1.2.1.4 Tumour histological type

Growth pattern, or histological type of the tumour, is used to classify invasive breast cancer and is a significant predictor of outcome. The majority of invasive breast cancers are classified as invasive ductal carcinoma of no special type as they do not exhibit specific histological characteristics (Ellis et al., 1992). The prognostic value of histological type can be grouped into four: excellent, good, poor, and very poor prognosis (Galea et al., 1992) with invasive cribriform, tubular, tubulo-lobular and mucinous has 10 years survival of >80% (Fisher et al., 2001), while invasive papillary, classic lobular and medullary cancers have a worse prognosis (Soerjomataram et al., 2008).

1.2.1.5 Lymphovascular invasion

Lymphovascular invasion (LBVI) including both lymphatic vessel invasion (LVI) and blood vessel invasion (BVI), has been defined as the presence of tumour cells within an endothelial-lined space in the area surrounding the invasive carcinoma (Rosai, 1993). It is an important step in the complex process of tumour metastasis and an important criterion for cancer therapy. Lymphovascular invasion is associated with other poor prognostic factors including increased tumour size and grade, and with axillary lymph node involvement (Lee et al., 2006a; Colleoni et al., 2007; Ejlertsen et al., 2009; Rakha et al., 2012). It is a significant prognostic marker (Lee et al., 2006a; Kato et al., 2003; Ejlertsen et al., 2009; Mohammed et al., 2011; Rakha et al., 2012), and increases the risk of local and distant recurrence (Cianfrocca and Goldstein, 2004; Truong et al., 2005; Rakha et al., 2012). For node negative patients, breast cancer mortality was >50% higher in women with positive LBVI compared to women with no LBVI (Lee et al., 2006a).

However, there is a lack of a routine standardised and objective assessment method to reliably examine LBVI and differentiate between LVI and BVI. Therefore, lymphovascular

invasion has been systematically reviewed and will be discussed in more detail in chapters 4.0 and 5.0.

1.2.1.6 Hormone receptors

Oestrogen and progesterone are steroid hormones crucial for breast development and have an important role in breast differentiation and tumorigenesis. They deliver their impact through their nuclear receptors, the oestrogen receptor (ER) and progesterone receptor (PR) (Anderson et al., 2002; Allred, 2008). ER is expressed in 70% - 95% of invasive lobular carcinomas, and in 70% - 80% of invasive ductal carcinomas, and PR is expressed in 60%-70% of invasive breast carcinomas (Lal et al., 2005). For the last two decades, immunohistochemistry (IHC) evaluation of hormone receptors has been the standard of practice (Figure 1.2). Different cut-off values have been used to determine hormone receptor status, however, recent guidelines by the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) have recommended that 1% positive tumour nuclei can identify patients who would benefit from hormonal therapy (Hammond et al., 2010).

The presence of these receptors is a very powerful predictor of breast cancer hormone therapies and has significantly improved the clinical outcomes of patients with hormone receptor-positive tumours. Hormonal treatment response rate for ER and PR positive tumours is approximately 80% (Bundred, 2001; Rampaul et al. 2001). Although the prognostic value of ER/PR receptors in breast cancer is considerably weak, previous studies have shown survival advantages among women with hormone receptor-positive tumours relative to women with hormone receptor-negative tumours (Anderson et al., 2001; Bundred, 2001; Dunnwald et al., 2007; Mohammed et al., 2012a).

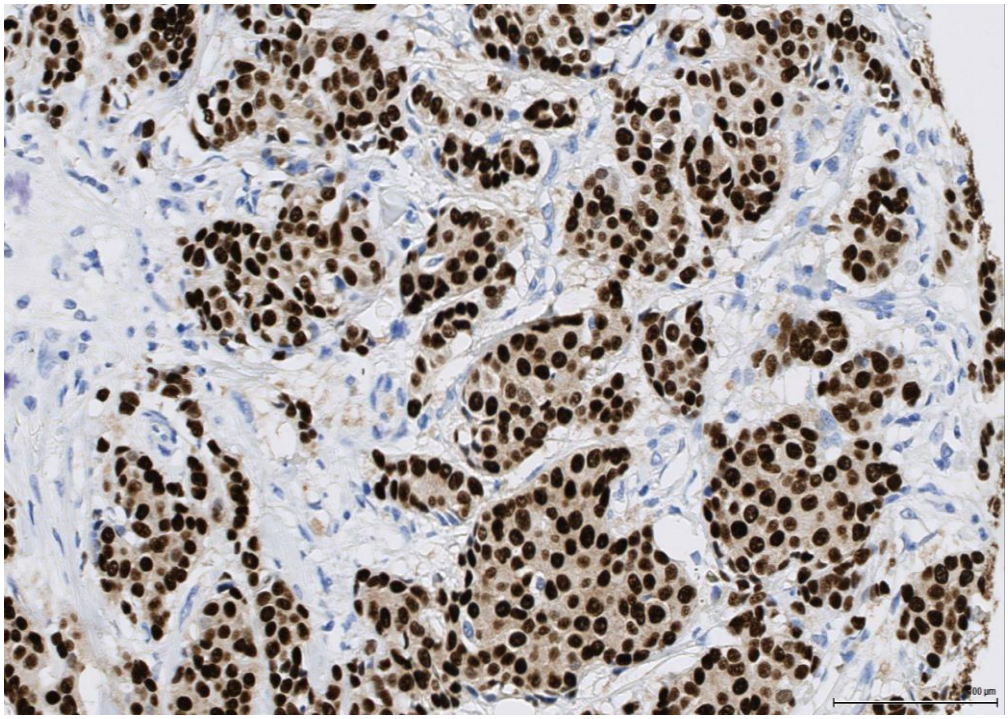


Figure 1-2 Immunohistochemical Staining for Oestrogen Receptor

This invasive ductal carcinoma shows that the majority of tumour cells are strongly positive for oestrogen receptor. Original magnification x20, scale 100µm.

1.2.1.7 Human epidermal growth factor receptor type 2 (Her-2)

The human proto-oncogene Her-2 (c-erbB-2 / *neu*) is located on chromosome 17 and encodes the transmembrane glycoprotein $p185^{HER2}$ which exhibits tyrosine kinase activity homologous to the epidermal growth factor receptor (Ross et al., 2009; Sauter et al., 2009). It is amplified and/or overexpressed in 15-30% of human breast cancer (Slamon et al., 1987) and associated with aggressive tumour features, such as high histological grade, negative ER status, increased recurrence and mortality rates (Romond et al., 2005; Pritchard et al., 2006; Hayes et al., 2007; Wolff et al., 2007). The prognosis of patients with a Her-2 positive tumour is significantly worse than that of patients with a Her-2 negative tumour (Romond et al., 2005; Pritchard et al., 2006; Francis et al., 2006; Santin et al., 2008) however, its prognostic significance is not sufficient and is principally used to predict the response to anti-Her-2 therapy, Trastuzumab or Herceptin.

IHC and fluorescent in situ hybridization (FISH) are the most commonly used methods to evaluate Her-2 status in breast cancer (Figure 1.3). Using formalin fixed paraffin-embedded tumour tissues, IHC detects Her-2 protein at the cell membrane whereas FISH quantify Her-2 gene in the tumour cells (Downs-Kelly et al., 2005; Mohammed et al., 2012b). The most recent guidelines for Her-2 testing and scoring from ASCO and CAP have recommended the following: a score of 3+ on IHC stain (intense, uniform circumferential staining) in ≥10 % of invasive carcinoma cells, in situ hybridization indicating more than six gene copies per nucleus, or a FISH gene ratio (ratio of Her-2 gene signals to chromosome 17 signals) equal to or greater than 2 indicates a positive result (Figure 1.3). A score of 0 or 1+ on IHC staining, a FISH result of less than 4 Her-2 gene copies per nucleus, or a FISH gene ratio of less than 2.0 indicates a negative result. Equivocal or weakly positive cases (a score of 2+ on IHC staining) are then referred to FISH test (Wolff et al., 2007).

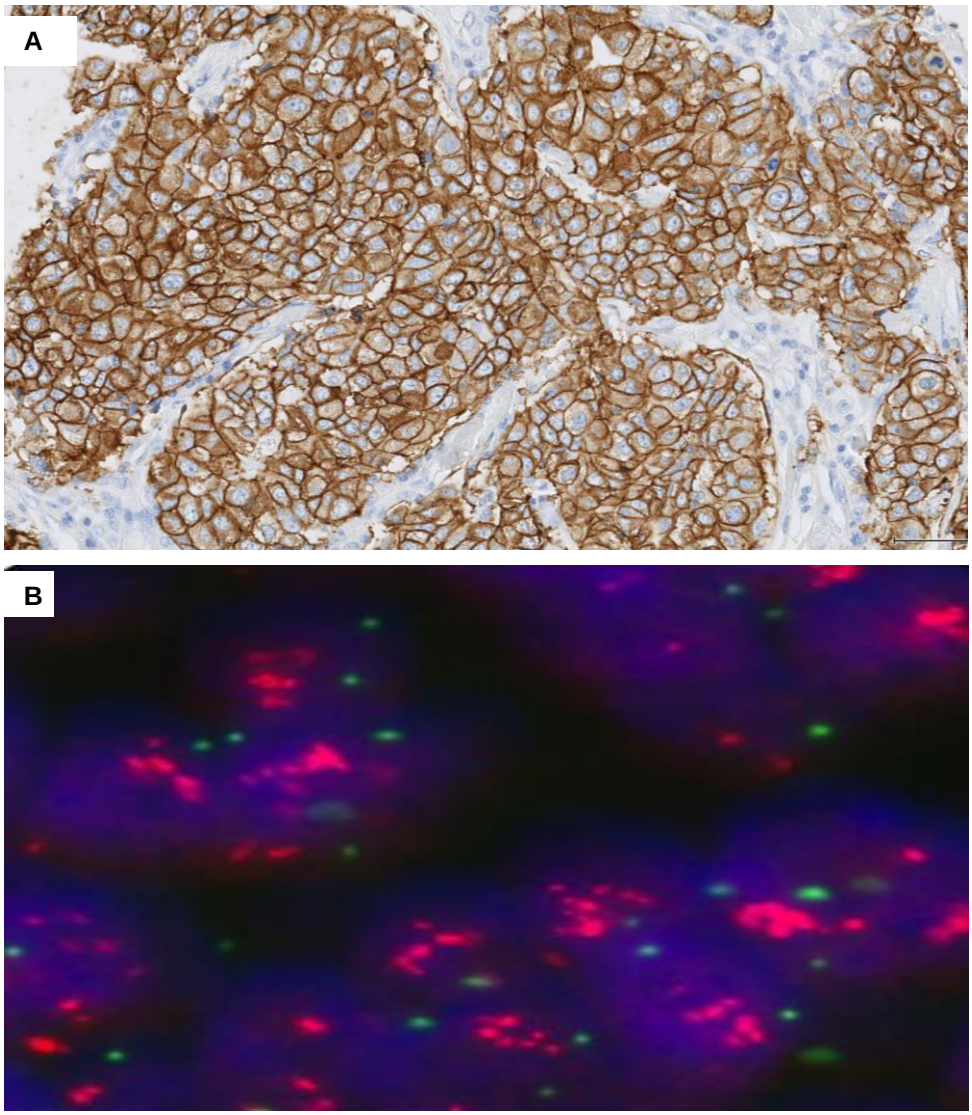


Figure 1-3 Immunohistochemical staining for Her-2 protein and fluorescent in situ hybridization for the Her-2 gene.

(A) positive staining is defined as intense complete membranous staining. (B) An increased number of Her-2 genes amplification (red signals) is evident compared to 1 or 2 copies of chromosome 17 centromere sequences (green signals). This tumour is classified as Her-2 gene-amplified carcinoma.

1.2.1.8 Tumour proliferation

Tumour proliferation is one of the hallmarks of cancer, indicating imbalance of cell proliferation and cell death. Different markers have been used to evaluate tumour proliferation including S-phase fraction, thymidine labeling index, mitotic count, and IHC assays for Ki67, cyclins and mitosin (Bundred, 2001; Cianfrocca and Goldstein, 2004; van Diest et al., 2004; Luporsi et al., 2012). High S-phase fraction is strongly correlated with high tumour grade, ER negativity, and Her-2 expression, all are adverse prognostic factors. Mitotic count, which is the number of mitoses in a given tumour area, has been combined in all grading systems of breast cancer and is considered the simplest tool to evaluate tumour proliferation (Bundred, 2001; Cianfrocca and Goldstein, 2004; van Diest et al., 2004). The prognostic value of mitotic count has been previously reported (van Diest et al., 2004) however, it has some technical and analytical difficulties.

Ki67, which represent tumour proliferation during the active phases of cell cycle, is the most commonly used proliferative marker (Morabito et al., 2003; Urruticoechea et al., 2005; Mohammed et al., 2012c). It is an independent prognostic factor in breast cancer, with a high level of Ki67 index is associated with a poor survival outcome (Yerushalmi et al., 2010; Dowsett et al., 2011). It is also directly associated with tumour size, lymph node involvement, histological grade, and Her-2 overexpression (Morabito et al., 2003).

1.2.1.9 Tumour necrosis

Necrosis is a type of cell death that lacks the features of apoptosis and autophagy in that it is often associated with the non-physiologically regulated cause of cell loss and can lead to local inflammation (Golstein and Kroemer, 2007). The most common cause of necrosis during tumour development is obviously inadequate oxygen and nutrient supply (metabolic stress) of fast-growing tumour cells (Proskuryakov and Gabai, 2010). Necrosis is a common histological feature and one of the established prognostic variables in many solid

organ tumours such as renal (Frank et al., 2002), lung (Swinson et al., 2002), colorectal (Pollheimer et al., 2010) and pancreatic (Hiraoka et al., 2010) cancers.

Regarding breast cancer, several studies dating back to 1970s have shown consistent evidence that tumour necrosis is associated with aggressive tumour features and poor prognosis (Carter et al., 1978; Fisher, et al., 1978; Shek and Godolphin, 1988; Gilchrist et al., 1993; Carlomagno et al., 1995; Kato et al., 1997; Leek et al., 1999; Kato et al., 2000; Ikpatt et al., 2002; Lee et al., 2006b; Richards et al., 2011).

A number of studies have reported tumour necrosis to predict survival dependent (Kato et al., 2000; Ikpatt et al., 2002) or independent of other high-risk characteristics (Shek and Godolphin, 1988; Gilchrist et al., 1993). More recently, necrosis has been associated with basal-like breast cancers (Colpaert et al., 2001; Fulford et al., 2006). However, there have been some conflicting results from reports that failed to find a relationship between tumour necrosis and breast cancer outcomes (Kato et al., 1997; Leek et al. 1999; Lee et al., 2006b) although all of these studies reported associations with high-risk features including high tumour grade, ER negativity and angiogenesis.

Grouped prognostic factors

Combination of some of the prognostic factors into prognostic indices has become part of clinical practice to help treatment decisions. These indices include TNM classification, Nottingham Prognostic Index, and Adjuvant! Online.

TNM staging consists of information on primary tumour size (T), axillary lymph node involvement (N), and the presence of distant metastasis (M) (Table 1.4). 79% of patients with localised breast cancer had survived 10 years following diagnosis compared to 53% of those with regional metastasis (Taylor et al., 2003), whereas only 3.4% patients presenting with distant metastasis (stage: M1) had 10-year survival (Olivotto et al., 2003).

Nottingham Prognostic Index (NPI) combines tumour size, lymph node status and tumour grade (Kollias et al., 1997; Kollias et al., 1999; D'Eredita et al., 2001), the strongest prognostic factors making it a suitable model for prognosis of breast cancer (Soerjomataram et al., 2008). It is calculated using the following formula:

NPI = 0.2x tumour size (in cm) + lymph node status (1, 2, or 3) + histological grade (1, 2, or 3).

Patients with NPI <3.4 are stratified into good prognostic group with 80% Survive more than10 years, whereas patients with NPI between 3.4 and 5.4 are stratified into moderate prognostic group and those with NPI is > 5.4 are stratified into poor prognostic group with only 13% survive > 10 years (Kollias et al., 1997).

Adjuvant Online is a web-based (www.adjuvantonline.com) statistical program and a tool for assessing the risk of recurrence and survival within 10 years of diagnosis. It is used for patients with early breast cancer to decide the most appropriate treatment (chemotherapy, hormonal therapy or none) based on well-established factors including patient's age,

menopausal status, involved lymph nodes, tumour size, tumour grade and ER status. Estimates provided by Adjuvant! have been shown to correlate closely with actual clinical outcomes in population- based cohorts (Olivotto et al., 2005).

Table 1-4 TNM stage grouping for breast cancer (American Joint Committee on breast cancer staging)

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

Tis Carcinoma in situ, * T1 includes T1mi (Tumour ≤ 1 mm in greatest dimension),

** T0 and T1 tumours with nodal micrometastases only (N1mi) are excluded from Stage IIA and are classified Stage IB.

Emerging Molecular and Genomic Markers

The molecular nature of breast cancers was first examined in 2000 by Perou and colleagues, demonstrated that the expression pattern of a set of genes within the tumour determine the molecular signature of breast cancer and that intrinsic signatures predict the clinical outcome of the disease (Perou et al., 2000; Sørlie et al., 2001). Five molecular subtypes of breast cancers, including luminal A, luminal B, Her-2 positive, basal, and normal-like, was described. Luminal A tumours have the best prognosis and basal and Her-2 positive tumours have the worst prognosis (Sørlie et al., 2001, Goldhirsch et al., 2011).

Features of molecular subtypes of breast cancer are shown in Table 1.5. Luminal types of breast cancers express high levels of ER and luminal epithelial genes. Luminal B tumours have a more aggressive phenotype, with higher proliferation rate compared to luminal A tumours. Luminal B tumours also include those tumours that express ER and have Her-2 overexpression or amplification. On the other hand, Her-2 positive breast cancers exhibit Her-2/neu gene amplification but do not express ER-related genes. The basal-like tumours frequently do not express the three key receptors ER, PR, and Her-2 and called “triple negative” tumours, but also expresse basal cytokeratins 5/6 and 17. They tend to be infiltrating ductal carcinomas with a high mitotic index (Eroles et al., 2012).

The clinical course of these intrinsic subtypes vary substantially. Luminal B breast cancers usually need to be treated more aggressively than luminal A, with both hormonal therapy and chemotherapy. Her-2 positive breast cancers respond very well to Her-2 targeted therapy. Basal type of breast cancers has a poor prognosis and currently there are no specific targeted therapies for them (Reis-Filho and Tutt, 2008). Normal breast like tumours presenting an intermediate prognosis between luminal

and basal-like and usually do not respond to neo-adjuvant chemotherapy. However, there are doubts about their real existence and some researchers believe they could be a technical artifact from high contamination with normal tissue during the microarrays (Eroles et al., 2012).

There are currently a few multigene prognostic and predictive tests for breast cancer including the Oncotype DX and MammaPrint genomic tests.

The Oncotype DX test is a reverse transcription polymerase chain reaction (RT-PCR)-based assay provides analysis of a panel of 21genes expression in formalin-fixed paraffin-embedded tumour tissues. It was originally designed to predict the likelihood of disease recurrence within 10 years of the initial diagnosis for women with early stage node negative ER positive breast cancer. Several distinct groups of genes are included, among them genes related to ER, PR, Her-2, invasion, proliferation, and other biological processes (Goldstein et al., 2008). The Oncotype DX is the most widely used molecular test in the clinical setting for making treatment decisions and is recommended by the St. Gallen Consensus (Gnant et al., 2011).

Based on the expression levels of these 21 genes, a score called breast cancer Recurrence Score (RS) is produced to stratify breast cancer patients into three risk groups; low risk (RS <18), intermediate risk (RS 18–30), and high risk (RS ≥31), with 10-year distant recurrence rates of 6.8 %, 14.3 %, and 30.5 %, respectively (Goldstein et al., 2008). The Oncotype DX test was later developed to be used for hormone receptor-positive breast cancer patients with up to three positive lymph nodes (Albain et al., 2010). The general consensus is that for patients with a low RS hormonal therapy without systemic chemotherapy is sufficient whereas, for patients

with a high RS hormonal therapy in addition to systemic chemotherapy is required to reduce risk of distant recurrence (Gnant et al., 2011).

Table 1-5 Features of the proposed molecular subtypes of breast cancer

Molecular subtype	Frequency	Cell of origin	ER/PR/Her-2	Proliferation rate	Histological grade	prognosis
Luminal A	50-60%	Luminal epithelial cell	ER and/or PR +ve, Her-2 -ve	Low	Low	Excellent
Luminal B	10-20%	Luminal epithelial cell	a) Her-2-ve, ER and/or PR +ve b) Her-2+ve, ER and/or PR +ve	a) High b) Any	Intermediate/ High	Intermediate/ Bad
Her-2 positive	10-15%	Late luminal progenitor	Her-2 +ve ER -ve and PR -ve	High	High	Bad
Normal breast-like	5-10%	Luminal epithelial cell	ER -ve/+ve Her-2 -ve	Low	Low	Intermediate/ good
Basal-like	10-20%	Basal/myoepithelial cell/ bipotent progenitor	ER -ve, PR -ve, and Her-2 -ve	High	High	Bad

Adapted from (Eroles et al., 2012)

Other gene expression-based platforms have been developed such as MammaPrint, the wound-response or healing model, the twogene expression ratio (HOXB13:IL17BR), the Rotterdam 76 gene signature, and the genomic grade index (also known as Map-Quant Dx). MammaPrint is already FDA approved however, the other gene expression signatures are less well known (Ma et al., 2004). Although gene profiling is useful to predict which patients with breast carcinoma would benefit from adjuvant systemic therapy, limitations such as difficulties in reproducing the specific gene sets, the expense of testing, and standardisation of reporting remains a challenge (Cianfrocaa and Goldstein, 2004).

Many other potential prognostic and predictive features, related to different tumour characteristics or biological processes such as cell-cycle regulators (p53, c-myc, cyclins), protease (urokinase, cathepsin D), and metastasis-related proteins (laminin 67 kDa receptor, nm23), have shown some promise in breast cancer, but have not yet achieved a definite role in patient management (Bundred, 2001).

1.2.2 Patient characteristics

1.2.2.1 Age at diagnosis and comorbidity

Young women with breast cancer tend to have more aggressive tumours and poor prognosis than older women (Kroman et al., 2000). Several previous studies reported that those aged 30 years old or less were associated with worse prognostic features such as high tumour grade, lymph node metastasis, vascular invasion, high proliferative rates, negative ER status, positive Her-2 status and poorer survival rate (Kollias et al., 1997; Kroman et al., 2000; Bundred, 2001; Sundquist et al., 2002; Morabito et al., 2003). This poor outcome improves as age increases, however, those older than 70 have poor outcome (Fisher et al., 2001; Arriagada et al., 2006). Furthermore, patient age is important for predicting response to chemotherapy and hormonal therapy, as menopausal status is an age-dependent factor. Adjuvant treatment helps to diminish the poor prognostic value of young age, whereas very old patients exhibit high mortality probably because they receive less extensive treatment; either related to the advanced age itself or the presence of serious concomitant diseases (Louwman et al., 2005).

1.3 Management of primary operable breast cancer

Optimal management of breast cancer should be provided through multidisciplinary teams (MDT), which include Diagnostic Team and Cancer Treatment Team. The purpose of the multidisciplinary team meetings is to provide tailored and appropriate treatment plans for all patients through coordinated multi-specialty care. According to the Association of Breast Surgery (ABS) guidelines, decisions of MDT meeting can be categorised as either diagnostic (where new cases are discussed prior to investigation for treatment), treatment planning (whereby the surgical treatment are discussed and appropriate adjuvant treatment options decided), or re-presentation (re-presentation of a patient with suspicious symptoms of recurrence) (ABS, 2009).

1.3.1 Diagnosis

For diagnosis of breast cancer, the ABS mandate, wherever possible, a non-operative breast cancer diagnosis by triple assessment (clinical and radiological assessment followed by core biopsy and/or fine needle aspiration). Fine needle aspiration cytology and core biopsy are simple techniques that require local anaesthesia only and accurately diagnose the breast lesions in at least 90% of cases (ABS, 2009).

1.3.2 Treatment

Treatment strategies for breast cancer in recent years have changed profoundly, with more treatment options and improvement in patient survival rates. These take into account the patients' age, presence of co-morbidity and clinicopathological characteristics. The main treatment for breast cancer includes surgery, radiotherapy, chemotherapy, hormone therapy, and biological therapy (targeted therapy) (Hammer et al., 2008; ABS, 2009).

1.3.2.1 Surgical treatment

Surgery remains the primary treatment option of a multicomponent treatment plan for breast cancer. The main goal is to remove the cancer, reduce the risk of local recurrence and to accurately define the stage of the disease. The surgical treatment of breast cancer consists of two main procedures: breast conservation (lumpectomy) or mastectomy. Both types are combined with an appropriate assessment of regional lymph nodes, for staging purpose and for adjuvant treatment guide, either by axillary lymph node dissection or by sentinel lymph node biopsy (Hammer et al., 2008; NICE, 2009). If there is proven axillary lymph node disease, pre-operatively axillary lymph node clearance should be undertaken; if there is no proven disease, the optimal axillary procedure is sentinel lymph node biopsy (ABS, 2009; NICE, 2009).

Data obtained from prospective clinical trials have demonstrated no survival differences between patients with early stage breast cancer based on whether they were treated with breast conservation therapy or mastectomy (Veronesi et al., 2002; Fisher et al., 2002).

However, accurate pre-operative assessment of the tumour extent and size is essential for deciding whether breast conservation surgery is an alternative option to mastectomy. This is made by clinical examination, mammography and ultrasound. Under-estimation of the true extent of the disease may result in a considerable number of patients, particularly those with invasive lobular cancer, dense breast pattern on mammography, discrepancy between the clinical and radiological estimated extent of disease. Selective use of magnetic resonance imaging should be discussed at the MDT meeting to plan surgical treatment for those patients (ABS, 2009; NICE, 2009).

1.3.2.1.1 Breast conservation surgery

Breast conservation therapy involves the resection of the primary tumour with adequate normal breast tissue surrounding the cancer to reduce tumour burden and obtain negative margins (Hammond, 2008). Breast conservation is generally followed by radiation therapy. Lumpectomy and radiotherapy is preferred for most women with early breast cancer; most stage I and II invasive carcinomas, and for some patients with larger tumours down-staged by neoadjuvant therapy. There is no age limit for this operation, however a previously irradiated breast is not suitable for conservation (Sainsbury et al., 2000; Singletary, 2001; Vinh-Hung and Verschraegen, 2004).

Patients undergoing breast conservation surgery should routinely have malignant tumours excised with microscopically clear circular margins. Although there is still a debate regarding acceptable margin, NICE (National Institute for Clinical Excellence) have previously recommended a minimum margin of 2 mm (NICE, 2002). More recently, the consensus from both the St Gallen 2013 and the ABS 2015 meetings was that 1 mm clear radial margin is acceptable (Goldhirsch et al., 2013; ABS, 2015). However, additional surgery to obtain clear margins should be recommended if, after MDT meeting discussion, the margin of excision is considered to be inadequate (ABS, 2009).

1.3.2.1.2 Mastectomy

Mastectomy is a second surgical option for patients with breast cancer. Mastectomy can be divided into three main approaches: 1) modified radical mastectomy, 2) simple mastectomy, 3) skin-sparing mastectomy (Hammer et al., 2008; Krag et al., 2010; Giuliano et al., 2011). It is indicated in cases where breast conservation is not appropriate. These are prior radiation therapy to the breast or chest wall, widespread disease that is multifocal, located in more than one quadrant and cannot be removed through a single incision with

negative margins, positive margin, tumours beneath or involving the nipple, after local recurrence in patients with previous conservation surgery, prophylaxis, and patient preference (Sainsbury et al., 2001; Mandal, 2012).

Modified radical mastectomy involves complete removal of the breast and its associated structure including the skin, areola, nipple, and level I and II axillary lymph nodes with preservation of the pectoralis major and minor muscles (Cotlar et al., 2003; Sainsbury, 2004; Loukas et al., 2011).

Simple mastectomy involves removal of the breast tissue, with no dissection of the axilla, except for the axillary tail that may include a few lymph nodes of the low anterior group. Simple mastectomy may be followed by radiotherapy to the axilla because no pathological staging of the axillary lymph nodes is performed with this procedure (Sainsbury 2004; Hammer et al., 2008). Removal of the nipple and areola is performed in both modified radical mastectomy and simple mastectomy.

Skin-sparing mastectomy is performed when a patient is undergoing immediate breast reconstruction. The purpose is to remove all breast tissue, along with the nipple-areola complex, while preserving as much viable skin as possible to achieve better cosmetic outcome (Cunnick and Mokbel, 2004). The nipple-areola complex could be also preserved with increased experience (Hammer et al., 2008). Several studies now demonstrate the improved aesthetic outcomes of skin or nipple-sparing mastectomy (Benediktsson and Perbeck, 2008; Gerber, 2009).

1.3.2.1.3 Breast reconstruction

Breast reconstruction and/or oncoplastic surgery is becoming increasingly important due to changes in patient expectations and demand. It aims to restore the normal shape and, to

some extent, consistency of the breast after wide local excision or after mastectomy. Breast reconstruction may be performed at the same time of the mastectomy (immediate) or later (delayed) (ABS at BASO, 2007). There are two basic categories for breast reconstruction; the implant based technique (using a silicone or saline implant) and the autologous reconstruction (using latissimus dorsi or transverse rectus abdominis myocutaneous flaps; either with 'pedicle' or 'free' flap). Many women may also need surgery to the contra-lateral breast to achieve symmetry using a variety of techniques, such as reduction mammoplasty or mastopexy approach and transfer of local-regional flaps (Grotting et al., 2003; Berry et al., 2010; Piper et al., 2015). Reconstruction of a nipple-sparing mastectomy with a variety of techniques leaves the patient with an outcome that is cosmetically and oncologically equivalent to that with lumpectomy, but usually without the need for radiation therapy (Benediktsson and Perbeck, 2008; Gerber, 2009; Endara et al., 2013).

Immediate breast reconstruction has the advantage of preserving the maximum of breast skin and the inframammary fold. However, there is a potential in individual patients for complications to result in the delay of adjuvant treatment. On the other hand, delayed reconstruction avoids any potential delay of adjuvant treatment and the detrimental effects of radiotherapy or chemotherapy on the reconstruction. However, delayed reconstruction requires replacement of a larger amount of breast skin where mastectomy flaps may be thin, scarred, or irradiated. Breast reconstruction contraindications include; non-resectable local chest wall disease, rapidly progressive systemic disease, patients who have serious co-morbidity or psychologically unsuitable (ABS at BASO, 2012).

1.3.2.2 Radiation therapy.

Radiotherapy is a common treatment which uses radiation to destroy breast cancer cells after conservation surgery. The goal is to eradicate the residual microscopic foci of cancer cells and reduce the risk of disease recurrence. Current indications for post mastectomy

radiotherapy (PMRT) include axillary nodal involvement of 4 or more nodes, tumour of 5 cm or more, T4 disease and positive surgical margins (NICE, 2009; Vilarino-Varela et al., 2009; Goldhirsch et al., 2013).

Radiotherapy is usually given as external beam radiotherapy; (40Gy in 15 fractions) as standard practice for patients with early invasive breast cancer following breast conservation surgery or mastectomy. External breast boost is offered to the site of local excision to patients with early invasive breast cancer and a high risk of local recurrence, following breast conservation surgery with clear margins and whole breast radiotherapy. Adjuvant radiotherapy to the supraclavicular fossa is given to patients with early breast cancer with ≥ 4 positive lymph nodes, and patients with early breast cancer with 1-3 positive lymph nodes if they have other poor prognostic factors e.g. T3/T4, histological grade 3 tumours, multifocality and LVI (NICE, 2009; West of Scotland Cancer Network, 2012).

Analysis of clinical trials by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) has shown that radiotherapy, after breast conservation, reduced the 10-year risk of any recurrence (locoregional or distant) from 35%-19%, and reduced breast cancer death rate from 25%-21% (EBCTCG, 2011). However, currently, there are insufficient recommendations for PMRT to women with 1-3 positive nodes. Individual data analysis of randomised trials showed that PMRT reduces both recurrence and breast cancer mortality for women with 1-3 positive nodes (EBCTCG, 2014).

1.3.2.3 Hormone therapy

Hormone therapy or endocrine therapy stops or reduces the growth of hormone positive breast cancer by blocking the production of oestrogen hormone or interfering with its ability to reach the tumour cells. Endocrine treatment is indicated as the principal

treatment for patients with ER positive and/or PR positive carcinomas (Pritchard, 2003; Sweetland, 2004).

Hormone therapy includes two main types: selective oestrogen receptor modulator or anti-oestrogen drugs such as tamoxifen, raloxifene and toremifene, and drugs that block oestrogen production or aromatase inhibitors (AI) (letrozole, anastrozole (reversible) and exemestane (irreversible). Anti-oestrogen drugs bind to oestrogen receptors and block their effect, whereas AI block the activity of an enzyme called aromatase that converts adrenal androgens into oestrogens in the muscle, liver and fat tissue. The selection of hormonal therapy depends on the source of oestrogen production. Before menopause oestrogen and progesterone are naturally produced by the ovaries and via the peripheral aromatase enzyme in postmenopausal women (Pritchard, 2003; Sweetland, 2004).

Tamoxifen has been the gold standard for more than 30 years, and is mainly used for ER positive premenopausal and postmenopausal women in the adjuvant and advanced metastatic settings (Pritchard, 2003). Tamoxifen is both an antagonist and a partial agonist of the oestrogen receptor (Wakeling et al., 1989) and its agonist action may become exaggerated over time leading to impairment in its anticancer activity and resulting in tamoxifen resistance (Norris et al., 1999; Ali and Coombes, 2002).

Aromatase inhibitors are an established treatment for postmenopausal women with early and metastatic breast cancer. In 2002, the ATAC trial was the first and by far the largest trial used anastrazol as adjuvant treatment for early breast cancer (ATAC trialist group, 2002). AIs are used as first line therapy, or as a second line therapy in patients who develop tamoxifen resistance (Campos, 2004; Clemons et al., 2004), and have generally superior efficacy and tolerability compared to tamoxifen (Cuzick et al., 2010). AIs given either for 5 years or for 2–3 years after 2–3 years of tamoxifen, produce greater reductions

in recurrence than 5 years of tamoxifen alone (Dowsett et al., 2010). However, the substantial benefits of AIs are associated with a significant, but manageable, increase in osteoporotic fractures and bone mineral loss (Perez et al., 2009).

The clinical management guidelines of West of Scotland Cancer Network recommend that premenopausal women with early ER positive breast cancer be offered adjuvant endocrine therapy with tamoxifen for 5 years with no further endocrine therapy or tamoxifen for additional 5 years. For postmenopausal women with early ER positive breast cancer, adjuvant endocrine therapy is offered as either tamoxifen for 5 years, an aromatase inhibitor for 5 years, tamoxifen initially for 5 years followed by an aromatase inhibitor for up to a further 3 years, or tamoxifen for 2.5 years followed by an aromatase inhibitor for up to 5 years (West of Scotland Cancer Network, 2012).

Bisphosphonate therapy reduces the risk of skeletal-related events in patients with bone metastases and can inhibit bone loss associated with AIs in postmenopausal women with early breast cancer (Brufsky et al., 2007; Bundred et al., 2008).

In the present thesis, patients were recruited between 1995 and 1998 and the only hormonal therapy available was tamoxifen. However, AIs were not introduced to routine practice by 1998. Therefore, none of the patients on the present thesis have received AIs.

1.3.2.4 Adjuvant Chemotherapy

Chemotherapy has the ability to destroy clinically undetectable micrometastasis after primary surgery and is usually recommended for women at significant risk of recurrence and relapse. The choice of chemotherapy regimen may be individualized based upon cancer-related factors such as the underlying risk of recurrence and the projected relative and absolute benefits from chemotherapy, as well as patient related factors such as age,

comorbidities, and risk tolerance (Hortobagyi, 2001). Chemotherapy agents include alkylating agents (cyclophosphamide), antimetabolites (5-fluorouracil, capecitabine, methotrexate), taxanes (paclitaxel, docetaxel), anthracyclines (doxorubicin, epirubicin) and mitotic inhibitors (vincristine) (Bergh et al., 2001).

Adjuvant chemotherapy may increase 10-year survival by 6% for node negative to 12% for node positive premenopausal women, and by 2%–6% in women aged over 50 (Bergh et al., 2001). Data from the Early Breast Cancer Trialists' Collaborative Group regarding polychemotherapy usage found that survival benefit was seen in the first 5 years with additional benefit during the second 5 years. Chemotherapy produced reduction in recurrence and increased survival was found in all groups analysed with more prominent effect in premenopausal women and those with ER negative tumours (EBCTCG, 2005).

Cyclophosphamide, methotrexate, and fluorouracil (CMF) and standard anthracycline based regimens reduce recurrence rates over 8 years by 30%, and breast cancer mortality rates by 20—25% (EBCTCG, 2012). Comparison of CMF with AC (Doxorubicin and cyclophosphamide) showed no difference in breast cancer mortality (EBCTCG, 2012).

1.3.2.5 Biological therapy

Trastuzumab or Herceptin is the most commonly used biological and targeted treatment for breast cancer. It is a humanised monoclonal antibody against the extracellular domain of the Her-2 receptor, and used in the adjuvant setting for treatment of Her-2 positive breast cancer (Vogel and Franco, 2003; Ross et al., 2003). Trastazumab has been originally approved by the FDA to treat breast cancer in September 1998 and expanded beyond the metastatic setting to treat patients with early breast cancer in 2006.

Clinical trials have shown that trastuzumab has improved both disease-free and overall survival in patients with early Her-2 positive breast cancer with node positive or high risk node negative breast cancer, when given in combination with or in sequence to adjuvant chemotherapy (Piccart-Gebhart et al., 2005; Romond et al., 2005).

The current optimal management and the gold-standard treatment care for patients with Her-2 positive tumours is one year of trastuzumab, which has been shown to be more effective than 6 months regimen (Gelber et al., 2012). Trastuzumab is also given in combination with adjuvant chemotherapy (taxan based chemotherapy is the preferred option) for patients with Her-2 positive and node positive tumour (West of Scotland Cancer Network, 2012). However, due to the age of the present thesis cohort (1995-1998), trastuzumab was not available for breast cancer treatment at that time and none of the patients on the present cohort have received this treatment.

1.3.2.6 Neoadjuvant therapy

The neoadjuvant (pre-operative) therapy to breast cancer is established as a therapeutic approach for selected high-risk breast cancers, tumours ≥ 2cm and for locally advanced disease. Neoadjuvant therapy could be radiotherapy, endocrine therapy, or chemotherapy. This approach offers some advantages, such as reduction in tumour size and down staging the disease, reduction in the extent of surgery and testing the efficacy of treatment given to patients (Thompson and Thompson, 2012).

Nearly half of the patients receiving neoadjuvant treatment may become suitable for breast conservation instead of mastectomy (Thompson and Thompson, 2012). Recent meta-analysis and clinical trials have demonstrated that neoadjuvant aromatase inhibitors have a better clinical and ultrasound response and a higher rate of breast conservation (Seo et al., 2009; Chia et al., 2010). In terms of survival, neoadjuvant chemotherapy is as effective as

adjuvant chemotherapy for locally advanced disease (Deo et al., 2003; Makhoul and Kiwan, 2011; Le Ray et al., 2012).

1.4 The tumour microenvironment in breast cancer

The tumour microenvironment is composed of several different cell types including non-immune cells such as fibroblasts, endothelial cells, bone marrow-derived cells, adipocytes, and immune cells such as marcrophages, natural killer cells, neutrophil and T lymphocytes, as well as blood and lymphatic vessels in a scaffold of extracellular matrix (ECM) (Kim et al., 2005; Hu and Polyak, 2008; Joyce and Pollard, 2009; Hanahan and Weinberg, 2011; Cirri and Chiarugi, 2012) (Figure 1.4). The non-cellular components of the tumour microenvironment including the ECM contains growth factors, proteases, protease inhibitors and other signalling molecules that play important roles in stromal reactions. Stromal cells surround and cross-talk with tumour cells and are key contributors in promoting the 'hallmarks' of cancer cells (Hanahan and Coussens, 2012). Importantly, these cells supply the tumour with molecules and growth factors essential for stimulation of blood vessels formation that provide the tumour cells with oxygen and nutrients (Pietras and Ostman, 2010).

1.4.1 The role of tumour stroma and stromal fibroblasts

The role of tumour microenvironment is becoming more recognised in breast cancer and every component plays an important role. The interactions between cancer cells and the tumour microenvironment have been shown to play a crucial role in overall tumour growth from initiation to progression (Coussens and Werb, 2002; Grivennikov et al., 2010; Pietras and Ostman, 2010). It has been reported that both the gene and protein expression profiles of the tumour stroma play an important role in breast cancer progression (Finak et al., 2008; Lin et al., 2008).

