**Charles University in Prague**
**Faculty of Medicine, Hradec Kralove**

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**Pathology**

**Morphologic and molecular characterization of triple negative breast carcinoma for identification of clinically relevant subtypes**

Morfologická a molekulární typizace triple-negativních karcinomů prsu – jako prostředek pro identifikaci klinicky relevantních subtypů

**MUDr. Ayotunde Folakemi Sobande**

Supervisor: prof. Aleš Ryška MUDr. PhD.

Hradec Králové, 2014          Date of defence: ...........................

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**Hradec Králové, 2014**

*'Apart from hypothesis-generating scientific research, a breast cancer classification should correlate with clinical outcome of patients or predict efficacy to therapy'*

**Jumppanen M**, Breast Cancer Research 2007

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## 1. Abbreviations

A: Anthracycline (doxorubicin)

AC: Anthracycline (doxorubicin) Cyclophosphamide

ACC: adenoid cystic carcinoma

AC-T: Anthracycline (doxorubicin) Cyclophosphamide plus Taxane

AD: axillary dissection

ADP: adenosine diphosphate

AT: Anthracycline (doxorubicin) Taxane

BLBC: basal-like breast cancer

CD: cluster of differentiation

CK: cytokeratin

CMF: Cyclophosphamide Methotrexate Fluorouracil

DAB: Diaminobenzidine

DCIS: ductal carcinoma in situ

EGFR: epidermal growth factor receptor

ER: oestrogen receptor

FDA: United States Food and Drug Administration

FEC: Fluorouracil Epirubicine Cyclophosphamide

FISH: fluorescence in situ hybridization

G:C: gene copy to chromosome ratio

GEP: gene expression profile

Gy: gray (unit of absorbed radiation)

HER2: human epidermal growth factor receptor 2

HER2:CEP17: HER2 gene to chromosome 17 centromere ratio

HMW: high molecular weight keratin

HR: hormone (oestrogen and progesterone) receptor

IHC: immunohistochemistry

IRS: immunoreactive score

ISH: in situ hybridization

ME: mastectomy

MYC: Myc proto-oncogene

NOS: not otherwise specified

NPI: Nottingham Prognostic Index

P53: protein 53

P63: protein 63

PARP: Poly (ADP-ribose) polymerase

pCR: pathologic complete response

PCR: polymerase chain reaction

PR: progesterone receptor

RNA: ribonucleic acid

RT PCR: reverse transcription polymerase chain reaction

RT: radiotherapy

S-100: S-100 protein

SEER: surveillance, epidemiology and end results

SERMs: selective oestrogen receptor modulators

SMA: smooth muscle actin

SOX2: SRY (sex determining region Y)-box 2

T: Taxane

T: tumour (size)

TDLU: terminal ducto-lobular unit

TK: tyrosine kinase

TN: triple negative

TNBC: triple negative breast carcinoma

TNM: tumour, node, metastasis (staging system)

VEGF: vascular endothelial growth factor

WHO: World Health Organization

XeNA: Capecitabine plus docetaxel

## 2. Introduction

Invasive breast carcinoma is a group of malignant epithelial tumours thought to arise from cells of the terminal ducto-lobular units of the breast. These malignancies differ in clinico-pathologic phenotype, molecular signature and quite possibly, etiopathogenesis. As a whole, breast carcinoma is the most common malignancy in women worldwide, accounting for 22% of all female cancers. It is also the most common cause of cancer death in women worldwide. Incidence varies widely geographically however many reports show that all over the world, breast cancer incidence seems to be on the rise.

Thanks to the implementation of breast cancer screening programs and the introduction of specific tailored treatment modalities, a significant decline in mortality has been observed in recent years; particularly in the western world.

A problem is posed by breast cancers that are currently un-classifiable using routine prognostic and predictive markers. These so called 'triple negative' tumours (owing to their lack of expression of ER, PR and HER2) are grouped together despite the clear heterogeneity within this category. There is currently no standard approach to management of these tumours or any widely accepted evidence-based and clinically relevant way to sub-classify them.

Triple negative breast carcinomas are usually associated with poor prognosis and yet paradoxically, reports show that they respond very well to neoadjuvant chemotherapy. The intense investigative efforts focused on this entity are thus justified and the search for morphological and molecular prognostic and predictive markers as well as specific molecules for targeted therapy continues.