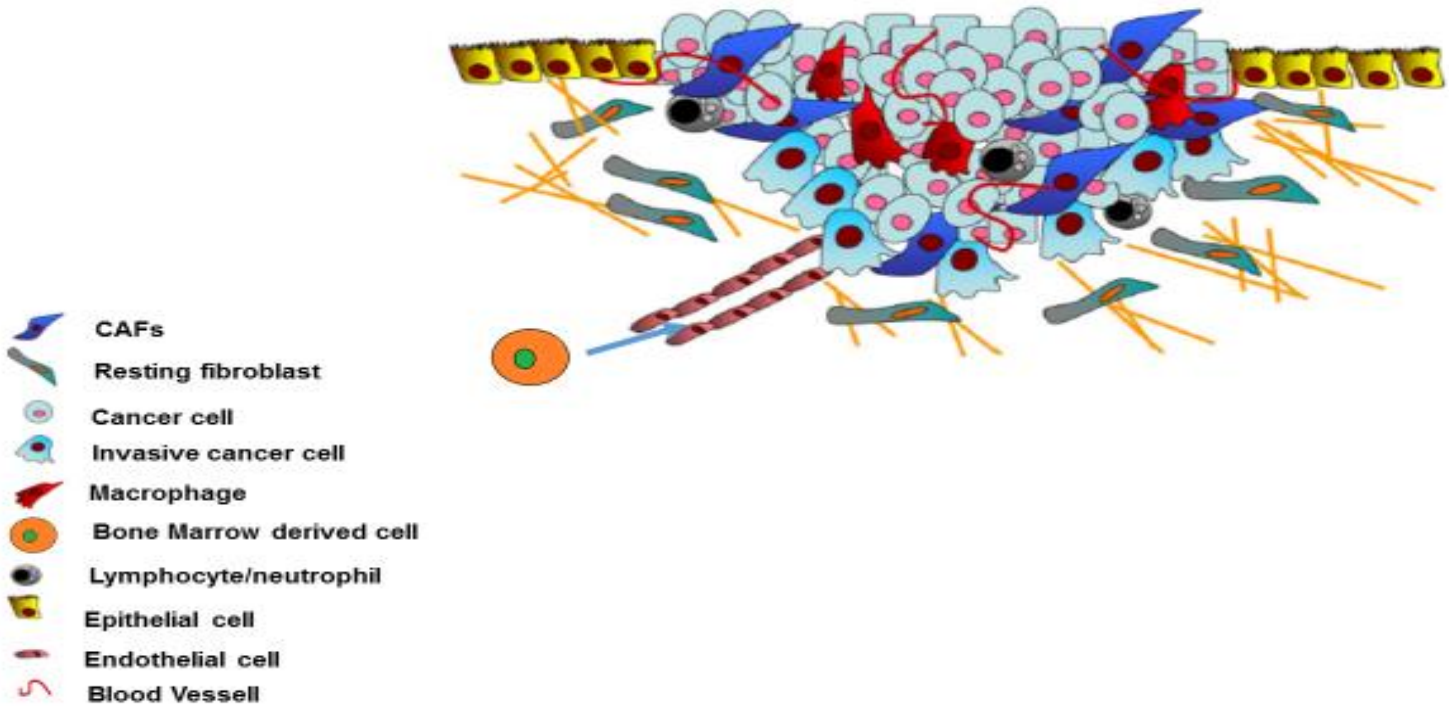


Figure 1-4 The primary tumour microenvironment

Cancer cells are surrounded by different stromal cells including bone marrow-derived cells, endothelial cells of the blood and lymphatic circulation, fibroblasts and cancer-associated fibroblasts (CAFs) and both innate and adaptive infiltrating immune cells. Modified from (Cirri and Chiarugi, 2012).

Furthermore, gene expression array data have shown that the activation of tumour associated stroma begins relatively early in the process of tumour development, even when the tumour epithelium is pre-invasive (Ma et al., 2009). In fact, automated analysis of breast cancer haematoxylin and eosin (H&E) slides has revealed that the stromal compartment of breast tumours contains more prognostic information than the epithelial component (Beck et al., 2011). Study of the tumour to stroma ratio indicate that expansion of tumour stroma is an important phenomenon influencing the prognostic outcome of solid organ tumours including breast cancer, with worse prognosis reported in patients with tumours that contained more than 50 % stroma (Mesker et al., 2007; de Kruijf et al., 2011).

It was shown that fibroblasts from tumour compartment, compared to fibroblasts derived from areas that were not intimately associated with invasive breast carcinoma, significantly increased the growth of epithelium and provided better support for cancer growth (Orimo et al., 2005). In particular, cancer-associated fibroblasts (CAFs) play an important role in all phases of tumour progression, supporting the influence of tumour microenvironment on the tumour's invasive behaviour (Chang et al., 2005; Cirri and Chiarugi, 2012). Previous studies have reported that the proliferative activity of tumour-stromal fibroblasts plays a very important role in the loco-regional and distant organ metastasis of breast cancer (Hasebe et al., 2000; Hasebe et al., 2001).

CAFs are the most abundant stromal cells of many tumours, including breast carcinomas (Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010). CAFs are the main producer of ECM proteins (i.e. collagens, fibronectin) and are the major player of ECM remodelling as they produce proteases and other enzymes involved in the post-transcriptional modification of ECM proteins themselves (Cirri and Chiarugi, 2012). Collagen cross linking of ECM is predominantly catalyzed by lysyl oxidase (LOX), expressed in fibroblasts during the early stages of breast carcinogenesis, whilst in a later stage LOX is also induced in hypoxic

carcinoma cells, promoting aggressive growth (Santhanam et al., 2010). Hence, ECM remodelling promoted by LOX activity positively affects tumour cell migration and invasion. Indeed, in a mouse model of breast carcinoma, treatment with LOX inhibitors led to a decrease of ECM cross-links, preventing ECM stiffening and delaying tumour progression (Levental et al., 2009).

Recently, emerging evidence supports the notion that the tumour associated stroma significantly facilitates tumour metastasis (Joyce and Pollard, 2009; Sethi and Kang 2011; Valastyan and Weinberg, 2011). In particular, CAF secrete growth factors, angiogenic factors, and inflammatory factors, whereby CAFs interact with cancer cells and collaborate with other components of the tumour stroma. These signalling molecules effectively mediate neo-angiogenesis, as well as proliferation, survival, motility and invasion of cancer cells (Kalluri and Zeisberg, 2006; Polanska et al., 2010).

Stromal cells also contribute to metastatic colonisation of circulating tumour cells in distant organs, in addition to the signalling molecules secreted by primary tumour cells e.g. vascular endothelial growth factor (VEGF) (Psaila and Lyden, 2009). Therefore, the local stromal environmental factors may support a pre-metastatic niche formation, facilitating homing and colonisation of circulating tumour cells. In particular, in breast cancer pulmonary metastases, bone marrow-derived cells that express VEGF receptor 1, endothelial cells, CAFs and platelets and signalling molecules such as fibronectin, and matrix-metalloproteinase 9 are all involved in mediating the formation of a pre-metastatic niche (Kaplan et al., 2005). In addition, it has been reported that metastatic cells bring CAFs originating from the primary tumours to the metastatic site, providing protection to circulating tumour cells from apoptosis in the bloodstream and support their growth once arriving at the metastatic site (Duda e al., 2010). Furthermore, monocytes, and tumour

associated macrophages (TAMs) also promote metastatic colonisation (Horimoto et al., 2012).

Another feature of the tumour microenvironment as potential predictive and prognostic marker is a histological phenomenon called tumour budding (the presence of individual cells and small clusters of tumour cells in the tumour stroma at the tumour invasive front) (Ueno et al, 2002; Prall et al, 2005). It has received much recent attention, particularly in the setting of colorectal cancer and has been considered as an independent adverse prognostic factor in colorectal cancer that may allow for stratification of patients into risk categories (Ueno et al, 2002; Prall et al, 2005; Prall et al., 2007; Lugli et al, 2009; Lugli et al., 2012, Kye et al., 2012; Mitrovic et al., 2012). In breast cancer however, the prognostic value of tumour budding remains unclear.

1.4.2 Local host inflammatory infiltrate

Numerous host cells of the immune system are recruited to and activated in the microenvironment of a developing tumour (Figure 1.5). The human immune system consists of the innate (natural) and the adaptive (acquired) immune systems which are both tightly linked together in a complex network of soluble factors (humoral innate system). Innate immune cells consist of granulocytes (neutrophils, basophils, and eosinophils), dendritic cells (DCs), macrophages, natural killer cells (NK cells) and mast cells. Adaptive immune cells are represented by T- and B-lymphocytes that express antigen-specific receptors and Immunoglobulin. The two major T-lymphocyte subsets are helper T-lymphocytes (CD4+) and cytotoxic-T lymphocytes (CD8+) which are required for cell-mediated immunity. The other subtypes of T-lymphocyte are regulatory T-lymphocytes (FOXP3+) and memory T-lymphocytes (CD45RO+) (Whiteside 2003; de Visser and Coussens, 2005; Medzhitov, 2007). Activation of the complement system, represented by a

complex network of more than 30 serum proteins and cell surface receptors, is a central event during innate immune defence after pathogenic tissue assault (de Visser and Coussens, 2005).

In the presence of a growing tumour, NK cells and macrophages activate each other via the reciprocal production of interferon gamma (INF-γ) and interleukin 12 (IL-12), promoting further tumour cell killing via apoptosis and reactive oxygen and nitrogen intermediates (Dunn et al. 2004; Bui and Schreiber 2007). DCs ingest tumour cell debris produced by tumour death, and then migrate to regional lymph nodes where T helper 1 (T_H1) cells activated by specific DCs stimulate maturation of CD8+ T-lymphocytes. Tumour-specific CD4+ and CD8+ T-lymphocytes infiltrate the tumour site and attack the antigen-bearing tumour cells that remain at the site. Some tumour cells can escape and continue to grow and expand in an uncontrolled manner (Dunn et al., 2004; Bui and Schreiber, 2007).

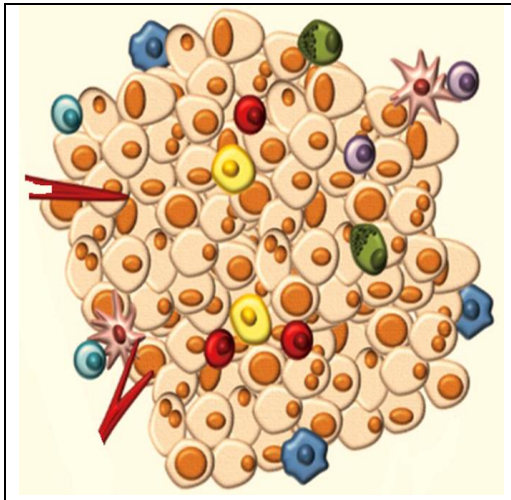
Accumulating data has demonstrated the impact of host-related parameters on cancer survival. Immune-classification of inflammatory infiltrates has a prognostic value and could identify patients at high-risk of tumour recurrence. Among the immune scoring methods are Klintrup-Mäkinen grade (Klintrup et al., 2005) and Immunoscore (Galon et al., 2012).

Klintrup–Mäkinen grade (K-M) is a simple grading scheme for estimation of the inflammatory reaction at the invasive margin using H&E pathological sections. It is a reproducible tool and reflects the general inflammatory response of the host to the tumours. It has been reported to provide prognostic information independent of cancer stage or grade, and categorises patients into low and high risk subgroups. Patients with low K-M score (low grade inflammatory reaction) have poor prognosis and form a potential target group for adjuvant therapy (Klintrup et al., 2005). High grade inflammatory reaction

predicts longer survival in patients with colorectal (Klintrup et al., 2005; Roxburgh et al, 2009) and breast cancer (Mohammed et al., 2012e).

The immunoscore is derived from the immune contexture and is based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), and detected using IHC both in the core of the tumour and in the invasive margin (Pages et al., 2009; Galon et al., 2012). It has been proposed as a clinically useful prognostic marker for cancer patients. The immunoscore provides a score ranging from Immunoscore 0 (I0) when low densities of both cell types are found in both regions, to Immunoscore 4 (I4) when high densities are found in both regions. The advantages of this test are: first, it appears to be a strong prognostic factor for cancer specific survival and overall survival; and second, it has a biological meaning as it reflects the adaptive immune response to tumours. Immunoscore as a new approach for cancer classification provides a target for novel therapeutic approaches, including immunotherapy (Galon et al., 2014).

The pronounced tumour inflammatory cell that infiltrates in and around the tumour is thought to represent the host in-situ anti-tumour immune response for many cancers including breast and has been described as a seventh “hallmark” of cancer (Colotta et al. 2009; Hanahan and Weinberg 2011). Previous reviews of literature have revealed the paradoxical roles of innate and adaptive immune cells as a causal player in breast carcinogenesis and prognostic outcome. The outcome of an immune response toward a growing breast neoplasm is largely determined by the type of immune response elicited (DeNardo and Coussens, 2007; Mohammed et al., 2012d) (Figure 1.5).



Cancer cell

Immune Cell		Role in cancer
	Myeloid Derived suppressor	Limited
		Suppress T cell functions Recruit immunosuppressive immune cells
	Natural Killer	Release cytotoxic cytokines Directly cytotoxic to cancer cells
		Limited
	Dendritic cell	Release cytotoxic cytokines Antigen presentation to T cells
		Suppress T cell functions Promote tumour growth and progression
	Macrophage	Release cytotoxic cytokines Antigen presentation to T cells
		Promote angiogenesis, tumour proliferation, Chemotaxis, invasiveness, and metastasis
	T lymphocytes (CD8+,CD4+)	Directly lyse cancer cells Release cytotoxic cytokines
		Release tumour promoting cytokines
	T regulatory	Restore homeostasis to decrease chronic inflammation
		Suppress anti-tumour immune responses Stimulate inflammatory cytokines production

Anti-tumour immune response
Pro-tumour immune response

Figure 1-5 Infiltration of immune cells into tumour.

Multiple immune cells of the tumour microenvironment, based on the context, can have variable functions to promote (gray highlight), or in some cases oppose, tumour progression. Modified from (Janet et al., 2014).

A recent review of literature has reported that generalised lymphocytic or non-specific peri-tumoural inflammatory infiltrate is associated with improved survival. In particular, there would appear to be consistent evidence that a dense CD3+ T-lymphocytic infiltrate is associated with improved survival in breast cancer. However, the prognostic value vary according to individual lymphocyte subsets (Mohammed et al, 2012d). The evidence is particularly strong for cytotoxic T-lymphocytes (CD8+) and its association with improved survival in patients with breast cancer (Baker et al., 2011; Ladoire et al., 2011; Mahmoud et al., 2011; Mohammed et al, 2012d), indicating its role in supporting anti-tumour immune response. Other subgroups from the adaptive arm of the immune system including memory-T-lymphocytes (CD45R0+) (Scholl et al., 1994; Scholl, 1996) and T-regulatory lymphocytes (FOXP3+) can increase breast cancer recurrence and negatively influence survival (Bates et al., 2006; Gobert et al., 2009; de Kruijf et al., 2010; Ladoire et al., 2011). Mohammed et al, 2012b), indicating its role in supporting pro-tumour immune responses.

Majority of studies examined the role of B-lymphocytic (CD20+) infiltrate in breast cancer survival reported its associated with improved cancer specific survival (Schmidt et al., 2008; Mahmoud et al., 2012d), indicating a role for humoral immune responses in cancer suppression.

Evidence suggests that innate immune cells, such as mast cell and macrophages, also appear to have a role in predicting prolonged survival outcome, whereas the evidence for dendritic cells is conflicting (Mohammed et al, 2012d). However, TAMs have long been known to promote cancer, partly through their ability to secrete angiogenic, metastatic and growth factors (Leek et al., 1996; Leek and Harris, 2002; Valkovic et al., 2002; Ostrand-Rosenberg, 2009).

Other cellular components such as plasma cells, natural killer cells, and eosinophils infiltrates are rare in malignant breast tumours, with limited involvement in cytotoxicity or tumour progression (O'Sullivan and Lewis, 1994). Few studies have examined the prognostic value of these cellular infiltrate in breast cancer (Hamlin, 1968; Aaltomaa et al., 1992; Iwamoto et al., 2003; Dabiri et al., 2004; Rajput et al., 2008) and most of these studies reported that high cellular infiltrate was associated with improved outcome in breast cancer.

The immune cells infiltrate produce tumour-promoting or tumour-suppressor cytokines including interleukins such as IL-6, IL-12 and IL-17, and interferons through activation of various transcription factors including signal transducer and activator of transcription (STAT) (Grivennikov et al., 2010). Signalling pathways including STAT1 and STAT3 appear to mediate stromal-tumour interactions in processes involved in tumour regulation (Grivennikov et al., 2010).

1.5 The Signal Transduction and Activation of Transcription Signalling Pathway

The signal transducer and activator of transcription (STAT) pathway transmits information received from extra-cellular polypeptides via trans-membrane receptors to the nucleus where STATs bind to specific DNA sequences and thereby regulate target genes expression and ultimately phenotypes (Darnel et al., 1994; Darnell, 1997; Bromberg and Darnell, 2000). There are seven members of STAT proteins in mammalian cells (STAT1, STAT2, STAT3, STAT4, STAT5a & b, STAT6) (Darnell, 1997). Activation of STATs in normal cells is usually temporary and critical for a variety of biological processes such as organ genesis, immune response, inflammation, regulation of cell differentiation, growth, and apoptosis (Hirano et al., 2000; Smithgall et al., 2000).

While all seven STAT-family members have been shown to be expressed in breast cancer cell lines, only STATs 1, 3, 5a, and 5b are expressed in breast cancer tissues (Clevenger, 2004). Variety of cytokines ligands, particularly interleukin-6 (IL-6) and interferons (IFNs) (Darnell, 1997) and large numbers of growth factors that exhibit tyrosine kinase activity, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) can activate STAT proteins (Ruff-Jamison et al., 1994; Zhong et al., 1994; Bromberg and Darnel, 2000; Bowman et al., 2000). Each STAT family protein responds to a defined set of cytokines, and each also regulates a group of specific genes (Table 1.6). Many cytokine receptors lack intrinsic tyrosine kinase domain and they instead rely on activation of receptor-associated tyrosine kinases such as members of the Janus kinase (JAK) family and SRC tyrosine kinases (Darnell, 1998; Ihle, 2001; Heinrich et al., 2003) or non-receptor tyrosine kinases such as SRC and ABL (Reddy et al., 2000) to initiate the STAT signalling cascade.

Table 1-6 STAT family activators and target genes

STAT	Key activators	Main target genes	Example genes
STAT1	IFNγ, IFNα and IFNβ	T_H1-type immunostimulatory, and pro-apoptosis	*TBX21, CD80, CD40, IL-12, CDKN1A* and several caspases
STAT3	IL-6, IL-10, IL-23, IL-21, IL-11, LIF and OSM	T_H17-type, anti-apoptosis, pro-proliferation, angiogenic, and metastatic	*IL-17*, *IL-23*, *BCL-XL*, *BCL-2*, *MCL-1*, *CCND1* and *VEGF*

IFN, interferon; T_H, T helper; TBX21, T-box 21; IL, interleukin; CDKN1A, cyclin-dependent kinase inhibitor 1A; LIF, leukaemia inhibitory factor; OSM, oncostatin M; *BCL*, B-cell lymphoma, MCL-1, Myeloid cell leukemia 1; CCND1, cyclin D1; VEGF, vascular endothelial growth factor. From (Yu et al., 2009).

1.5.1 STAT Structure and Activation of STAT pathway.

STAT proteins consist of various different functional domains (Figure 1.6). The amino-terminal (N-term) domain enables the interaction between two STAT dimers and facilitate tetramerisation. This interaction can stabilise the binding of the two STAT dimers to adjacent sites in DNA and enhance STAT activity on certain promoters. The coiled-coil domain mediates the interactions with regulatory proteins and other transcription factors. The DNA-binding domain is required for direct contact with STAT-binding sites in gene promoters, and to recognise the cognate binding sequences. The SRC-homology 2 (SH2) domain mediates reciprocal interactions between one STAT monomer and the phosphotyrosine residue (pY) of another to form active STAT dimer, which is essential for the binding site with DNA. The carboxy-terminal domain or transactivation domain is a mediator of the transcriptional activation of target genes. It is also having a serine residue for phosphorylation (pS) that regulate the transcriptional activity of this domain (Clevenger 2004; Yu et al., 2004).

Figure 1-6 Structural map of the STAT protein.
PY, phosphotyrosine residue and PS, phosphoserine residue.

As shown in Figure. 1.7, transmembrane receptor activation by ligands results in the activation of receptor-associated tyrosine kinases or activation of intrinsic receptor tyrosine kinase activity such as the JAKs or Src tyrosine kinases. Subsequently these tyrosine kinases phosphorylate the cytoplasmic portion of the receptor to provide docking sites for the recruitment of cytoplasmic monomeric STAT proteins via their SH2 domains. Once STATs are recruited to activated tyrosine kinases, they become themselves substrates for tyrosine phosphorylation (Darnell et al., 1994; Ihle, 2001; Reddy et al., 2000; Bromberg and Darnel 2000; Bowman et al., 2000). Although receptor-associated tyrosine kinases such as JAKs and Src kinase can cooperate in STAT activation by both growth factor and cytokine receptors, oncogenic forms of non-receptor tyrosine kinases, such as Src and Abl, can also phosphorylate STATs independently of receptor engagement (Bowman et al., 2000; Danial et al., 2000). Phosphorylation of STAT monomers enables them to dimerise via reciprocal phosphotyrosine-SH2 domain interactions (Darnell et al., 1994; Darnel yy1997) and further translocate into the nucleus, where the dimers bind to specific DNA-response elements and directly regulate gene expression (Darnell et al., 1997; Reddy et al., 2000; Bromberg and Darnell, 2000; Ihle, 2001; Buettner et al., 2002; Yu et al., 2004).

Constitutive activation of STATs is associated with permanent changes in the expression of genes that control fundamental cellular processes and lead to oncogenesis. STATs, in particular STAT3 and STAT5 are proposed to contribute to tumour development and progression through up-regulation of genes encoding apoptosis inhibition, cell cycle regulation, and induction of angiogenesis (Figure 1.7) (Buettner et al., 2002; Clevenger, 2004; Alvarez et al., 2005; Hodge et al., 2005; Yu et al., 2009). In contrast, STAT1 has been associated with the suppression of tumour growth (Bromberg and Darnel, 2000; Lynch et al., 2007).

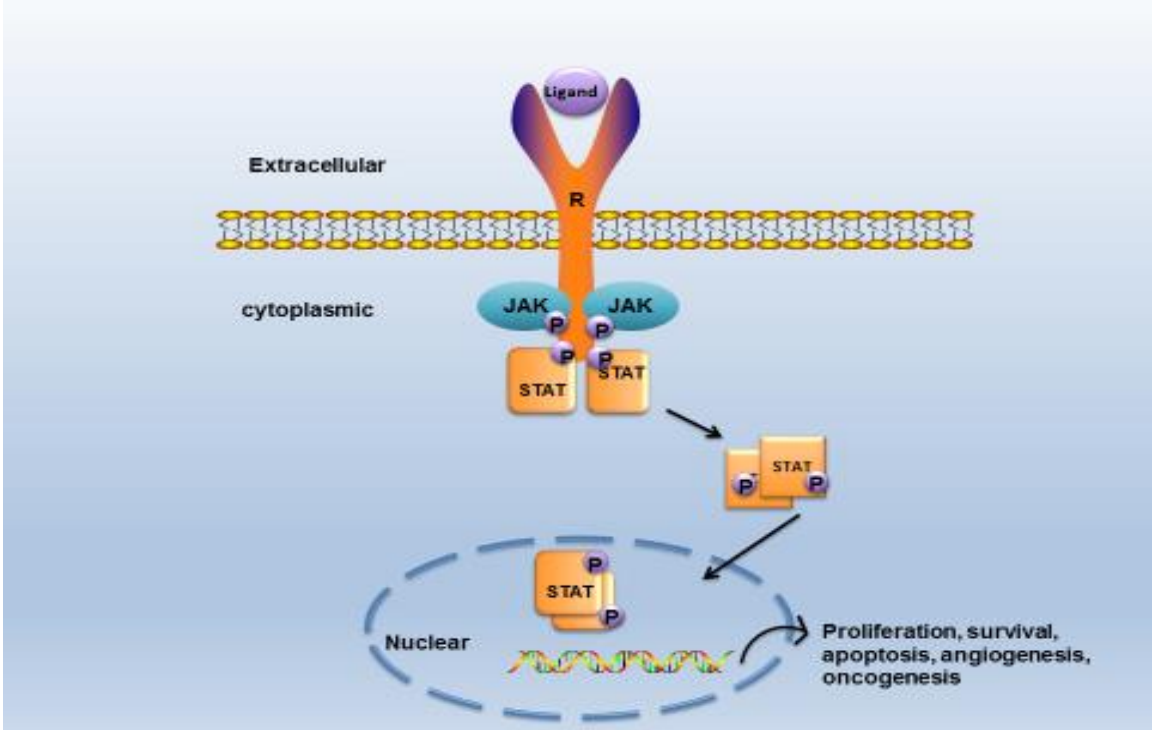


Figure 1-7 STATS signalling pathway

Binding of ligands (growth factors or cytokines) to their receptors (R) leads to activation of intrinsic receptor tyrosine-kinase activity or of receptor-associated kinases, such as the Janus kinase (JAK), in this figure, or Src tyrosine kinases. These tyrosine kinases subsequently phosphorylate the cytoplasmic tails of the receptor to provide docking sites for the recruitment of monomeric STATs. Subsequently, STATs themselves become tyrosine phosphorylated, dimerised and translocated to the nucleus, where they directly regulate gene transcription of biological processes. Among these is proliferation, survival, apoptosis, angiogenesis and oncogenesis.

In normal untransformed mammary glands, it is not surprising that STAT family members modulate mammary gland development during pregnancy, lactation and involution since these proteins play important roles in regulating cell proliferation and apoptosis (Philp et al., 1996; Watson 2001). With regard to carcinogenesis, several studies have demonstrated that elevated activities of STATs proteins are frequently found in variable human tumour cell lines (Li and Shaw, 2000; Kazansky and Rosen, 2001; Garcia et al., 2001; Buettner et al., 2002; Alvarez et al., 2005) and in a wide variety of human tumours, including breast cancers (Widschwendter et al., 2002; Dolled-Filhart et al., 2003; Gritsko et al., 2006; Yamashita et al., 2006).

1.5.2 Role of STAT1 in breast cancer and stromal immune responses.

STAT1 was the first STAT protein to be discovered and is mainly activated in response to Type I and II IFNs, and upon activation of their receptors, STAT1 mediates the transcription of genes encoding proteins with anti-proliferative, anti-viral and immune regulatory properties. The activity of STAT1 is controlled by phosphorylation at pY 701 and pS 727 within the transactivation domain of the protein (Darnell et al., 1997; Buettner et al., 2002; Yu et al., 2004; Schindlr et al., 2007; Stark and Darnell, 2012).

Several lines of evidence implicate STAT1 as an anti-proliferative and a pro-apoptotic molecule. Early studies, using mouse models, demonstrated that STAT1-deleted mice were more susceptible to tumour development than controls (Kaplan et al., 1998, Shankaran et al., 2001). Activation of STAT1, in response to IFN-γ, has been shown to be associated with inhibition of proliferation of both mouse and human tumour cells via up-regulation of cyclin-dependent kinase inhibitor p21 expression and down-regulation of c-Myc promoter expression (Ramana et al., 2000). In addition, STAT1 induces apoptosis by up-regulation of caspases 2 and 3 expression or the expression of inducible nitric oxide

synthase (Battle and Frank, 2002; Kim and Lee, 2007). The suppressing role of STAT1 in cancer is further supported by its ability to inhibit angiogenesis and tumour metastasis in mouse models (Huang et al., 2002).

STAT1 as a key transcriptional factor of IFN signalling plays an important role in innate immune response by protecting the host from virus infections and other pathogens (Schindlr et al., 2007; Stark and Darnell, 2012). IFNs signal through STAT1 do not only increase anti-tumour immune responses through the activation of natural killer cells and macrophages but also through activation of adaptive immune mediators; T_H1 and cytotoxic CD8+T-lymphocytes (Yu et al., 2009).

Targeted disruption of STAT1 results in viable mice with compromised innate immunity and are highly susceptible to infection (Durbin et al., 1996; Meraz et al., 1996). In addition, lack of STAT1 significantly increases the incidence of spontaneous mammary tumour development in mice (Schneckenleithne et al., 2011). The increased rate of tumour formation in STAT1 deficient mice (STAT1−/−) was previously attributed to impaired immune surveillance of tumours because these mice failed to respond to IFN-γ and displayed reduced natural killer activity (Dunn et al., 2006). In turn, lack of STAT1 could potentially promote tumour cell survival due to the loss of IFN-dependent tumour surveillance system (Buettner et al., 2002).

Recent evidence for the role of STAT1 in the tumour microenvironment has further supported the immune surveillance role in breast cancer. Using immunohistochemistry in a large cohort of patients, selective down-regulation of STAT1 protein was more prominent in the tumour cells compared with the surrounding stroma and infiltrating lymphocytes (Chan et al., 2012). In addition, STAT1 sustained efficient cytotoxic T-lymphocyte mediated immune response and was essential for full functioning CD8+

cytotoxicity (Schneckenleithne et al., 2011). Thus, the ability of STAT1 to control the function of immune cells may play a crucial role in regulation of tumourigenesis.

In normal circumstances, STAT1 is regulated during the different stages of mammary gland development and its expression and activity is detected only in virgin animals, or during early pregnancy and late involution (Watson, 2001), but not through pregnancy and lactation (Philp et al., 1996).

Recent studies, using different experimental approaches, support the anti-tumour function of STAT1 in mammary tumourigenesis (Klover et al., 2010; Raven et al., 2011; Chan et al., 2012). Tumour initiated by Her-2 in STAT1−/− female mice developed breast tumours ~6 weeks earlier than their STAT1+/+ counterparts (Ravan et al., 2011) and the overall disease latency was significantly enhanced in STAT1-deficient mice being 49.4 weeks compared with 62.4 weeks in STAT1-proficient animals (Klover et al., 2010). Non-tumourigenic and tumourigenic mammary epithelial STAT1−/− cells in vivo were capable of increasing proliferation rate and were found to form significantly thicker mammosphere layers in the mammary acini (Schneckenleithne et al., 2011). In addition, Chan and colleagues demonstrated that STAT1 deficient mice spontaneously developed mammary adenocarcinomas displaying ER +ve / PR +v tumour cells similar to that of human luminal breast cancer, and that STAT1 expression was frequently lost during breast cancer progression. Induction of wildtype STAT1 into STAT1 deficient mammary tumour cells lead to apoptosis (Chan et al., 2012), indicating a cell autonomous or a tumour cell-specific function of STAT1 independent of STAT1-mediated transcription. Furthermore, the anti-tumour activity of the milk protein α-casein, in preventing breast cancer growth and metastases has been found to require activation of STAT1 (Bonuccelli et al., 2012).

However, STAT1 has been also implicated in cancer development including mammary tumours. This is based on the observation that total and phosphorylated STAT1 (ph-STAT1) is elevated in human breast cancer (Widschwendter et al., 2002; Sheen-Chen et al., 2007; Yau et al., 2010; Magkou et al., 2012) and is associated with variable prognostic outcomes. STAT1 DNA binding activity and Y705 phosphorylation in invasive breast carcinomas was associated with improved survival independent of other prognostic variables (Widschwendter et al., 2002). Increased STAT1 mRNA levels were shown to be part of a molecular signature associated with better prediction of the metastatic outcome for patients with hormone receptor negative and triple negative breast cancers (Yau et al., 2010). In contrast, assessment of STAT1 levels by Charpin and colleagues in larger cohort, reported an association between STAT1 expression and worse survival (Charpin et al., 2009). Also, ph-STAT1 was associated with advanced stage and worse survival in premenopausal women (Magkou et al., 2012). However, one report, using total STAT1 protein, found no association between level of total STAT1 and breast cancer survival (Sheen-Chen et al., 2007).

Overall, it is clear that a conclusion about the prognostic value of STAT1 in breast cancer cannot be made due to rarity and inconsistency of evidence available.

1.5.3 Role of STAT3 in breast cancer and stromal immune responses

STAT3 is a major member of STAT family and one of the most established intracellular signalling molecules. It represents as a central effector of the local inflammatory response in cells and was first named acute phase response factor as it was reported to regulate the expression of many genes involved in the acute phase response to tissue injury and infection (Pensa et al., 2009). Since then, STAT3 has been found to be activated by tyrosine phosphorylation (pY705) in response to IL-6 and other inflammatory cytokines (Berishaj et al., 2007) (Figure 1.7). The activity of STAT3 is also controlled by phosphorylation at serine residue (pS727) within the transactivation domain of the protein by members of the mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinase families (Decker, 2000).

Several line of evidence from different human cell types and murine implement STAT3 as an oncogene because it up-regulate target gens involved in proliferation (cyclin D1, c-Myc, c-Fos and MEK5), angiogenesis (VEGF MMP-2, MMP-10, MMP-1 and KIF-8) and apoptosis (BCL-2, BCL-XL, MCL-1, and survivin), reflecting the role of STAT3 in cell cycle, cell invasion, angiogenesis and cell survival (Niu et al., 2001;Buettner et al., 2002; Yu et al., 2004; Alvarez et al., 2005; Hsieh et al., 2005; Germain and Frank, 2007).

Early studies, in model systems, have shown a direct role of STAT3 in oncogenesis using a constitutively active STAT3 mutant, which transforms fibroblasts in culture and allows the transformed cells to form tumours (Bromberg et al., 1999). STAT3 inhibition is associated with anti-tumour effect and suppression of proliferation of tumour cells with activated STAT3 (Rivat et al., 2004; Gao et al., 2005; Xi et al., 2005), supporting direct contribution of STAT3 in tumours pathogenesis, rather than serving only as a marker of tumorigenesis. Inhibiting STAT3 in tumours can also induce apoptosis in addition to cell cycle arrest (Niu

et al., 1999; Niu et al., 2001). In contrast, activated STAT3 in human multiple myeloma cell supported survival of tumour cells and prevented apoptosis by upregulating expression of the anti-apoptotic protein BCL-XL (Catlett-Falcone et al., 1999).

The role of STAT3 in cancer progression is further supported by its regulatory role on cell migration. STAT3 can affect the migration of tumour cells not only via its transcriptional regulation of genes involved in cell migration but also through its transcription-independent interaction with focal adhesion molecules. Phosphorylated STAT3 was found to localise to the migrating protrusions and focal adhesions in migrating cells (Silver et al., 2004; Jia et al., 2005). STAT3 also can inhibit the tubulin binding protein Stathmin which promotes microtubule depolymerisation (Ng et al., 2006).

Based on its role in regulating IL6-JAK/STAT3-dependent inflammation and immunity, studies have identified STAT3 as an important molecule in regulating immune responses in the tumour microenvironment (Catlett-Falcone et al., 1999; Yu et al., 2007; Grivennikov et al., 2009; Mantovani et al., 2008; Kortylewski et al., 2009; Wang et al., 2009; Yu et al., 2009). STAT3 is implicated in mediating pro-tumour immune responses and inducing pathways underlying cancer inflammation (Heinrich et al., 2003; Wang et al., 2009; Yu et al., 2009). It is also an important activator of many genes that are crucial for immunosuppression (Bronte-Tinkew et al., 2009; Ernst et al., 2008). STAT3 on the one hand promotes pro-oncogenic inflammatory pathways, including nuclear factor-κB (NF-κB) and IL-6-JAK pathways, while on the other hand opposes STAT1- and NF-κB-mediated T_H1 anti-tumour immune responses (Yu et al., 2007; Yu et al., 2009).

In innate immune cells, STAT3 signalling is required for the immunosuppressive and tumour-promoting effects of myeloid-derived suppressor cells and tumour associated macrophages (Cheng et al., 2003; Kortylewski et al., 2005; Kortylewski et al., 2009).

STAT3 also mediates T regulatory cell expansion, an important negative regulator of T_H1-type CD4+ T-lymphocytes and CD8+ T-lymphocytes (Kortylewski et al., 2005; Matsumura et al., 2007; Kortylewski et al., 2009) in tumours and is central for the development of T_H17 T-lymphocytes (Chen et al., 2006) which can promote tumour growth (Wang et al., 2009; Yu et al., 2009). Thus, STAT3 plays a critical role in determining the nature of cancer-associated inflammation.

STAT3 plays an important role in the normal development of the mammary gland. STAT3 mRNA levels are high in the mammary epithelium of adult virgins and remain elevated till early pregnancy (Philp et al., 1996, Watson, 2001). It is necessary for the cell death that occurs during the involution and remodelling process after cessation of lactation (Pensa et al., 2009). At weaning, an apoptotic program mediated by STAT3 is initiated to clear the mammary gland of its excess cellular burden. During the first phase of involution, levels of ph-STAT3 are elevated in the mammary gland, and this rapid activation of STAT3 is essential for involution to proceed (Chapman et al., 1999; Humphreys et al., 2002). During the second phase of involution, STAT3 induces an immune response and modulates macrophages and mast cells into an alternate state required for to clear epithelial cells (Hughes et al., 2012; Kreuzaler et al., 2011).

However, STAT3 activation can also promote breast cancer formation and progression (Burke et al., 2001; Garcia et al., 2001). Constitutive activation of STAT3 is required for enhancing transformation or blocking apoptosis in breast cancer cell lines and tissues (Bromberg and Darnell, 2000; Garcia et al., 1997; Page et al., 2000; Garcia et al., 2001). In mice model, constitutive activation of STAT3C allele could enhance the rat *Neu* oncogene tumourigenic activity and trigger development of earlier onset tumour with more aggressive features and metastatic potential than wild-type mice (Barbieri et al., 2010). STAT3 has also been shown to promote epithelial-to-mesenchymal transition and cell

invasion in breast cancer tissue (Sullivan et al., 2009). In addition, inhibition of STAT3 with various pharmacological agents suppresses tumour growth, recurrence and invasion in breast cancer cell lines as well as in a human-xenograft model (Yang et al., 2012; Zhang et al., 2012).

In addition, STAT3 activation is frequently observed in breast tissue and more than 60% of primary tumours display constitutive pY705 STAT3 (Alvarez et al., 2005). Previous studies of ph-STAT3 in invasive breast cancers showed positive correlations between increased levels of ph-STAT3 expression and metastasis to regional lymph nodes (Hsieh et al 2005), Her-2 positivity, surviving, and incomplete response to neoadjuvant chemotherapy (Diaz et al., 2006); all are poor prognostic features. STAT3 is also serine phosphorylated in about 60% of breast tumours and is associated with ER-ve tumours, increased stage and increased tumour size (Yeh et al., 2006).

However, very little is known about the prognostic value of STAT3 in breast cancer, and this value remains controversial. Tissue microarray studies have reported significant relationship between ph-STAT3 and improved survival in 346 node negative breast cancers (Dolled-Filhart et al., 2003), 721 patients with low grade tumours (Sato et al., 2011), and 125 node positive breast cancers (Sonnenblick et al., 2012). In contrast, automated aassessment of ph-STAT3 levels in more than 900 specimens, found negative association between ph-STAT3 and survival (Charpin et al., 2009) consistent with findings of Sheen-Chen and colleagues (Sheen-Chen et al., 2008), whereas a study of 571 breast cancers documented no association between STAT3 expression and survival (Yamashita et al., 2006).

It is clear that conclusion about the prognostic value of STAT3 as well as STAT1 in breast cancer cannot be made due to rarity and inconsistency of evidence available. The

mechanisms behind the relationship between STAT1 and STAT3 and cancer survival have yet to be clarified.

1.6 Hypothesis and aims

Breast cancer is the most frequent female cancer worldwide, and the second leading cause of cancer deaths in women. Although breast cancer survival rates are better than many other major cancers in women, it is still relatively poor in certain subtypes, such as triple negative disease.

It would appear, in patients with breast cancer, that the information of the relationship between the tumour-based factors and the host-based factors and the relationship with disease outcome is limited. The larger impact of the tumour microenvironment on tumour growth and progression is increasingly evident. Despite this, the determination of optimal treatment of cancer is now solely based on characteristics of the tumour cells itself (size, grade, involved lymph node, hormone receptor and Her-2 status).

The importance of the stromal vasculature and the prognostic value of lymphovascular invasion have been established as a risk factor of relapse and death for high risk patients with node negative breast cancer but yet there is no standardised method for the detection or discrimination between lymphatic and blood vessel invasion.

It would appear that by ignoring the stromal compartment, valuable information about breast cancer progression and metastasis is lost. In fact, it has already been well documented in various studies that carcinoma cells and the surrounding stromal cells co-evolve with each other during the course of tumour progression. Signalling molecular pathways, like STATs pathways, seem to mediate the interaction between phenotypic features of the tumour and the host, presumably influencing or supressing breast cancer progression.

Therefore, the aims of this thesis were to examine:

1. The role of lymphatic and blood vessel invasion in predicting survival and methods of detection in patients with primary operable breast cancer.

2. The relationship between the tumour stroma percentage, clinicopathological characteristics and outcome in patients with invasive ductal breast cancer.

3. The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer.

4. The relationship between total and phosphorylated STAT1 and total and phosphorylated STAT3 tumour cell expression, components of the tumour microenvironment and survival in patients with invasive ductal breast cancer.

Chapter 2 Patients and Methods

2.1 Patient cohorts

Patients presenting with invasive breast cancer at Glasgow Royal Infirmary, Western Infirmary, Victoria and Stobhill Hospitals, all located in the West of Scotland, between 1995 and 1998 were included in the thesis studies (total n=621). The clinicopathological data available on the database were patients' age, histological tumour type, involved lymph node status, tumour size, tumour grade, lymphovascular invasion, type of surgery and use of adjuvant treatment. This pathological information was retrieved from the pathology routine reports. Other data comprising ER status, PR status, Her-2 status, Ki67 proliferative index, CD68+ macrophage infiltrate, CD4+ T-lymphocyte infiltrate, CD8+ T-lymphocyte infiltrate and CD138+ plasma cell infiltrate was also included and was performed as previously described (Mohammed et al., 2012a; Mohammed et al., 2012b; Mohammed et al., 2012c; Mohammed et al., 2013). General peri-tumoural inflammatory infiltrate and tumour necrosis were also included and were performed as previously described (Ikpatt et al., 2002; Klintrup et al., 2005; Mohammed et al., 2012e) (n=474). In addition, general peri-tumoural inflammatory infiltrate and tumour necrosis were scored according to Klintrup and Ikpatt criteria (n=147) by the author (FG).

The surgical treatment for patients of the present thesis cohort was either breast conservation followed by radiotherapy, or mastectomy followed by radiotherapy, according to the individual patient's indication. The percentage of patients who received either of the surgical options is given in the results section of every chapter. Following surgery, patients received either hormone therapy, hormone therapy and/or chemotherapy, or did not receive any adjuvant treatment. The present thesis cohort was recruited between 1995 and 1998, and the only hormone therapy received was tamoxifen. None of the

patients have received aromatase inhibitors or trastuzumab, as these treatment options were established after 1998.

After surgery, patients were routinely followed-up (3 months, 6 months and then annually for up to five years) with clinical examination and mammography surveillance. Ultrasound, MRI and CT scanning are provided for patients with suspicion of recurrence.

Information on date and cause of death was checked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st of May 2013 and that served as the censor date. Cancer specific survival was measured from the date of primary surgery until the date of death from breast cancer.

Exclusion criteria: Patients were excluded from the study if; clinical follow-up data was incomplete, tissue blocks were not available or had insufficient tumour tissue as determined by the pathologist. To maximise group homogeneity and to limit the potential confounding effects of other tumour types on the analysis in the studies, ductal breast cancers only were included.

Institutional Review Board approval for use of human tissue in this study was given by the Research Ethics Committee of the West Glasgow University Hospitals NHS Trust.

2.2 Methods

2.2.1 Use of full-section haematoxylin and eosin slides.

Full-section H&E slides stained according to standard protocol were retrieved and used to assess general peri-tumoural inflammatory infiltrate and tumour necrosis on a high definition computer monitor. H&E sections were also used to assess and review the presence of lymphovascular invasion (as described on chapter 4.0), tumour stroma percentage (as described on chapter 5.0) and tumour budding (as described on chapter 6.0).

2.2.1.1 Assessment of general tumour inflammatory infiltrate:

Briefly, tumours were scored on a 4 point scores based on appearances at the edge of tumour invasion. A score of 0, signified there was no inflammatory cells at the tumour's invasive margin; score 1, indicated a mild and patchy inflammatory cells; score 2, denoted a prominent band-like inflammatory reaction at the invasive margin and score 3, revealed a florid cup-like inflammatory infiltrate at the invasive edge with frequent destruction of cancer cell islands (Figure 2.1).

For reproducibility, a total of 60 tumour specimens were scored independently for peri-tumoural general inflammatory infiltrate by two observers (FG. and ZM) and the interobserver intraclass correlation coefficient (ICCC) was 0.96. FG then scored all sections for analysis. These scores were then subsequently classified as low (score 0 and 1) and high (score 2 and 3).

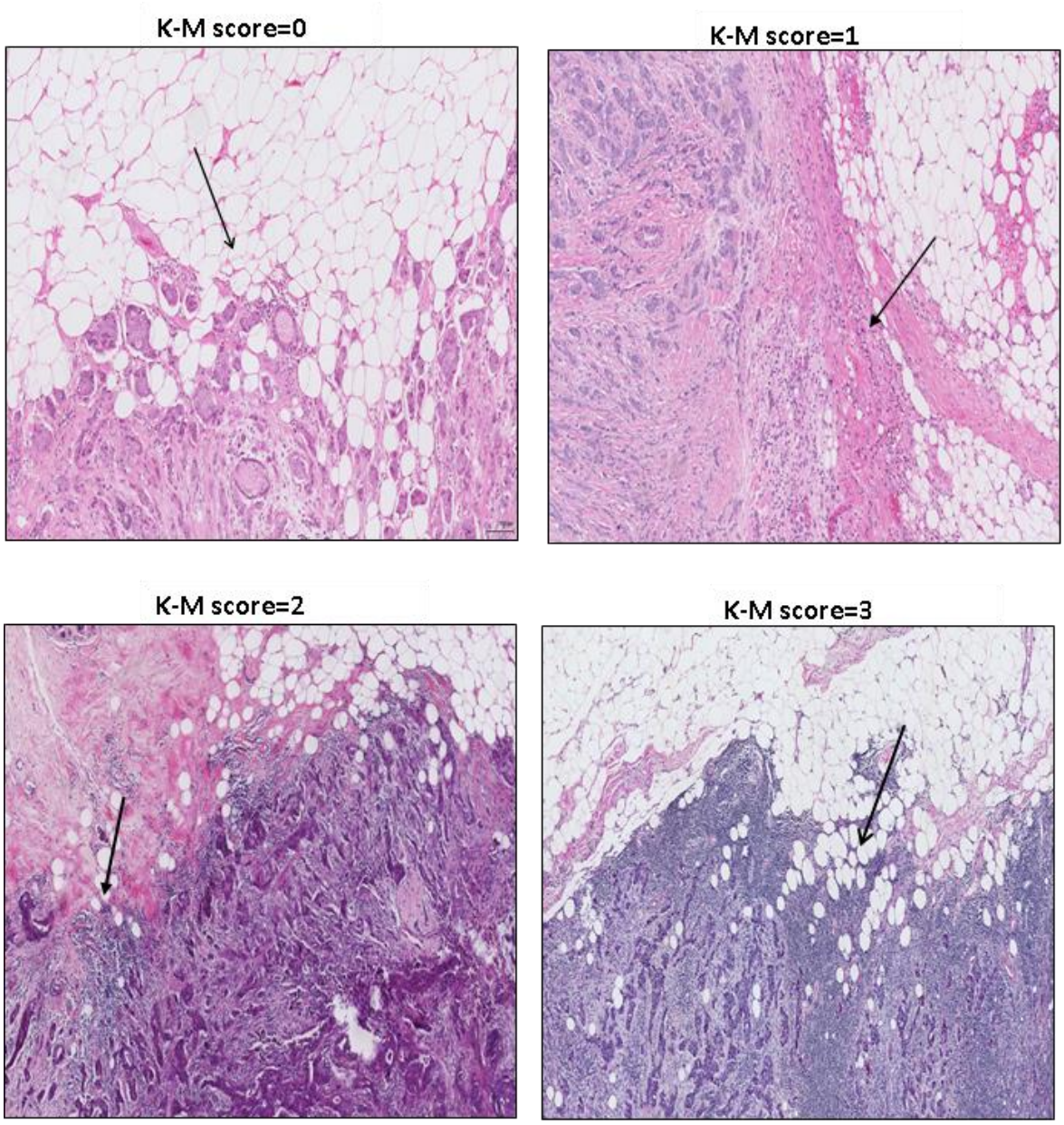


Figure 2-1 H&E sections shows scoring of general peri-tumoural inflammatory infiltrate at the invasive margin according to Klintrup–Mäkinen (K-M score).

K-M=0 no inflammatory cells, K-M=1 mild inflammatory cells, K-M=2 band-like inflammatory infiltrate, K-M=3 cup-like inflammatory infiltrate. 10x objective and 100µm scale.

2.2.1.2 Assessment of tumour necrosis

Assessment of tumour necrosis was performed on H&E scanned sections and was adapted from Ikpatt and colleagues (Ikpatt et al., 2002). The sections were examined at low power (×10) for evidence of tumour necrosis. The extent of necrosis was assessed semiquantitatively at high power (×40 magnification) as 0 = absent (only single-cell death identifiable); 1 = mild or focal (necrosis in <25% of field); 2 = moderate (necrosis in 25-50% of field) and 3 = extensive (confluent necrosis in >50% of field) (Figure 2.2).

A total of 65 tumour specimens were scored independently for tumour necrosis by two observers (FG and JJG) blind to patient outcome and the other observer's score. ICCC was 0.87. FG then scored the rest of slides for analysis. Subsequently, these scores were then classified into two grades as low (0 and 1) and high (2 and 3).

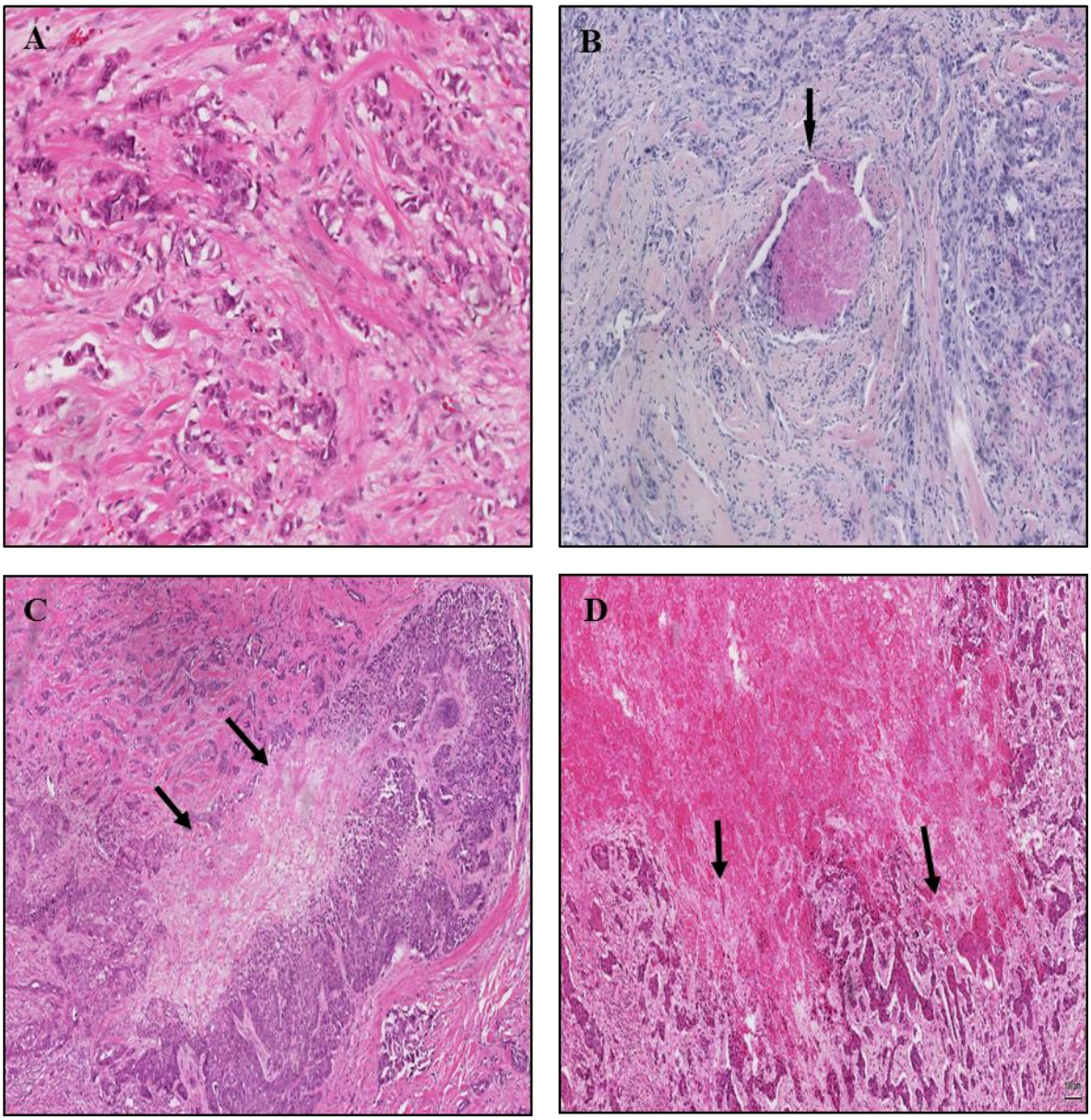


Figure 2-2 Examples of the tumour necrosis grades

A: absent (none), B: focal (less than 25%), C: moderate (25%–50%) and D: extensive (more than 50%). Original magnification X20 and 100μm scale.

2.2.2 Use of immunohistochemistry and tissue microarray

Formalin-fixed paraffin-embedded tissue blocks of the primary tumours were retrieved from pathology archives of Glasgow Royal Infirmary and Stophill hospital and utilised for immunohistochemistry (IHC) staining. Two consecutive full sections of 2.5 µm thick from each block were cut for staining with lymphatic endothelial marker D2-40 and Factor VIII as described in chapter 4.0.

Formalin-fixed paraffin-embedded tissue blocks of the primary tumours were also used to construct tissue microarrays (TMAs) utilised for IHC staining of total STAT1, ph-STAT1, total STAT3 and ph-STAT3 as described in chapter 7.0.

TMA construction

For TMA construction, tumour-rich areas on full H&E stained sections were identified and marked by a qualified pathologist (JJG), and were matched to formalin-fixed paraffin-embedded blocks. The procedure for construction of TMAs was as previously described (Kononen et al. 1998; Tovey et al. 2006). Three different 0.6mm^2 cores from each carcinoma were punched from a formalin-fixed paraffin-embedded whole section and distributed in three pre-prepared holes in three new recipient paraffin array blocks (Beecher Instruments, Silver Spring, Maryland, USA) (175 to 188 cores per block). These new blocks can contain a multitude of histospots with known coordinates to allow easy linkage to clinicopathological data, and can be sectioned up to numerous times for multiple analyses. Cores of prostate, colon, lung, Liver, Heart, kidney were also included in the tissue microarray as controls.

ER and PR status were assessed on TMA using IHC with Dako (Glostrup, Denmark) ER antibody and Leica (Wetzlar, Germany) PR antibody as previously described (Mohammed

et al, 2012a), and scored according to the American Society of Clinical Oncology and College of American Pathologists guidelines with cut-off value of 1% positive tumour nuclei (Hammond et al., 2010). Her-2 status were assessed on TMA using IHC with Hercep test Her-2 antibody (monoclonal rabbit anti-human, DAKO, Glostrup, Denmark) as previously described (Mohammed et al, 2012b), and scored as the following: 3+ score was regarded as positive; 2+ score was regarded as equivocal, leading to referral for Her-2 FISH; and 0 and 1+ scores were regarded as negative. Ki67 proliferation index was assessed on TMA using IHC with DAKO anti-Ki67 (Monoclonal mouse anti-human, Ki67 antigen, DAKO, Glostrup, Denmark) (Mohammed et al, 2012c).

IHC staining on TMA sections were also previously used for the assessment of macrophages, helper and cytotoxic T- lymphocytes, and plasma cells using CD68, CD4, CD8 and CD138 antibodies respectively (Mohammed et al, 2013).

2.2.3 Slide scanning and scoring

The H&E tumour sections and IHC stained slides were scanned using Hamamatsu NanoZoomer Digital Pathology 2.0-HT scanner (Welwyn Garden City, Hertfordshire, UK) at objective magnification x20. Visualization and image analysis assessment was carried out using SlidePath Digital Image Hub, version 3.0 and 4.0.1, (SlidePath, Leica Biosystems, Milton Keynes, UK) which is a secure, web-enabled digital slide management system.

Chapter 3 The role of lymphatic and blood vessel invasion in predicting survival and methods of detection in patients with invasive ductal breast cancer

3.1 Introduction

The process by which breast cancer kills patients is primarily through progression, in particular metastatic disease. Approximately 10% of newly diagnosed breast cancer patients have locally advanced and/or metastatic disease at the time of presentation (Li et al., 2003b; Sant, 2001). More than 40% of node negative carcinoma patients will eventually experience later recurrence and/or metastasis (EBCTCG, 1998).

Metastatic breast carcinoma exhibits a great deal of variability in its clinical presentation and behaviour. The prognosis is generally poor with a median overall survival of approximately 2 to 3 years (Ali et al., 2003; Bernard-Marty et al., 2004). In some patients, depending on the site of metastasis and treatment given, survival may range from a few months to several years (Insa et al., 1999; Dufresne et al., 2008). However, over period of time these metastatic cells residing in distant organs often relapse, corrupt the local microenvironment and acquire the ability to develop into macro-metastases (Horimoto et al., 2012).

One of the very early steps of metastatic spread is penetration of tumour cells into lymphatic and/or blood vessels in and around the primary tumour. The prognostic significance of lymphovascular invasion (LBVI) has been described more than four decades ago (Teel, 1964). Since then, several independent studies have investigated the prognostic significance of LBVI in breast cancer in both lymph node negative and positive tumours.

In 1999, the College of American Pathologists (CAP) consensus accepted peri-tumoural LBVI as prognostic factor of local failure and reduced overall survival in breast cancer,

and recommended that vascular invasion should be assessed in peri-tumoural breast tissue. However, not all commentators agreed on its clinical importance (Fitzgibbons et al., 1999). At the 9th St Gallen meeting in January 2005, LBVI accepted as sufficiently reliable to define risk category of relapse and death form the disease in patients with node negative breast cancer. The consensus from the meeting was that the presence of LBVI defined intermediate risk and its absence defined low risk for node negative disease. The importance of LBVI in patients with node positive cancers was considered uncertain, and more studies were still required (Goldhirsch., 2005). In addition, extensive peri-tumoural LBVI was categorised, at the 11th St Gallen meeting (2009), to stratify patients with early breast cancer for chemotherapy induction (Goldhirsch., 2009).

Based on the CAP consensus (1999) and both St Gallen meetings, there was no agreement on the need for specific stains to identify vascular spaces or the necessity to distinguish LVI from BVI (Fitzgibbons et al., 1999; Goldhirsch., 2005; Goldhirsch., 2009). However, one of the major challenges in the field has been to distinguish LVI and BVI on H&E stained sections from retraction artifacts caused by tissue handling and fixation (Saigo and Rosen, 1987; Bettelheim et al., 1984; Van den Eynden., 2006; Hoda et al., 2006).

Therefore, the aim of the present systematic review was to examine the prognostic significance of LVI and BVI separately and together (LBVI), and how they are detected.

3.2 Materials and Methods

The review of published literature was undertaken according to a pre-defined protocol. The primary area of interest was the relationship between the lymphovascular invasion (either general, lymphatic vessel invasion or blood vessel invasion) and outcome (cancer specific, relapse free and overall survival) in patients with primary operable breast cancer. A literature search, using appropriate key words (breast cancer, lymphovascular / lymphatic / blood vessel invasion and survival) was made of the US National Library of Medicine (MEDLINE), the Excerpta Medica database (EMBASE), the Cochrane Database of Systematic Reviews (CDSR) and the Database of Abstracts and Reviews (DARE) for articles reporting the prognostic value of lymphovascular invasion (May 1964 to August 2012).

From this search, the titles and abstracts were examined and if relevant, the full text papers were obtained. Studies in which sample size was ≤ 100 patients, median/mean follow-up was not reported or less than 5 years, and studies not available in English language were excluded. Where there were duplicate publications of the same patient dataset from same centre were only the most recent study was considered. The bibliographies of all included articles were subsequently hand searched to identify additional studies. It was taken that vascular invasion was ascertained in H&E sections if no other detection method was specified. All papers included in the review were examined by FG. For each group of studies, a weighted average for the invasion rate using H&E, H&E and/or classical staining and immunostaining was calculated by multiplying the invasion rate reported in the study by the number of patients in the study. The product of this multiplication was added to the products of other studies in the group and the total was divided by the total number of all the patients in the group studies.

3.3 Results

3.3.1 Study selection process

The study selection process is summarised in Figure 3.1. The initial literature search returned 227 articles of potential interest. After title and abstract review, full text was obtained for 129 studies. Hand searching bibliographies identified 25 additional studies. Of these, 95 were excluded (32 did not examine the prognostic value of lymphovascular invasion, 7 were review articles, 24 had sample size ≤ 100 patients, 13 had follow-up less than 5 years, 8 were not available in English and 11 were multiple publications). A total of 59 independent studies (62,514 patients) were included in the present review (Figure 3.1).

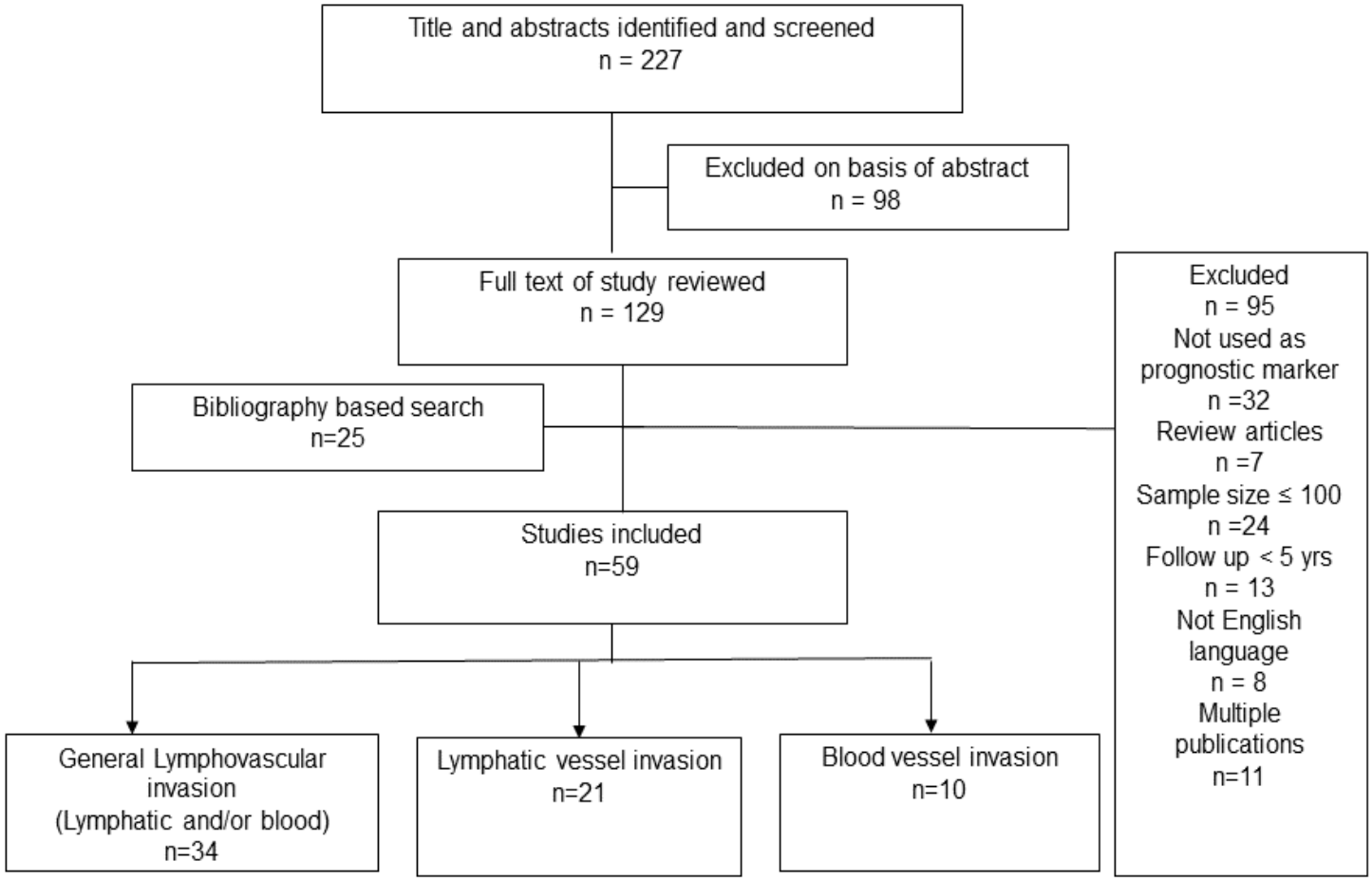


Figure 3-1 Flow chart depicting the study selection process

3.3.2 The prognostic value of general lymphovascular invasion (LBVI) in primary operable breast cancer

There were 32 published studies (Table 3.1), comprising data on 43,311 patients, that have reported the presence of LBVI was associated with an unfavorable outcome, primarily relapse free and overall survival, in primary breast cancer (Bettelheim et al., 1984; Kolliase et al., 1990; Lee et al., 1990; Pinder et al., 1994; Magee et al., 1996; de Mascarel et al., 1998; van Tienhoven et al., 1999; McCready et al., 2000; Jmor et al., 2002; Millis et al., 2002; Woo et al., 2002; Kuru et al., 2003; Neri et al., 2005; Shen et al., 2005; Trudeau et al., 2005; Truong et al., 2005; Dinshaw et al., 2006; Lee et al., 2006a; Beinart et al., 2007; Mohammed et al., 2007; Grasic-Kuhar et al., 2008; Ejlertsen et al., 2009; Vial et al., 2009; Ragage et al., 2010; Thike et al., 2010; Viale et al., 2010; Panet-Raymond et al., 2011; Ovcaricek et al., 2011; Sabatier et al., 2011; Yi et al., 2011; Freedman et al., 2012; Rakha et al., 2012).

The earliest report of this group observed an association between LBVI and reduced overall survival in node negative and node positive patients that persisted after adjustment for T stage and lymph node status (Bettelheim et al., 1984).

Seventeen of these studies, comprising data on 30,462 patients, reported prognostic value of the LBVI independent of T stage and lymph node status (Pinder et al., 1994; Van Tienhoven et al., 1999; McCready et al., 2000; Jmor et al., 2002; Woo et al., 2002; Shen et al., 2005; Dinshaw et al., 2006; Beinart et al., 2007; Grasic-Kuhar et al., 2008; Ejlertsen et al., 2009; Viale et al., 2009; Thike et al., 2010; Mohammed et al., 2011; Panet-Raymond et al., 2011; Sabatier et al., 2011; Yi et al., 2011; Rakha et al., 2012).

Ten studies reported prognostic value of the LBVI in patients with node negative breast cancer (Lee et al., 1990; Magee et al., 1996; de Mascarel et al., 1998; Millis et al., 2002;

Kuru et al., 2003; Trudeau et al., 2005; Truong et al., 2005; Lee et al., 2006; Viale et al., 2010; Rakha et al., 2012).

One report of these showed a trend with overall survival (*P*=0.07) in multivariate analysis (Millis et al., 2002). Two studies reported prognostic value of the LBVI in node positive breast cancer (Neri et al., 2005; Ragage et al., 2010) independent of T stage, grade, and number of involved lymph nodes. Four studies reported prognostic value of the LBVI in triple negative disease (Ovcaricek et al., 2011) independent of T stage and lymph node status (Viale et al., 2009; Thike et al., 2010; Sabatier et al., 2011).

There were two published studies (Table 3.1), comprising data on 570 patients, reported that the presence of LBVI was not associated with overall or relapse free survival in primary breast cancer (Kim et al., 1998; Camp et al., 2000) though both studies were of small sample size.

The majority of the studies (32/34) reported that LBVI was a predictor of poor overall, relapse free or cancer specific survival. Most of the studies (32/34) detected the presence of the LBVI, primarily in peri-tumoural area by reviewing H&E stained sections. Only 2/34 of these reports used immunostaining with a lymphatic marker (D2-40/podoplanin) and blood vascular markers (CD34 and CD31) and both were independent predictors of outcome (Table 3.1). The overall average of LBVI rate was (24%) using H&E and (35%) using immunostaining. The rate of LBVI, as detected by H&E was variable (9-50%), and less variable using immunostaining (32-41%). This would suggest that LBVI using immunostaining is more reliable than that using nonspecific staining.

In conclusion, there is good evidence from the majority of studies that the presence of LBVI predicts poorer survival independent of T stage and lymph node status in patients with primary breast cancer. In particular, LBVI provides independent prognostic

information in subgroup of patients with node negative tumors. Most of these studies were conducted on a large number of patients and recent studies tended to use specific staining. Giving that the weighted average of LBVI rate, using immunostaining, was higher and the range was narrower, immunostaining appears to be a more reliable approach to identify LBVI in patients with primary operable breast cancer.

Table 3-1 General lymphovasular invasion (LBVI) in patients with primary operable breast cancer

Author	Patients n. (LBVI %)	Follow-up months	LN status	Location	Technique	Comment
Bettelheim et al., 1984	232 (50)	65	mixed	Peri-tumoural	H&E	Predicts poorer survival in all patient, node negative but not node positive patients
Lee et al., 1990	221 (24)	120	-ve LN	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Pinder et al., 1994	709 (23)	204	mixed	All section	H&E	Predicts poorer OS independently
Magee et al., 1996	708 (14)	96	-ve LN	N/D	H&E	Predicts poorer OS independently
de Mascarel et al., 1998	1320 (20)	103	-ve LN	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Kim et al., 1998	280 (48)	96	mixed	N/D	H&E	No significant association with RFS
Kollias et al., 1999	318 (N/D)	120	mixed	All section	H&E	Predicts poorer OS in small size tumour ≤1 cm
van Tienhoven et al., 1999	133 (42)	74	mixed	N/D	H&E	Predicts poorer OS independently
Camp et al., 2000	290 (9)	103	-ve LN	N/D	H&E	No significant association with OS
McCready et al., 2000	156 (35)	59	mixed	N/D	H&E	Predicts poorer RFS independently
Jmor et al., 2002	113 (35)	60	mixed	N/D	H&E	Predicts poorer OS independently in younger women ≤35yr
Millis et al., 2002	477 (19)	226	-ve LN	N/D	H&E	Predicts poorer OS
Woo et al., 2002	1258 (28)	144	mixed	Peri-tumoural	H&E	Predicts poorer OS independently
Kuru et al., 2003	384 (11)	70	-ve LN	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Neri et al., 2005	376 (50)	103	+ve LN	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Shen et al., 2005	120 (23)	59	mixed	N/D	H&E	Predicts poorer CSS after ipsilateral tumour recurrence independently

Truong et al., 2005	763 (28)	84	-ve LN	Peri-tumoural	H&E	Predicts poorer OS independently
Trudeau et al., 2005	415 (26)	96	-ve LN	Peri-tumoural	H&E	Predicts poorer CSS and RFS independently
Dinshaw et al.,2006	1022 (27)	53	mixed	N/D	H&E	Predicts poorer overall survival independently
Lee et al., 2006	2760 (19)	82	-ve LN	Peri-tumoural	H&E	Predicts poorer CSS and RFS independently
Beinart et al., 2007	771 (12)	60	mixed	N/D	H&E	Predicts poorer OS independently in patients with bilateral cancer
Mohammed et al., 2007	177	96	mixed	Intra-tumoural Peri-tumoural	CD34	Predicts poorer OS and RFS independently
	H&E (19)				CD31	
	IHC (32):				D2-40	
	LVI (31)					
	BVI (1)					
Grasic-Kuhar et al., 2008	1035 (15)	204	mixed	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Ejlertsen et al., 2009	16121 (15)	96 OS	mixed	Peri-tumoural	H&E, D2-40	Predicts poorer OS and RFS independently
		72 RFS			CD34, CD31	
Viale et al., 2009	284 (24)	204	mixed	Peri-tumoural	H&E	Predicts poorer OS independently in triple negative disease
Ragage et al., 2010	374 (46)	126	+ve LN	Peri-tumoural	H&E	Predicts poorer RFS independently in patients with Her-2-ve/HR+ve tumour
Thike et al., 2010	653 (45)	84	mixed	N/D	H&E	Predicts poorer OS independently in triple negative disease
Viale et al., 2010	2754 (23)	108	-ve LN	Peri-tumoural	H&E	Predicts poorer RFS
Ovcaricek et al., 2011	269 (25)	71	mixed	N/D	H&E	Predicts poorer OS and RFS in triple negative disease

Panet-Raymond et al., 2011	269 (22)	137	mixed	N/D	H&E	Predicts poorer overall and CSS independently after ipsilateral breast tumour recurrence
Sabatier et al., 2011	101 (41)	60	mixed	Peri-tumoural	D2-40, CD31	Predicts poorer RFS independently in triple negative disease
Yi et al., 2011	3728 (N/D)	78	mixed	N/D	H&E	Predicts poorer CSS independently
Freedman et al., 2012	1,478 (29)	68	mixed	N/D	H&E	Predicts poorer OS
Rakha et al., 2012	3812 (30)	85	mixed	Peri-tumoural	H&E	Predicts poorer CSS and RFS independently

Follow-up (mean/median), N/D: not described, RFS relapse free survival, OS overall survival, Her-2 human epidermal growth factor receptor-2, HR hormonal receptor, CSS cancer specific survival.

3.3.3 The prognostic value of lymphatic vessel invasion (LVI) in primary operable breast cancer

There were nineteen published studies (Table 3.2), comprising data on 12, 893 patients, reported that the presence of LVI was associated with reduced survival, primarily relapse free survival, in primary breast cancer (Clayton, 1991; Clemente et al., 1992; Neville, 1992; Gasparini 1994; Genta et al., 1994; Nixon et al., 1994; Lauria et al., 1995; D'Eredita et al., 2001; Fisher et al., 2001; Kato et al., 2003; Schoppmann et al., 2004; Dinshaw et al., 2005; Arnaout-Alkarain et al., 2007; Yamauchi et al., 2007; Gudlaugsson et al., 2011; Kurebayashi et al., 2012; Tezuka et al., 2007; Mohammed et al., 2011; Matsunuma et al., 2012).

Six of these studies reported prognostic value of LVI independent of T stage and lymph node status (Nixon et al., 1994; Lauria et al., 1995; Schoppmann et al., 2004; Dinshaw et al., 2005; Yamauchi et al., 2007; Kurebayashi et al., 2012). One of these studies examined the prognostic significance of LVI in three different areas (intra-tumoural area, non-tumoural area and advanced tumoural area) using H&E and D2-40 staining, reported that LVI predicted poorer relapse free survival independently regardless of the area examined or stain used, and that number of LVI identified gradually increased from the intra-tumoural area to the non-tumoural area (Yamauchi et al., 2007).

Six studies reported prognostic value of the LVI in patients with node negative breast cancer (Clayton, 1991; Neville, 1992; Lauria et al., 1995; Arnaout-Alkarain et al., 2007; Gudlaugsson et al., 2011; Mohammed et al., 2011) five of them were independent of T stage (Clayton, 1991; Neville, 1992; Lauria et al., 1995; Gudlaugsson et al., 2011; Mohammed et al., 2011).

Two studies, comprising data on 1056 patients, reported that the presence of LVI was not associated with cancer specific survival in primary breast cancer (Rosen et al., 1991;

Saimura et al., 1999), both studies used homogenous group of patients with node negative tumours though sample size were relatively small.

The majority of the studies (19/21) reported that LVI was a predictor of poor relapse free, overall or cancer specific survival. Most of these studies (15/21) detected the presence of LVI in peri-tumural area using H&E stained sections. Six recent studies used immunostaining, four of which reported independent prognostic value (Table. 3.4). The overall weighted average of LVI was 33% using H&E and 25% using immunostaining. The rate of LVI using H&E was wide ranging from 10-49% and was narrower using immunostaining ranging from 21-42%.

In conclusion, there is good evidence from the majority of studies (19/21) that the presence of LVI predicted poorer outcomes in patients with primary operable breast cancer. Further, LVI provides independent prognostic information in subgroup of patients with node negative breast cancer. Half of the studies were conducted on relatively large number of patients and reported a high rate of LVI. Giving that the weighted average of LVI was similar using H&E and IHC and that the rate of LVI was narrower using immunostaining, immunostaining appears to be more reliable approach to identify LVI in patients with primary operable breast cancer.

Table 3-2 Lymphatic vessel invasion (LVI) in primary operable breast cancer

Author	Patients n. (LVI %)	Follow up months	Lymph node status	Location	Technique	Comment
Clayton, 1991	378 (20)	182	-ve LN	Peri-tumoural	H&E	Predicts poor CSS independently
Rosen et al., 1991	293 (12)	238	-ve LN	N/D	H&E	No significant association with survival
Clemente et al., 1992	506	66	-ve LN	Peri-tumoural	H&E	Predicts poorer OS and RFS in both routine and re-viewed reports
Neville et al., (1992)	1203 (42)	60	-ve LN	Peri-tumoural	H&E	Predicts poorer RFS independently
Gasparini et al., (1994)	254 (10)	62	-ve LN	Peri-tumoural	H&E	Predicts poorer RFS independently
Genta et al., (1994)	318 (49)	102	mixed	Peri-tumoural	H&E	Predicts poorer OS and RFS independently
Nixon et al., (1994)	1398 (29)	99	mixed	N/D	H&E	Predicts poorer RFS independently
Lauria et al., (1995)	1408 (34)	76	mixed	Peri-tumoural	H&E	Predicts increased risk of death in both node negative and positive subgroups
Saimura et al., (1999)	763 (35)	74	-ve LN	N/D	H&E	No significant association with survival
D'Eredita et al., (2001)	402 (15)	120-192	mixed	Peri-tumoural	H&E	Predicts poorer OS
Fisher et al., (2001)	1036 (N/D)	180	mixed	N/D	H&E	Predicts poorer OS
Kato et al., (2003)	509 (23)	108	mixed	N/D	H&E	Predicts poorer OS and RFS
Schoppmann et al., (2004)	374 (28)	268	mixed	Intra-tumoural Invasive area	D2-40	Predicts poorer OS and RFS independently
Dinshaw et al., (2005)	1022 (27)	60	mixed	All section	H&E	Predicts poorer OS and RFS

						independently
Arnaout-Alkarain et al., (2007)	303 D2-40 (27) H&E (17.5)	89	-ve LN	Invasive area	H&E D2-40, CD31	Predicts poorer OS
Tezuka et al., (2007)	131 D2-40 (42), H&E (51)	69	mixed	Peri-tumoural Intra-tumoural	H&E, D2-40, CD34	Predicts poorer RFS
Yamauchi et al., (2007)	151 H&E: Intra-tumoural area (13) Non tumour area (34) Advance tumour area (23) D2-40: Intra-tumoural area (20) Non tumour area (46) Advance tumour area (26)	101	mixed	Intra-tumoural Non tumour area Advance tumour area	H&E,D2-40	Predicts poorer RFS independently
Gudlaugsson et al., (2011)	240 (21)	117	-ve LN	Intra-tumoural Peri-tumoural	D2-40, p63	D2-40+ve/p63-ve predicts poorer OS independently when only combined with high PPH3 in older women
Mohammed et al., (2011)	1005 (21)	107	-ve LN	Intra-tumoural Peri-tumoural	CD34, CD31, D2-40	Predicts poorer OS and RFS independently
Kurebayashi et al., (2012)	261 (3) ≥4 LVI/specimen	99	mixed	Peri-tumoural	H&E	Predicts poorer RFS and CSS particularly with PR -ve and high Ki67

Matsunuma et al., (2012)	1994(45) All (30) extensive	112	+ve LN	N/D	H&E	Predicts poorer RFS independently

Follow-up (Mean/median), N/D: not described, CSS cancer specific survival, OS overall survival, RFS relapse free survival, PPH3 phosphohistone H3, PR progesterone receptor, Ki67 tumour proliferation index.

3.3.4 The prognostic value of blood vessel invasion (BVI) in primary operable breast cancer

There were seven published studies (Table 3.3), comprising data on 4073 patients, reported that the presence of BVI was associated with reduced survival in patients with primary breast cancer (Friedell et al., 1965; Kister et al., 1966; Ruiz et al., 1973; Sampat et al., 1977; Lauria et al., 1995; Fisher et al., 2001; Kato et al., 2003). Two of these studies reported that the presence of BVI predicted poorer survival with long-term follow up independent of the T stage and lymph node status (Fisher et al., 2001; Kato et al., 2003). A recent study of more than one thousand patients with node negative breast cancer, using vascular markers CD34 and CD31, reported BVI in only 7 cases (<1%), but there were no specific features associated with the characterisation of these cases (Mohammed et al., 2011).

Two studies (Table 3.3), comprising data on 611 patients, reported that the presence of BVI was not associated with survival in patients with primary breast cancer (Rosen et al., 1991; Genta et al., 1994). Though the first study used homogenous group of patients with node negative tumours, both studies were relatively small.

Majority of the studies (8/10) detected the presence of BVI using H&E and/or classical staining (Verhoeff technique, Weigert's resorcin fuchsin, van Gieson) for elastic fibers surrounding blood vessels. Two recent studies (2/10) used immunostaining. One of these reported that BVI was an independent predictor of outcome (Table 3.4). The earliest reports of these studies reported prognostic value of BVI independent of nodal status using univariate analysis and small sample size. The overall weighted average of the BVI rate was relatively similar using H&E and/or classical staining (16%) and immunostaining (10%). The rate of BVI using H&E and/or classical staining was variable ranging from (4-46%) and (1-29%) using immunostaining. In conclusion, the prognostic value of the

presence of BVI and the best method of detection in primary operable breast cancer remains unclear.

Table 3-3 Blood vessels invasion (BVI) in patients with primary operable breast cancer

Author	Patients n. (BVI %)	Follow up months	Lymph node status	Location	Technique	Comment
Friedell et al (1965)	153 (46)	60	mixed	N/D	Verhoeff technique	Predicts poorer 5-yr survival rate in presence or absence of +ve LN
Kister et al., (1966)	328 (21)	120	mixed	N/D	Verhoeff technique	Predicts poorer OS in the presence but not in the absence of +ve LN
Ruiz et al., (1973)	394 (46)	60	mixed	N/D	Verhoeff technique	Predicts poorer 5-yr survival rate in LN –ve disease
Sampat et al., (1977)	242 (N/D)	60	mixed	N/D	H&E Weigert's resorcin fuchsin	Predicts poorer survival in presence or absence of +ve LN
Rosen et al., (1991)	293 (19)	238	-ve LN	N/D	van Gieson and modified Hart's stains	No significant association with survival
Genta et al., (1994)	318 (14)	102	mixed	Pri-tumoural	H&E	No significant association with survival
Lauria et al., (1995)	1408 (4)	76	mixed	Pri-tumoural	H&E	Predicts poorer OS
Fisher et al., (2001)	1039 (N/D)	180	mixed	N/D	H&E	Predicts poorer OS independently
Kato et al., (2003)	509 (29)	108	mixed	N/D	Factor VIII, Elastica	Predicts poorer OS and RFS independently
Mohammed et al., (2011)	1005 (<1)	107	-ve LN	Intra-tumoural Peri-tumoural	CD34, CD31 D2-40	Analysis was not possible

Follow-up (Mean/median), ND: not described, OS overall survival, CSS cancer specific survival, RFS relapse free survival, RR relative risk.

Table 3-4 Stains reporting method of detection of LBVI, LVI and BVI and their prognostic value

	Stain	Studies (n)	Prognostic studies (n)	Non prognostic studies (n)	Total
General Lymphovascular invasion	H&E	32	30	2	34
	D2-40/Podoplanin	2	2		
	CD34 & CD31				
Lymphatic vessel invasion	H&E	15	13	2	21
	Podoplanin/D2-40	6	6		
Blood vessel invasion	H&E and/or Classical stains (Verhoeff technique, Weigert's resorcin fuchsin, van Gieson)	8	6	2	10
	Factor VIII & Elastica	1	1		
	CD34 & CD31	1	Analysis was not possible		

3.4 Discussion

From the present review, there is robust evidence that general LBVI and LVI are powerful prognostic factors of poorer survival in patients with primary operable breast cancer. Also, immunostaining appears to detect LBVI and LVI more reliably than H&E. However, the prognostic role of BVI and the optimal detection method, when specifically examined, remains unclear. This would suggest that general LBVI is mostly lymphatic vessel invasion rather than blood vessel invasion and that this is the main rout of breast cancer spread.

Further, the present review provides robust evidence that the presence of LBVI and LVI is independent high risk factor in patients with node negative breast cancer. This prognostic effect would suggest that the process of lymphovascular invasion or lymphatic vessel invasion, in itself, is sufficiently valuable to be incorporated into the existing staging systems. However, reliable standardised methods are required for optimal risk assessment.