## 2.1. Breast cancer

### 2.2.1. Breast microanatomy and the cell of origin theory

The human mammary gland is comprised of a branching network of ducts that end in clusters of terminal ducto-lobular units (TDLUs) (1). In the normal breast TDLU, two distinct cell layers are identified lining the tubular structures, an inner luminal epithelial layer with secretory function and an outer myoepithelial layer adjacent to the basement membrane with contractile function (1) (Fig. 1). This layered architecture is found throughout the breast – from nipple to terminal lobules (2). Mammary stem cells comprise a third group and these cells lack a specific anatomical location; they may be present both basally and luminally (2). This third group of cells was discovered when it was found that functional TDLUs can be generated after transplanting isolated cells from different parts of the mammary gland to cleared mouse mammary fat pads (3). Mammary stem cells give rise to both luminal and myoepithelial cells via a series of lineage-restricted intermediaries (4). These three different cell types may be distinguished from one another immunohistochemically. Luminal cells express low molecular weight keratins such as CK7, 8, 18 and 19 (3, 5) (Fig. 2). Myoepithelial cells express smooth muscle actin, calponin, S-100, p63, p-cadherin, CK5/6, CK14 and CD10 (3, 6, 7) (Figs. 3-4). CK5 and CK14 however, have also been demonstrated in luminal cells (2). Cells that co-express CK14 and CK19 are reported to express high levels of stem cell-associated genes (5). Thus, while to some extent it is possible to distinguish between these three cell types, there is an overlap in the expression of certain proteins.

It is possible to conclude that the heterogeneity of the cell population comprising the normal TDLU contributes to the heterogeneity observed in the epithelial tumours arising from the breast; different types of breast carcinomas could develop from different cell populations in the TDLU.  This is the basis of the prevailing concept in the field of breast cancer research; that different breast cancer subtypes arise from distinct breast epithelial cells at varied stages of differentiation that serve as the 'cell of origin' (4, 7). To some extent results of gene expression profiling did support this theory with the identification of luminal and basal subtypes. The differences in subtypes were thought to reflect differences in mutation profiles as well as differences in cell of origin (4). It is uncertain as to how

closely the genetic profile resembles the phenotypic reality, and more importantly, to what extent it affects the course of the disease and patients' outcome.
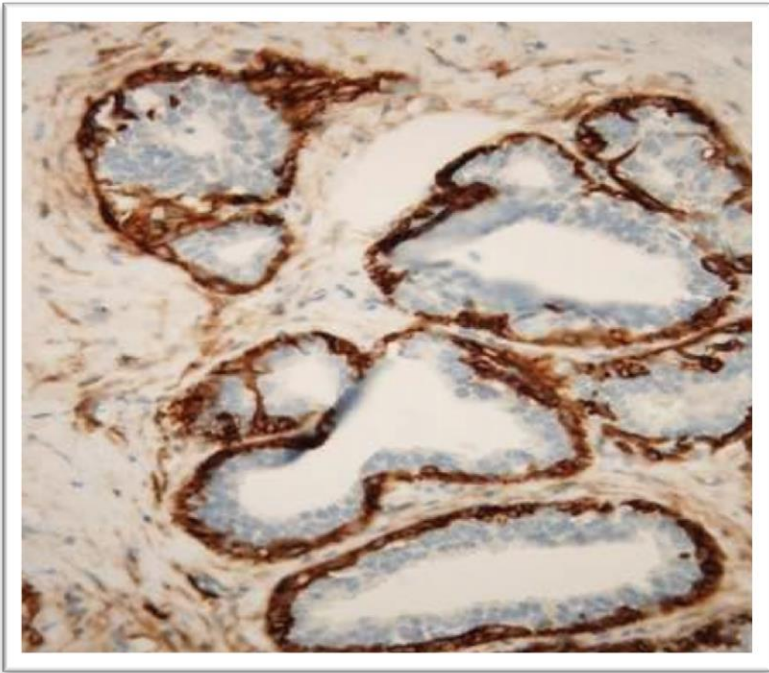
**Figure 1 Normal breast TDLU with 2 distinct layers of cells lining tubular structures**
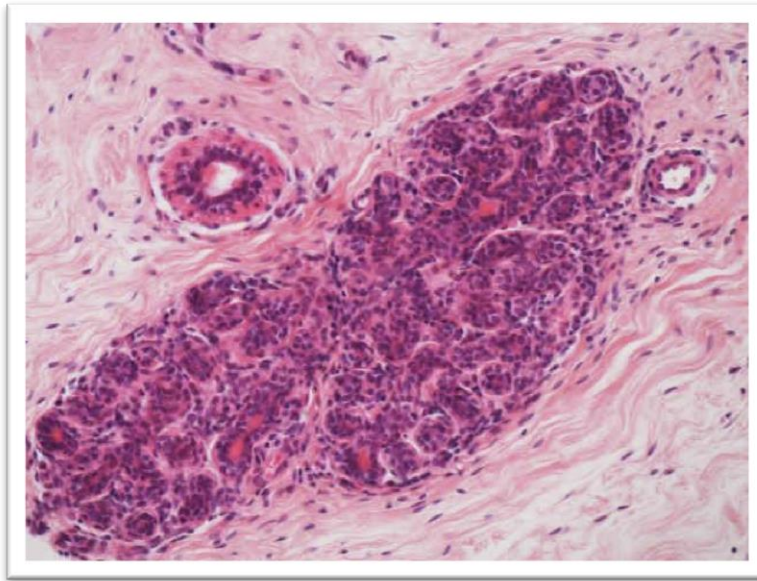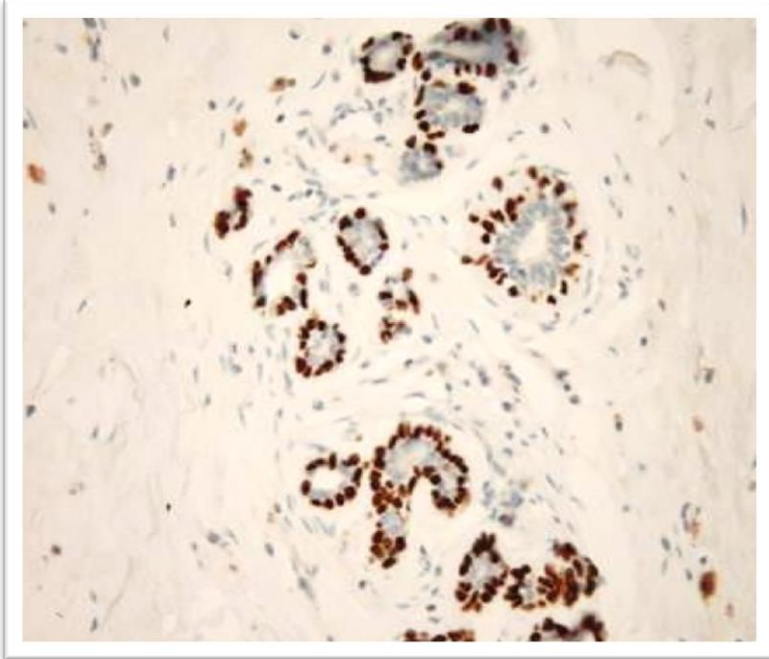


**Figure 2 CK18 staining cytoplasm of luminal cells of TDLU**
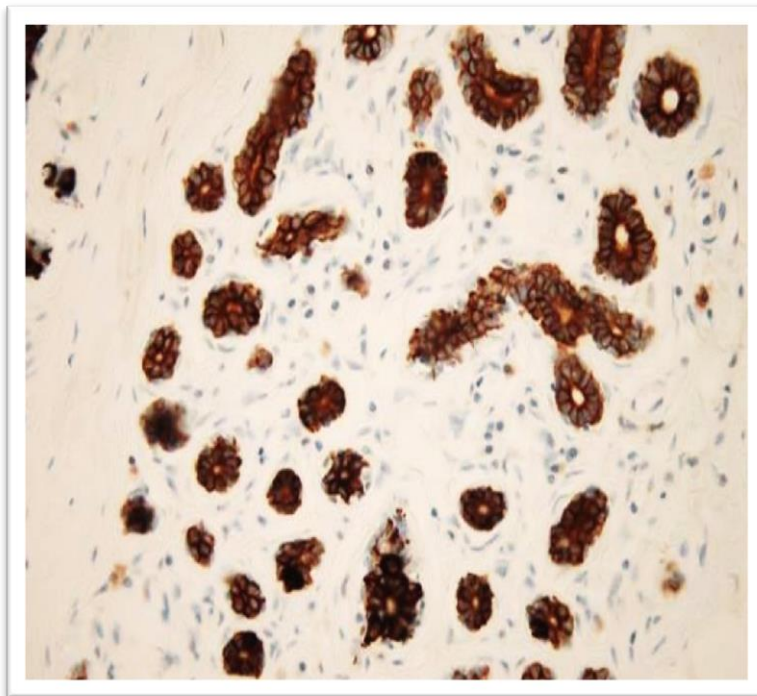
**Figure 3 CD10 staining basally located cells of TDLU**