The prognostic value of LBVI has been reported using H&E staining in the majority of studies. These studies have not discriminated between the types of vessel invasion whether lymphatic or blood vessel and have inconsistently used the terms vascular or lymphovascular invasion. For example, the American Joint Committee on Cancer (AJCC) staging guidelines (2005) has used the term lymphovascular invasion to indicate both lymphatic and blood vessel invasion (TNM Atlas, 2005). This clearly may be confusing as these terms may indicate involvement of lymphatic or lymphatic and blood vessels. This is in large part due to the routine use of H&E slides to assess lymphovascular invasion. H&E approach has lower rate of detection in some studies as low as 9%. This could be, in part, attributed to the inter-observer variability (Gilchrist et al., 1982) or the difficulty to distinguish lymphatic from blood vessels especially for small collapsed vessels or vessels completely filled with tumour cells. Another challenge, long recognised, on H&E sections

is how to distinguish lymphatic and blood vessels from stromal retraction artifacts caused by tissue handling and fixation (Saigo and Rosen, 1987; Bettelheim et al., 1984; Van den Eynden., 2006; Hoda et al., 2006). Although AJCC mandates distinguishing between lymphatic and blood vessel invasion, these guidelines lack a routine standardised and objective pathological assessment method to reliably differentiate them.

There was a substantial improvement in consistency in reporting the rate of breast cancer cases with LBVI and LVI using immunostaining (32-41% and 21-42%, respectively). Such improvement has been documented with lymphatic (e.g. D2-40 or podoplanin) and blood vessel (e.g. CD34 and CD31) endothelial markers. Moreover, these markers do not only discriminate retraction artifacts from LVI and BVI but also distinguish between lymphatic vessels and blood vessels, allowing specifically study of LVI and BVI (Saigo and Rosen, 1987; Schoppmann et al., 2001; Mohammed et al., 2009; Van den Eynden., 2006).

In theory, invasion into lymphatic vessels and blood vessels may lead to different consequences: LVI may be predictive of lymph node metastasis, whereas BVI may be the source of systematic spread. Indeed, the presence of LVI has been correlated with presence of lymph node involvement, local recurrence and poor survival in breast cancer (Rosen et al., 1981a; Lee et al., 1990; Rosen et al., 1991; Ghadha et al., 1994; Leitner et al., 1995; Mohammed et al., 2011). The impact of LVI is mainly seen in patients with node negative breast cancer, therefore, identification of LVI particularly using D2-40 could objectively identify node negative patients at higher risk of recurrence who might benefit from adjuvant chemotherapy. BVI is also associated with metastatic spread. Early studies correlated BVI with a high rate of recurrence and metastasis (Sampat, et al., 1977; Rosen et al., 1981b; Weigand et al., 1982). However, further work is required to confirm such findings using modern staining techniques.

Since BVI correlates with the occurrence of systematic spread, and patients without BVI have better recurrence free and overall survival (Lauria et al., 1995; Fisher et al., 2001; Kato et al., 2003), identifying BVI objectively may be an important step forward in identifying patients at higher risk of systemic spread, including patients with triple negative cancers. However, it is clear from the present review that BVI is currently assessed inconsistently (4%-46%) using H&E or classical histostaining. In terms of immunostaining use to detect BVI, there are few studies have attempted to identify BVI using such approach. Kato and colleagues used Factor VIII in addition to Elastica van Gieson stain and reported high rate of BVI up to 29% (Kato et al., 2003). Another study using D2-40 and CD34 to distinguish between LVI and BVI reported higher rate of BVI, 36% (Van den Eynden., 2006). In contrast, more recent studies by Mohammed and colleagues reported as low incidence of BVI as 1% using D2-40, CD34 and CD31 (Mohammed et al., 2007; Mohammed et al., 2011). As Factor VIII has been found to be occasionally reactive to lymphatic endothelium, the high rate in Kato et al., study may result from LVI being counted as BVI. Van den Eynden and colleagues used D2-40 to distinguish LVI however, CD34 has been found reactive to stromal cell and connective tissue surrounding tumour nest that may give false impression of BVI. It remains to be determined whether the introduction of immunostaining for BVI will improve prediction of outcome in patients with primary operable breast cancer.

3.5 Summary

To date, numerous studies have examined the prognostic role of general LBVI, LVI and BVI in primary operable breast cancer. The majority of studies (49/59) used H&E and classical histochemistry to identify LVI and BVI. Only 10 recent studies used immunostaining of endothelium lining lymphatic and blood vessels and were able to show clear differences between LVI and BVI.

Although, most of the studies included in the present review used H&E to identify lymphatic and blood vessels that may be optimal, this reflects current practice in most pathology departments. Thus, the present review provides clear information about the method of detection of LVI and BVI and how these methods influence the clinical outcomes.

The present review clearly indicates that the IHC technique with appropriate antibodies facilitates the objective assessment of LVI and BVI in patients with primary operable breast cancer. Therefore, future work should see the application of this approach to the routine clinical pathology assessment of these patients.

Chapter 4 Immunohistochemical detection improves the prognostic value of lymphatic and blood vessel invasion in invasive ductal breast cancer

4.1 Introduction

Breast cancer is the most frequently diagnosed malignancy among women and the majority of patients present with early stage disease. Many patients with node negative tumours are at high risk of local and/or distant metastasis and would benefit from adjuvant systemic therapy. Lymphatic and blood vessel invasion are of prognostic significance and is primarily used to make decisions for lymph node negative patients with borderline tumour sizes (Cianfrocca and Goldstein, 2004). Lymphovascular invasion shows a clear relation with nodal status (Pinder et al., 1994; Lauria et al., 1995; Mohammed et al., 2007; Ejlertsen et al., 2009; Rakha et al., 2012). In node negative breast cancer, LBVI has been found to be associated with local recurrence (Pinder et al., 1994; Veronesi et al., 1995; Sandquist et al., 2000; Voogd et al., 2001, Mohammed et al., 2011), distant metastasis and poor survival (Lee et al., 2006a; Trudeau et al., 2005; Mohammed et al., 2011). Node negative patients with positive LBVI had more than 30% recurrence rate (Neville et al., 1992) and higher breast cancer mortality (53%) compared with patients with no lymphovascular invasion (29%) (Lee et al., 2006a).

10-20% of breast cancer is basal like with the majority being triple negative tumours (negative for ER, PR and Her-2 overexpression). This aggressive subtype is more likely to be associated with younger age, higher grade and advanced tumour stage with poor survival (Perou et al., 2000; Sørlie et al., 2001; Carey et al., 2006; Rakha et al., 2006; Bauer et al., 2007). Currently, there are no specific treatment guidelines for triple negative breast cancer (Reis-Filho and Tutt, 2008). In addition, neither currently used clinicopathological factors nor molecular profiling techniques are able to subdivide this

subgroup of patients with respect to prognosis (Sotiriou and Pusztai, 2009; Fan et al., 2006, Fulford et al., 2006).

Lymphovascular invasion including lymphatic and blood vessel invasion is an important prognostic factor that stratifies risk of local failure and help decisions about systemic therapy. Numerous studies have reported that LBVI and LVI are powerful prognostic factors of poorer survival in patients with early breast cancer using both H&E and IHC approaches (chapter 3.0). While immunohistochemistry (IHC) appears more reliable to detect LBVI and LVI than H&E, the prognostic role of BVI and the optimal detection methods remain unclear (chapter 3.0).

Therefore, the aim of the present study was to examine the prognostic value of different assessment methods of lymphovascular invasion in patients with invasive ductal breast cancers, and in particular node negative and triple negative diseases.

4.2 Patients and methods

4.2.1 Patients

360 patients with primary operable invasive ductal breast cancer whose samples were successfully stained for D2-40 and FVIII from patients described in section 2.1 were included in this study.

4.2.2 Methods

Lymphovascular invasion was assessed as part of the routine pathological work-up at the pathology department using H&E sections. The assessment of ER, PR and Her-2 was performed as previously described in sections 2.2.2.

4.2.2.1 Immunohistochemistry of D2-40 and FVIII

For visualization of lymphatic and blood vessels, 2 consecutive samples of 2.5 μm thick sections from each block (one block/case) were stained for the lymphatic endothelial marker D2-40 (Covance, Monoclonal Antibody, SIG-3730, USA) diluted 1:100 and Factor VIII (Mouse Monoclonal Antibody, NCL-L-Vwf, Leica, Newcastle, UK) diluted 1:100. Sections were dewaxed in xylene and rehydrated through descending concentrations of ethanol. For antigen retrieval of Factor VIII, sections were microwaved for 14 minutes in sodium citrate buffer (pH 6). Endogenous hydrogen peroxidase activity was blocked with 3% H2O2 for 15 minutes. Non-specific binding was blocked by incubation with 10% horse serum for 30 minutes. Sections were subsequently incubated with the respective primary antibody; 60 minutes at room temperature for D2-40 and 30 minutes at 25ºC for Factor VIII. Sites of binding were detected using the Envision technique (Dako, code K5007) and with 3-30 diaminobenzidine (Vector, code SK 4001, Burlingame, CA, USA), as chromogenic substrate, according to the manufacturer's instruction. Slides were counterstained with haematoxylin and were dehydrated and mounted with DPX. Two full

sections of tonsil tissue were used as positive and negative controls for each antibody. The procedure as above was applied for positive controls. For negative controls, primary antibodies were omitted.

4.2.2.2 Slide scanning and assessment

Routine H&E sections, D2-40 and Factor VIII stained sections for the 360 patients were scanned at objective magnification x20 as previously described in Section 2.2.3. Assessment of $LBVI_{H\&E,}$ $LVI_{D2\text{-}40}$ and $BVI_{FactorVIII}$ were carried out on a computer monitor using the Slidepath Tissue IA system version 3.0 (Slidepath, Leica Biosystems).

LBVI on H&E sections ($LBVI_{H\&E}$) was reviewed centrally and blinded to the pathology report. For the assessment of $LVI_{D2\text{-}40}$ and BVI_{FVIII}, serial sections similar to that of H&E sections, from each block were stained with D2-40 and Factor VIII. $LBVI_{H\&E}$, $LVI_{D2\text{-}40}$ and BVI_{FVIII} were identified at peri-tumoural, invasive front or intra-tumoural areas. $LBVI_{H\&E}$ was identified using criteria previously described (Davis et al., 1985), as the presence of tumour cell emboli within a vessel space, which was identified by associated fibrin clot and/or an endothelial cell lining. $LVI_{D2\text{-}40}$ was identified by tumour cells within D2-40-positively stained vessels, while BVI_{FVIII} was counted only when tumour cells were identified in D2-40-negative, Factor VIII-positive vessels. A total of 30% of H&E and IHC stained sections for LBVI, LVI and BVI were independently scored by two observers (FG, ZM) blinded to patient outcome and the other observer's score. The inter class correlation coefficient (ICCC) of $\geq$0.84 was obtained for H&E, D2-40 and Factor VIII indicated excellent agreement, and FG scored all the slides and this data was used in the analysis.

4.2.2.3 Statistical analysis

Consistency between the observers was analysed using the ICCC. Interrelationships between variables were assessed using contingency table analysis with X^2 test for trend as appropriate. Univariate and multivariate survival analysis were performed using the Kaplan-Meier analysis and Cox proportional hazards model with a stepwise backward elimination to derive a final model of variables with a significant independent relationship with survival. All statistical analyses were 2-sided with significance defined as a *P* value <0.05. All statistical analysis was performed using the SPSS software version 19 (SPSS Inc., Chicago,IL, USA).

4.3 Results

4.3.1 Clinicopathological characteristics and $LBVI_{H\&E}$, $LVI_{D2\text{-}40}$ and BVI_{FVIII} in the whole cohort, in node negative patients and in triple negative patients

The clinical and pathological characteristics of the 360 patients are shown in Table 4.1. Majority of patients were older than 50 years (65%), had tumours size ≤2 cm (51%), had grade III carcinoma (52%) and no axillary lymph node involvement (57%). A total of 212 patients (59%) had ER positive tumours and 192 patients (53%) had PR negative tumours. Two hundred eighty nine patients (80%) had Her-2 negative tumours with 28% of patients had triple negative tumours. 81 (23%) patients received tamoxifen, 144 (40%) received chemotherapy, and 45 (13%) received both. Eighty nine patients (24%) experienced recurrences. Of these patients, 17 (5%) had local recurrence, 67 (19%) had distant recurrence and five patients had both.

$LBVI_{H\&E}$ was readily identified when tumour cells invaded into large vessels and especially when lymphatic vessels were accompanied by adjacent blood vessels, however, invasion into small lymphatic or blood vessels as well as stromal artifact could be difficult to assess (Figure 4.1). D2-40 stained vessels were usually clear and readily assessed. $LVI_{D2\text{-}40}$ was identified by the presence of tumour emboli in vessels that showed D2-40 positivity of the endothelium. Although D2-40 was positive in myoepithelial cells of breast ducts in some cases, this was readily distinguished from lymphatic endothelium by morphological characteristics (Figure 4.1E).

Table 4-1 The clinicopathological characteristics of patients with invasive ductal breast cancer (n=360).

Clinicopathological characteristics	Patients, n (%)
Age (≤50/ >50 years)	125(35%)/235(65%)
Size (≤20/ 21-50/ >50 mm)	185(51%)/162(45%)/13(4%)
Grade (I / II / III)	48(13%)/124(34%)/188(52%)
Involved lymph node (-ve/+ve)	206(57%)/154(43%)
ER status (no/yes)	148(41%)/212(59%)
PR status (no/yes)	192(53%)/168(47%)
Her-2 status (no/yes)	289(80%)/71(20%)
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	130(36%)/230(64%)
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)[a]	81(23%)/45(13%)/144(40%)/83(23%)
Tumour recurrence (no/local/distant/both)	271(75%)/17(5%)/67(19%)/5(1%)
Alive/cancer death/non cancer death	189(53%)/97(27%)/74(21%)

a number of patients when incomplete data available

D2-40 staining was helpful in identifying small lymphatic emboli and lymphatic vessels obscured by tumour cells (Figure 4.1). Blood vessels were intensely and continuously positive for Factor VIII. Factor VIII staining of lymphatic endothelium was faint or negative (Figure 4.2). $LVI_{D2\text{-}40}$ was generally more extensive than BVI_{FVIII} and lymphatic tumour emboli were larger than blood vessel emboli.

$LBVI_{H\&E}$ was reported in 102/360 (28%) patients, $LVI_{D2\text{-}40}$ was present in 127/360 (35%) patients and BVI_{FVIII} was present in 59/360 (16%) patients. Eighty nine (25%) patients had LVI only, whereas twenty one (6%) patients had BVI only, and thirty eight (10%) had both LVI and BVI. $LBVI_{IHC}$ ($LVI_{D2\text{-}40}$ + BVI_{FVIII}) was present in 148 (41%) patients. In node negative patients (206), $LBVI_{H\&E}$ was present in 41 (20%), $LVI_{D2\text{-}40}$ was present in 53 (26%) and BVI_{FVIII} was present in 21 (10%). In triple negative patients (102), $LBVI_{H\&E}$ was present in 31 (30%), $LVI_{D2\text{-}40}$ was present in 36 (35%) and BVI_{FVIII} was present in 14(14%).

While $LBVI_{H\&E}$ was strongly associated with $LBVI_{IHC}$ (P<0.001), 80 (22%) patients in whom $LBVI_{H\&E}$ had not been identified were positive for $LVI_{D2\text{-}40}$ and/or BVI_{FVIII}. Also, in 34 patients (9%) in whom $LBVI_{H\&E}$ had been identified, IHC was negative for both $LVI_{D2\text{-}40}$ and BVI_{FVIII}.

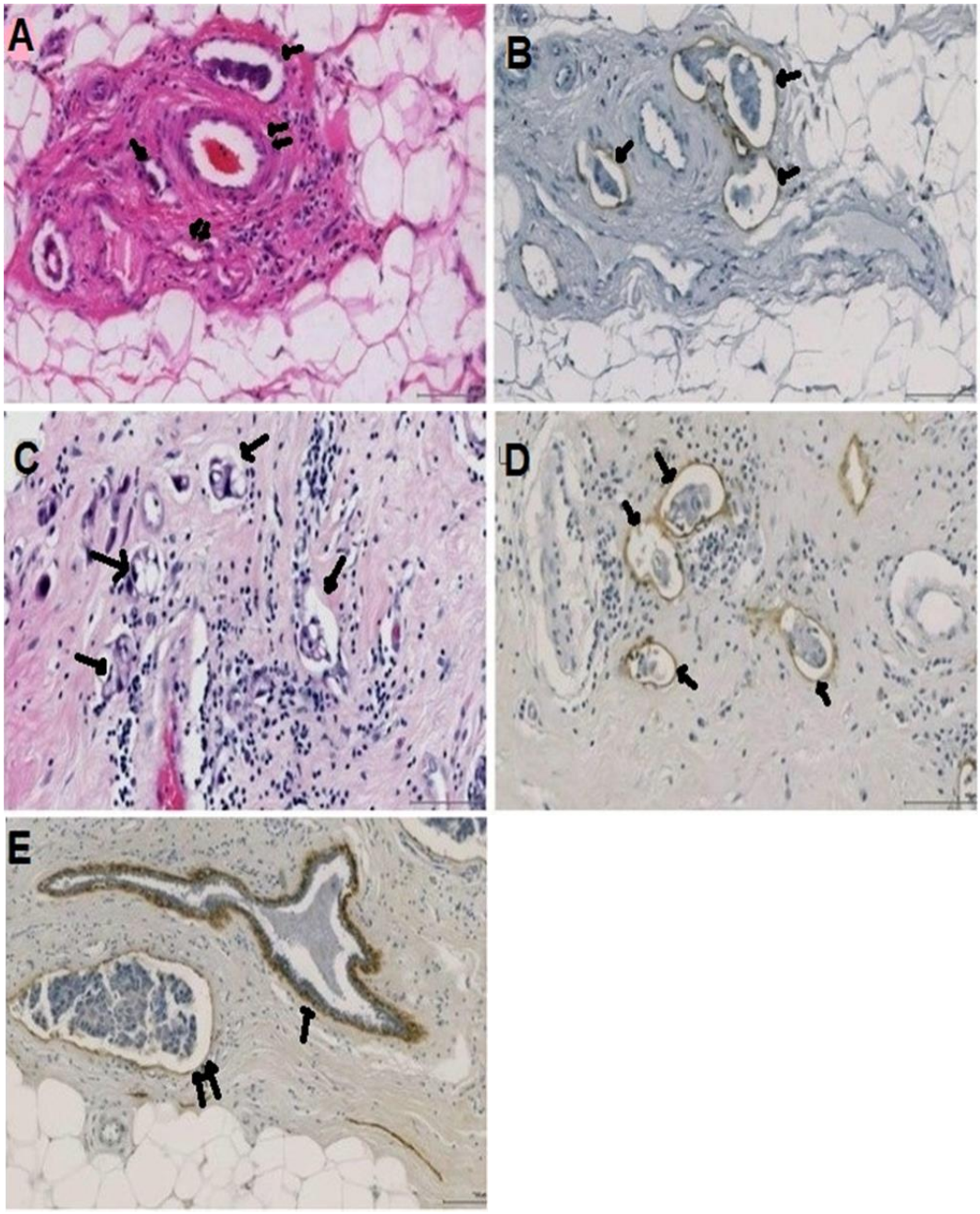


Figure 4-1 Examples of LVI in invasive breast cancer sections stained with H&E and D2-40.

A: H&E conspicuous carcinoma emboli in large and small vascular spaces (single arrows) accompanying structurally identified blood vessels (double arrows). B: similar section stained with D2-40 confirming that these are LVI (arrows). C: carcinoma emboli in small vessels (arrows) that could not be characterised on H&E section. D: similar section stained with D2-40 confirming that these are LVI (arrows). (Scale bar 100 μm). E: pattern of D2-40 staining in normal breast duct myoepithelium (single arrows) and how it is different from that of lymphatic endothelium (double arrows). (Scale bar100 μm).

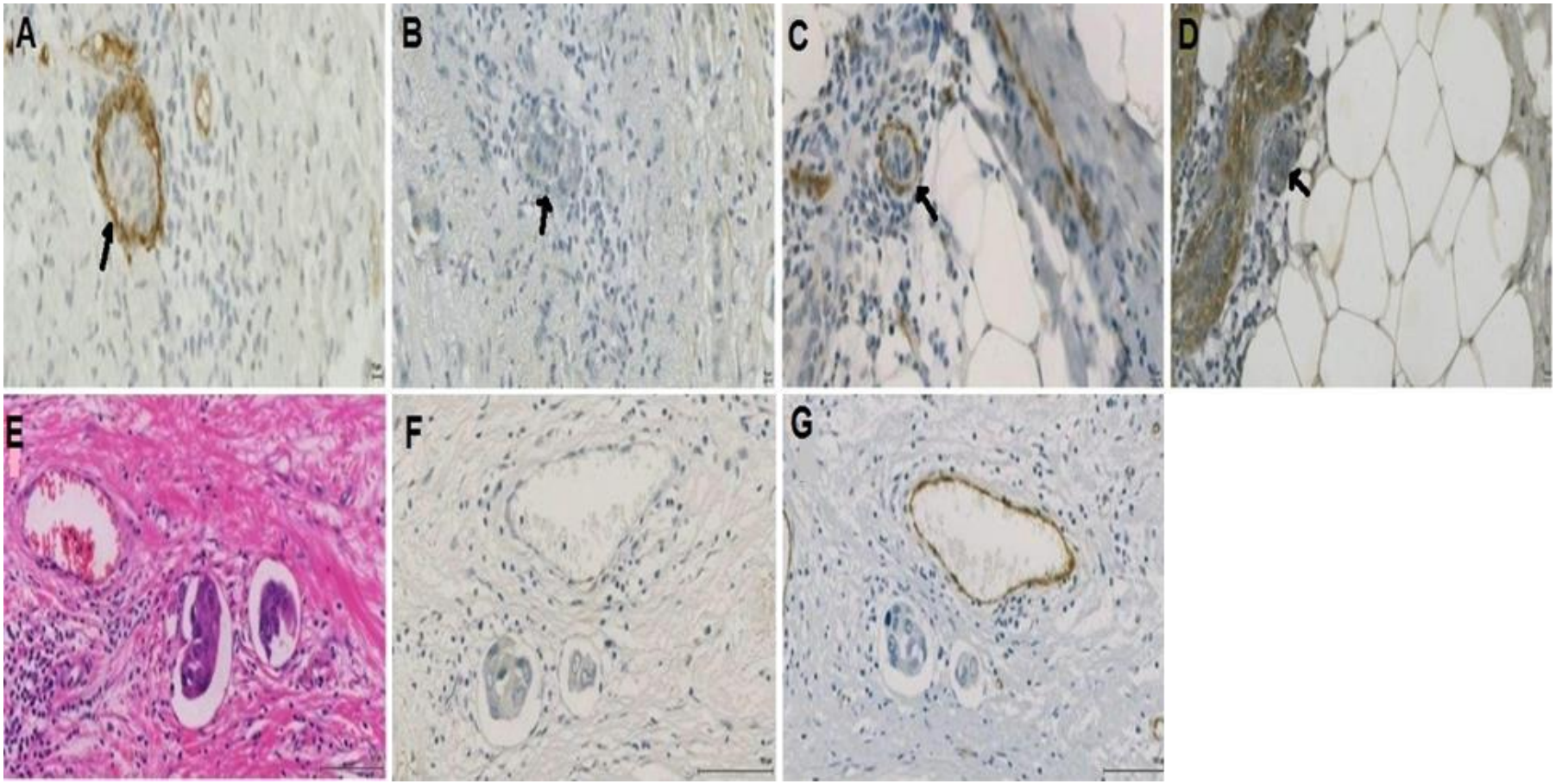


Figure 4-2 Examples of BVI in invasive breast cancer (sections stained with Factor VIII, D2-40 and H&E).

A & C: carcinoma cells within Factor VIII-positive vessels. These are negative for D2-40 (B & D), indicating BVI. E-G show consecutive sections stained with H&E. (E) showing tumour cells inside endothelial lining space, however, D2-40 (F) and Factor VIII (G) are both negative suggesting stromal artifact (note the positive staining of blood vessel with Factor VIII). (Scale bar 10µm).

As shown in Table 4.2, the presence of $LBVI_{H\&E}$ was associated with large tumour size (P<0.001), high tumour grade (P=0.028), involved lymph node (P<0.001), tumour recurrence (P<0.001) and locoregional treatment (P=0.002). No association was seen with hormonal status, Her-2 status and systemic therapy. In node negative patients, tumour size (P=0.008), locoregional treatment (P=0.045) and tumour recurrence (P=0.001) were significantly associated with $LBVI_{H\&E}$. In triple negative patients, the presence of $LBVI_{H\&E}$ was associated with tumour size (P=0.006), involved lymph node (P =0.003), locoregional treatment (P=0.017) and tumour recurrence (P= 0.012).

Table 4.3 shows that the presence of $LVI_{D2\text{-}40}$ was associated with younger age (P=0.006), large tumour size (P=0.024), high tumour grade (P<0.001), involved lymph node (P<0.001), locoregional treatment (P=0.017) and tumour recurrence (P<0.001). In node negative patients, the presence of $LVI_{D2\text{-}40}$ was associated with younger age (P=0.008) large tumour size (P=0.019) and high tumour grade (P=0.002), Her-2 positivity (P=0.032) and tumour recurrence (P<0.001). In triple negative patients, the presence of $LVI_{D2\text{-}40}$ was associated with younger age (P=0.034), involved lymph node (P=0.001) and tumour recurrence (P<0.001).

Table 4.4 shows that the presence of BVI_{FVIII} was associated with large tumour size (P<0.001), high tumour grade (P=0.044), involved lymph node (P<0.001), Her-2 positivity (P=0.003) and tumour recurrence (P<0.001). In node negative patients, BVI_{FVIII} was only significantly associated with larger tumour size (P=0.012) and tumour recurrence (P<0.001). In triple negative patients, the presence of BVI_{FVIII} was significantly associated with involved lymph node (P=0.019) and tumour recurrence (P=0.001).

Table 4-2 The relationship between clinicopathological characteristics and lymphovascular invasion ($LBVI_{H\&E}$) in patients with invasive ductal breast cancer

All patients (n=360)	**$LBVI_{H\&E}$ -ve n=258(72%)**	**$LBVI_{H\&E}$ +ve n=102(28%)**	**(*P*-value)**
Age (≤50/ >50 years)	86/172	39/63	0.379
Size (≤20/ 21-50/ >50 mm)	147/106/5	38/56/8	<0.001
Grade (I / II / III)	38/95/125	10/29/63	0.028
Involved lymph node (-ve/+ve)	165/93	41/61	<0.001
ER status (no/yes)	100/158	48/54	0.150
PR status (no/yes)	131/127	61/41	0.122
Her-2 status (no/ yes)	211/47	78/24	0.254
Tumour recurrence (no/local/distant/both)	213/7/36/2	58/10/31/3	<0.001
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	106/152	24/78	0.002
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	63/29/96/66	18/16/48/17	0.820
Alive/cancer death/non cancer death	148/54/56	41/43/18	0.158
Cancer specific survival (months)[a]	178(171-188)	138(121-155)	<0.001
Node negative patients (n=206)	**n=165(80%)**	**n=41(20%)**	
Age (≤50/ >50 years)	51/114	16/25	0.322
Size (≤20/ 21-50/ >50 mm)	103/60/2	17/22/2	0.008
Grade (I / II / III)	29/60/76	5/13/23	0.233
ER status (no/yes)	69/96	18/23	0.809
PR status (no/yes)	90/75	23/18	0.858
Her-2 status (no/ yes)	138/27	30/11	0.123
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	77/88	12/29	0.045
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	46/9/51/58	9/9/11/11	0.660

Tumour recurrence (no/local/distant/both)	143/6/15/1	27/2/10/2	0.001
Alive/cancer death/non cancer death	104/23/38	20/12/79	0.365
Cancer specific survival (months)[a]	190(181-199)	168(146-190)	0.010
Triple negative patients (n=102)	**n=71(70%)**	**n=31(30%)**	
Age (≤50/ >50 years)	30/41	17/14	0.073
Size (≤20/ 21-50/ >50 mm)	44/26/1	12/15/4	0.006
Grade (I / II / III)	1/11/59	0/5/26	0.804
Involved lymph node (-ve/+ve)	50/21	12/19	0.003
Tumour recurrence (no/local/distant/both)	59/1/11/0	18/2/11/0	0.012
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	34/37	7/24	0.017
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	8/3/41/18	4/6/17/4	0.083
Alive/cancer death/non cancer death	46/15/10	15/14/2	0.561
Cancer specific survival (months)[a]	176(159-192)	126(97-177)	0.016

a=Mean (95%CI)

Table 4-3 The relationship between clinicopathological characteristics and lymphatic vessel invasion (LVI_{D2-40}) in patients with invasive ductal breast cancer

All patients (n=360)	**LVI $_{D2-40-ve}$ n=233 (65%)**	**LVI $_{D2-40+ve}$ n=127 (35%)**	**(*P*-value)**
Age (≤50/ >50 years)	69/164	56/71	0.006
Size (≤20/ 21-50/ >50 mm)	129/97/7	56/65/6	0.038
Grade (I / II / III)	41/87/105	7/37/83	<0.001
Involved lymph node (-ve/+ve)	153/80	53/74	<0.001
ER status (no/yes)	89/144	59/68	0.129
PR status (no/yes)	113/120	79/48	0.013
Her-2 status (no/ yes)	193/40	96/31	0.099
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	94/139	36/91	0.024
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	62/25/80/63	19/20/64/20	0.384
Tumour recurrence (no/local/distant/both)	199/5/28/1	72/12/39/4	<0.001
Alive/cancer death/non cancer death	141/39/53	48/58/21	0.059
Cancer specific survival (months)[a]	186(177-194)	134(120-149)	<0.001
Node negative disease (n=206)	**n=153 (74%)**	**n=53 (26%)**	
Age (≤50/ >50 years)	42/111	25/28	0.008
Size (≤20/ 21-50/ >50 mm)	96/55/2	24/27/2	0.019
Grade (I / II / III)	33/53/67	1/20/32	0.002
ER status (no/yes)	62/91	25/28	0.400
PR status (no/yes)	80/73	33/20	0.210
Her-2 status (no/ yes)	130/23	38/15	0.032
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	71/82	18/35	0.116
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	46/11/41/54	9/7/21/15	0.418
Tumour recurrence (no/local/distant/both)	137/4/11/1	33/4/14/2	<0.001
Alive/cancer death/non cancer death	99/18/36	25/17/11	0.266
Cancer specific survival (months)[a]	198(190-206)	153(131-174)	0.001
Triple negative patients (n=102)	**n=66(65%)**	**36(35%)**	
Age (≤50/ >50 years)	24/42	21/15	0.034
Size (≤20/ 21-50/ >50 mm)	38/25/3	18/16/2	0.485

Grade (I / II / III)	1/11/54	0/5/31	0.493
Involved lymph node (-ve/+ve)	48/18	14/22	0.001
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	27/39	14/22	0.842
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	9/5/34/18	3/4/24/4	0.850
Tumour recurrence (no/local/distant/both)	58/0/8/0	19/3/14/0	<0.001
Alive/cancer death/non cancer death	44/11/11	17/18/1	0.702
Cancer specific survival (months)[a]	175(163-197)	125(94-139)	0.001

a=Mean (95% CI)

Table 4-4 The relationship between clinicopathological characteristics and blood vessel invasion (BVI_{FVIII}) in patients with invasive ductal breast cancer

All patients (n=360)	**BVI_{FVIII} -ve n=301(84%)**	**BVI_{FVIII}+ve n=59(16%)**	**(*P*-value)**
Age (≤50/ >50 years)	104/197	21/38	0.848
Size (≤20/ 21-50/ >50 mm)	168/123/10	17/39/3	<0.001
Grade (I / II / III)	45/104/152	3/20/36	0.044
Involved lymph node (-ve/+ve)	185/116	21/38	<0.001
ER status (no/yes)	139/162	32/27	0.258
PR status (no/yes)	156/145	36/23	0.196
Her-2 status (no/ yes)	250/51	39/20	0.003
Locoregional treatment (Lumpectomy+ radiotherapy/ mastectomy+radiotherapy)	114/187	16/43	0.075
Systemic treatment (hormonal/hormonal + chemotherapy/ chemotherapy/none)	70/38/118/70	11/7/26/13	0.442
Tumour recurrence (no/local/distant/both)	243/13/42/3	28/4/25/2	<0.001
Alive/cancer death/non cancer death	179/60/62	10/37/12	<0.001
Cancer specific survival (months)[a]	181(173-189)	93(73-112)	<0.001
Node negative disease (n=206)	**n=185 (90%)**	**n=21 (10%)**	
Age (≤50/ >50 years)	59/126	8/13	0.566
Size (≤20/ 21-50/ >50 mm)	113/69/3	7/13/1	0.012
Grade (I / II / III)	33/64/88	1/9/11	0.294
ER status (no/yes)	79/106	8/13	0.686
PR status (no/yes)	100/85	13/8	0.494
Her-2 status (no/ yes)	154/31	14/7	0.064
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	84/101	5/16	0.059
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	50/17/54/62	5/1/8/7	0.760
Tumour recurrence (no/local/distant/both)	158/8/16/3	12/0/9/0	<0.001
Alive/cancer death/non cancer death	120/25/40	4/10/7	0.003
Cancer specific survival (months)[a]	194(186-202)	110(75-146)	<0.001
Triple negative patients (n=102)	**n=88(86%)**	**n=14(14%)**	
Age (≤50/ >50 years)	40/48	5/9	0.498
Size (≤20/ 21-50/ >50 mm)	52/32/4	4/9/1	0.052
Grade (I / II / III)	1/12/75	0/4/10	0.281
Involved lymph node (-ve/+ve)	58/30	4/10	0.008
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	37/51	4/10	0.342

Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	12/7/50/19	0/2/8/3	0.177
Tumour recurrence (no/local/distant/both)	71/3/14/0	6/0/8/0	0.001
Alive/cancer death/non cancer death	59/18/11	2/11/1	0.019
Cancer specific survival (months)[a]	179(162-191)	61(31-91)	<0.001

a=Mean (95% CI)

4.3.2 Survival analysis of $LBVI_{H\&E}$, $LVI_{D2\text{-}40}$ and BVI_{FVIII} in the whole cohort, in node negative patients and in triple negative patients

The minimum follow-up of survivors was 142 months; median follow-up of survivors was 168 months. During follow up 171 patients died, 97 died of their cancer. The presence of $LBVI_{H\&E}$, $LVI_{D2\text{-}40}$ and BVI_{FVIII} were analysed with 15years follow-up data using the Kaplan–Meier analysis and Cox regression.

Kaplan–Meier curves showed increased risk of death with $LBVI_{H\&E}$, $LVI_{D2\text{-}40}$ and BVI_{FVIII} in the whole cohort, node negative and triple negative patients (Figure 4.3-4.5). Univariate analysis indicated that $LBVI_{H\&E}$ was significantly associated with cancer specific survival in the whole cohort (P<0.001), node negative (P=0.010) and in triple negative patients (P=0.016). The Presence of $LVI_{D2\text{-}40}$ was strongly and significantly associated with cancer specific survival in the whole cohort (P<0.001), in node negative patients (P=0.001) and in triple negative patients (P<0.001). The presence of BVI_{FVIII} was strongly and significantly associated with cancer specific survival in the whole cohort, node negative and triple negative patients (all P<0.001) (Table 4.5).

On multivariate survival analysis for the whole cohort, tumour size (P=0.017), tumour grade (P=0.026), LN status (P=0.016), $LVI_{D2\text{-}40}$ (P=0.022) and BVI_{FVIII} (P<0.001) remained independently associated with cancer specific survival. On multivariate survival analysis for node negative patients, tumour size (P=0.038), $LVI_{D2\text{-}40}$ (P=0.008) and BVI_{FVIII} (P=0.001) remained independent predictors of shorter cancer specific survival. On multivariate survival analysis for triple negative patients, tumour size (P<0.001), $LVI_{D2\text{-}40}$ (P=0.014) and BVI_{FVIII} (P<0.001) remained independently associated with cancer specific survival (Table 4.5).

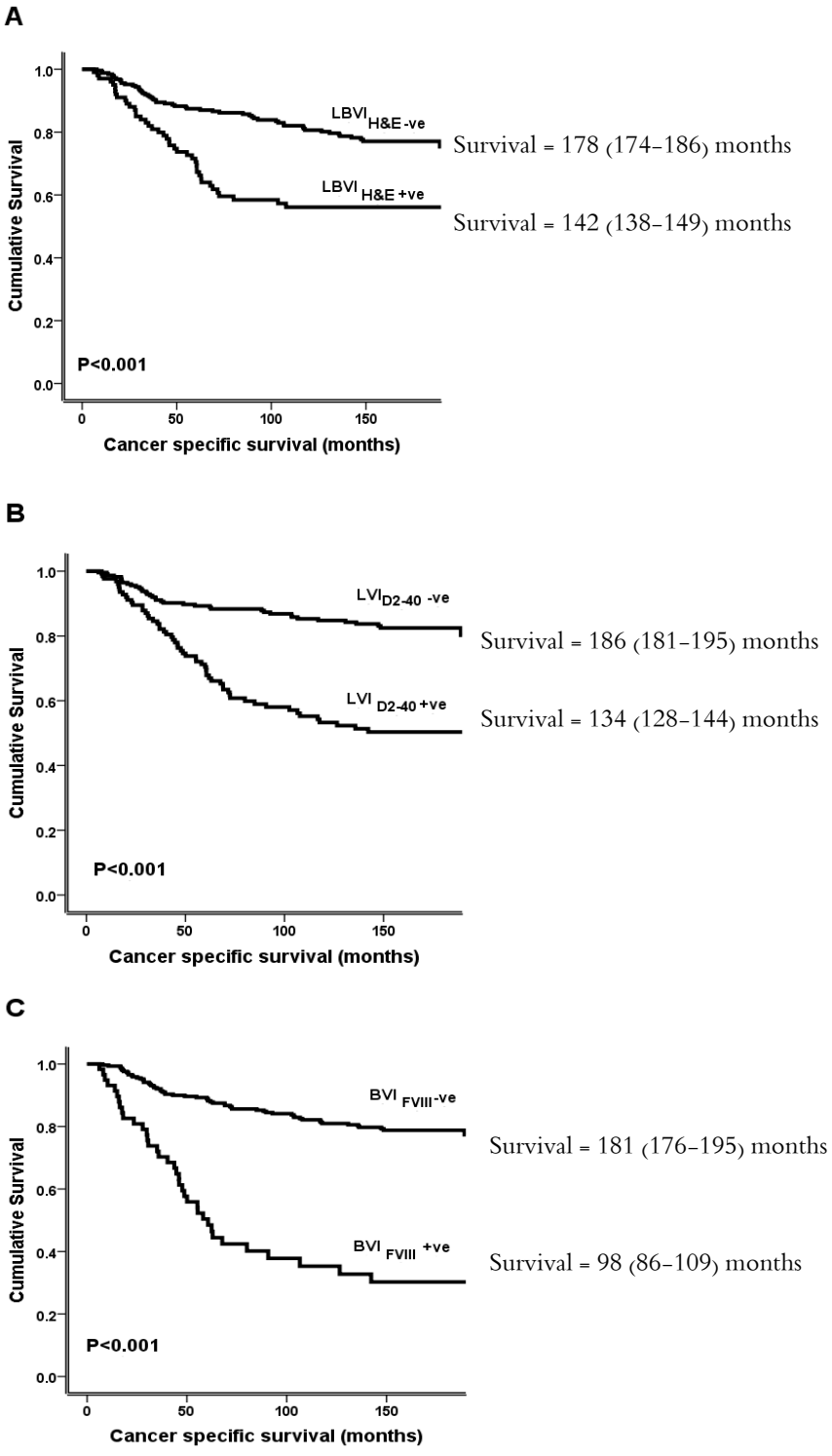


Figure 4-3 Comparison of Kaplan-Meier survival curves (Log rank) of cancer specific survival for (A) $LBVI_{H\&E}$, (B) LVI_{D2-40} and (D) BVI_{FVIII} in the whole cohort.

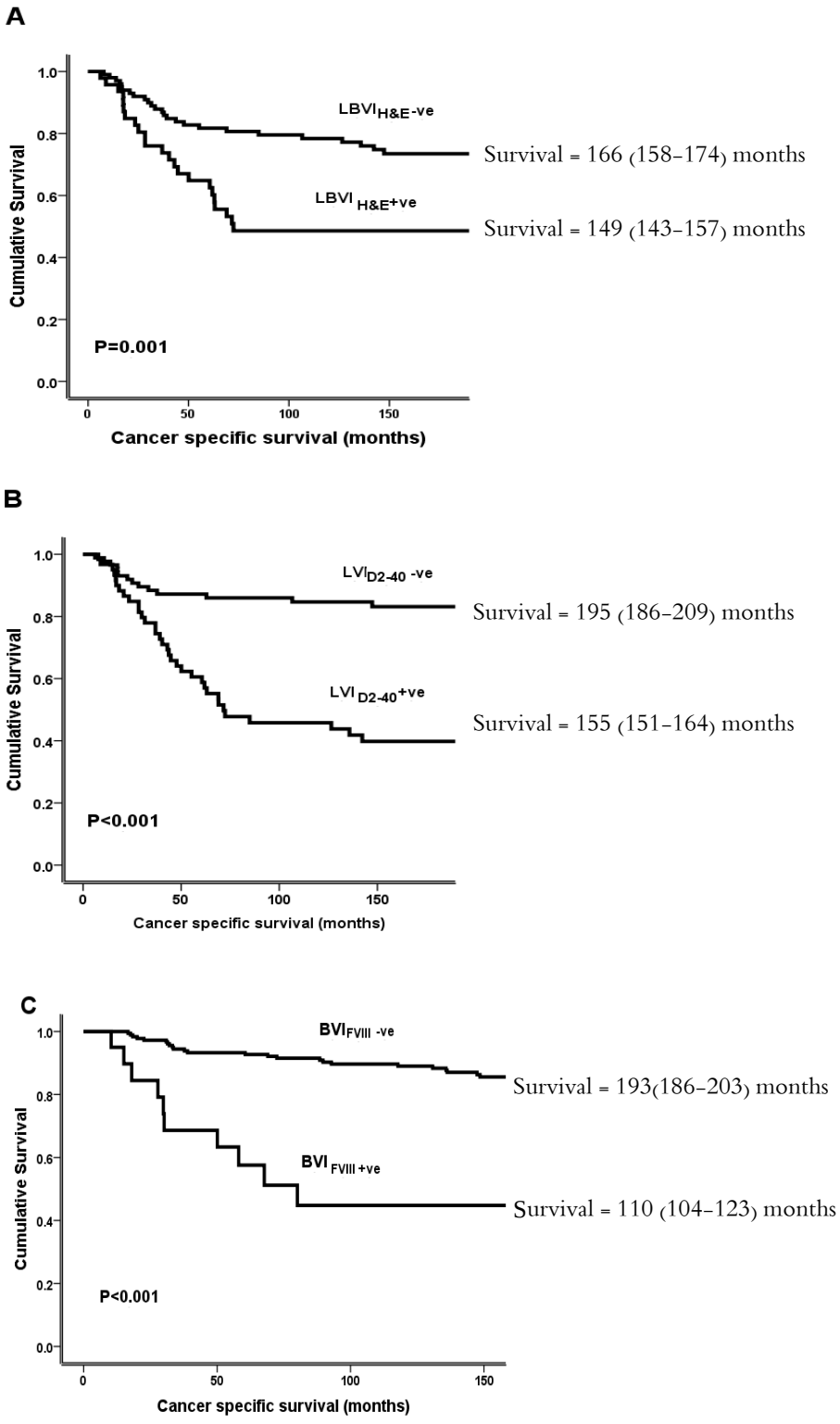


Figure 4-4 Comparison of Kaplan-Meier survival curves (Log rank) of cancer specific survival for (A) $LBVI_{H\&E}$, (B) $LVI_{D2\text{-}40}$ and (D) BVI_{FVIII} in node negative patients.