**Figure 4 p63 staining nuclei of basally located cells of TDLU**

## 2.1.2. Epidemiology of breast cancer

Breast carcinoma is the most common malignancy in women worldwide, and the number one cause of cancer death in women (8, 9). Yearly, over 1 million new cases are diagnosed (8, 10). Less than 1% of breast carcinomas are diagnosed in men (11). Significant geographical and racial differences exist in breast cancer incidence, probably largely related to environmental and socioeconomic factors (12, 13). The highest age standardized incidence rate is recorded in Belgium (109.2 per 100,000) and the lowest rates (as low as 8 per 100,000) are recorded in African and Asian countries (14). Japan remains the only high-income country showing low incidence rates (15, 16). According to GLOBOCAN data from 2008, the Czech Republic ranks 26th in incidence of breast cancer with an age standardized rate of 70.9 (17). In most African countries cervical cancer remains the most common malignancy in women however in some North African populations, breast cancer incidence rates are similar to those of European countries (8).

Trends of breast cancer incidence show dynamic changes and in general, incidence appears to be increasing worldwide. Rising incidences are reported in many Asian countries, particularly in India, Singapore and Taiwan (18, 19). A further increase in certain developing countries is anticipated with increasing life expectancy (19). An exception to the general trend of increasing incidence of breast cancer is observed in the United States where there has been a decline in incidence from 2002/2003 primarily in women aged 50-69 years (10).

Breast carcinoma may be diagnosed at any age but the mean age at diagnosis is 56 years thus it typically affects postmenopausal women. Over 90% of cases in the Czech Republic occur in patients over 50 years of age (17). Mean age at diagnosis however, varies geographically; it is lower in developing countries than in Europe and North America. In India for example, a majority of cases are diagnosed in premenopausal women with 45 years being the age at which incidence peaks (20). Also, Saudi women are typically 46-50 years of age at diagnosis (21). In African countries, breast cancer incidence rates are also higher in premenopausal women (22). These observations imply that there is a true genetic

influence on breast cancer development. The biology of breast carcinoma also appears to vary geographically as hormone receptor positivity in Taiwanese populations appears to be associated with younger age (18). In African and Western populations however, it is triple negativity and HER2 positivity that are associated with young age at diagnosis (18).

Despite the rising incidence, a decline in breast cancer mortality has been observed in many high income countries for the past few decades. This improvement is partially due to the benefits of early detection offered by national mammographic screening programs and also to new possibilities of improving personalized therapy (20).

## 2.1.3 Classification of breast carcinoma (historical and current approaches)

**Histopathology**

Traditionally breast cancer is classified based on histological appearance. The World Health Organization Classification of Tumours recognizes 31 different histological subtypes of invasive breast carcinoma (Table 1) (15). Invasive ductal carcinoma NOS (not otherwise specified) is by far the most common subtype of breast carcinoma, accounting for up to 75% of cases (23), followed by lobular carcinoma comprising 5-15% of invasive breast tumours (15). The other 'special' histological types are rare however significant as some of them are associated with particular patterns of clinical behaviour and prognosis. Medullary carcinoma for example is associated with excellent prognosis despite its aggressive histological appearance (24) and adenoid cystic carcinoma in the breast, unlike its head and neck counterpart is also associated with good prognosis (25, 26).