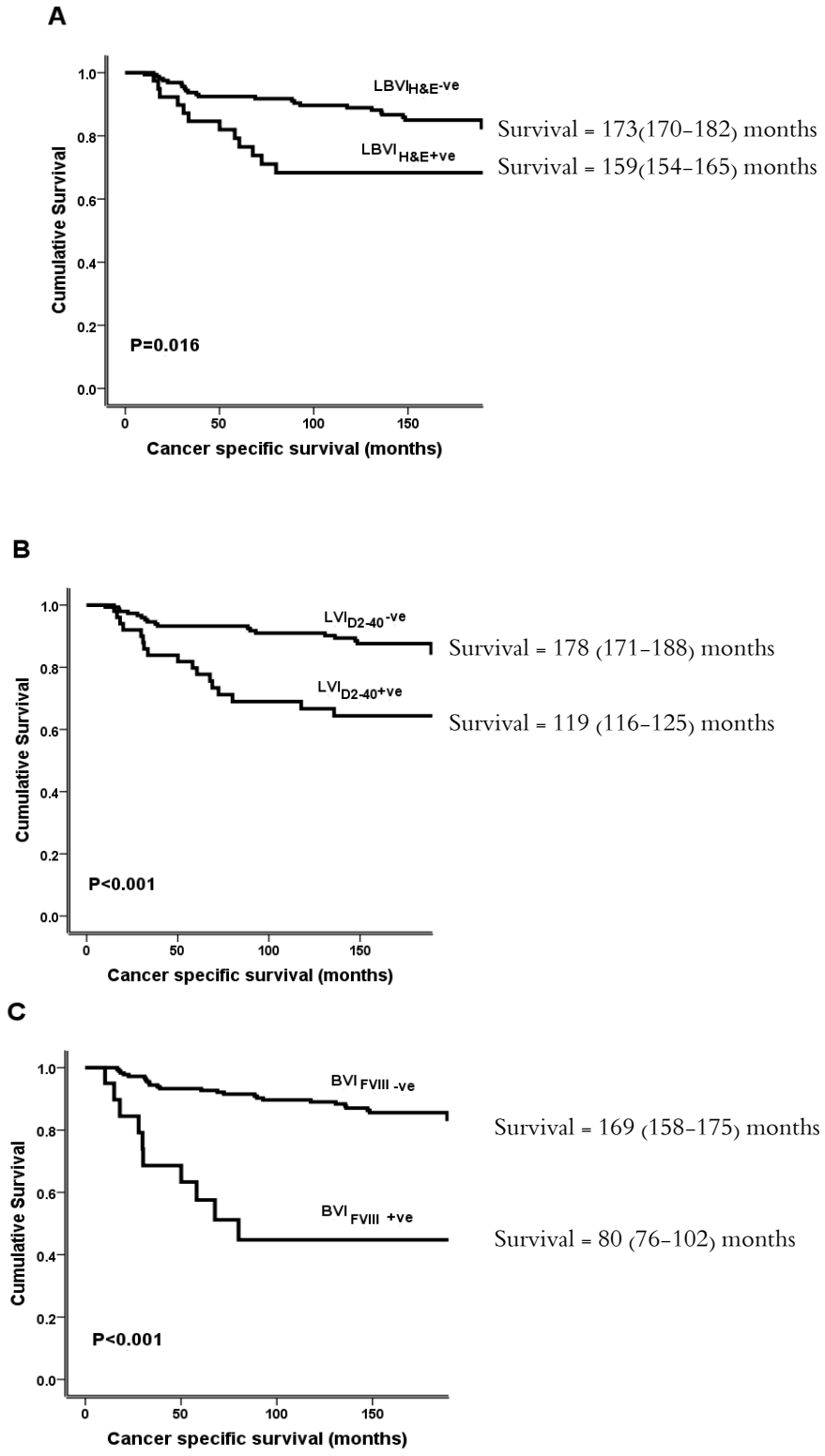


Figure 4-5 Comparison of Kaplan-Meier survival curves (Log rank) of cancer specific survival for (A) $LBVI_{H\&E}$, (B) $LVI_{D2\text{-}40}$ and (D) BVI_{FVIII} in triple negative patients.

Table 4-5 The relationship between clinicopathological characteristics and cancer specific survival in patients with invasive ductal breast cancer

All patients (n=360)	**Univariate analysis**		**Multivariate analysis**	
	Hazard ratio (95% CI)	***P*-value**	**Hazard ratio (95% CI)**	***P*-value**
Age (≤50/ >50 years)	0.97(0.64-1.45)	0.861		
Size (≤20/ 21-50/ >50 mm)	2.16(1.52-3.05)	<0.001	1.55(1.08-2.24)	0.017
Grade (I / II / III)	1.84(1.31-2.57)	<0.001	1.49(1.05-2.24)	0.026
Involved lymph node (-ve/+ve)	2.83(1.87-4.28)	<0.001	1.72(1.01-2.68)	0.016
ER (no/yes)	0.68(0.45-1.01)	0.055		0.541
PR (no/yes)	0.64(0.43-0.97)	0.033		0.316
Her-2 status (no/ yes)	1.34(0.84-2.14)	0.216		
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	2.01(1.27-3.19)	0.003		0.115
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	1.11(0.93-1.34)	0.229		
$LBVI_{H\&E}$ (absent/present)	2.39(1.61-3.54)	<0.001		0.173
$LVI_{D2\text{-}40}$ (absent/present)	3.31(2.19-4.97)	<0.001	1.71(1.08-2.69)	0.022
BVI_{FVIII} (absent/present)	5.12(3.38-7.78)	<0.001	3.19(2.01-5.04)	<0.001
Node negative patients (n=206)				
age (≤50/ >50 years)	0.69(0.36-1.36)	0.290		
Size (≤20/ 21-50/ >50 mm)	2.33(2.32-3.31)	0.007	1.92(1.04-3.59)	0.038
Grade (I / II / III)	1.64(1.64-2.74)	0.061		0.230
ER (no/yes)	0.83(0.43-1.62)	0.594		

PR (no/yes)	0.81(0.41-1.59)	0.812		
Her-2 status (no/ yes)	2.11(1.03-4.31)	0.040		0.368
LBVI $_{H\&E}$ (absent/present)	2.43(1.21-4.89)	0.010		0.645
LVI $_{D2-40}$ (absent/present)	3.24(1.67-6.29)	0.001	2.30(1.15-4.57)	0.008
BVI$_{FVIII}$ (absent/present)	6.03(2.87-13.77)	<0.001	4.23(2.96-9.71)	0.001
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	2.10(0.01-4.39)	0.047		0.162
Systemic treatment (hormonal/hormonal + chemotherapy/chempotheray/none)	0.83(0.39-1.72)	0.544		
Triple negative patients (n=102)				
age (<50/ >50 years)	1.13(0.54-2.36)	0.739		
Size (≤20/ 21-50/ >50 mm)	3.07(1.72-5.43)	<0.001	3.23(1.71-6.08)	<0.001
Grade (I / II / III)	0.70(0.33-1.50)	0.364		
Involved lymph node (-ve/+ve)	3.99(1.81-8.79)	0.001		0.099
LBVI $_{H\&E}$ (absent/present)	2.48(1.12-5.15)	0.016		0.179
LVI$_{D2-40}$ (absent/present)	3.44(1.62-7.32)	<0.001	2.62(1.25-5.28)	0.014
BVI $_{FVIII}$ (absent/present)	7.38(3.43-16.11)	<0.001	3.79(1.74-6.08)	<0.001
Locoregional treatment (Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	1.98(0.87-4.48)	0.100		
Systemic treatment (hormonal/hormonal + chemotherapy/chemotherapy/none)	0.53(0.89-1.34)	0.579		

4.4 Discussion

The results of the present study show that LBVI$_{H\&E}$, LVI$_{D2\text{-}40}$ and BVI$_{FVIII}$ all predicted tumour recurrence and cancer specific survival in an observational cohort of patients with early breast cancer. These results make a case for routine clinical assessment of lymphatic and blood vessel invasion by IHC to ascertain LVI and BVI.

In the present study, the proportion of patients with LBVI$_{H\&E}$ (28%) was consistent with most previous studies of breast cancer compared with (22-48%) in the literature, (20%) compared with (11-28%) for patients with node negative tumour, and (30%) compared with (24-45%) for patients with triple negative tumour (chapter 3). Similarly, in terms of the association between LBVI$_{H\&E}$ and other well-established high risk features such as tumour size, LN status, tumour grade, and breast cancer recurrence and survival are consistent with previous studies. Therefore, the present cohort is consistent with previous reports in which the prognostic value of LBVI$_{H\&E}$ has been established.

In the present study, the proportion of patients with LVI$_{D2\text{-}40}$ (35%) was consistent with most previous studies using a similar approach (28-46%), (26%) compared with (21-27% %) for patients with node negative tumour, and (35%) compared with (26-41%) for patients with triple negative tumour (chapter 3). LVI$_{D2\text{-}40}$ was associated with other well-established high risk features such as tumour size, LN status, tumour grade, and with tumour recurrence. In addition, the presence of LVI$_{D2\text{-}40}$ was significantly associated with increased locoregional treatment.

Furthermore, the presence of LVI$_{D2\text{-}40}$ provided independent prognostic information not only in the whole cohort but also in the subgroup of patients with lymph node negative and triple negative breast cancer. These results are consistent with recent studies that assessed LVI objectively using D2-40 (Shoppmann et al., 2004; Mohammed et al., 2007; Yamauchi et al., 2007; Mohammed et al., 2011). Thus, the present study confirms that D2-40

staining is a practical and effective way of identifying endothelial cells lining lymphatic vessels in patients with early breast cancer, in particular node negative disease. These findings suggest that $LVI_{D2\text{-}40}$ might usefully be incorporated into the routine clinical pathological staging of patients with breast cancer.

In the present study, the proportion of patients with BVI (Factor VIII) was lower than that of previous studies by Kato and colleagues that used a similar approach (16%) compared to (27-29%) in the whole cohort and (10%) compared to (18%) in node negative patients (Kato et al., 2000; Kato et al., 2002; Kato et al., 2003). Given that Kato and colleagues did not use a specific lymphatic marker such as D2-40 to differentiate between lymphatic and blood vessels and that Factor VIII has been found to be occasionally reactive to lymphatic endothelium, it may be that the higher rate reported by Kato and co-workers reflects LVI being assessed as BVI. Moreover, the present cohort would not explain the large discrepancy between the present BVI rate and that reported by Mohammed and colleagues (Mohammed et al., 2007; Mohammed et al., 2011) of only 0.7% of cases. Clearly, further prospective work is required across multiple centres to standardise the reporting of BVI, an important determinant of outcome in primary operable ductal breast cancer.

The results of the present study show for the first time the significance of BVI in triple negative breast cancer. This is an important finding, because currently used clinicopathologic and molecular markers, including the recent multigene assays, have a limited prognostic value in this molecular subtype. Most of these tumours are of high grade and exhibit poor prognosis gene signatures (Fan et al., 2006; Desmedt et al., 2008; Wirapati et al., 2008). Triple negative tumours have also been found to metastasise to the brain and lung suggesting that this subtype may prefer haematogenous spreading (Luck et al., 2008). Thus, objective assessment of BVI may provide additional independent prognostic information for this clinically important subgroup, in whom risk stratification and decisions about systemic therapy need to be determined.

The results of the present study suggest that BVI is less frequent than LVI in breast cancer, consistent with previous studies (Lee et al., 1990; Lauria et al., 1995; Kato et al., 2003; Van den Eynden et al., 2006). This would suggest that LVI is potentially a more important route of breast cancer spread. However, results of the present study show that twenty one of 206 patients (10%) without lymph node metastases had BVI. Blood vessel invasion in patients without lymph node metastases may explain the subsequent development of metastatic disease.

It is recognised that D2-40 may stain myoepithelial cells of the normal breast ducts and ductal carcinoma in situ (DCIS) especially in small ducts completely filled by solid-pattern DCIS (Schoppmann et al., 2001; Kaiserling et al., 2004; Arigami et al., 2005). There is evidence that p63 staining may be useful in distinguishing D2-40 positive myoepithelium. However, this would increase the complexity of the present approach for routine clinical pathological analysis. Moreover, with awareness that myoepithelium may also be immunoreactive largely obviates this problem. Specifically, the tumour growth pattern enables distinction of ductal carcinoma in situ from lymphovascular invasion. Also, the myoepithelium is discontinuous in small ducts whereas the endothelial lining of the lymphatic vessels is continuous and the myoepithelial cells of larger ducts are larger than the endothelial cells of lymphatic vessels (Arnaout-Alkarain et al., 2007). Finally, the distribution of the stain for the myoepithelial cells is recognised to be patchy and the intensity less than that of the adjacent lymphatic endothelium (Rabban and Chen, 2008). Therefore, increase in sensitivity of detection of lymphatic vessel invasion may be reasonably attributed to the demarcation of lymphatic endothelium that stains positively for D2-40 around the tumour emboli and although, D2-40 may also bind to myoepithelium of breast ducts, it is not difficult to distinguish between myoepithelial reactivity and endothelial staining of the vessels.

Factor VIII has been previously reported as a blood vessel endothelial marker in breast cancer and is consistently found in normal endothelial cells in blood vessels. While it occasionally stains endothelial cells in lymphatics, staining of lymphatic endothelium is usually faint and discontinuous (Martin et al., 1987; Saigo and Rosen, 1987; Kao et al., 2002). Some studies have suggested that the vascular marker CD31 may be superior to factor VIII for blood vessels staining (Horak et al., 1992; Fox et al., 1994). However, another study reported that the higher sensitivity of CD31 of vascular endothelium did not yield results more discriminating for predicting survival outcome than results produced with factor VIII (Gasparini et al., 1994).

In the present study, although the value of lymphovascular invasion detected using IHC was significantly correlated with the value of lymphovascular invasion detected using H&E (P<0.001), $LBVI_{H\&E}$ had a 22% false negative and a 9% false positive compared with that of $LBVI_{IHC}$. In those patients, adjuvant chemotherapy treatment, to reduce the risk of cancer recurrence, would have been missed, which may affect patients' outcome negatively. In addition, patients with false positive results might have been offered unnecessary cytotoxic treatment. This would indicate that the frequency of detection of lymphovascular invasion increased using IHC, and that the IHC detection method has more sensitivity and specificity than that of H&E.

These lesions were difficult to identify on the H&E sections due to invasion into small lymphatic or blood vessels or due to vessels that had been obscured by tumour cells. Thirty four patients had tumours that were $LBVI_{H\&E}$ positive, were negative for both $LVI_{D2\text{-}40}$ and BVI_{FVIII}. A recognised explanation for such a discrepancy is that stromal retraction artifacts, caused by tissue handling and fixation, on H&E sections cause false positives (Bettelheim et al., 1984; Saigo and Rosen, 1987, Hoda et al., 2006; Van den Eynden et al., 2006). In addition, the H&E approach has considerable inter-observer variability and lower overall detection rate in most previous studies (chapter 3.0).

The results of the present and previous studies point to a substantial improvement in the consistency of reporting and an increase in the rate of detection of LBVI, LVI and BVI in patients with breast cancer cases using an IHC approach (Bettelheim et al., 1984; Saigo and Rosen, 1987; Schoppmann et al., 2001; Arnaout-Alkarain et al., 2007; Mohammed et al., 2007).

A limitation of the present study was that intra- and peri-tumoral lymphovascular invasion were not separately analysed owing to the small number of cases with intra-tumoural foci compared to that of peri-tumoural ones. This precluded meaningful analysis of each component but was unlikely to materially influence the concordance between the detection of $LBVI_{H\&E}$ and $LBVI_{IHC}$. Nevertheless, the results are of interest and make a case for further studies of routine clinical assessment of lymphatic and blood vessel invasion by IHC to ascertain LVI and BVI.

In summary, the results of the present study show that IHC for D2-40 and Factor VIII defined lymphatic and blood vessel invasion with greater sensitivity and specificity than H&E, and improved detection of LVI and BVI in early invasive breast cancer. Moreover, the prognostic significance of the $LVI_{D2\text{-}40}$ and BVI_{FVIII} was superior to that of $LBVI_{H\&E}$ and this was consistent throughout analysis of sub-cohorts. Therefore, these results make the case for their assessment in routine clinical and pathological practice and to be incorporated into the existing staging systems.

Chapter 5 The relationship between the tumour stroma percentage, tumour microenvironment and survival in patients with invasive ductal breast cancer

5.1 Introduction

Breast cancer is a heterogeneous disease with different responses to treatment and variable outcomes. Therefore, there is still a need for new prognostic and predictive markers helpful of selecting patients with high risk and aggressive diseases who might benefit from adjuvant and targeted therapy.

Like other sloid tumour, development and progression of breast cancer is not solely dependent on the intrinsic properties of cancer cells but also on the interaction between the tumour and the surrounding microenvironment (Colotta et al., 2009; Hanahan and Weinberg, 2011). Recent evidence suggests that the tumour stroma itself is now increasingly appreciated, influencing tumour growth, angiogenesis and dissemination. Tumour stroma is thought to promote tumourigenesis by different mechanisms including remodelling of the ECM, regulation of the tumour immune response, and alterations in stromal regulatory pathways affecting the motility and aggressiveness of cancer cells (Kim et al., 2005; Hu and Polyak, 2008; Cirri and Chiarugi, 2012; Criscitiello et al., 2014).

It has been reported that tumour stroma has prognostic value in patients with colorectal (Mesker et al., 2007; West et al., 2010; Huijbers et al., 2013; Park et al., 2014) and esophageal cancers (Staal et al., 2010; Wang et al., 2012). Also, the percentage of tumour stroma has been recently reported to have prognostic value in patients with triple negative (de Kruijf et al., 2011; Moorman et al., 2012) and node negative breast cancer (Dekker et al., 2013). Indeed, assessment of the proportion of tumour stroma using routine pathological specimens may act as a surrogate for tumour stroma activity and its subsequent effect on survival.

It is not clear, however, whether the effect of an expanded tumour stroma on survival is independent of host local inflammatory responses and other components of the tumour microenvironment. Moreover, the relationship between tumour stroma, host and tumour characteristics remain unknown. Therefore, the aim of the present study was to examine the relationship between the percentage of tumour to stroma, host inflammatory response, clinicopathological characteristics and outcome in patients with early breast cancer, in particular node negative and triple negative disease.

5.2 Patients and methods

5.2.1 Patients

361 patients with primary operable invasive ductal breast cancer, whose routine haematoxylin and eosin sections were available from patients described in section 2.1 were included in this study.

5.2.2 Methods

The assessment of ER, PR, Her-2, Ki67 proliferative index, CD68+ macrophage infiltrate, CD4+ T-lymphocyte infiltrate, CD8+ T-lymphocyte infiltrate was performed as previously described in sections 2.2.2.

Scanned routine H&E sections for the 361 patients were used to score general peri-tumoural inflammatory infiltrate using Klintrup–Mäkinen grade and tumour necrosis as previously described in chapter 2.0.

The assessment of lymphatic and blood vessel invasion was performed as previously described in chapter 4.0.

5.2.2.1 Slide scanning and scoring

Routine H&E sections for the 361 patients were scanned at objective magnification x20 as previously described in section 2.2.3.

5.2.2.2 Aassessment of tumour stroma

Assessment of tumour stroma percentage (TSP) on H&E scanned slides was carried out at the most invasive tumour area according to previously described criteria (Mesker et al., 2007). As Slidepath provides different levels of magnification similar to a conventional microscope, the most invasive tumour area to be analysed was identified visually and

selected using a ×4 or ×5 magnifications. The magnification was then set to 10x at the selected area where both stroma and tumour tissue were available. Tumour cells must be present at all borders of the image field (north–east–south–west) (Figure 5.1). When necrotic and mucinous tissue was present within the selected area, the mucinous and necrotic tissue was visually excluded form the scoring. Scoring percentages were given per tenfold (10, 20, 30% etc.).

Cut-off at 50% TSP was used as described in previous reports (Mesker et al., 2007; de Kruijf et al., 2011) that is; stroma low tumours were the presence of tumour stroma in ≤50 % of tumour area (Figure 5.1A) whereas, stroma high tumours were the presence of tumour stroma in more than 50 % of tumour area (Figure 5.1B).

A total of 40 specimens were independently estimated for TSP by two observers (FG and JE) blinded to patient outcome and the other observer's score. The ICCC was 0.83 indicating excellent agreement. The author FG then scored the rest of slides.

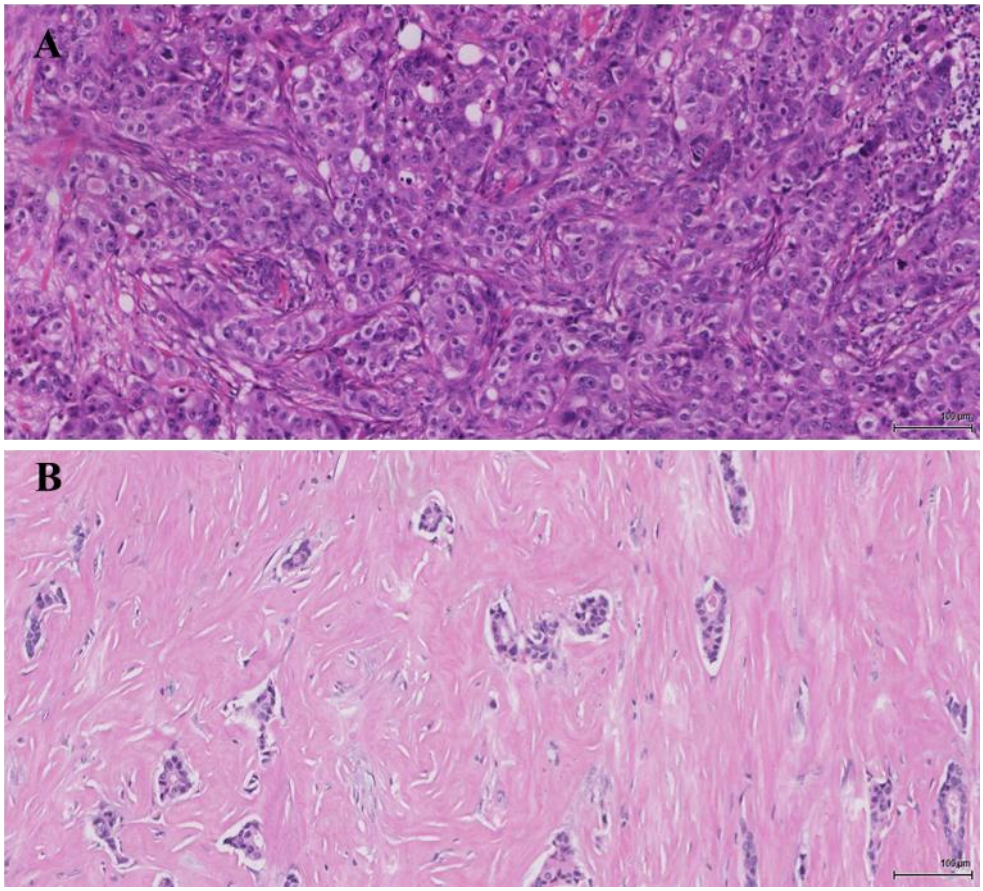


Figure 5-1 H&E stained sections of invasive ductal breast tumours showing examples of tumour stroma percentage

(A) tumour with low stroma (10%); (B) tumour with high stroma (80%). 10x objective and 100µm scale.

5.2.2.3 Statistical analysis

Consistency between the observers was analysed using the ICCC value using Reliability Analysis. Inter-relationships between variables were assessed using contingency table analysis with the chi-squared test for trend as appropriate. Univariate and multivariate survival analysis were performed using the Kaplan-Meier analysis and Cox proportional hazards model with calculation of hazard ratios (HR) and 95% confidence interval (95% CI). A stepwise backward procedure was used to derive a final model of the variables that had a significant independent relationship with survival. All statistical analyses were 2-sided and significance defined as *P*-value <0.05. All statistical analysis was performed using the SPSS software version 19 (SPSS Inc., Chicago,IL, USA).

5.3 Results

5.3.1 Clinicopathological characteristics

Table 5.1 shows clinicopathological characteristics of patients (n=361). The majority were older than 50 years (65%), had a grade III carcinoma (53%) equal or smaller than 2 cm (51%) with no axillary lymph node involvement (57%). The majority had ER positive tumours (59%), PR negative tumours (54%) and Her-2 negative tumours (80%). 36% had LVI, 16% had BVI and the majority had a high-grade tumour necrosis (64%). 60% had low-grade general peri-tumoural inflammatory infiltrate with the cellular inflammatory infiltrates (CD68+macrophage infiltrate, CD4+T-lymphocyte infiltrate and CD8+T-lymphocyte infiltrate) presented as tertiles (Table 5.1). In all, 81 (22%) patients received only tamoxifen, 144 (40%) received only chemotherapy and 45 (13%) received both.

5.3.2 Tumour stroma percentage (TSP)

For all patients TSP was evaluated on one section derived from the most invasive part of the tumour. The tumour specimens showed variety in TSP ranging from very solid tumours with little stromal involvement (Figure 5.1A) to tumours with large areas of stromal proliferation scattered with single and grouped tumour cells (Figure 5.1B). In total, 252 (70%) patients had low TSP (≤50% stroma) and 109 (30%) patients had high TSP (>50% stroma). In node negative patients, 153(74%) patients had low TSP and 54 (26%) had high TSP. In triple negative patients, 81 (79%) patients had low TSP and 22 (21%) had high TSP.

Table 5-1 The clinicopathological characteristics of patients with invasive ductal breast cancer (n=361)

Clinicopathological characteristics	Patients (n%)
Age (≤50/ >50 years)	125(35%)/236(65%)
Size (≤20/ 21-50/ >50 mm)	185(51%)/163(45%)/13(4%)
Grade (I / II / III)	48(13%)/124(34%)/189(53%)
Involved lymph node (-ve/+ve)	207(57%)/154(43%)
ER status (no/yes)	149(41%)/212(59%)
PR status (no/yes)	193(54%)/168(47%)
Her-2 status (no/yes)	290(80%)/71(20%)
Lymphatic vessel invasion (no/yes)	233(65%)/128(36%)
Blood vessel invasion (no/yes)	302(84%)/59(16%)
Tumour necrosis (low/high)	131(36%)/230(64%)
Klintrup–Mäkinen grade (low/high)	215(60%)/146(40%)
CD68+macrophage infiltrate [a]	82(23%)/115(32%)/103(29%)
CD4+T-lymphocyte infiltrate	132(37%)/112(31%)/117(32%)
CD8+T-lymphocyte infiltrate	121(34%)/118(33%)/122(34%)
Tumour stroma percentage (≤50%/>50%)	252(70%)/109(30%)
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	130(36%)/231(64%)
Systemic adjuvant therapy (hormonal/hormonal + chemotherapy/chemotherapy/none) [a]	81(22%)/45(13%)/144(40%)/84(23%)
Tumour recurrence (no/yes)	272(75%)/89(25%)
Alive/cancer death/non cancer death	189(52%)/97(27%)/75(21%)

a Number of patients when incomplete data available

5.3.3 Association of TSP with clinicopathological variables and outcome

The relationship between TSP, clinicopathological variables and host inflammatory response is shown in tables 5.2-5.4. Patients with high TSP were old age women (*P*=0.035), had more involved lymph node (0.049), Her-2 positive tumours (*P*=0.029), low-grade general peri-tumour inflammatory infiltrate (*P*=0.034), low CD68+macrophage infiltrate (*P*<0.001), low CD4+ (*P*=0.023) and low CD8+ T-lymphocytes infiltrate (*P*=0.017), had tumour recurrence (*P*=0.015) and shorter cancer specific survival (*P*=0.001). In node negative patients (n=207), a high TSP was associated with low CD68+ macrophage infiltrate (*P*=0.001), low CD4+ (*P*=0.040) and low CD8+ T-lymphocytes infiltrate (*P*=0.016), and shorter cancer specific survival (*P*=0.005). In triple negative patients (n=103) a high TSP was associated with increased tumour size (*P*=0.017), high tumour grade (*P*=0.014), low CD8+ T-lymphocyte (*P*=0.048) and shorter cancer specific survival (*P*=0.041). A high TSP was not associated with hormonal status, LVI, BVI and tumour necrosis.

To examine the association between the expansion of tumour stroma and tumour proliferation, a sub-analysis of the relationship between the TSP and Ki67 index in different patient groups was performed (Table 5.5). Only 59% of patients from the whole cohort, 44% from node negative group and 65% from triple negative group had Ki67 information available. There was no significant statistical difference between TSP high and low groups in all sub-cohorts.

Table 5-2 The relationship between clinicopathological characteristics and TSP in patients with invasive ductal breast cancer (n=361)

	TSP≤50% n=252(70%)	**TSP>50% n=109(30%)**	**(*P*-value)**
Age (≤50/ >50 years)	96/156	29/80	0.035
Size (≤20/ 21-50/ >50 mm)	136/108/8	49/55/5	0.109
Grade (I / II / III)	35/77/140	13/47/49	0.289
Involved lymph node (-ve/+ve)	153/99	54/55	0.049
ER status (no/yes)	107/145	42/67	0.487
PR status (no/yes)	135/117	58/51	0.950
Her-2 status (no/ yes)	210/42	80/29	0.029
Lymphatic vessel invasion (no/ yes)	163/89	70/39	0.933
Blood vessel invasion (no/ yes)	215/87	37/22	0.195
Tumour necrosis (low/high)	91/161	40/69	0.915
Klintrup–Mäkinen grade (low/high)	141/111	74/35	0.034
CD68+macrophage infiltrate (tertiles)	40/84/80	42/31/23	<0.001
CD4+T-lymphocyte infiltrate (tertiles)	66/111/75	36/54/19	0.023
CD8+T-lymphocyte infiltrate (tertiles)	71/80/101	73/46/26	0.017
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	93/159	37/72	0.591
Systemic adjuvant therapy (hormonal/ hormonal + chemotherapy/chemotherapy/none)	55/35/97/61	30/21/35/23	0.104
Tumour recurrence (no/yes)	199/53	73/36	0.015
Alive/cancer death/non cancer death	151/55/46	38/42/29	<0.001
Cancer specific survival (months)[a]	176(168-186)	144(128-160)	<0.001

a=Mean (95%CI)

Table 5-3 The relationship between clinicopathological characteristics and TSP in node negative patients (n=207).

	TSP ≤50% n=153(74%)	**TSP >50% n=54(26%)**	**(*P*-value)**
Age (≤50/ >50 years)	54/99	13/41	0.131
Size (≤20/ 21-50/ >50 mm)	90/60/3	30/23/1	0.709
Grade (I / II / III)	26/45/82	8/28/18	0.123
ER status (no/yes)	68/85	20/34	0.345
PR status (no/yes)	87/66	27/27	0.385
Her-2 status (no/ yes)	128/25	41/13	0.208
Lymphatic vessel invasion (no/yes)	112/41	41/13	0.857
Blood vessel invasion (no/yes)	139/47	14/7	0.426
Tumour necrosis (low/high)	62/91	21/33	0.488
Klintrup–Mäkinen grade (low/high)	89/64	39/15	0.068
CD68+macrophage infiltrate (tertiles)	27/49/46	24/13/10	0.001
CD4+T-lymphocyte infiltrate (tertiles)	36/67/50	17/28/9	0.040
CD8+T-lymphocyte infiltrate (tertiles)	41/52/60	22/20/12	0.016
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	65/88	24/30	0.803
Systemic adjuvant therapy (hormonal/hormonal + chemotherapy/ chemotherapy/none)	35/12/55/49	15/8/16/15	0.251
Recurrence status (no/yes)	130/23	41/13	0.133
Alive/cancer death/non cancer death	102/20/31	124/35/48	0.002
Cancer specific survival (months)[a]	192(183-201)	164(144-184)	0.005

a=Mean (95%CI)

Table 5-4 The relationship between clinicopathological characteristics and TSP in triple negative patients with invasive ductal breast cancer (n=103)

	TSP≤50% n=81(79%)	**TSP>50% n=22(21%)**	**(*P*-value)**
Age (≤50/ >50 years)	38/43	7/15	0.208
Size (≤20/ 21-50/ >50 mm)	48/31/2	8/11/5	0.017
Grade (I / II / III)	0/10/71	1/6/15	0.014
Involved lymph node (-ve/+ve)	52/29	11/11	0.228
Lymphatic vessel invasion (no/yes)	51/30	15/7	0.563
Blood vessel invasion (no/yes)	71/10	17/5	0.223
Tumour necrosis (no/yes)	21/60	5/17	0.761
Klintrup–Mäkinen grade (low/high)	32/49	10/12	0.124
CD68+macrophage infiltrate (tertiles)	15/16/25	8/2/5	0.132
CD4+T-lymphocyte infiltrate (tertiles)	13/33/35	7/8/7	0.123
CD8+T-lymphocyte infiltrate (tertiles)	20/17/44	8/8/6	0.048
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	30/51	11/11	0.273
Systemic adjuvant therapy (hormonal/ hormonal+ chemotherapy/ chemotherapy/none)	9/6/47/4	3/3/11/5	0.202
Tumour recurrence (low/high)	83/32	21/15	0.119
Alive/cancer death/non cancer death	52/20/9	9/9/4	0.076
Cancer specific survival (months)[a]	176(167-185)	147(133-163)	0.041

a=Mean (95% CI)

Table 5-5 The inter-relationship between TSP and Ki67 in patients with invasive ductal breast cancer

	TSP≤50%	**TSP>50%**	**(*P*-value)**
All patients (n=214)	**n=115(76%)**	**n=36(24%)**	
Ki67 (low/high)	122/27	54/11	0.833
Node negative patients (n=120)	**n=89(74%)**	**n=31(26%)**	
Ki67 (low/high)	75/14	28/3	0.407
Triple negative patients (n=99)	**n=76(77%)**	**n=23(23%)**	
Ki67 (low/high)	61/15	19/4	0.803

The minimum follow-up of survivors was 142 months and the median follow-up was 168 months. During follow-up 89 patients developed recurrence (25%), 172 patients died, 27% died of their cancer.

The 15-year cancer specific survival rate was 79% *v* 21% in the TSP low group *v* TSP high group. Kaplan Meier survival curves show that high TSP was significantly associated with poorer cancer specific survival in the whole cohort ($P<0.001$), in node negative patients ($P=0.005$) and in triple negative patients ($P=0.041$) (Figure 5.2 A-C). In multivariate survival analysis, a high TSP was associated with reduced cancer specific survival independent of other variables in the whole cohort (HR 1.85, 95% CI 1.18-2.91, $P=0.007$) (Tables 5.6) and in node negative patients (HR 3.32, 95% CI 1.43-7.75, $P=0.005$) (Tables 5.7) but not in triple negative patients ($P=0.555$) (Table 5.8).

The relationship between TSP, clinicopathological characteristics and survival in patients who underwent adjuvant systemic treatment was examined. In total, 270 (75%) patients from the whole cohort, 135 (65%) with node negative patients and 79 (77%) with triple negative patients received adjuvant systemic treatment. In the whole cohort (Table 5.6), a high TSP was associated with shorter cancer specific survival following adjuvant treatment in univariate analysis (HR 2.04, 95% CI 1.29-3.22, $P=0.002$). On multivariate analysis, a high TSP was associated with reduced cancer specific survival (HR 1.89, 95% CI 1.13-3.16, $P=0.016$), independent of LVI, BVI, tumour necrosis and CD68+T-lymphocyte infiltrate. In node negative patients, a high TSP showed a trend towards shorter cancer specific survival ($P=0.071$) (Table 5.7). In triple negative patients, a high TSP was not associated with shorter cancer specific survival following adjuvant treatment ($P=0.257$) (Table 5.8).

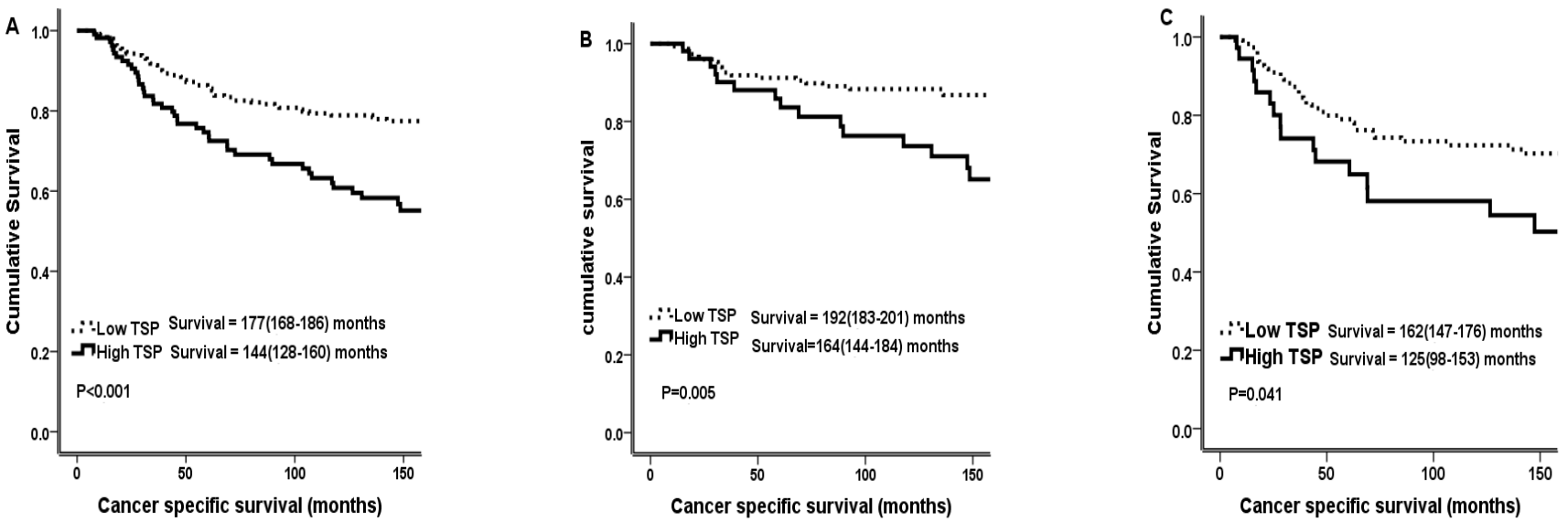


Figure 5-2 Kaplan-Meier survival curves (Log rank) of cancer specific survival for tumour stroma percentage.

In (A) the whole cohort, (B) node negative patients and (C) triple negative patients.