After a morphologic type is assigned to a tumour, grading must be done to assess the degree of differentiation as histological subtype alone is a poor predictor of biological behaviour and a wide spectrum of differentiation exists, particularly for ductal carcinomas, ranging from very well differentiated tumours that closely resemble benign breast tissue to dedifferentiated malignancies. Grade is recognized as a powerful prognostic factor as studies show a significant association between grade and survival in invasive breast cancer (15). It is considered to be one of the strongest prognostic factors in operable breast cancer (27). Low grade tumours are associated with low recurrence risk, high grade tumours with high risk of recurrence, and prediction of outcome for intermediate or moderately differentiated tumours is more ambiguous (27).

The Nottingham combined histological grading system (also known as the Nottingham or the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system) is the most widely used grading system for breast cancer (28, 29, 30). There is an international consensus that the Nottingham grading system should be the gold standard for breast cancer grading (28). Tubule formation, nuclear pleomorphism and mitotic activity are taken in to account in the assessment of tumour grade (Table 2) (15).

**Table 1 WHO histological classification of breast carcinoma (15)**

| Malignant Epithelial Tumours of the Breast | |
|---|---|
| **Invasive ductal carcinoma, not otherwise specified** | 8500/3 |
|     Mixed type carcinoma | |
|     Pleomorphic carcinoma | 8022/3 |
|     Carcinoma with osteoclastic giant cells | 8035/3 |
|     Carcinoma with choriocarcinomatous features | |
|     Carcinoma with melanotic features | |
| **Invasive lobular carcinoma** | 8520/3 |
| **Tubular carcinoma** | 8211/3 |
| **Invasive cribriform carcinoma** | 8201/3 |
| **Medullary carcinoma** | 8510/3 |
| **Mucinous carcinoma and other tumours with abundant mucin** | |
|     Mucinous carcinoma | 8480/3 |
|     Cystadenocarcinoma and columnar cell mucinous carcinoma | 8480/3 |
|     Signet ring carcinoma | 8490/3 |
| **Neuroendocrine tumours** | |
|     Solid neuroendocrine carcinoma | |
|     Atypical carcinoid tumour | 8249/3 |
|     Small cell / oat cell carcinoma | 8041/3 |
|     Large cell neuroendocrine carcinoma | 8013/3 |
| **Invasive papillary carcinoma** | 8503/3 |
| **Invasive micropapillary carcinoma** | 8507/3 |
| **Apocrine carcinoma** | 8401/3 |
| **Metaplastic carcinomas** | 8575/3 |
|     Pure epithelial metaplastic carcinomas | 8575/3 |
|         Squamous cell carcinoma | 8070/3 |
|         Adenocarcinoma with spindle cell metaplasia | 8572/3 |
|         Adenosquamous carcinoma | 8560/3 |
|         Mucoepidermoid carcinoma | 8430/3 |

| | |
|---|---|
| Mixed epithelial/mesenchymal metaplastic carcinomas | 8575/3 |
| **Lipid-rich carcinoma** | 8314/3 |
| **Secretory carcinoma** | 8502/3 |
| **Oncocytic carcinoma** | 8290/3 |
| **Adenoid cystic carcinoma** | 8200/3 |
| **Acinic cell carcinoma** | 8550/3 |
| **Glycogen-rich clear cell carcinoma** | 8315/3 |
| **Sebaceous carcinoma** | 8410/3 |
| **Inflammatory carcinoma** | 8530/3 |

**Table 2 Assessment of histological grade in breast carcinomas (15)**

| Feature | Score |
|---|---|
| **Tubule and gland formation** | |
| Majority of tumour (>75%) | 1 |
| Moderate degree (10-75%) | 2 |
| Little or none (<10%) | 2 |
| **Nuclear pleomorphism** | |
| Small, regular uniform cells | 1 |
| Moderate increase in size and variability | 2 |
| Marked variation | 3 |
| **Mitotic counts** | |
| Dependent on microscope field area | 1-3 |
| | |
| **Grade 1 – well differentiated** | 3-5 points |
| **Grade 2 – moderately differentiated** | 6-7 points |
| **Grade 3 – poorly differentiated** | 8-9 points |

**TNM Stage**

The TNM classification system (edited by the International Union against Cancer) is used to indicate anatomical extent of disease (31). It is the most widely used staging system for malignant disease (32). The classification takes into account size of the invasive component of the primary tumour, its relationship to surrounding structures and/or its specific features e.g. skin ulceration and presence of tumour cells in dermal lymphatic vessels (inflammatory carcinoma) (T), regional lymph node status (N) and presence of distant metastasis (M) (Table 3). The information provided by staging helps direct treatment and is of prognostic value (33). Figure 5 shows distribution of breast carcinoma by stage for women diagnosed in the Czech Republic (17).