Table 5-6 The relationship between clinicopathological characteristics and cancer specific survival in the whole cohort

	Univariate analysis		**Multivariate analysis**	
	Hazard ratio(95% CI)	*P*-value	Hazard ratio(95% CI)	*P*-value
All patients (n=361)				
Age (<50/ >50 years)	0.97 (0.64-1.46)	0.881		
Size (≤20/ 21-50/ >50 mm)	2.17 (1.54-3.07)	<0.001		0.120
Grade (I / II / III)	1.85 (1.3-2.58)	<0.001	1.49 (1.02-2.20)	0.042
Involved lymph node (-ve/+ve)	1.97 (1.51-2.56)	<0.001	1.75 (1.08-2.83)	0.023
ER status (no/yes)	0.68 (0.45-1.01)	0.055		0.346
PR status (no/yes)	0.64 (0.42-0.96)	0.033		0.762
Her-2 status (no/ yes)	1.44 (0.88-2.35)	0.145		
Lymphatic vessel invasion (no/yes)	3.01 (2.39-4.95)	<0.001	2.14(1.31-3.49)	0.002
Blood vessel invasion (no/yes)	4.98(3.39-7.72)	<0.001	3.49(2.07-5.91)	<0.001
Tumour necrosis (low/high)	1.97 (1.29-2.99)	0.002	2.65 (1.50-4.68)	0.001
Klintrup–Mäkinen grade (low/high)	1.15 (0.77-1.73)	0.482		
CD68+T-lymphocyte infiltrate (tertiles)	0.73 (0.55-0.96)	0.025	0.51 (0.38-0.69)	0.001
CD4+T-lymphocyte infiltrate (tertiles)	0.46 (0.23-1.70)	0.075		0.321
CD8+T-lymphocyte infiltrate (tertiles)	0.64 (0.49-0.82)	<0.001	0.62 (0.51-0.89)	0.014
Tumour stroma percentage (≤50%/>50%)	1.89 (1.26-2.82)	<0.001	1.85 (1.18-2.91)	0.007
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	2.01(1.27-3.19)	0.003		0.621

Systemic adjuvant therapy (hormonal/hormonal + chemotherapy/ chemotherapy/none)	1.15 (0.71-1.87)	0.573		
Systemic adjuvant therapy(n=270)				
Size (≤20/ 21-50/ >50 mm)	1.44 (0.96-2.15)	0.080		0.876
Grade (I / II / III)	1.66 (1.13-2.43)	0.010		0.223
Involved lymph node (-ve/+ve)	1.78 (1.31-2.40)	<0.001		0.187
PR status (no/yes)	0.47 (0.27-0.83)	0.009		0.530
Lymphatic vessel invasion (no/yes)	3.78 (2.34-6.08)	<0.001	3.21 (1.82-5.65)	<0.001
Blood vessel invasion (no/yes)	4.61 (2.87-6.08)	<0.001	4.29 (1.45-4.96)	<0.001
Tumour necrosis (low/high)	2.53 (1.41-4.52)	0.002	2.68 (1.45-4.96)	0.003
CD68+T-lymphocyte infiltrate (tertiles)	0.63 (0.46-0.86)	0.004	0.47 (0.33-0.68)	<0.001
CD4+T-lymphocyte infiltrate (tertiles)	0.89 (0.67-1.19)	0.456		
CD8+T-lymphocyte infiltrate (tertiles)	0.78 (0.56-1.03)	0.841		
Tumour stroma percentage (≤50%/>50%)	2.04 (1.29-3.22)	0.002	1.89 (1.13-3.16)	0.016
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	1.93 (1.15-3.26)	0.013		0.725

Table 5-7 The relationship between clinicopathological characteristics and cancer specific survival in patients with node negative breast cancer (n=207)

	Univariate analysis		**Multivariate analysis**	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio(95% CI)	*P*-value
Age (<50/ >50 years)	0.70 (0.36-1.36)	0.290		
Size (≤20/ 21-50/ >50 mm)	2.32 (1.25-4.31)	0.007	1.88 (0.95-3.72)	0.070
Grade (I / II / III)	1.64 (0.97-2.73)	0.062		0.170
ER (no/yes)	0.83 (0.43-1.62)	0.595		
PR (no/yes)	0.81 (0.41-1.59)	0.548		
Her-2 status (no/ yes)	2.11 (1.03-4.31)	0.040		0.676
Lymphatic vessel invasion (-ve/+ve)	3.32 (1.67-6.29)	0.001	2.31(1.11-4.84)	0.026
Blood vessel invasion (no/yes)	5.38 (2.57-12.28)	<0.001	4.15(3.38-19-63)	0.001
Tumour necrosis (low/high)	1.97 (1.48-8.59)	0.005	3.08 (1.21-7.83)	0.018
Klintrup–Mäkinen grade (low/high)	1.47 (0.76-2.86)	0.255		
CD68+macrophage infiltrate (tertiles)	0.68 (0.43-1.07)	0.096		0.055
CD4+T-lymphocyte infiltrate (tertiles)	0.88 (0.59-1.32)	0.520		
CD8+T-lymphocyte infiltrate (tertiles)	0.89 (0.59-1.33)	0.558		
Tumour stroma percentage (≤50%/>50%)	2.24 (1.29-4.97)	0.005	3.32 (1.43-7.75)	0.005
Locoregional therapy.(Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	2.11 (1.01-4.39)	0.047		0.256

Systemic adjuvant therapy (hormonal /hormonal + chemotherapy /chemotherapy/none)	1.18 (0.57-2.42)	0.657		
Systemic adjuvant therapy (n=135)				
Size (≤20/ 21-50/ >50 mm)	1.02 (0.46-2.28)	0.954		
Grade (I / II / III)	1.27 (0.70-2.29)	0.431		
Her-2 status (no/ yes)	2.34 (1.00-5.43)	0.050	3.25 (0.94-6.47)	0.081
Lymphatic vessel invasion (no/yes)	3.10 (1.02-5.36)	0.001	3.66 (1.43-9.36)	0.007
Blood vessel invasion (no/yes)	4.87 (2.10-15.45)	0.001	12.4 (3.91-38.98)	0.002
Tumour necrosis (no/yes)	2.34 (0.87-6.28)	0.090	3.46 (1.08-11.09)	0.037
CD68+macrophage infiltrate (tertiles)	0.49 (0.28-0.87)	0.014	0.44 (0.23-0.82)	0.010
Tumour stroma percentage (≤50%/>50%)	2.12 (0.94-4.78)	0.071	2.54 (0.93-6.96)	0.078
Locoregional therapy.(Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	1.84 (0.79-4.31)	0.158		

Table 5-8 The relationship between clinicopathological characteristics and cancer specific survival in patients with triple negative breast cancer (n=103)

	Univariate analysis		**Multivariate analysis**	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio(95% CI)	*P*-value
Age (<50/ >50 years)	1.13 (0.54-2.36)	0.740		
Size (≤20/ 21-50/ >50 mm)	3.07 (1.73-5.48)	<0.001	2.79 (1.43-5.45)	0.002
Involved lymph node (-ve/+ve)	3.99 (1.82-8.79)	0.001	2.26 (0.96-5.37)	0.063
Grade (I / II / III)	0.70 (0.34-1.51)	0.364		
Lymphatic vessel invasion (no/yes)	3.44(1.62-7.31)	<0.001	2.43(1.63-5.77)	0.023
Blood vessel invasion (no/yes)	4.73(3.42-10.09)	<0.001	3.75 (1.75-9.48)	0.002
Tumour necrosis (low/high)	5.74 (1.36-24.16)	0.017	3.59 (0.82-15.75)	0.089
Klintrup–Mäkinen grade (low/high)	1.12 (0.53-2.34)	0.770		
CD68+macrophage infiltrate (tertiles)	0.71 (0.42-1.18)	0.183		
CD4+T-lymphocyte infiltrate (tertiles)	1.07 (0.65-1.76)	0.803		
CD8+T-lymphocyte infiltrate (tertiles)	0.94 (0.61-1.43)	0.756		
Tumour stroma percentage (≤50%/>50%)	1.06 (1.03-1.12)	0.041		0.555
Locoregional therapy.(Lumpectomy+ radiotherapy/mastectomy + radiotherapy)	1.98 (0.88-4.48)	0.100		
Systemic adjuvant therapy (hormonal/ hormonal + chemotherapy/	0.914 (0.56-1.39)	0.678		

chemotherapy/none)				
Systemic adjuvant therapy (n=79)				
Size (≤20/ 21-50/ >50 mm)	1.81(0.92-3.62)	0.085		0.252
Involved lymph node (-ve/+ve)	4.17 (1.64-10.59)	0.003		0.131
Lymphatic vessel invasion (no/yes)	4.05 (1.06-9.87)	0.002	3.13 (1.26-7.65)	0.014
Blood vessel invasion (no/yes)	7.93(3.36-18.69)	<0.001	6.12 (2.53-14.09)	0.001
Tumour necrosis (low/high)	4.04 (0.95-17.23)	0.060		0.186
CD8+T-lymphocyte infiltrate (tertiles)	0.78 (0.53-1.12)	0.178		
Tumour stroma percentage (≤50%/>50%)	1.72 (0.68-4.36)	0.257		
Locoregional therapy.(lumpectomy+ radiotherapy/mastectomy + radiotherapy)	2.63 (1.04-6.69)	0.042	2.72 (1.23-5.98)	0.013

5.4 Discussion

The results of the present study show that high TSP was consistently associated with low tumour inflammatory infiltrate. Furthermore, TSP was associated with poorer outcome in the whole cohort and in patients with node negative and triple negative disease with long term follow-up. Taken together the present results highlight the importance of the stroma in the tumour microenvironment and its impact on outcome.

Consistent with previous reports of the role of tumour stroma in breast cancer (de Kruijf et al., 2011; Moorman et al., 2012; Dekker et al., 2013) survival in the present study was significantly shorter in patients with high TSP tumours. However, TSP was not independently associated with survival in triple negative patients in the present study, whereas de Kruijf and co-workers reported that TSP was an independent prognostic factor. The difference between these findings might be attributed to the differences in cohorts size, patients' characteristics or might be due to treatment regimen undertaken though in both studies patients did not receive neoadjuvant treatment. Irrespective, from previous work it was not clear whether the effect of an expanded tumour stroma on survival was independent of host inflammatory response and other components of the tumour microenvironment.

Although the interrelationships between the tumour stroma, tumour microenvironment and gross pathological characteristics are likely complex, the tumour stroma percentage remained independently and more strongly associated with reduced cancer specific survival. These results confirm the importance of tumour-host factors, such as the tumour microenvironment in determining oncological outcome.

In particular, node negative patients with high TSP had a more than triple times higher risk of breast cancer death compared to those with low TSP, independent and comparable to that of tumour size, LVI and necrosis. Furthermore, survival was also significantly shorter

in patients who received adjuvant therapy and had high-TSP tumours. Thus, in addition to identifying patients at high risk, TSP may also select patients less likely to benefit from standard therapy and who should be considered for additional adjuvant treatment, potentially targeted at the stroma itself (Engels et al., 2012).

Despite recognition of the importance of the tumour stroma in cancer progression, its relationship with other components of the tumour microenvironment has yet to be fully characterised. In the present study, increased amount of tumour stroma was associated with a low-grade tumour inflammatory infiltrate, as measured by the Klintrup–Mäkinen score and by macrophages and T-cell subtypes. This is consistent with the recent observation that a high TSP trended toward a low general peri-tumoural inflammatory infiltrate in patients with colorectal cancer (Park et al., 2014). However, the underlying mechanism is still unclear.

The interactions between breast stroma and inflammatory cells are not fully understood. It has previously been proposed that the tumour stroma may prevent effective tumour infiltration by immune cells (Ueno et al., 2004). The results from cell line experiments would also support our findings, namely that fibroblasts and myofibroblasts can modulate the ability of lymphocytes and macrophages to invade a tumour and may prevent penetration of immune cells within tumours, creating a physical barrier against an immune reaction while promoting tumour growth and progression, due to their contractile properties and their associated extracellular matrix (Lieubeau et al., 1999).

In the present study, although the cell markers of both innate and adaptive immune cells were examined, the effect of TSP on survival remained independent of local inflammatory responses, suggesting the presence of other mechanisms rather than a direct effect on immune cells. Indeed, tumour stroma may promote the development of a pro-tumour rather than anti-tumour immune infiltrate (Fridman et al., 2011). Stroma-associated

fibroblasts or CAFs may also induce suppression of the immune response and produce immunosuppressive molecules such as TGF-β and VEGF, suggesting that CAFs may promote cancer immunoescape (Yaguchi et al., 2011; Engels et al., 2012). This may implicate certain cell signalling pathways such as the common cell signalling pathway associated with inflammation; the JAK-STAT pathway (Yu et al., 2007; Yu et al., 2009). Therefore, further characterisation of the tumour inflammatory cells infiltrate and their association with tumour stroma and JAK-STAT signalling are warranted.

The main potential limitation of the present study was that direct investigation of the effect of tumour stroma on the infiltration of inflammatory cells was not carried out. This would require either cell line or animal models. Although cell line or animal models do have the advantage of allowing direct investigation of the effect of tumour microenvironment on inflammatory cell infiltration, they often lack clinical relevance to the patient with breast cancer. In particular, based on such models, progress on immunotherapy for breast cancer has been slow over the last 4 decades. The present study highlights the importance of the amount of tumour stroma on immunological response in patients with invasive ductal breast cancer.

In conclusion, the results of the present study show that a high tumour stroma percentage in primary operable invasive ductal breast cancer was associated with recurrence and shorter long-term survival. Implementing this simple and reproducible parameter in routine pathological examination may help optimize risk stratification in patients with invasive ductal breast cancer. The present study findings suggest that high TSP enables tumour cells to evade the immune surveillance and promote tumour progression.

Chapter 6 The relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer

6.1 Introduction

With more molecular and genomic measurements of breast cancer have emerged, it is clear for such disease that the need to effectively stratify patients according to likely outcome remains important. This should be done against a comprehensive characterisation of the tumour and its microenvironment.

Recently, the tumour budding which refers to detachment of single or cluster of up to five cancer cells scattered in stroma at the invasive front of tumour (Ueno et al., 2002; Prall et al., 2005; Lugli et al., 2009) has been proposed as an important determinant of progression and survival in a number of solid cancers (Hase et al., 1993; Ueno et al., 2002; Prall et al., 2005; Choi et al., 2007; Koike et al., 2008; Masugi et al., 2010; Taira et al., 2012; Koyuncuoglu et al., 2012). In particular, tumour budding is thought to be an early step in cancer metastasis as postulated to be linked to the process of epithelial-mesenchymal transition (EMT) (Masugi et al., 2010; Zlobec and Lugli., 2010; Taira et al., 2012; Koyuncuoglu et al., 2012, Lugli et al., 2012; Liang et al., 2013; Dawson and Lugli., 2015), which is a crucial step during carcinoma progression and metastasis (Kalluri and Weinberg, 2009).

In breast cancer, there is still limited information about the role of tumour budding (Liang et al., 2013; Salhia et al., 2015). Liang and colleagues has reported the significance of budding in small breast cancer cohort (n=160) with limited follow-up and only reported budding effect on overall survival but not cancer specific survival (Liang et al., 2013). The second report examined the association of tumour budding and clinicopathological characteristics however, survival analysis was not reported (Salhia et al., 2015)

Moreover, it is not clear, whether the effect of an increased tumour budding on survival is independent of host inflammatory response and other components of the tumour microenvironment. Therefore, the present study aims to examine the relationship between tumour budding, the tumour microenvironment and survival in patients with invasive ductal breast cancer.

6.2 Patients and method

6.2.1 Patients

474 patients with primary operable invasive ductal breast cancer, whose routine haematoxylin and eosin sections were available from patients described in section 2.1 were included in this study.

6.2.2 Methods

Assessment of ER, PR, Her-2, Ki67 proliferation index, CD68+ macrophage infiltrate, CD4+ T-lymphocyte infiltrate, CD8+ T-lymphocyte infiltrate and CD138+ plasma cell infiltrate was performed as previously described in chapter 2.0. Scanned routine H&E sections for the 474 patients were used to score general peri-tumoural inflammatory infiltrate and tumour necrosis as previously described in chapter 2.0.

The assessment of lymphatic and blood vessel invasion was performed as previously described in chapter 4.0. Tumour stroma percentage (TSP) was also assessed on scanned H&E sections for the 474 patients as previously reported in chapter 5.0.

The molecular subtypes were defined as follows: Luminal A: oestrogen (ER) and/or progesterone receptor (PR) positive, Her-2 negative, low proliferative index (≤15%); Luminal B: hormone receptor positive, Her-2 positive/or high proliferative index (>15%); Her-2 subtype: Her-2 positive and hormone receptor negative, any proliferative index; triple negative: Her-2 negative, hormone receptor negative, any proliferative index.

6.2.2.1 Slide scanning and scoring

Routine haematoxylin and eosin sections for the 474 patients were scanned at objective magnification x20 as previously described in chapter 2.0.

6.2.2.2 Assessment of tumour budding

Scanned routine H&E sections for the 474 patients were used to score tumour budding at the deepest tumour invasion margin according to previously published protocol (Ueno et al, 2002). At ×5 magnification, an area representative of the tumour invasive margin was selected. A grid of 0.385 mm^2 size at five highest budding areas was drawn. Using a × 20 magnification a tumour budding was counted. A bud was identified as an isolated single cancer cell or a group of up to five cancer cells (Ueno et al., 2002; Prall et al., 2005; Lugli et al., 2009) (Figure 6.1). The highest bud count per field was used as the number of buds. Areas of necrosis or mucin were excluded from the field.

To ensure reliability, co-scoring of 60 randomly selected cases was carried out by (FG) and consultant pathologist (JJG). The inter-observer intraclass correlation coefficient (ICCC) for the raw continuous scores was 0.81 ($P<0.001$). All the slides were then scored by (FG).

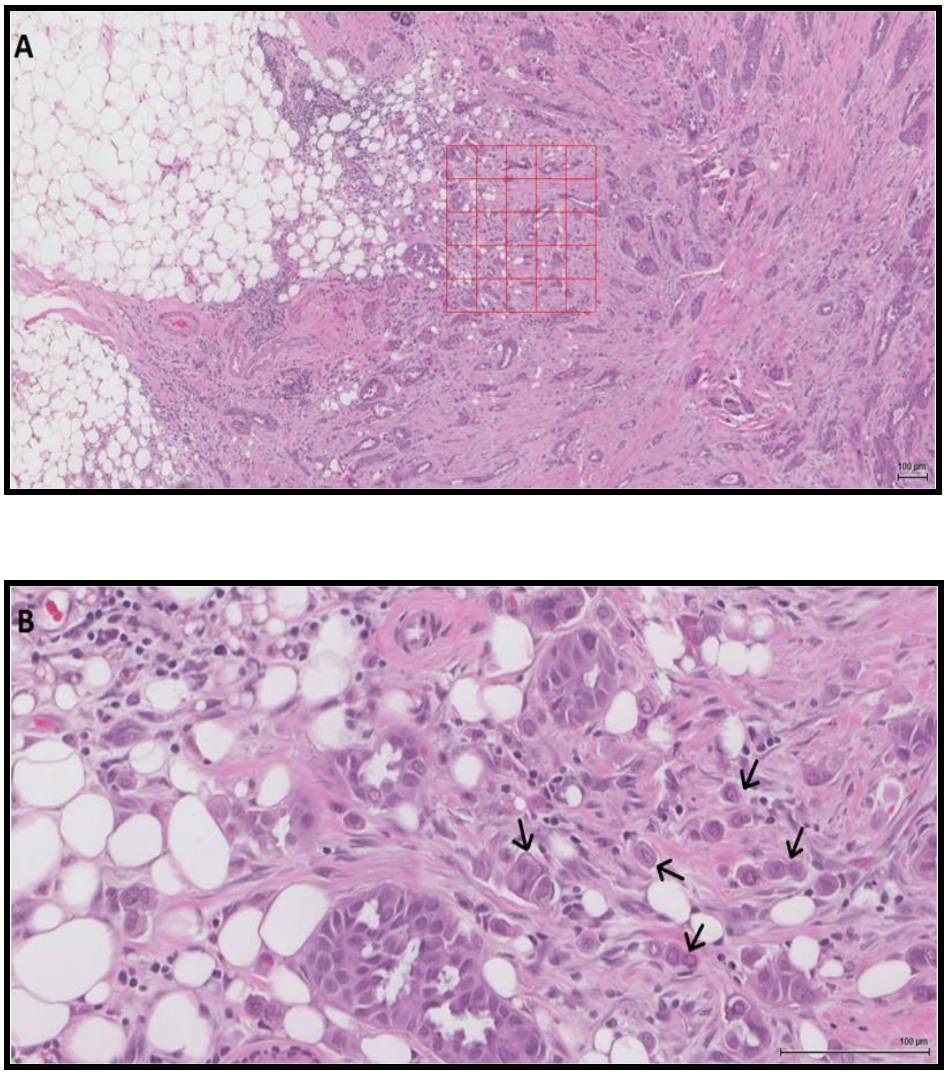


Figure 6-1 H&E stained sections of invasive ductal breast cancer showing examples of tumour budding.

(A) Shows a grid of high tumour budding area at the invasive margin, (B) shows single and clusters of tumour budding (arrows). Original magnification x20, scale 100 µm.

6.2.2.3 Statistical analysis

To identify the cut-off value of tumour budding for survival analysis, the highest budding count per 5 fields were split into tertiles and survival analysis between each group using Kaplan–Meier log-rank test was performed (Figure 6.2) (Choi et al., 2007; Sy et al., 2010). Subsequently, the first and second tertiles (highest tumour budding count was ≤20) were considered as low budding group and the third tertile (highest tumour budding was **>**20) was considered as high budding group. To simplify all further analysis, patients were subsequently grouped into low tumour budding (≤20) and high tumour budding (>20).

When ROC analysis was carried out with cancer specific survival as an end-point, the optimal number of tumour buds was between 15 (sensitivity=0.55, specificity=0.70) and 20 buds (sensitivity =0.63, specificity = 0.60) per 5 fields. Therefore, the threshold was set at 20 buds. At this threshold the AUC was 0.625, (P<0.001). This was consistent with the threshold derived from the plot of the tertiles (see Figure 6.2).

Consistency between the observers was analysed using the ICCC value. The relationships between variables were assessed using contingency table analysis with the x^2 test for linear trend. Kaplan–Meier analysis was used to examine the effect of tumour budding on cancer specific survival. Univariate survival analysis was performed using Cox proportional hazards regression. Variables with P-value of <0.1 were entered into a multivariable model using a backwards conditional method for all patients, node negative patients, and those who have low TSP and high K-M score. All statistical analyses were two-sided and significance defined as P-value <0.05. All statistical analysis was performed using the SPSS software version 22 (IBM SPSS, IL, USA).

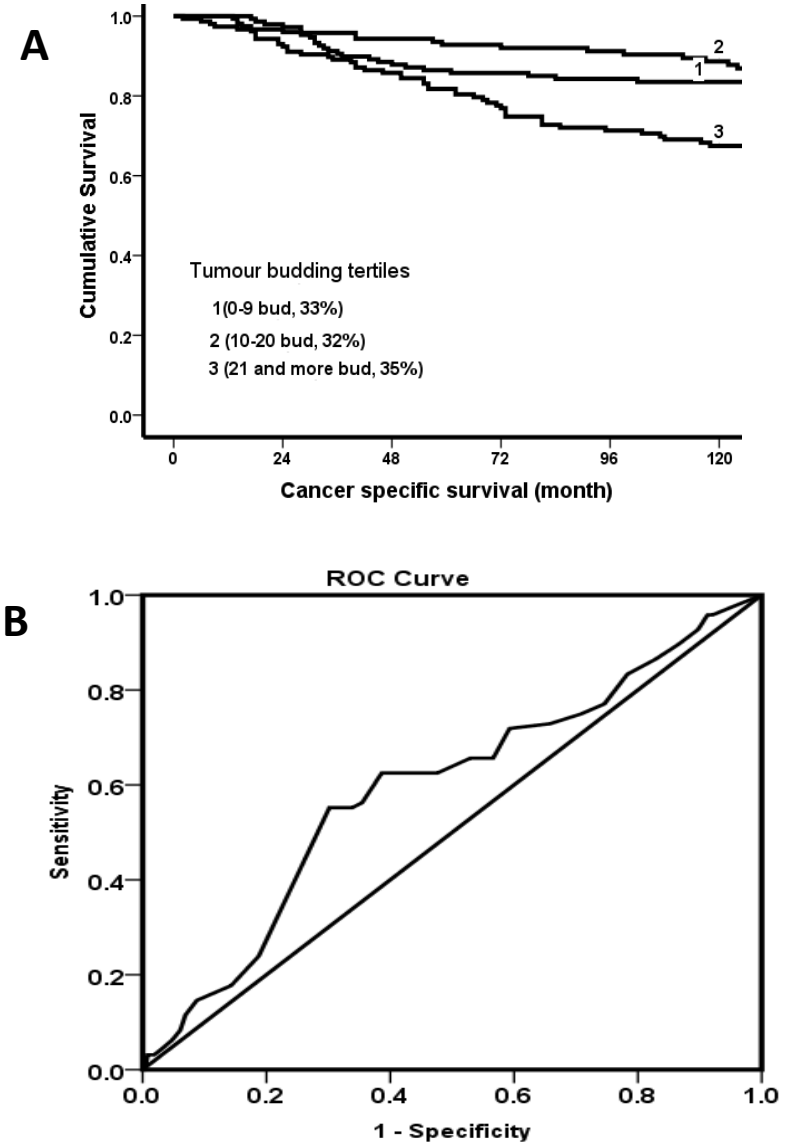


Figure 6-2 Kaplan-Meier survival curve (Log rank) and Roc curve for tumour budding.

A-Shows Kaplan-Meier curve according to tumour budding turtiles. First tertile (1) includes patients with the highest budding count/5 fields = 0-9 buds. Second tertile (2) includes patients with the highest budding count/5 fields = 10-20 buds. Third tertile (3) includes patients with the highest budding count/5 fields ≥ 21 buds. B-Shows Receiver operating characteristic (ROC) curve aiding the selection of the cut-off score for the low-grade versus high-grade budding. The optimal number of tumour buds was between 15 (sensitivity=0.55, specificity=0.70) and 20 buds (sensitivity =0.63, specificity = 0.60).

6.3 Results

Table 6.1 summarises clinicopathological characteristics of patients (n=474). The majority of patients (70%) were older than 50 years, had small tumour size ≤20mm (60%), had grade II and III tumours (80%) and negative lymph node (54%). The majority had ER positive (69%) tumours, PR positive tumours (61%) and Her-2 negative tumours (80%). 182 (38%) had lumpectomy and radiotherapy, and 292 (62%) had mastectomy and radiotherapy. 243 (51%) patients received tamoxifen only, 101 (21%) patients received adjuvant chemotherapy only, and 95 (20%) had both. 221 (47%) of patients had luminal A tumours, 111 (23%) had luminal B tumours, 31 (7%) had Her-2 positive and 78 (18%) had triple negative tumours. A high tumour budding was identified in 167 (35%) patients.

The relationship between tumour budding, clinicopathological characteristics, local host inflammatory response and TSP is presented in Table 6.2. Tumour budding was not significantly associated with age, size, grade, necrosis, Ki67 and BVI. A high tumour budding was associated with ER positive status (P=0.003), lymph node positive tumours (P=0.009), presence of LVI (P<0.001), and high TSP (P=0.001). A high tumour budding was inversely associated with local tumour inflammatory response as measured by the Klintrup–Mäkinen grade (P=0.002), but not by macrophage, plasma cells and T-cell lymphocyte subtypes.

The relationship between clinicopathological characteristics and tumour budding in node negative patients is presented in Table 6.3. A high tumour budding was associated with presence of LVI (P<0.001) and inversely associated with local inflammatory response as measured by the Klintrup–Mäkinen grade (P=0.038). A high tumour budding showed a trend towards an association with TSP (P=0.080).

Table 6-1 The clinicopathological characteristics of patients with invasive ductal breast cancer (n=474)

	Patients, n (%)
Age (≤50/ >50 years)	140(30%)/334(70%)
Size (≤20/ 21-50/ >50 mm)	283(60%)/178(38%)/13(3%)
Grade (I / II / III)	94(20%)/190(40%)/190(40%)
Involved lymph node (-ve/+ve) [a]	257(54%)/212(45%)
ER status (no/yes) [a]	141(30%)/330(69%)
PR status (no/yes) [a]	180(38%)/289(61%)
Her-2 status (no/ yes) [a]	381(80%)/74(16%)
Lymphatic vessel invasion (no/yes)	327(69%)/147(31%)
Blood vessel invasion (no/yes)	419(88%)/55(12%)
Tumour necrosis (low/high)	226(48%)/248(52%)
Ki67 index (low/high) [a]	345(73%)/106(22%)
Klintrup–Mäkinen grade (low/high)	345 (73%)/129 (27%)
CD68+ (low/moderate/high) [a]	145(31%)/153(32%)/153(32%)
CD4+ (low/moderate/high) [a]	207(44%)/90(19%)/157(33%)
CD8+ (low/moderate/high) [a]	151(32%)/145(31%)/158(33%)
CD138+(low/moderate/high) [a]	254(54%)/55(12%)/143(30%)
Tumour stroma percentage (low/high)	320(68%)/154(32%)
Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	182(38%)/292(62%)
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none)	243(51%)/95(20%)/101(21%)/27(6%)
Alive/cancer death/non cancer death [a]	275(58%)/96(20%)/90(19%)

a Number of patients when incomplete data available.

Table 6-2 The relationship between clinicopathological characteristics and tumour budding in patients with invasive ductal breast cancer (n=474)

	Tumour budding≤20 n=307(65%)	**Tumour budding>20 n=167(35%)**	**(*P*-value)**
Age (≤50/ >50 years)	99/208	41/126	0.080
Size (≤20/ 21-50/ >50 mm)	186/114/7	97/64/6	0.469
Grade (I / II / III)	62/108/137	32/82/53	0.099
Involved lymph node (-ve/+ve)	180/124	77/88	0.009
ER status (no/yes) [a]	105/199	36/131	0.003
PR status (no/yes)	126/176	54/113	0.054
Her-2 status (no/ yes)	245/44	136/30	0.429
Lymphatic vessel invasion (no/yes)	232/75	94/73	<0.001
Blood vessel invasion (no/yes)	271/36	150/17	0.610
Tumour necrosis (low/high)	138/169	88/79	0.107
Ki67 index (low/high)	225/64	120/42	0.364
Klintrup–Mäkinen grade (low/high)	209/98	136/31	0.002
CD68+ (low/moderate/high)	99/88/101	46/65/52	0.708
CD4+ (low/moderate/high)	132/53/104	75/37/53	0.675
CD8+ (low/moderate/high)	97/79/113	54/66/45	0.173
CD138+(low/moderate/high)	164/27/97	90/28/46	0.687
Tumour stroma percentage (low/high)	224/83	98/69	0.001
Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	118/189	64/103	0.891
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none)	148/92/72/18	95/33/29/9	0.096
Alive/cancer death/non cancer death	199/43/55	76/53/35	0.002
Cancer specific survival (months)[a]	159 (153-164)	136 (127-145)	<0.001

a=Mean (95% CI)

Table 6-3 The relationship between clinicopathological characteristics and tumour budding in patients with node negative invasive ductal breast cancer (n=257)

	Tumour budding≤20 n=180(70%)	**Tumour budding>20 n=77(30%)**	**(*P*-value)**
Age (≤50/ >50 years)	49/131	23/54	0.666
Size (≤20/ 21-50/ >50 mm)	125/54/1	56/20/1	0.696
Grade (I / II / III)	45/62/73	18/41/18	0.137
ER status (no/yes)	57/122	18/59	0.173
PR status (no/yes)	74/103	23/54	0.072
Her-2 status (no/ yes)	151/21	66/10	0.835
Lymphatic vessel invasion (-ve/+ve)	153/27	50/27	<0.001
Blood vessel invasion (no/yes)	162/18	69/8	0.925
Tumour necrosis (low/high)	93/87	49/28	0.078
Ki67 index (low/high)	136/34	62/12	0.488
Klintrup–Mäkinen grade (low/high)	133/47	66/11	0.038
CD68+ (low/moderate/high)	65/52/52	26/26/23	0.747
CD4+ (low/moderate/high)	80/31/59	38/14/24	0.623
CD8+ (low/moderate/high)	60/46/64	26/31/19	0.313
CD138+(low/moderate/high)	102/15/52	45/15/15	0.393
Tumour stroma percentage (low/high)	224/83	98/69	0.080
Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	84/96	37/40	0.832
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none	105/20/36/17	54/6/8/8	0.142
Alive/cancer death/non cancer death	126/15/33	45/17/15	0.184
Cancer specific survival (months)[a]	167 (162-173)	150 (138-168)	0.001

a= Mean (95%CI)

The median survival of survivors was 164 months, with 96 deaths from breast cancer and 90 non-cancer deaths. 13(3%) patients do not have survival data and were excluded from all survival analysis. Mean cancer specific survival was shorter in patients with high tumour budding compared with those with low tumour budding (136 versus 159 months, $P<0.001$) (Figure 6.3A).

The relationship between tumour budding, clinicopathological characteristics and cancer specific survival is presented in Table 6.4. On univariate analysis, a high tumour budding was associated with shorter CSS ($P<0.001$). On multivariate analysis, a high tumour budding was associated with reduced CSS (HR 2.21, 95% CI 1.41-3.47, $P=0.001$), independent of PR status, nodal status, tumour necrosis, LVI, BVI, CD8+ T-lymphocyte infiltrate, CD138+ plasma cell infiltrate, TSP and locoregional treatment.

In node negative patients, a high tumour budding was associated with shorter mean CSS compared with a low tumour budding (150 versus 167 months, $P=0.001$) (Figure 6.3B). On multivariate survival analysis (Table 6.3), a high tumour budding was associated with reduced CSS (HR 3.18, 95% CI 1.46-6.93, $P=0.004$), independent of PR status, tumour necrosis, LVI, and TSP.

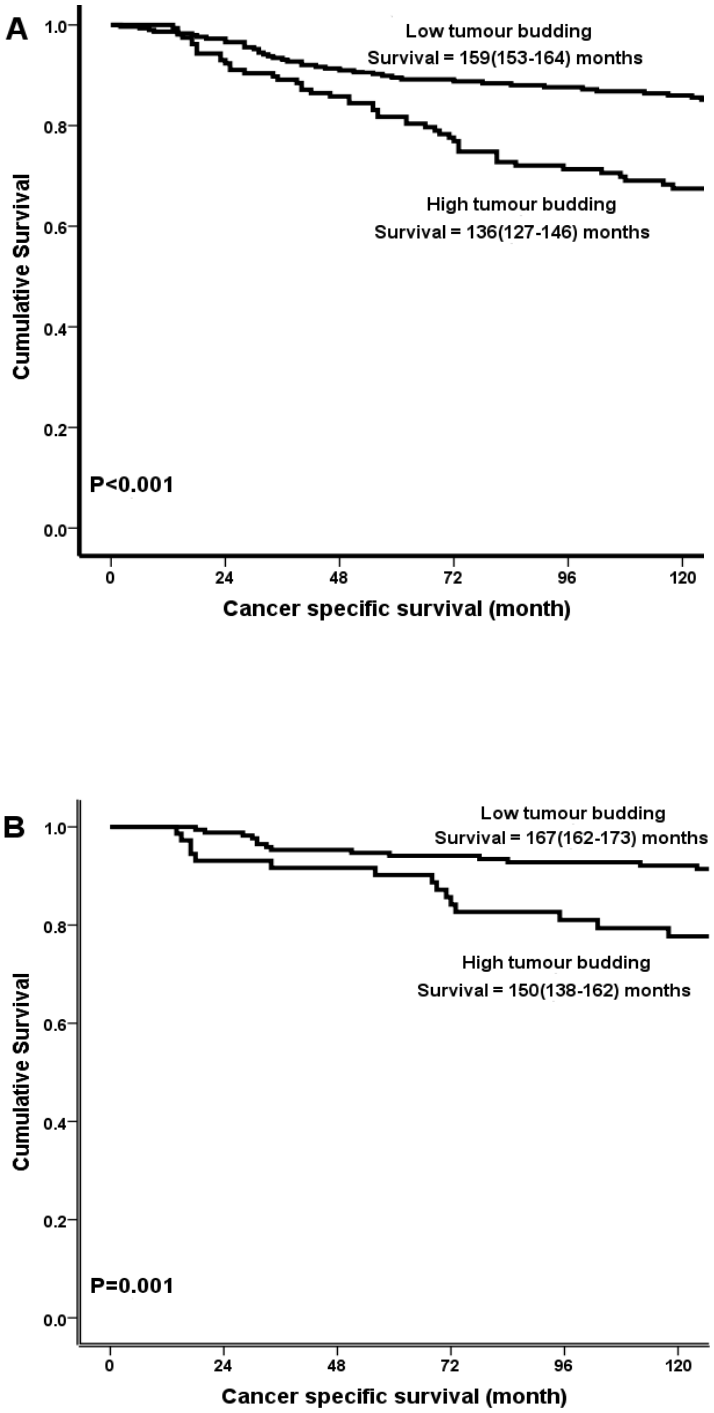


Figure 6-3 Kaplan-Meier survival curves (Log rank) of cancer specific survival (A) in all patients, and (B) in patients with node negative tumours.

In order to account for the high TSP and low cellular inflammatory infiltrate effects, sub-group survival analyses were performed based on low TSP and high Klintrup–Mäkinen grade (Figure 6.4 and Table 6.5). In stroma low patients, a high tumour budding was associated with shorter mean cancer specific survival compared with a low tumour budding (144 versus 162 months, *P*=0.002) (Figure 6.4A). On multivariate survival analysis, a high tumour budding was associated with reduced cancer specific survival (HR 2.44, 95% CI 1.35-4.40, *P*=0.003), independent LVI, BVI, tumour necrosis, CD68+ macrophage infiltrate and locoregional treatment (Table 6.5).

In patients with high Klintrup–Mäkinen grade, a high tumour budding was associated with shorter mean cancer specific survival compared with a low tumour budding (110 versus 151 months, *P*=0.003) (Figure 6.4B). On multivariate survival analysis, a high tumour budding was associated with reduced cancer specific survival (HR 2.56, 95% CI 1.23-5.36, *P* = 0.012) LVI, BVI, CD68+ macrophage infiltrate and TSP (Table 6.5).

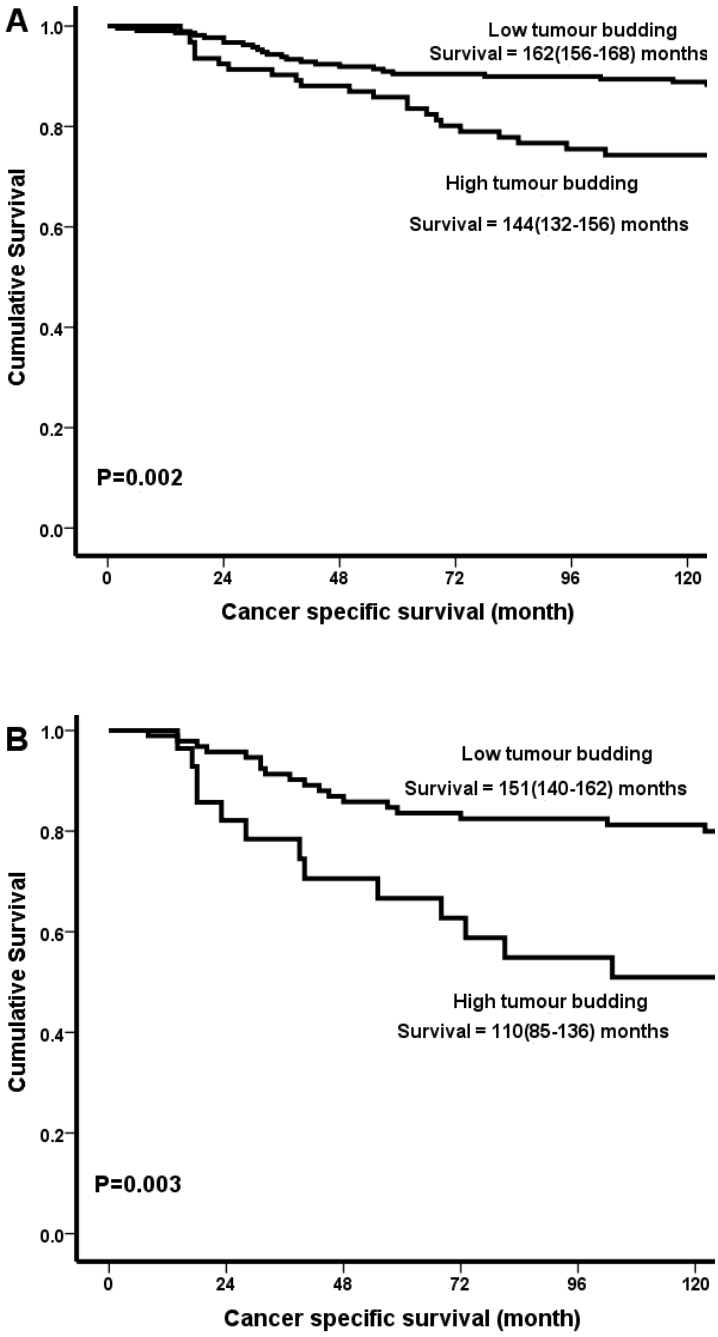


Figure 6-4 Kaplan-Meier survival curves (Log rank) of cancer specific survival (A) in patients with low TSP, and (B) in patients with high Klintrup–Mäkinen score.

Table 6-4 The relationship between clinicopathological characteristics and cancer specific survival in patients with invasive ductal breast cancer

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	***P*-value**	**Hazard ratio(95% CI)**	***P*-value**
All patients (n=461)				
Age (<50/ >50 years)	1.22(0.77-1.91)	0.397		
Size (≤20/ 21-50/ >50 mm)	2.11(1.49-2.97)	<0.001		0.324
Grade (I / II / III)	1.87(1.38-2.53)	<0.001		0.276
Involved lymph node (-ve/+ve)	2.76(1.80-4.23)	<0.001	1.48(1.12-4.93)	0.106
ER status (no/yes)	0.62(0.41-0.93)	0.021		0.200
PR status (no/yes)	0.54(0.36-0.81)	0.003	0.53(0.35-0.80)	0.004
Her-2 status (no/ yes)	2.02(1.27-3.22)	0.003		0.959
Tumour necrosis (low/high)	1.97(1.48-8.59)	0.005	3.45 (2.01-5.92)	<0.001
Lymphatic vessel invasion (no/yes)	4.14(2.75-6.29)	<0.001	1.98(1.23-3.16)	0.004
Blood vessel invasion (no/yes)	3.39(2.14-5.39)	<0.001	2.04(1.22-3.41)	0.006
Klintrup–Mäkinen grade (low/high)	1.48(0.96-2.26)	0.069		0.868
CD68+ (low/moderate/high)	0.86(0.67-1.09)	0.222		
CD4+ (low/moderate/high)	1.00(0.80-1.25)	0.983		
CD8+ (low/moderate/high)	0.69(0.54-0.88)	0.004	0.88(0.82-0.99)	<0.001
CD138+(low/moderate/high)	1.38(1.11-1.71)	0.003	1.03(1.02-1.06)	0.001
Tumour stroma percentage (low/high)	2.19(1.46-3.27)	<0.001	1.65(1.08-2.51)	0.020
Tumour budding (low/high)	2.53(1.69-3.78)	<0.001	2.21(1.41-3.47)	0.001

Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	2.34(1.47-3.75)	<0.001	2.05(1.24-3.39)	0.005
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none	1.24(1.01-1.51)	0.033		0.508
Node negative patients (n=251)				
Age (<50/ >50 years)	1.22(0.55-2.71)	0.632		
Size (≤20/ 21-50/ >50 mm)	2.49(1.25-4.97)	0.010		0.276
Grade (I / II / III)	1.67(1.03-2.72)	0.038		0.894
ER status (no/yes)	0.48(0.24-0.97)	0.040		0.806
PR status (no/yes)	0.39(0.19-0.81)	0.010	0.36(0.17-0.75)	0.006
Her-2 status (no/ yes)	1.75(0.72-4.26)	0.221		
Tumour necrosis (low/high)	3.75(1.73-8.11)	0.001	3.45(1.25-7.83)	0.003
Lymphatic vessel invasion (no/yes)	4.67(2.33-9.36)	<0.001	3.14(1.44-6.83)	0.004
Blood vessel invasion (no/yes)	3.95(1.77-8.80)	0.001	2.38(0.98-5.79)	0.055
Klintrup–Mäkinen grade (low/high)	1.45(0.67-3.13)	0.347		
CD68+ (low/moderate/high)	0.52(0.39-1.35)	0.643		
CD4+ (low/moderate/high)	1.04(0.63-1.21)	0.872		
CD8+ (low/moderate/high)	0.653(0.324-1.15)	0.132		
CD138+(low/moderate/high)	1.13(0.48-1.63)	0.625		
Tumour stroma percentage (low/high)	1.46(1.84-3.66)	0.014	1.91(1.45-5.21)	0.011
Tumour budding (low/high)	2.83(1.46-5.86)	0.003	3.18(1.46-6.93)	0.004
Locoregional treatment (lumpectomy	1.88(0.98-3.91)	0.089		0.439

+radiotherapy/mastectomy+radiotherapy)		
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none	0.98(0.71-1.38)	0.947

Table 6-5 The relationship between clinicopathological characteristics and cancer specific survival in patients with low TSP and high K-M score

	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	***P*-value**	**Hazard ratio(95% CI)**	***P*-value**
Stroma low patients (n=311)				
Age (≤50/ >50 years)	1.04(0.59-1.86)	0.885		
Size (≤20/ 21-50/ >50 mm)	2.73(1.69-4.40)	<0.001		0.503
Grade (I / II / III)	1.93(1.27-2.94)	0.002		0.422
Involved lymph node (-ve/+ve)	2.52(1.45-4.39)	0.001		0.114
ER status (no/yes)	0.52(0.30-0.92)	0.023		0.232
PR status (no/yes)	0.65(0.32-1.06)	0.072		0.345
Her-2 status (no/ yes)	1.96(1.04-3.70)	0.036		0.337
Lymphatic vessel invasion (no/yes)	4.59(2.63-8.02)	<0.001	1.99(1.06-3.74)	0.031
Blood vessel invasion (no/yes)	5.49(3.13-9.64)	<0.001	3.70(1.96-6.81)	<0.001
Tumour necrosis (low/high)	4.59(2.30-9.14)	<0.001	3.95(1.89-8.22)	<0.001
Klintrup–Mäkinen grade (low/high)	1.64(0.93-2.08)	0.076		0.566
CD68+ (low/moderate/high)	0.99(0.99-1.00)	0.028	0.94(0.92-0.99)	0.002
CD4+ (low/moderate/high)	0.01(0.09-1.01)	0.337		
CD8+ (low/moderate/high)	0.99(0.99-1.02)	0.243		
CD138+ (low/moderate/high)	1.01(0.99-1.01)	0.092		0.888
Tumour budding (low/high)	2.29(1.32-3.95)	0.002	2.44(1.35-4.40)	0.003
Locoregional treatment (lumpectomy	3.41(1.65-7.00)	0.001	3.37(1.53-7.40)	0.002

+radiotherapy/mastectomy+radiotherapy)				
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none	1.35(1.04-1.77)	0.026		0.507
High Klintrup–Mäkinen grade patients (n=123)				
Age (≤50/ >50 years)	0.73(0.36-1.45)	0.365		
Size (≤20/ 21-50/ >50 mm)	1.86(0.93-3.74)	0.081		0.238
Grade (I / II / III)	1.75(0.71-4.32)	0.226		
Involved lymph node (-ve/+ve)	2.35(1.09-5.09)	0.030		0.235
ER status (no/yes)	0.60(0.29-1.23)	0.165		
PR status (no/yes)	0.49(0.22-1.10)	0.496		
Her-2 status (no/ yes)	1.22(0.59-2.53)	0.598		
Lymphatic vessel invasion (no/yes)	6.25(2.65-14.49)	<0.001	5.21(2.16-12.58)	<0.001
Blood vessel invasion (no/yes)	3.94(1.89-8.20)	<0.001	2.91(1.32-6.39)	0.008
Tumour necrosis (low/high)	23.78(0.16-34.34)	0.213		
CD68+ (low/moderate/high)	0.95(0.92-0.99)	0.004	0.96(0.93-0.99)	0.003
CD4+ (low/moderate/high)	0.99(0.99-1.01)	0.300		
CD8+ (low/moderate/high)	0.99(0.98-0.99)	0.006		0.139
CD138+ (low/moderate/high)	1.01(0.99-1.01)	0.164		
Tumour stroma percentage (low/high)	2.62(1.26-5.45)	0.010	2.35(1.10-5.06)	0.027
Tumour budding (low/high)	2.82(1.39-5.72)	0.003	2.56(1.23-5.36)	0.012
Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	2.74(1.13-6.66)	0.026		0.198

Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/none	1.14(0.78-1.66)	0.500

When survival analysis for tumour budding was performed across the different molecular subtypes, a high tumour budding was associated with shorter mean cancer specific survival compared with a low tumour budding in luminal B patients (155 versus 114 months, $P<0.001$), and triple negative patients (154 versus 94 months, $P<0.001$), whereas lumnal A and Her-2 +ve tumours showed no significant difference in mean cancer specific survival between high and low tumour budding groups (Figure 6.4).

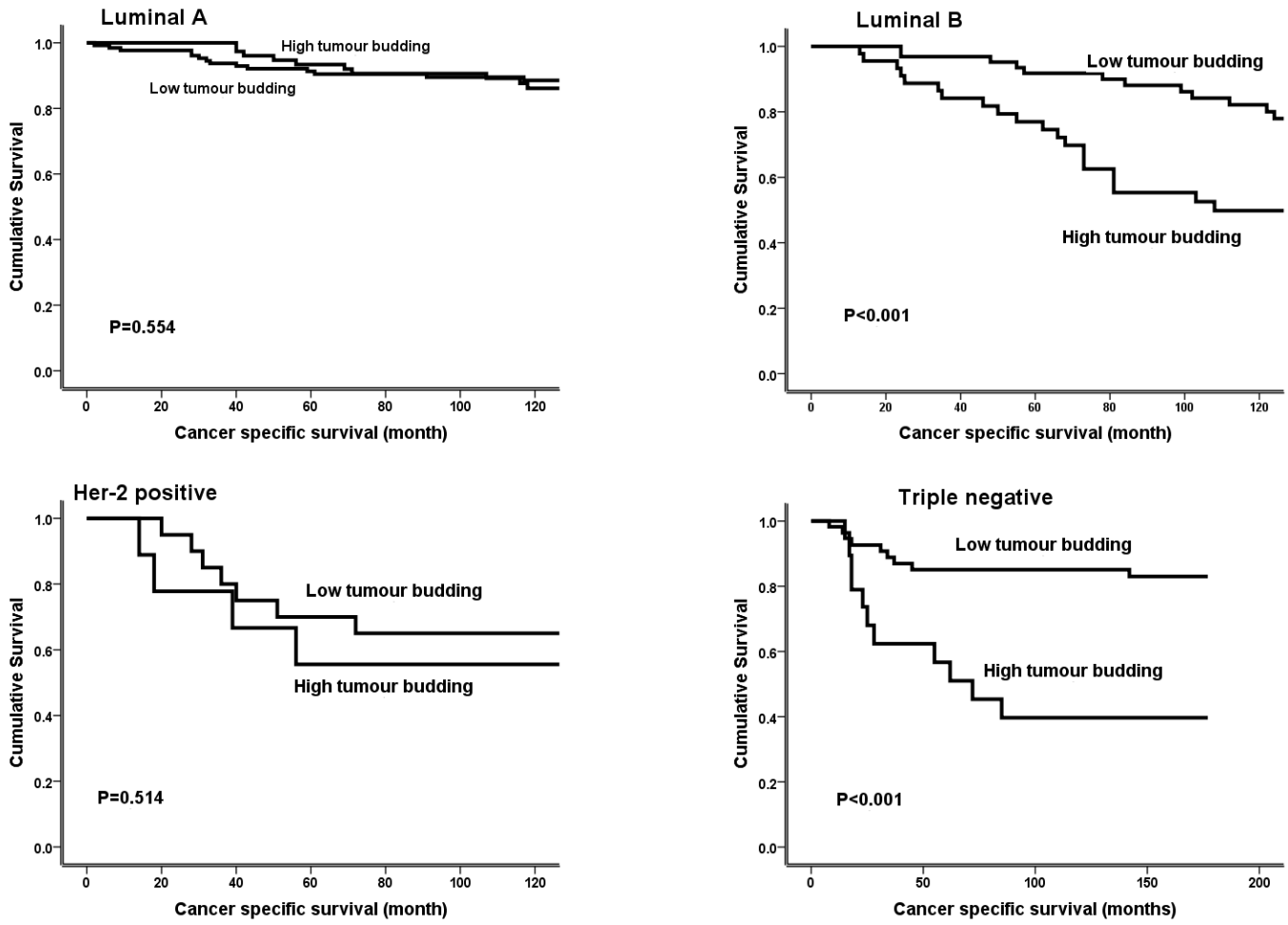


Figure 6-5 Kaplan-Meier survival curves (Log rank) of cancer specific survival in different molecular subtypes.

Only luminal B (mean survival; 155 versus 114 months) and triple negative (mean survival; 154 versus 94 months) subgroups show significant association between high tumour budding and reduced cancer specific survival.

6.4 Discussion

In the present study high tumour budding was associated with more tumour stroma and a weaker inflammatory cell infiltrate and was independently associated with reduced cancer specific survival. These results suggest a complex relationship between tumour budding and the tumour microenvironment and disease progression in patients with invasive ductal breast cancer.

Few studies have examined the prognostic value of tumour budding in breast cancer (Liang et al., 2013; Salhia et al., 2015). The prognostic value and method of assessment of tumour budding in colorectal cancer has recently been reviewed by van Wyk and colleagues. They concluded that IHC did not improve the detection rate or the prognostic value of tumour budding over that of H&E (van Wyk et al, 2015). Therefore, in the present study, the H&E approach was used.

In the present study, examination of tumour budding was reproducible (ICCC=0.81). Patients, in the present study, were divided into three budding groups based on tertiles. The cut-off considered the best discriminator of cancer specific survival (Choi et al, 2007; Sy et al., 2010) was between groups 2 and 3, and yielded a cut-off consistent with previous reports (16-25 buds) (Prall et al., 2005; Wang et al., 2009). Furthermore, in the present study tumour budding was found in 35% of patients and is consistent with previous report in patients with breast cancer (Liang et al., 2013).

The results of the present study showed that high grade budding was significantly associated with ER positive tumours. These results are consistent with the recent observations of Salhia and colleagues using a pan-cytokeratin stain to assess tumour budding (Salhia et al., 2015). The basis of these observations is not clear. However, it was recently reported that oestrogen is involved in EMT in breast cancer cell lines with stem cell properties (Sun et al., 2014) and that oestrogen is involved in disruption of tight

junction and increased cell motility (Sanchez et al., 2010; Jime´nez-Salazar et al., 2014). Therefore, this may suggest that ER positive tumours with high tumour budding may be undergoing a higher degree of EMT and as a result more metastatic potential. If this were to be the case, then it might be expected that anti-oestrogen treatment would reduce the degree of budding in those patients.

Despite being associated with lymph node metastasis and lymphatic vessel invasion, tumour budding was not associated with blood vessel invasion. The basis of this observation was not clear however, tumour buds might find their way of metastasis through invasion into lymphatic vessels than blood vessels, as it is the major route of metastasis in breast cancer (Mohammed et al., 2009). In the present study, there was a lack of any perceived association between tumour budding and tumour size, grade, necrosis, or Ki67 in all cohort and in sub-group analysis. Previous breast and colorectal cancers studies reported that budded cells to display lower proliferation activity rather than high proliferative activity (Palmqvist et al., 2000; Liang et al., 2013; Dawson and Lugli., 2015). This may suggest that detachment and dissociation of tumour cells are not influenced by increased tumour size, its differentiation or proliferation activities.

Although the interrelationships between the tumour budding, tumour microenvironment and gross pathological characteristics are likely complex, tumour budding remained independently associated with cancer specific survival in different patient sub-groups. In high risk patients with node negative disease, tumour budding was significantly associated with reduced cancer specific survival alongside with tumour necrosis, LVI and BVI. Indeed, the present results further confirm the importance of both tumour and host-based factors of the tumour microenvironment in determining cancer outcome.

Although there is now increased appreciation of the importance of the tumour budding in cancer progression and survival in several previous reports (Hase et al., 1993; Ueno et al.,

2002; Prall et al., 2005; Koike et al., 2008; Masugi et al., 2010; Taira et al., 2012; Koyuncuoglu et al., 2012; Liang et al., 2013), its relationship with other components of the tumour microenvironment has yet to be fully characterised. It was of interest that the present study found an association between tumour budding and increased amount of tumour stroma percentage. Earlier reports in colorectal cancer have shown an association between tumour budding and the presence of an immature stroma and a high density of stromal myofibroblasts (Ueno et al., 2004). Furthermore, tumour stroma has been implicated to facilitate EMT, which is one of the features of budded cells (Masugi et al., 2010; Zlobec and Lugli, 2010; Taira et al., 2012; Koyuncuoglu et al., 2012, Lugli et al., 2012; Liang et al., 2013), and metastasis of tumour cells into normal tissue (De Wever and Mareel, 2003; Hemmings, 2013). Therefore, the present finding may support an important role of the tumour stroma in facilitating tumour cell de-differentiation and dissemination, perhaps providing suitable energy substrate and reducing the build-up of metabolic waste (Koukourakis et al., 2006).

Of interest, the present study has reported the relationship between tumour budding and local host inflammatory infiltrate. There was a low peri-tumoural general inflammatory infiltrate, as measured by Klintrup–Mäkinen score but not by individual subtypes of innate or adaptive immune cells, in patients with high grade tumour budding. This may suggest that tumour budding may promote the development of a pro-tumour rather than anti-tumour immune response. It is of interest that the prognostic value of the ratio of CD8+ T-lymphocytes and budding has recently reported in primary operable colorectal cancer and showed that a high tumour budding and a low CD8+ T-lymphocytes index was associated with tumour progression and worse survival (Lugli et al., 2009), confirming the pro-tumour impact of the tumour budding. However, when we examined CD8+/budding index in the present breast cancer cohort, the CD8+/budding index did not show additional

prognostic value to that of tumour budding alone. Therefore, further work is required to establish the prognostic value of the CD8+/ budding index in patients with cancer.

Given that tumour budding has independent prognostic value in patients with primary operable invasive ductal breast cancer, it would be of interest to examine the prognostic value of intra-tumoural budding (ITB) since if this was the case then it may be applied to the initial diagnostic biopsy samples to better predict likely outcome and plan treatment prior to surgery. For example, if ITB was strongly associated with lymph node metastases, then it may be that the corresponding sentinel lymph nodes should be analysed carefully on frozen sections in preoperative biopsies. Indeed, Zlobec and colleagues reported that ITB in preoperative biopsies predicts the presence of lymph node and distant metastases in colorectal cancer patients (Zlobec et al., 2014). However, Salhia and colleagues reported that, in breast cancer, ITB in preoperative core biopsies was associated with blood vessel invasion but not with lymphatic and nodal invasion (Salhia et al., 2015). Nevertheless, prospective studies comparing the prognostic value of tumour budding in preoperative core biopsies and resection specimens would be of great interest.

As breast cancer is heterogeneous, variation in stromal biology may exist between breast cancer subtypes, and may possibly influence on outcome. High tumour budding was significant predictor of poor survival in patients with luminal B and triple negative tumours. Luminal B tumours have a more aggressive phenotype, with higher proliferation rate compared to luminal A tumours. Luminal B tumours also include those tumours with Her-2 overexpression or amplification and they needed to be treated more aggressively than Luminal A (Eroles et al., 2012; Wu and Sahin., 2016). For Basal like or triple negative breast cancers, currently there are no specific targeted therapies (Eroles et al., 2012; Kumar and Aggarwal., 2016; Wu and Sahin., 2016). Therefore, the detection of tumour buds at the invasive front may therefore represent an additional prognostic indicator that may help

better understanding of the malignant progression of the molecular subtypes and could be a potential target for their treatment.

Taken together, the present results suggest that tumour budding may promote disease progression through a direct effect on local and distant invasion into lymph nodes and lymphatic vessels. Indeed, budded cells have been shown to display epithelial mesenchymal transition-like molecular phenotype in several cancers (Masugi et al., 2010; Zlobec and Lugli, 2010; Taira et al., 2012; Koyuncuoglu et al., 2012, Lugli et al., 2012; Liang et al., 2013), which is an early and critical step in cancer metastasis (Kalluri and Weinberg et al, 2009). Interstingly, in breast cancer, budded tumour cells at the invasive margin show reduced expression of membranous E-cadherin, and increased expression of cytoplasmic vimentin (Liang et al., 2013), essential phenotypic features of EMT (De Crane and Berx, 2013). Indeed, results of the present study would indicate that the detection of tumour buds at the invasive front might therefore represent a morphologic link between tumour progression, lymphatic invasion, spread of tumour cells to regional lymph nodes, and the establishment of metastatic dissemination.

The results of the present study suggest that tumour budding should be incorporated into routine clinical practice. However, in order for that to occur it has to be shown to be a reliable measure. Although several studies have confirmed the prognostic value of tumour budding, several different methods have been described (Hase et al., 1993; Ueno et al., 2002; Prall et al., 2005; Wang et al., 2009). Therefore, there is a need for a standardised method to assess tumour budding in patients with cancer. In particular, if the standardised assessment of the tumour budding can reliably be performed in routine pathological sections and can offer useful prognostic information for clinicians and this would form the platform for the integration of tumour budding into existing staging systems.

With reference to patients with breast cancer, to date, tumour budding has been rarely examined and therefore the results of the present study need to be externally validated. Furthermore, whether tumour budding could be used as an additional morphological feature to stratify ER positive into a high and low risk category has also to be validated.

In conclusion, the present study provides comprehensive assessment of the associations between tumour budding and the tumour microenvironment and, in a mature cohort of patients with long term follow-up, further confirms the prognostic relevance of assessment of the tumour microenvironment in patients with invasive ductal breast cancer. Assessment of the tumour budding utilising routine pathological slides is relatively simple and may be readily incorporated into routine clinical pathology reporting to improve risk stratification, in particular for patients with node negative breast cancer.

Chapter 7 The relationship between total and phosphorylated-STAT1 and STAT3 tumour cell expression, components of tumour microenvironment and survival in patients with invasive ductal breast cancer

7.1 Introduction

Components of the tumour microenvironment including tumour stroma and tumour inflammatory cell infiltrates are now recognised to play a key role in cancer progression and represent interactions between the tumour and the host (Hanahan and Weinberg, 2011; McAllister and Weinberg, 2014). The underlying mechanism of the interaction between the different components of tumour microenvironment is not clear. Cross-talk between signalling pathways determines how a cell integrates the environmental signals received, ultimately translating them in transcriptional regulation of specific sets of genes (Schindler et al., 2007). Signal transducers and activators of transcription family (STATs) has been recognised to act downstream signalling of cytokine and growth factor receptors (Schindler et al., 2007; Stark and Darnell, 2012) and may therefore plausibly play a central role in determining the phenotypic characteristics of the tumour and the host.

The IL-6/Janus-activated kinase can trigger tyrosine phosphorylation of both STAT1 and STAT3 through homo- or hetero-dimerisation of the signal transduction subunit gp130 (Heinrich et al., 2003; Regis et al., 2008). STATs detect a variety of signals at the cell membrane and transduce them to the nucleus directly affecting gene regulation of cell growth, survival, differentiation, and motility. STAT1 is a central mediator of both type I and type II interferon (Darnell et al., 1997; Buettner et al., 2002; Yu et al., 2004), however both IFNs can in addition activate STAT3 (Regis et al., 2008).

STAT1 and STAT3 employ a complex interaction on both tumour cells and the tumour microenvironment including immune infiltrates. STAT1 and STAT3 are thought to play opposite roles in tumourigenesis and the set of target genes is mostly distinct (Avalle et al.,

2012). STAT1 is considered as a growth suppressor based on its role as a pro-apoptotic and anti-proliferative molecule (Bromberg and Darnell, 2002; Schindlr et al., 2007; Stark and Darnell, 2012; Koromilas and Sexl et al., 2013). STAT3 is well established as an oncogene involves in mammary epithelial cell growth and differentiation. However, it also behaves as a tumour suppressor (Turkson and Jove, 2000; Ecker et al., 2009). Early studies have shown that STAT3 is essential in mammary gland epithelial cell apoptosis and involution (Chapman et al., 2000; Sutherland et al., 2006).

Nevertheless, studies on STAT-deficient cells/animals have revealed the existence of reciprocal STAT1 to STAT3 regulatory mechanisms which represent the cross regulation between the two molecules (Avalle et al., 2012). Increased and prolonged phosphorylation of STAT1 in response to gp130 cytokines occurs in several systems upon STAT3 gene inactivation (Regis et al., 2008; Avalle et al., 2012).

Despite the fact that several experimental studies suggest that STAT1 and STAT3 play a critical role in breast cancer tumorigenesis, the prognostic value of these proteins in patients with breast cancer remains unclear (Table 7.1). Five studies have examined the prognostic value of STAT1 in breast cancer, using either total STAT1 or phosphorylated STAT1 (ph-STAT1). An initial analysis by Widschwendter et al using Western blotting and DNA binding technique, reported an independent association between high ph-STAT1 activation and improved overall and cancer specific survival (CSS) (Widschwendter et al., 2002). In contrast, IHC of ph-STAT1, found that ph-STAT1 in premenopausal women was associated with poor overall survival but not in postmenopausal women. However, co-expression of Ph-STAT1 with ER or PR was associated with longer CSS in postmenopausal women (Magkou et al., 2012). Studies measuring total STAT1 have also reported conflicting results. IHC of total STAT1 by Sheen-Chen et al and Huang et al reported no association between total STAT1 and outcome (Sheen-Chen et al., 2007;

Huang et al., 2014), whereas total STAT1 was a significant predictor of worse survival in one study (Charpin et al., 2009) (Table 7.1).

Ten studies have examined the prognostic value of STAT3 in breast cancer, using either total STAT3 or phosphorylated STAT3 (ph-STAT3) (Table 7.1). High total STAT3 was significantly associated with improved outcome in three studies (Dolled-Filhart et al., 2003; Sato et al., 2011; Huang et al., 2014), and with poor outcome in one study (Sheen-Chen et al., 2008). Ph-STAT3 expression was not associated with breast cancer survival in two studies (Widschwendter et al., 2002; Yamashita et al., 2006), and was associated with improved survival in large cohort of patients (Aleskandarany et al., 2016), patients with lymph node positive tumours (Sonnenblick et al., 2012), and patients treated with adjuvant chemotherapy (Sonnenblick et al., 2014). In contrast, ph-STAT3 was a significant predictor of worse survival in one study (Charpin et al., 2009) (Table 7.1). Therefore, given that clinical trials evaluating Il-6/JAK/ STAT inhibitors in breast cancer patients are under way (Lin et al., 2013), it would be important to determine the role of STAT1 and STAT3 in this disease.

Also, commensurate with their role in regulating cytokine-dependent inflammation and immunity, the relationship between STAT1 and STAT3 and components of the tumour microenvironment is not clear. Therefore, the aim of the present study was to examine the relationship between total and phosphorylated STAT1 and STAT3 tumour cell expressions, components of the tumour microenvironment and survival in a mature cohort of patients with invasive ductal breast cancer.

Table 7-1 Studies on the prognostic significance of STAT1 and STAT3 in breast cancer

References	Patients	Sample size	Follow-up	Protein examined	Association with outcome
STAT1 studies					
Widschwendter et al., 2002	N/S	53	6.8	ph-STAT1	associated with improved overall and CSS (multivariate analysis)
Sheen Chen et al., 2007	N/S	102	5.8	total STAT1	no association with overall survival
Charpin et al., 2009	N/S	924	6.5	total STAT1	associated with reduced CSS
Magkou et al., 2012	Premenopausal/ postmenopausal	165	7.5	ph-STAT1	in premenopausal women: associated with poor OS (univariate analysis) in postmenopausal women: co-expression with ER/or PR was associated with improved CSS (univariate analysis)
Huang et al., 2014	N/S	546	15	total STAT1	no significant association with CSS
STAT3 studies					
Widschwendter et al., 2002	N/S	53	6.8	ph-STAT3	no association with survival
Dolled-Filhart et al., 2003	LN -ve	255	5 & 20	total STAT3	associated with improved OS
Yamashita et al., 2006	N/S	506	7.5	ph-STAT3	no association with OS and CSS
Sheen-Chen et al., 2008	N/S	102	5	total STAT3	associated with reduced OS

Charpin et al., 2009	N/S	924	6.5	ph-STAT3	associated with reduced CSS
Sato et al., 2011	all, LN-ve/ LN+ve, low & high grade	721	>10	total STAT3	associated with improved OS in patients with low grade tumours (univariate analysis)
Sonnenblick et al., 2012	LN +ve	125	5 & 10	ph-STAT3	associated with improved OS
Sonnenblick et al., 2013	N/S	375	10	ph-STAT3	associated with improved OS in patients treated with adjuvant chemotherapy
Huang et al., 2014	N/S	546	15	total STAT3	associated with improved CSS (univariate analysis)
Aleskandarany et al., 2016	N/S	1270	N/A	Ph-STAT3	associated with improved CSS (multivariate analysis)

N/S: not specified invasive breast cancer, LN: lymph node, ph-STAT1 tyrosine phosphorylated STAT1, ph-STAT3: tyrosine phosphorylated STAT3, CSS: cancer specific survival.

OS: overall survival, Follow-up in years.

7.2 Patients and Methods

7.2.1 Patients

384 patients with primary operable invasive ductal breast cancer, whose samples were successfully stained for total and ph-STAT1, and total and ph-STAT3 from patients described in section 2.1, were included in this study.

7.2.2 Methods

TMA was utilised in this study and was constructed as previously described in chapter 2.0. The assessment of ER, PR, Her-2, CD68+ macrophage infiltrate, CD4+ T-lymphocyte infiltrate, CD8+ T-lymphocyte infiltrate and CD138+ plasma cell infiltrate was performed as previously described in chapter 2.0.

Scanned routine H&E sections for the 384 patients were used to score general peri-tumoural inflammatory infiltrate and tumour necrosis as previously described in chapter 2.0.

IHC were utilised to assess lymphatic and blood vessel invasion as previously described in chapter 4.0. Tumour stroma percentage and tumour budding were also assessed on scanned H&E sections for the 384 patients as previously reported in chapters 5.0 and 6.0.

The molecular subtypes were defined as follows: luminal A: ER and/or PR positive, Her-2 negative, low proliferative index (≤15%); luminal B: hormone receptor positive, Her-2 positive/or high proliferative index (>15%); Her-2 subtype: Her-2 positive and hormone receptor negative, any proliferative index; triple negative: Her-2 negative, hormone receptor negative, any proliferative index.

7.2.2.1 Immunohistochemistry of STAT1 and STAT3

Immunohistochemical expression of total STAT1, Y701 phosphorylated STAT1 (ph-STAT1), total STAT3 and Y705 phosphorylated STAT3 (ph-STAT3) were carried out using a previously constructed triplicate TMA blocks. Sections of 2.5 µm thickness from each TMA block were placed on silanized glass slides. Sections were dewaxed in xylene before being rehydrated using graded alcohols. Antigen retrieval for all STATs isoforms was performed using Tris-EDETA buffer (pH 8) for 20 minutes before cooling for 20 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 20 minutes before rinsing in water. Normal horse serum at dilution 1:10 was applied for 30 minutes at room temperature as a blocking solution. TMA sections were then incubated overnight at 4^{o}C with the primary antibodies as following: total STAT1 (STAT1 (42H3) Rabbit monoclonal antibody, code 9175, Cell Signaling Technology, USA) at a concentration of 1:100; ph-STAT1 (Rabbit PAb to STAT1 phosphoY701, code ab30645, Abcam, Cambridge) at a concentration of 1:150; total STAT3 (STAT3 Rabbit Ab, code 9132L, Cell Signaling Technology, USA) at a concentration of 1:200; Ph-STAT3 (Y705) antibody (P-STAT3 (Y705) Rabbit Ab, code 9131L, Cell Signaling Technology, USA) at a concentration of 1:200. Sections were then washed in TBS for ten minutes. Envision (Dako) was then added to the sections for 30 minutes at room temperature before washing in TBS for ten minutes. DAB substrate was added for five minutes until colour developed before washing in running water for ten minutes. Slides were then counterstained in haematoxylin for 60 seconds and blued with Scotts' tap water before being dehydrated through a series of graded alcohols. Cover slips were applied using distrene, plasticizer, xylene (DPX).

7.2.2.2 Slide scanning and scoring

Stained TMA sections for the 384 patients were scanned at objective magnification x20 as previously described in chapter 2.0. Assessment of total STAT1, ph-STAT1, total STAT3

and ph-STAT3 expression levels were performed by a single examiner (FG) blinded to clinical data at x20 magnification (total magnification x40) using the weighted histoscore.

Weighted histoscore: Examined protein expression levels were scored at each cellular location (cytoplasm and nuclei) separately. The weighted histoscore method assesses the staining intensity and the percentage of cells stained with that intensity for the full slide (Kirkegaard et al., 2006). It is calculated by (1 × % cells staining weakly positive) + (2 × % cells staining moderately positive) + (3 × % cells staining strongly positive). This gives a semiquantitative classification of staining intensity, with the maximum score being 300 (if 100% of cells stain strongly positive) and the minimum score being 0 (if 100% of cells are negative). The weighted histoscore method is a well-established method for scoring tissue that has heterogeneous staining. To ensure reproducibility of scoring, 15% of tumours for each antibody were co-scored by a second investigator (JE) blinded to other data. The intraclass correlation coefficient (ICCC) was 0.85 and 0.83 for cytoplasmic and nuclear total STAT1 respectively, and 0.82 and 0.87 for cytoplasmic and nuclear ph-STAT1 respectively, indicating good agreement. The ICCC was 0.79 and 0.80 for cytoplasmic and nuclear total STAT3 respectively, and 0.81 and 0.78 for cytoplasmic and nuclear ph-STAT3 respectively, indicating good agreement.

7.2.2.3 Statistical analysis

For the purpose of statistical analysis, patients were split into two groups on the basis of the mean of cytoplasmic and nuclear STAT1/STAT3 weighted histoscore as low cytoplasmic and low nuclear STAT1/STAT3 expression, and high cytoplasmic and high nuclear STAT1/STAT3 expression. In order to identify the impact of cellular STAT1/STAT3 expression at both cytoplasmic and nuclear location, an expression code was developed (STAT1/STAT3 tumour cell expression) as follows: patients with both low cytoplasmic and nuclear expression were classified as the low tumour cell expression

group, patients with either cytoplasmic or nuclear expression is low were classified as the moderate tumour cell expression group, and patients with both high cytoplasmic and high nuclear expression were classified as the high tumour cell expression group. These analyses have been applied for total and for ph-STAT1 separately, and total and ph-STAT3 separately.

Subsequently, the relationships between clinicopathological characteristics, ph-STAT1 tumour cell expression and ph-STAT3 tumour cell expression were examined using the Chi-square test for linear trend. The relationship between total and ph-STAT1 tumour cell expression, total and ph-STAT3 tumour cell expression and cancer specific survival was examined using Kaplan-Meier log-rank analysis. Univariate survival analysis was performed using Cox proportional hazards regression. Variables with *P*-value of <0.1 were entered into a multivariable model using a backwards conditional method. A *P*-value <0.05 was considered statistically significant. All analysis was performed using SPSS version 22.0 (IBM SPSS IL, USA).

7.3 Results

Total and ph-STAT1 and STAT3 expression in tumour cells were quantified using the weighted histoscore. The IHC staining of total and ph-STAT1 and STAT3 was homogenous in both the cytoplasm and nuclei of tumour cells, which is consistent with previous reports (Aleskandarany et al., 2016). The staining was also observed in the surrounding stromal cells (fibroblasts and infiltrating inflammatory cells) with variable degrees of positivity (Figure 7.1).

The histoscore of total STAT1 expression ranged from 0-200 within the cytoplasm and from 0-220 within the nucleus, with cytoplasmic and nuclear expression in 270 patients (70%) and 268 patients (70%) respectively. The histoscore for ph-STAT1 expression ranged from 0-190 within the cytoplasm and from 0-225 within the nucleus, with cytoplasmic and nuclear expression in 350 patients (91%) and 374 patients (97%) respectively. Total STAT1 cytoplasmic expression was not correlated with ph-STAT1 nuclear expression ($P=0.421$). Expression of total STAT1 and ph-STAT1 within the nucleus correlated strongly with their expression within the cytoplasm (all $P<0.001$).

The histoscore of total STAT3 expression ranged from 0-280 within the cytoplasm and from 0-293 within the nucleus, with cytoplasmic and nuclear expression in 375 patients (98%). The histoscore of ph-STAT3 expression ranged from 0-150 within the cytoplasm and from 0-250 within the nucleus, with cytoplasmic and nuclear expression in 359 patients (93%) and 376 patients (98%) respectively. Total STAT3 tumour cell expression correlated strongly with ph-STAT3 tumour cell expression ($P<0.001$). Expression of total STAT3 and ph-STAT3 within the nucleus correlated strongly with their expression within the cytoplasm (all $P<0.001$).

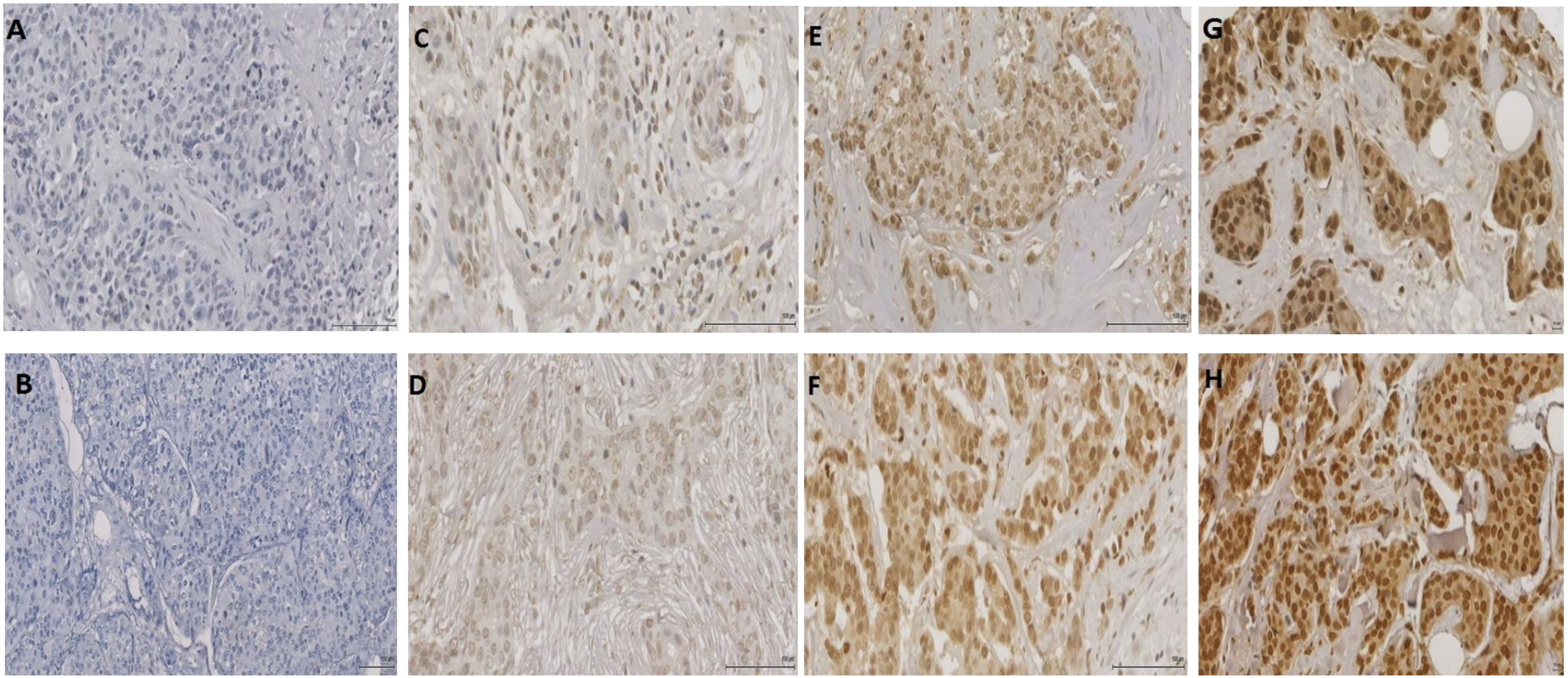


Figure 7-1 Sections of invasive ductal beast carcinomas showing IHC expression levels of ph-STAT1 (first row) and ph-STAT3 (second row). No appreciable expression was detected in the negative controls of ph-STAT1 (A) and ph-STAT3 (B). C-H show the staining intensity of the STAT1 and STAT3 expression as low (C and D), moderate (E and F), and strong (G and H). Original magnification, 20×. Scale bars = 100 μm (A-F), 10 μm (G and H).

The clinical and pathological characteristics of patients with invasive ductal breast cancer are shown in Table 7.2. The majority of patients aged 50 years or older (70%), had a tumour size ≤ 2 cm (61%), grade III carcinoma (43%) with negative axillary lymph node involvement (54%). The majority had ER positive tumours (68%), PR positive tumours (60%) and Her-2 negative tumours (79%), with high grade tumour necrosis (53%). 241 (63%) patients had mastectomy with radiotherapy, 194 (51%) patients received only hormonal therapy, 90 (23%) received only chemotherapy, and 70 (18%) received both. 174 (45%) patients had luminal A tumours, 92 (24%) had luminal B tumours, 30 (8%) had Her-2 positive tumours and 68 (18%) had triple negative tumours.

The relationship between the total and ph-STAT1 expression and the total, and ph-STAT3 expression within the nucleus and the cytoplasm is presented in Table 7.3. Total STAT1 cytoplasmic expression was not associated with ph-STAT3 at both nuclear and cytoplasmic compartments ($P>0.05$). Total STAT1 nuclear expression was not associated with ph-STAT3 cytoplasmic expression ($P>0.05$). Expression of ph-STAT1 within the nucleus and the cytoplasm was correlated strongly with total and ph-STAT3 expression within the nucleus and the cytoplasm (all $P<0.001$).

Table 7-2 The clinicopathological characteristics of patients with invasive ductal breast cancer (n=384)

Clinicopathological characterestics	Patients, n (%)
Age (≤50/ >50 years)	116(30%)/268(70%)
Size (≤20/ 21-50/ >50 mm)	233(61%)/142(37%)/9(2%)
Grade (I / II / III)	71(19%)/147(38%)/166(43%)
Involved lymph node (-ve/+ve)[a]	209(54%)/172(45%)
ER status (no/yes)	116(30%)/268(68%)
PR status (no/yes)[a]	152(40%)/230(60%)
Her-2 status (no/ yes)[a]	305(79%)/70(18%)
Lymphaic vessel invasion (no/yes)	254(66%)/130(34%)
Blood vessel invasion (no/yes)	340(88%)/44(12%)
Tumour necrosis (low/high)	183(48%)/201(52%)
Klintrup–Mäkinen grade (low/high)	272(71%)/112(29%)
CD68+ (low/moderate/high)[a]	116(30%)/129(34%)/124(32%)
CD4+ (low/moderate/high)[a]	160(42%)/75(20%)/136(35%)
CD8+ (low/moderate/high)[a]	124(32%)/119(31%)/128(33%)
CD138+(low/moderate/high)[a]	203(53%)/45(12%)/122(32%)
Tumour stroma percentage (low/high)	264(69%)/120(31%)
Tumour budding (low/high)	250(65%)/134(35%)
Locoregional treatment (lumpectomy + radiotherapy/mastectomy +radiotherapy)	143(37%)/241(63%)
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/ none)[a]	194(51%)/70(18%)/90(23)/24(6%)
Recurrence status (no/yes)[a]	285(74%)/95(25%)
Alive/cancer death/non cancer death	228(59%)/82(22%)/74(19%)

a Number of patients when incomplete data available.

Table 7-3 The relationship between ph-STAT1 and ph-STAT3 expression

	Total STAT1 cytoplasmic expression	**Total STAT1 nuclear expression**	**Ph-STAT1 cytoplasmic expression**	**Ph-STAT1 nuclear expression**
Total STAT3 cytoplasmic expression	0.027	0.003	<0.001	<0.001
Total STAT3 nuclear expression	0.026	0.007	<0.001	<0.001
Ph-STAT3 cytoplasmic expression	0.207	0.539	<0.001	<0.001
Ph-STAT3 nuclear expression	0.115	0.003	<0.001	<0.001

The relationship between ph-STAT1 and ph-STAT3 tumour cell expression and clinicopathological characteristics was shown in Table 7.4. Ph-STAT1 tumour cell expression was not associated with patient age, tumour size, Her-2 status, or the presence of lymphatic (LVI) and blood (BVI) vessel invasion. High ph-STAT1 tumour cell expression was positively associated with ER status (P=0.001), PR status (P=0.048), and negatively with increased tumour grade (P=0.015). Similarly, ph-STAT3 tumour cell expression was not associated with patient age, tumour size and Her-2 status, though borderline significant associations with reduced LVI (P=0.055) and BVI (P=0.052) were observed. High ph-STAT3 tumour cell expression was positively associated with ER status (P<0.001), PR status (P=0.015) and negatively with increased tumour grade (P<0.001).

Within the tumour microenvironment, high ph-STAT1 tumour cell expression was not associated with tumour stroma percentage (TSP) and tumour budding. High ph-STAT1 tumour cell expression was negatively associated with tumour necrosis (P=0.001), and was positively associated with the generalised inflammatory infiltrate as measured using Klintrup–Mäkinen (K-M) grade (P=0.007). Similarly, high ph-STAT3 tumour cell expression was not associated with TSP and tumour budding. High ph-STAT3 tumour cell expression was negatively associated with tumour necrosis (P=0.001) and cellular inflammatory infiltrate as measured using CD4+ helper T-lymphocytes (P=0.024). High ph-STAT1 and ph-STAT3 tumour cell expression were also significantly associated with reduced tumour recurrence (P=0.003 and P=0.001 respectively).

Table 7-4 The relationship between ph-STAT1 and ph-STAT3 tumour cell expression and clinicopathological characteristics (n=384)

	Ph-STAT1 tumour cell expression				Ph-STAT3 tumour cell expression			
	low n=127, 33%	**moderate n=136, 35%**	**high n=121, 32%**	***P* value**	**low n=154, 40%**	**moderate n=121, 32%**	**high n=109, 28%**	***P* value**
Age (≤50/ >50 years)	35/92	38/98	43/78	0.175	46/108	40/81	30/79	0.744
Size (≤20/21-50/>50 mm)	73/48/6	88/47/1	72/47/2	0.444	91/58/5	73/46/2	69/38/2	0.402
Grade (I / II / III)	17/43/67	29/53/54	25/51/45	**0.015**	14/60/80	25/47/49	32/40/37	**<0.001**
Lymph node status (-ve/+ve)	63/62	78/57	68/53	0.357	76/76	66/55	67/41	0.057
ER status (no/yes)	50/77	41/95	25/96	**0.001**	66/88	32/89	18/91	**<0.001**
PR status (no/yes)	57/70	56/79	39/81	**0.048**	70/83	49/72	33/75	**0.015**
Her-2 status (no/ yes)	99/26	102/29	104/15	0.105	120/31	94/25	91/14	0.173
Tumour necrosis (low/high)	45/82	69/67	69/52	**0.001**	60/94	57/64	66/43	**0.001**
Lymphatic vessel invasion (no/yes)	85/42	89/47	80/41	0.890	96/58	77/44	81/28	0.052
Blood vessel invasion (no/yes)	109/18	123/13	108/13	0.390	133/21	104/17	103/6	0.055
Klintrup–Mäkinen grade (low/high)	97/30	98/38	77/44	**0.007**	108/46	81/40	83/26	0.347
CD68+ (low/moderate/high)	40/47/33	40/39/54	36/43/37	0.514	49/53/45	42/33/42	25/43/37	0.183
CD4+ (low/moderate/high)	47/30/44	55/28/51	58/17/41	0.297	57/30/61	51/18/48	52/27/27	**0.024**
CD8+ (low/moderate/high)	46/38/37	39/51/44	39/30/47	0.179	52/43/53	36/39/42	36/37/33	0.785

CD138+(low/moderate/high)	65/14/42	74/14/45	64/17/35	0.613	90/15/42	61/12/44	52/18/36	0.109
Tumour strtoma percentage (low/high)	83/44	93/43	88/33	0.212	99/55	91/30	74/35	0.426
Tumour budding (low/high)	88/39	79/57	83/38	0.884	96/58	76/45	78/31	0.140
Locoregional treatment (lumpectomy+radiotherapy /mastectomy +radiotherapy)	47/80	52/84	44/77	0.920	55/99	43/78	45/64	0.385
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/ none)	58/23/37/8	70/27/28/10	66/20/25/6	0.102	72/26/48/7	57/24/30/7	65/20/12/10	0.060
Recurrence status (no/yes)	86/40	99/37	100/18	**0.003**	105/49	88/32	92/14	**0.001**

Bold indicates significant association

The median follow-up of survivors was 148 months, with 82 cancer-associated deaths and 74 non-cancer deaths. The relationship between total and ph-STAT1 tumour cell expression and CSS using Kaplan-Meier log rank test was examined (Figure 7.2). The total STAT1 tumour cell expression was not associated with CSS (*P*=0.435) (Figure 7.2A). High ph-STAT1 tumour cell expression was associated with improved CSS compared to low tumour cell expression (*P*=0.002) (Figure 7.2B). The mean survival of patients with low ph-STAT1 tumour cell expression was 140 months (95% CI 130-151 months) and 10-year survival rate was 68%, whereas the mean survival of patients with high expression was 162 months (95% CI 154-169 months) and 10-year survival rate was 84%.

The relationship between total and ph-STAT3 tumour cell expression and CSS using Kaplan-Meier log rank test was subsequently examined (Figure 7.3). High total STAT3 tumour cell expression was associated with CSS (*P*<0.001) (Figure 7.3A). High ph-STAT3 tumour cell expression was associated with improved CSS compared to patients with low tumour cell expression (*P*<0.001) (Figure 7.3B). The mean survival of patients with low expression was 139 months (95% CI 129-149 months) and 10-year survival was 62%, whereas the mean survival of patients with high expression was 170 months (95% CI 163-176 months) and 10-year survival was 80%.

The relationship between ph-STAT1 and ph-STAT3 tumour cell expression, clinicopathological characteristics, and CSS is presented in Table 7.5. In univariate analysis, both high ph-STAT1 (*P*=0.002) and ph-STAT3 (*P*<0.001) tumour cell expression were associated with improved CSS. In multivariate analysis, high ph-STAT1 tumour cell expression was not independently associated with CSS (*P*=0.193). In contrast, high ph-STAT3 tumour cell expression was independently associated with improved CSS (HR 0.64, 95% CI 0.64-0.90, *P*=0.010) independent of other variables, including nodal status, tumour necrosis, LVI, BVI, CD8+ T-lymphocyte infiltrate, CD138+ plasma cell infiltrate, and tumour budding (Table 7.5).

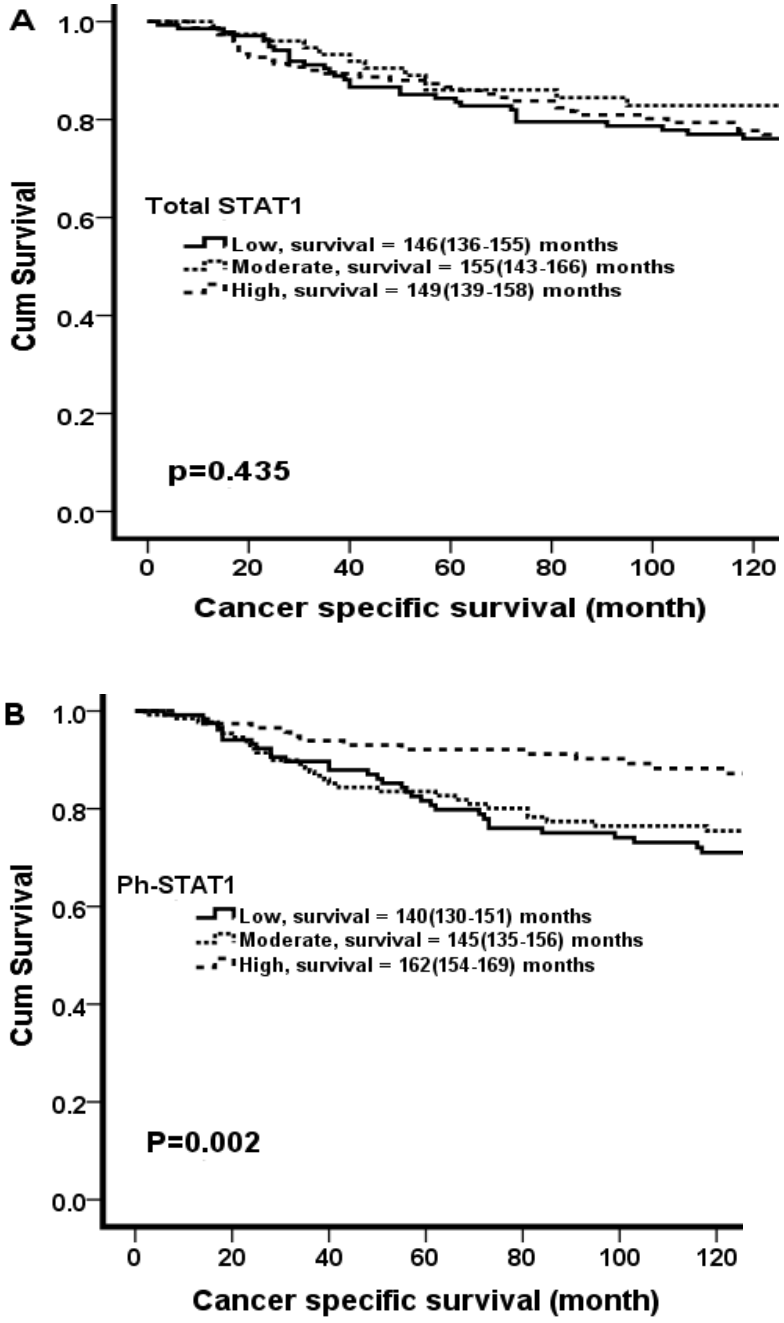


Figure 7-2 Kaplan-Meier survival curves (Log rank) of cancer specific survival.

(A) Total STAT1 tumour cell expression and (B) Ph-STAT1 tumour cell expression.

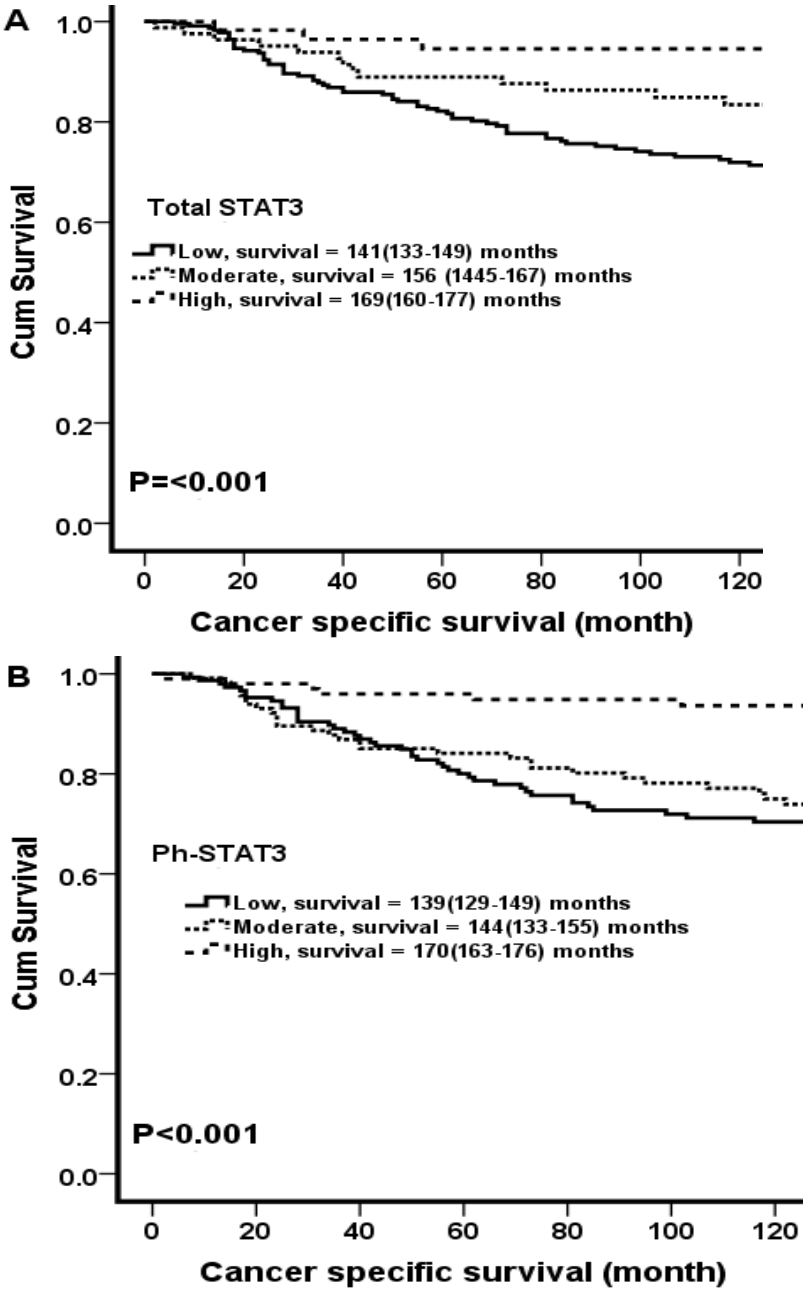


Figure 7-3 Kaplan-Meier survival curves (Log rank) of cancer specific survival.

(A) Total STAT3 tumour cell expression and (B) Ph-STAT3 tumour cell expression.

Due to the strong association observed between both ph-STAT1 and ph-STAT3 and tumour necrosis, the relationship between ph-STAT1 and ph-STAT3 tumour cell expression with CSS in patients with high tumour necrosis was subsequently examined (Table 7.6). In univariate analysis, high ph-STAT3 but not ph-STAT1 tumour cell expression was significantly associated with improved CSS. In multivariate analysis, high ph-STAT3 tumour cell expression was significantly associated with improved CSS (HR 0.69, 95% CI 0.51-0.95, *P*=0.030) independent of LVI, BVI, CD68+ macrophage infiltrate, CD8+ T-lymphocyte infiltrate, tumour budding and locoregional treatment (Table 7.6).

The relationship between ph-STAT1 and ph-STAT3 tumour cell expression and CSS using Kaplan-Meier log rank test, with relevance to different molecular subtypes, was examined (Figures 7.4 and 7.5). High ph-STAT1 was significantly associated with improved CSS in luminal A (n=174) tumours (*P*=0.007). High ph-STAT3 was significantly associated with improved CSS in luminal A (n=174) (*P*=0.005) and B (n=92) tumours (*P*=0.017). The small Her-2 positive subtype cohort (n=30) precluded meaningful analysis.

Table 7-5 The relationship between clinicopathological characteristics, ph-STAT1 and ph-STAT3 tumour cell expression and cancer specific survival in patients with invasive ductal breast cancer (n=384)

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
Age (≤50/ >50 years)	1.14(0.70-1.85)	0.604		
Size (≤20/ 21-50/ >50 mm)	2.21(1.52-3.23)	<0.001		0.475
Grade (I / II / III)	1.89(1.37-2.63)	<0.001		0.254
Involved lymph node (-ve/+ve)	3.85(2.37-6.24)	<0.001	1.90(1.10-3.29)	0.021
ER status (no/yes)	0.54(0.35-0.84)	0.006		0.141
PR status (no/yes)	0.58(0.38-0.90)	0.015		0.181
Her-2 status (no/ yes)	2.05(1.26-3.32)	0.004		0.272
Tumour necrosis (low/high)	5.87(3.26-10.67)	<0.001	4.42(2.31-8.45)	<0.001
Lymphatic vessel invasion (no/yes)	4.08(2.61-6.37)	<0.001	1.94(1.13-3.31)	0.015
Blood vessel invasion (no/yes)	3.28(1.98-5.43)	<0.001	1.79(1.02-3.14)	0.044
Klintrup–Mäkinen grade (low/high)	1.47(0.93-2.23)	0.099		0.526
CD68+ (low/moderate/high)	0.79(0.59-1.02)	0.069		0.101
CD4+ (low/moderate/high)	0.99(0.78-1.26)	0.982		
CD8+ (low/moderate/high)	0.62(0.47-0.82)	<0.001	0.58(0.42-0.80)	0.003
CD138+(low/moderate/high)	1.34(1.06-1.69)	0.014	1.65(1.25-2.18)	<0.001
Tumour stroma percentage (low/high)	2.17(1.40-3.35)	<0.001		0.096
Tumour budding (low/high)	2.46(1.59-3.78)	<0.001	1.88(1.17-3.03)	0.009

Ph-STAT1 tumour cell expression (low/moderate/high)	0.65(0.49-0.86)	0.002		0.193
Ph–STAT3 tumour cell expression (low/moderate/high)	0.54(0.40-0.74)	<0.001	0.64(0.64-0.90)	0.010
Locoregional treatment (lumpectomy +radiotherapy/mastectomy+radiotherapy)	2.62(1.55-4.42)	0.001		0.054
systemic treatment (hormonal/hormonal +chemotherapy/chemotherapy/none)	1.26(1.02-1.55)	0.020		0.408

Table 7-6 The relationship between clinicopathological characteristics, ph-STAT1 and ph-STAT3 tumour cell expression and cancer specific survival in patients with high grade necrosis (n=201)

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**
Size (≤20/ 21-50/ >50 mm)	1.66(1.09-2.50)	0.016		0.388
Grade (I / II / III)	1.17(0.78-1.73)	0.452		
Involved lymph node (-ve/+ve)	2.36(1.39-4.03)	0.002		0.188
ER status (no/yes)	0.77(0.48-1.24)	0.303		
PR status (no/yes)	0.78(0.49-1.27)	0.326		
Her-2 status (no/ yes)	1.19(0.71-2.02)	0.503		
Lymphatic vessel invasion (no/yes)	3.28(1.98-5.44)	<0.001	2.69(1.57-4.61)	<0.001
Blood vessel invasion (no/yes)	2.78(1.62-4.77)	<0.001		0.108
CD8+ (low/moderate/high)	0.51(0.38-0.69)	<0.001	0.55(0.40-0.76)	<0.001
CD138+(low/moderate/high)	1.18(0.92-1.52)	0.195		
Tumour stroma percentage (low/high)	2.14(1.32-3.47)	0.002		0.380
Tumour budding (low/high)	2.51(1.56-4.04)	<0.001	1.66(1.01-2.75)	0.048
Ph-STAT1 tumour cell expression (low/moderate/high)	0.83(0.63-1.12)	0.230		

Ph–STAT3 tumour cell expression (low/moderate/high)	0.65(0.46-0.90)	0.011	0.67(0.47-0.94)	0.021
Locoregional treatment (lumpectomy+radiotherapy / mastectomy +radiotherapy)	2.37(1.33-4.20)	0.003	2.03(1.10-3.73)	0.023
Systemic treatment (hormonal/hormonal+ chemotherapy/chemotherapy/ none)	1.11(0.86-1.43)	0.415		

Only significant variables on univariate analysis were used

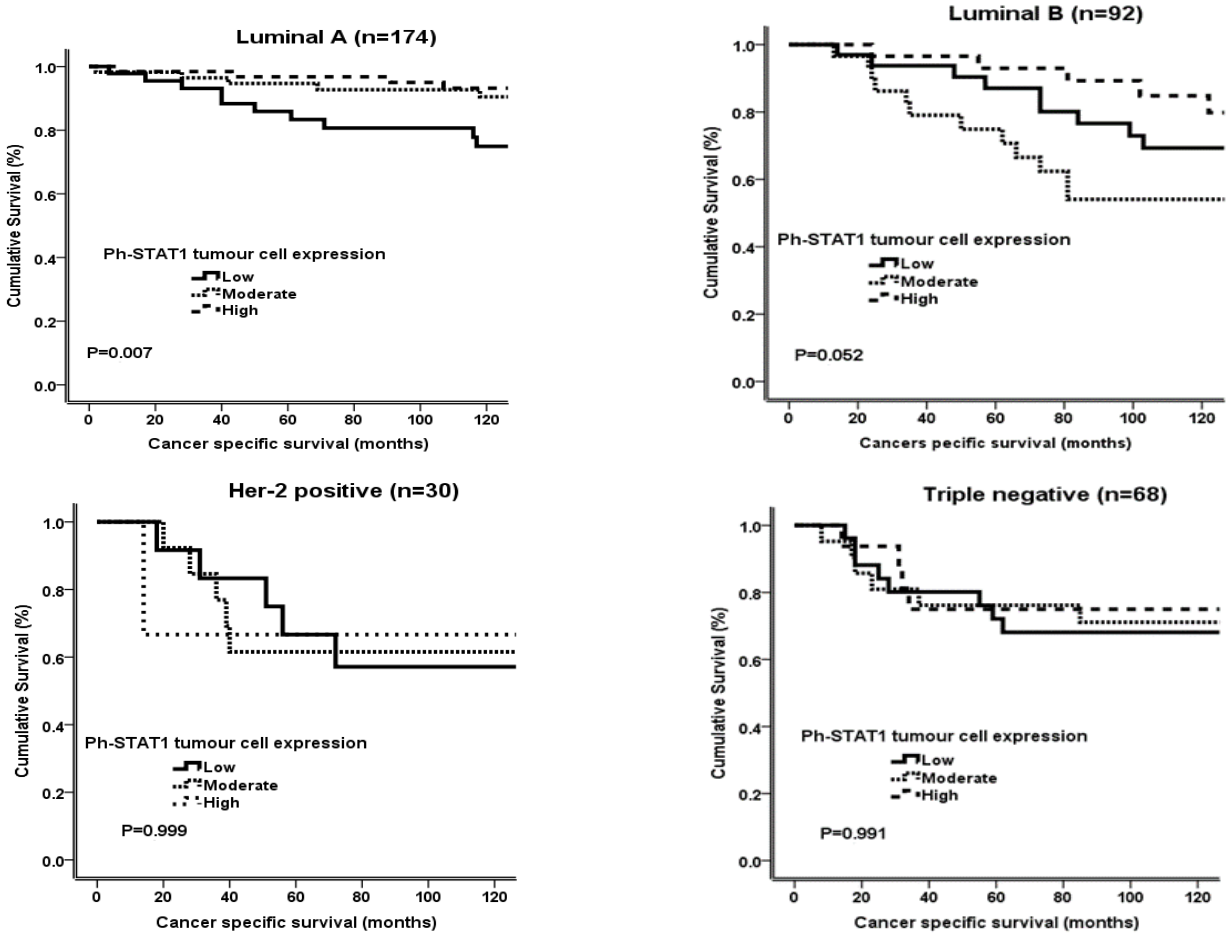


Figure 7-4 Kaplan-Meier survival curves (Log rank) of ph-STAT1 in different molecular subtypes.

Only luminal A shows significant association between high tumour cell expression of ph-STAT1 and improved cancer specific survival.

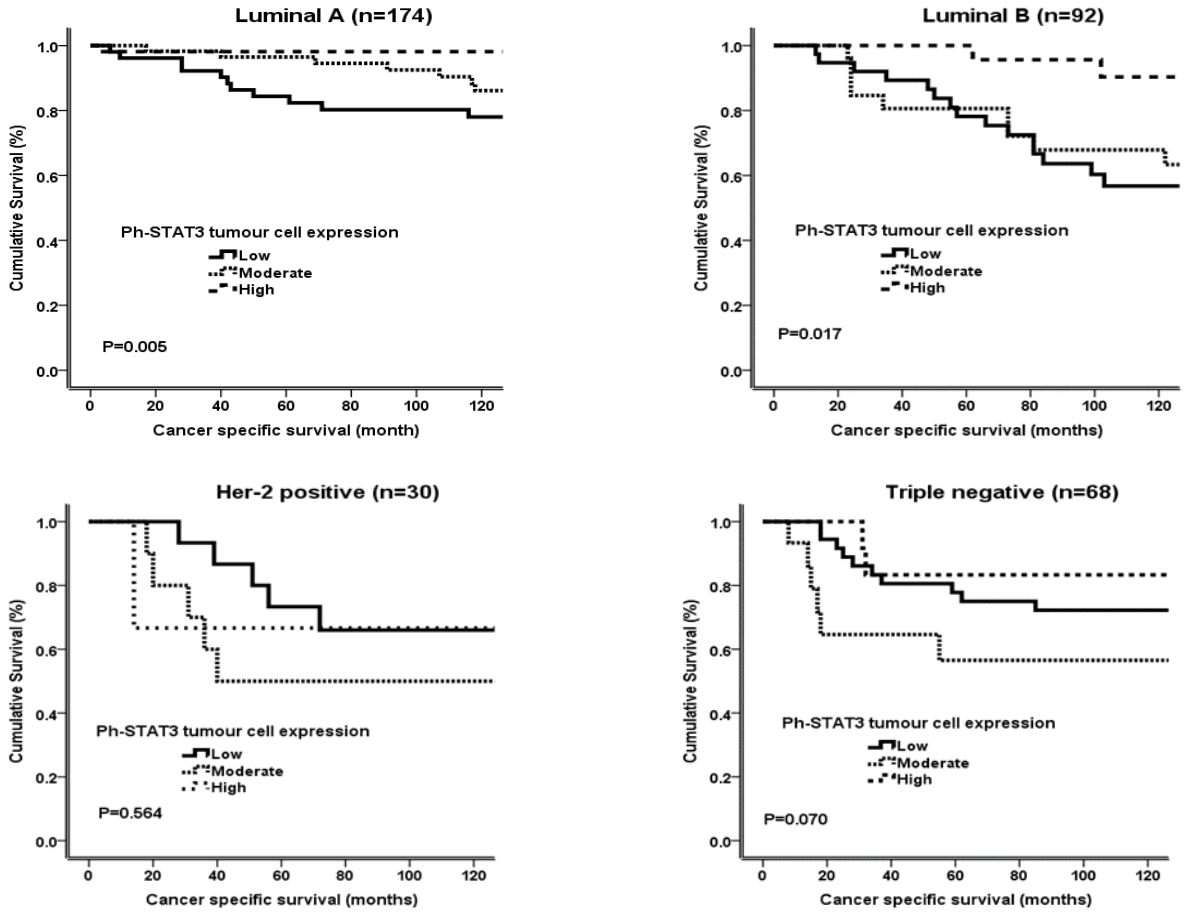


Figure 7-5 Kaplan-Meier survival curves (Log rank) of ph-STAT3 in different molecular subtypes.

Both Luminal A and Luminal B show significant association between high tumour cell expression of ph-STAT3 and improved cancer specific survival

7.3.1 Discussion

In the present study, increased tumour cell expression of both ph-STAT1 and ph-STAT3 was associated with improved survival and the phenotypic characteristics of the tumour, in particular the low tumour grade and lack of tumour necrosis. Therefore, activation of tumour STATs may be an important mechanism by which the tumour cells mitigate the development of an aggressive phenotype in patients with invasive ductal breast cancer.

To our knowledge, no previous study has conducted a comprehensive analysis of total and phosphorylated STAT1 and STAT3 expression in patients with ductal breast cancer. Ph-STAT1 and ph-STAT3 were strongly associated with each other independent of cellular location. In multivariate analysis, ph-STAT1 was independently associated with prolonged CSS, however when ph-STAT3 was also included in the model only ph-STAT3 remained independently associated with CSS. These results suggest that ph-STAT3 is the dominant STAT protein associated with improved survival in patients with invasive ductal breast cancer.

The observation that STAT1 is associated with improved survival may be explained by its role in promoting apoptosis and inhibition of proliferation (Koromilas and Sexl, 2013). STAT1 induces the apoptotic pathway by up-regulation of caspases 2 and 3 expression (Battle and Frank, 2002; Kim and Lee, 2007), and recently Magou and colleague has reported a positive association between ph-STAT1 and caspase 3 expression in primary breast cancer tissues (Magou et al., 2012). Furthermore, STAT1 has been reported to inhibit mammary tumours development in experimental models (Klover et al., 2010; Raven et al., 2011; Schneckenleithne et al., 2011; Chan et al., 2012). STAT3, in some contexts, also behaves as a tumour suppressor protein targeting genes involved in apoptosis and induction of growth arrest (Turkson and Jove, 2000; Ecker et al., 2009). In particular, STAT3 is activated during apoptotic involution of mammary gland (Chapman et al., 2000;

Sutherland et al., 2006; Scribner et al., 2011; Hughes et al., 2012) and suppression of brain tumours (de la Iglesia et al., 2008). Indeed, consistent with such a scheme, Sato and colleagues, in a large dataset of more than 700 patients, found that levels of ph-STAT3 were reduced over progression from normal breast epithelia to invasive and metastatic breast cancer (Sato et al., 2011). Furthermore, STAT3 has been shown to up-regulate tissue inhibitor of metalloproteinase-1 expression, which decreases invasiveness of breast cancer cells (Dien et al., 2006).

It should be noted that discrepancies in prognostic value of STAT1 and STAT3 between the different studies might be attributable to a combination of factors, such as different protein isoforms, different methods of detection as well as differences in patient cohorts and length of clinical follow-up. Also, it is plausible that STATs has prognostic value in certain subsets or molecular subtypes of breast cancer as well as different context of the tumour microenvironment. Indeed, cross-talk between STATs and ER signalling pathways has been reported by several laboratories. Oestrogen activates STAT1 in human osteoblasts and breast cancer cells by tyrosine phosphorylation, and promotes the formation of STAT1–DNA complexes (Kennedy et al., 2005). Also oestrogen receptors have been reported to up regulate STAT3 activation in response to 17_β oestradiol via a non-genomic pathway (Bjornstrom and Sjoberg 2002) and through direct protein interactions between ER and STAT3, occurring primarily through the DNA-binding domain of ER (Silva and Shupnik et al., 2007). Indeed, in the present study both STAT1 and STAT3 were directly associated with ER positive status and this relationship may provide a useful therapeutic target in patients with primary ductal breast cancer.

The present study reported an association between ph-STAT1, ph-STAT3 and the inflammatory cell infiltrate. High ph-STAT1 tumour cell expression was associated with up-regulation of local inflammatory infiltrate as evidenced by increased generalised inflammatory cell infiltrate (K-M grade). In contrast, high ph-STAT3 tumour cell

expression was associated with down-regulation of the local inflammatory infiltrate as evidenced by decrease in the CD4+ T-lymphocytes. In fact, in the present study, STAT1 and STAT3 were expressed in both the stroma associated fibroblasts and cells of the inflammatory infiltrate (Figure 7.1). Taken together, the results of present study would suggest an important role for STAT1 and STAT3 in regulating anti-tumour immunity in the breast tumour microenvironment (Yu et al., 2007; Yu et al., 2009). Such findings might be exploited to design therapies to counteract immune dysfunction and improve cancer immunotherapy (Avalle et al., 2012).

The present study reports for the first time a negative association between ph-STAT1 and ph-STAT3 expression and tumour necrosis. The basis of such an observation is not clear, however it is of interest that IFNγ-induced STAT1 activation has been previously shown to negatively regulate hypoxia-inducible factor-1 (HIF-1) α-dependent transcription in human glioblastoma cells lines (Hiroi et al., 2009), once more highlighting the opposing effects of STAT1 in tumours. HIF-1 is a master regulator of the transcriptional response to hypoxia (Semenza and Wang, 1992). Tumour hypoxia has been shown to be associated with a more clinically aggressive phenotype, resistance to therapy, angiogenesis and metastasis (Hockel and Vaupel, 2001; Harris, 2005). Therefore, further understanding of the molecular mechanism by which STAT1 down-regulates hypoxia-induced transcription may also lead to the development of a better therapeutic measure for cancer treatment.

The present study has found that in patients with high necrotic breast tumours, elevated ph-STAT3 expression was significantly associated with better survival, suggesting a protective role of STAT3 against tumour necrosis, which may further explain the association of STAT3 with good prognosis. Although the mechanism underlying this is not clear, hypoxic stress might influence STAT3 signalling, which in turn may down-regulate HIF-1 pathway. Indeed, constitutively active STAT3 acts as a master regulator of cell metabolism, inducing aerobic glycolysis via HIF-1 α transcriptional induction

(Demaria et al., 2010) as it is part of the complex signalling network that shapes the metabolic phenotype of tumour cells.

Of interest, in the present study the prognostic role of ph-STAT1 and ph-STAT3 tumour cell expression with relevance to different molecular subtypes was examined. Ph-STAT1 and Ph-STAT3 were significant predictors of prolonged cancer specific survival in luminal subtypes. This may indicate that the role of STATs in breast cancer may be driven by endocrine hormone and further support the cross-talk with ER. Previous reports have shown that patients with low proliferating luminal A tumours have higher ph-STAT3 expression compared to those with the luminal B tumours (Tell and Horvath, 2014). Furthermore, in ER negative, Her-2 positive tumours, no response was observed to trastuzumab in patients with STAT3 activation (Sonnenblick et al., 2015) and that JAK2 drives a JAK1/STAT3-independent signaling program in triple negative breast cancer (Balko et al., 2016), demonstrated that there are different activators and targets for STAT3 in different subgroups of breast cancer.

Taken together, the results of the present study would suggest that both STAT1 and STAT3 act as tumour suppressor proteins. STAT1 has long been implicated in growth suppression (Bromberg and Darnel, 2000; Lynch et al., 2007; Koromilas and Sexl et al., 2013) as loss of STAT1 function results in early development of breast tumours (Klover et al., 2010; Raven et al., 2011; Schneckenleithuer et al., 2011; Chan et al., 2012). Unlike other STAT members, loss of STAT3 function results in early embryonic lethality STAT3 (Inghirami et al., 2005) and suppression of tumour cells proliferation (Rivat et al., 2004; Gao et al., 2005; Xi et al., 2005), suggesting its crucial role as an oncogene. The mechanisms underlying STAT3 signalling pathway's diverse and sometimes opposing roles are still largely unknown. It would suggest that this pleomorphic role of STAT3 in breast cancer prognosis, as an oncogene or a tumour suppressor, may be a function of the setting or cellular context, in particular the tumour microenvironment and necrosis. It may

also suggest that there are other signal transduction pathways involved in the effect elaborated by tumour STAT3 expression. In addition, these results would indicate that STATs are central to the signalling networks in ductal breast cancer and that STAT3, in particular, has cross-talk with members of other pathways, such as the transcription factors HIF, and NFkB (Cairns et al., 2011; Mauro et al., 2011).

In conclusion, STAT1 and STAT3 tumour cell expression appears to be an important determinant of favourable outcome in patients with invasive ductal breast cancer. The present results suggest that STAT3 may affect disease outcome through direct impact on tumour cells counteracting aggressive tumour features as well as interaction with the surrounding microenvironment.

Of interest, several studies have examined the important role of STAT5 in breast cancer using both experimental and clinical data (Iavnilovitch et al., 2002; Nevalainen et al., 2004; Yamashita et al., 2006; Sultan et al., 2008; Peck et al., 2012). Therefore, it would be interesting to examine the role of STAT5 in breast cancer tumour microenvironment in future work.

Chapter 8 Discussion

In the present thesis a number of studies were carried out and their significance is discussed below.

In the present thesis it was shown that, in a review of lymphovascular invasion, the majority of studies used H&E and classical histochemistry to identify LVI and BVI reflecting current practice in most pathology departments. The prognostic significance of LBVI and LVI was well-documented and associated with aggressive features of breast tumours, the prognostic value and the optimal detection method of BVI was unclear. Only few recent studies used immunohistochemical staining of the endothelium lining lymphatic and blood vessels and were able to show clear differences between LVI and BVI.

In a prospective study, IHC for D2-40 and Factor VIII defined lymphatic and blood vessel invasion with greater sensitivity and specificity than H&E, improving detection of LVI and BVI in node negative and triple negative breast cancers. LVI and BVI, IHC compared with H&E, were more significantly associated with tumour recurrence and were independent predictors of cancer specific survival. In particular, in patients with node negative and triple negative tumours. Therefore, the results from these studies show that immunohistochemical detection of lymphatic and blood vessel invasion provides a superior assessment and may be useful for the objective assessment of LVI and BVI in routine clinical and pathological practice.

Given that LVI and BVI are important indicators of poor outcome in primary invasive ductal breast cancer, it may be that other markers of invasion such as peri-neural invasion will be also shown to have prognostic significance. If this was also observed then it may suggest that there is an underlying process promoting tumour invasion into these vessels.

This concept was examined in the subsequent chapters (5.0, 6.0, 7.0).

In the present thesis, tumour stroma percentage was associated with positive lymph node (an early sign of invasion), larger tumour size and grade and Her-2 positivity. In addition, TSP was consistently associated with low grade immune cell infiltrate in all subgroup analysis. Although the underlying biology of this association is not clear, stroma-associated fibroblasts (CAFs and myofibroblasts) may prevent penetration of immune cells within tumours, modulating the ability of lymphocytes and macrophages to invade a tumour while promoting tumour growth and progression, due to their contractile properties and their associated extracellular matrix (Lieubeau et al., 1999), and facilitate invasion into vessels. Increased expansion of tumour stroma was also an indicator of poor outcome independent of nodal status, lymphatic and blood vessel invasion and local inflammatory response.

On the basis of the above results, the relationship between the tumour microenvironment and tumour budding was examined. Tumour budding was associated with the key features of local and metastatic spread, positive lymph node and lymphatic vessel invasion. Of interest, there was an association between high tumour budding and increased amount of tumour stroma percentage and low local inflammatory infiltrate. Furthermore, high tumour budding was an indicator of reduced CSS independent of nodal status, LVI, BVI, inflammatory cells infiltrate and TSP. Therefore, in the context of the present comprehensive examination of the prognostic value of phenotypic features of tumour and the surrounding microenvirounment, it was found that tumour stroma and tumour budding provided prognostic value independent of well-established tumour characteristics, tumour necrosis, and components of the local tumour inflammatory cell infiltrate. Importantly, tumour stroma and budding were independent prognostic variables in node negative tumours indicating their importance as additional prognostic markers for early stage breast cancer, and that may aid risk stratification for those patients.

Tumour stroma has been implicated in facilitating epithelial-mesenchymal transition (one of tumour budding features) and metastasis of tumour cells into normal tissue (De Wever and Mareel, 2003; Hemmings, 2013). Therefore, it may be that tumour stroma is essential in facilitating tumour cell de-differentiation and escape from immune surveillance. On the other hand, tumour budding is an early and essential step for the tumour to metastasise. Therefore, the relationship between the tumour inflammatory cell infiltrate, tumour stroma and tumour budding might be linked to the process of epithelial-mesenchymal transition. In particular, accumulating evidence supports the hypothesis that tumour budding is driven by an EMT like process in the tumour microenvironment (Grigore et al., 2016; Koelzer et al., 2016). Of interest, in breast cancer, budded tumour cells at the invasive margin show reduced expression of membranous E-cadherin, and increased expression of cytoplasmic vimentin (Liang et al., 2013), essential phenotypic features of EMT (De Crane and Berx, 2013).

Developing microenvironment based prognostic score combining constituents of tumour microenvironment may help to define appropriate standardised approaches to the tumour microenvironment. For example, the very recently developed Glasgow Microenvironment Score, based on K-M grade and TSP, was independent predictor of cancer specific survival in patients with colorectal cancer (Park et al., 2015). Given the potential importance of the tumour microenvironment, characterisation of intracellular signalling pathways important in the tumour microenvironment is of considerable interest. One plausible signalling molecule that links tumour stroma, inflammatory cell infiltrate, and tumour budding is the STAT.

An investigation of STAT1 and STAT3 tumour cell expressions was carried out in chapter 7.0. Ph-STAT1 and Ph-STAT3 tumour cell expression were associated with reduced recurrence and prolonged survival in patients with invasive ductal breast cancer. In addition, Ph-STAT1 and Ph-STAT3 tumour cell expression was significantly associated

with good prognostic parameters including low tumour grade, ER and PR +ve status and low grade tumour necrosis.

In addition, a recent study on STAT3 by Aleskandarany and colleagues, using large dataset, reported similar results that ph-STAT3 was associated with good prognostic markers and good prognosis (Aleskandarany et al., 2016). This of particular interest especially a trial targeting STAT3 is under progression (Lin et al., 2013). However, with the concept that there are different molecular pathways interact with STATs, inhibition of only one of them may not be sufficient to obtain anti-tumour effect.

In breast cancer, it has long recognised that STAT1 plays a significant role as a tumour suppressor in consistent with our result. It is apparent from the present thesis results (and majority of previous reports) that STAT3 may also acts as a tumour suppressor in addition to its role as an oncogene. This pleomorphic role perhaps is a function of the cellular context (Ecker et al., 2009). The results of the present thesis show that STAT3 is an independent prognostic factor of prolonged survival in high grade tumour necrosis. Therefore, the suppressor role of STAT3 is, at least in part, a function of the tumour microenvironment.

The above observations of the present thesis point to the importance of the tumour microenvironment in promoting tumour budding, LVI and BVI. The observations from STATs work may suggest that an important driving mechanism for the above associations is the presence of tumour necrosis, probably secondary to hypoxia. Further work is needed to examine the interaction of other molecular pathways involved in the tumour microenvironment such as HIF and NFkB in patients with invasive ductal breast cancer. Also, it would be of interest to examine the role of STAT5 in the context of tumour microenvironment.

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Appendix

Immunohistochemistry

1-Reagents

Tris (hydroxymethyl) methylamine (Trizma Base)	(Fisher Scientific)
Sodium Chloride	(VWR)
EDTA, disodium Salt, dihydrate	(Sigma)
Citric acid	(Sigma)
Tri- sodium citrate	(Fisher Scientific
Xylene	(Fisher Scientific)
Alcohol solutions (100%, 90%, 70%)	(Fisher Scientific)
H202	(VWR)
Horse Serum	(Vector Laboratories)
Antibody dilutent	(DAKO)
DAKO kit (pen, AB solution, Envision)	(DAKO)
Haematoxylin	(VWR)
DPX Mountant	(VWR)

2-Buffers and Solutions

- Antigen retrieval buffers

1- Citrate Buffer (pH 6.0). Antigen retrieval buffer for FVIII

Citric acid	1.92g
Tri- sodium citrate	2.94g
Diluted H2O	1 litre

2- Tris Buffer (pH 8.0). Antigen retrieval buffer for total and ph-STAT1 and total and ph-STAT3.

EDTA, disodium Salt, dihydrate	0.37g
Tris (hydroxymethyl) methylamine	0.55g
Diluted H2O	1 litre

- **H2O2 (3%)**

H2O2	40ml
dH2O	360ml

- **Horse Serum (10 %)**

Horse serum	100 µl
TBS	1ml

- **TBS Buffer diluted pH(7.50) (10x stronger)**

Tris (hydroxymethyl) methylamine	300g
NaCl	438g
dH2O	5 litres

To Make 1X TBS (5 liters) from 10X:
500 ml of 10X and 5 liters of dH2O

- **DAB solution (chromagen 3,3 diaminobenzidine)**

DAB buffer	2 drops
DAB substrate	4 drops
DAB hydrogen Peroxide	2 drops
Ddiluted H2O	5ml

- **Scott Tap Water Substitute (S.T.W.S)**

Magnesium Sulphate – $MgSO_4$, $7H_2O$	40g
Sodium Hydrogen Carbonate – $NaHCO_3$	7g
Diluted H2O	2 litres

- **Mayer's Haematoxylin**

Haematoxylin	1g
Potassium Alum (Aluminium Potassium Sulphate)	50g
Sodium Iodate	0.2g
Citric Acid	1g
Chloral Hydrate	50g
Diluted H2O	1 litre
Glacial Acetic Acid	2 drops