**Table 3 TNM classification for breast cancer (15)**

| Primary tumour (T) | |
| --- | --- |
| **TX** | Primary tumour cannot be assessed |
| **T0** | No evidence of primary tumour |
| **Tis** | Carcinoma in situ |
| **Tis (DCIS)** | Ductal carcinoma in situ |
| **Tis (LCIS)** | Lobular carcinoma in situ |
| **Tis (Paget)** | Paget disease of the nipple with no tumour in the underlying breast parenchyma. |
| **T1** | Tumour ≤ 20 mm in greatest dimension |
| **T1mi** | Tumour ≤ 1 mm in greatest dimension |
| **T1a** | Tumour > 1 mm but ≤ 5 mm in greatest dimension |
| **T1b** | Tumour > 5 mm but ≤ 10 mm in greatest dimension |
| **T1c** | Tumour > 10 mm but ≤ 20 mm in greatest dimension |
| **T2** | Tumour > 20 mm but ≤ 50 mm in greatest dimension |
| **T3** | Tumour > 50 mm in greatest dimension |
| **T4** | Tumour of any size with direct extension to the chest wall and/or to the skin |

| | |
|---|---|
| | (ulceration or skin nodules) |
| **T4a** | Extension to chest wall, not including only pectoralis muscle adherence/invasion |
| **T4b** | Ulceration and/or oedema (including peau d'orange) of the skin and/or satellite nodules in ipsilateral breast |
| **T4c** | Both T4a and T4b, above |
| **T4d** | Inflammatory carcinoma |
| **Regional lymph nodes (N)** | |
| **pNX** | Regional lymph nodes cannot be assessed |
| **pN0** | No regional lymph node metastasis identified histologically. |
| **pN1** | Micrometastases; or metastases in 1-3 axillary lymph nodes and/or in internal mammary nodes, with metastases detected by sentinel lymph node biopsy but not clinically detected |
| **pN1mi** | Micrometastases (> 0.2 mm and/or > 200 cells, but none > 2.0 mm) |
| **pN1a** | Metastases in 1-3 axillary lymph nodes (at least 1 metastasis > 2.0 mm) |
| **pN1b** | Metastases in internal mammary nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected |
| **pN1c** | Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected |
| **pN2** | Metastases in 4-9 axillary lymph nodes or in clinically detected internal mammary lymph nodes in the absence of axillary lymph node metastases |
| **pN2a** | Metastases in 4-9 axillary lymph nodes (at least 1 tumour deposit > 2.0 mm) |
| **pN2b** | Metastases in clinically detected, internal mammary lymph nodes in the absence of axillary lymph node metastases |
| **pN3** | Metastases in $\geq 10$ ipsilateral axillary lymph nodes; or in infraclavicular lymph nodes; or in clinically detected, ipsilateral internal mammary lymph nodes in the presence of $\geq 1$ positive axillary lymph node; or in > 3 axillary lymph nodes with clinically negative, microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes |

| | |
|---|---|
| **pN3a** | Metastases in ≥ 10 axillary lymph nodes (at least 1 tumour deposit > 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes |
| **pN3b** | Metastases in clinically detected, ipsilateral internal mammary lymph nodes in the presence of ≥ 1 positive axillary lymph nodes; or in > 3 axillary lymph nodes and in internal mammary lymph nodes, with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected |
| **pN3c** | Metastases in ipsilateral supraclavicular lymph nodes |
| **Distant metastasis (M)** | |
| **M0** | No clinical or radiographic evidence of distant metastasis |
| **M1** | Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven > 0.2 mm |

**Table 4 Anatomic stage (prognostic) groups (15)**

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

**Figure 5 Stage distribution for women diagnosed with breast cancer in the Czech Republic (17)**
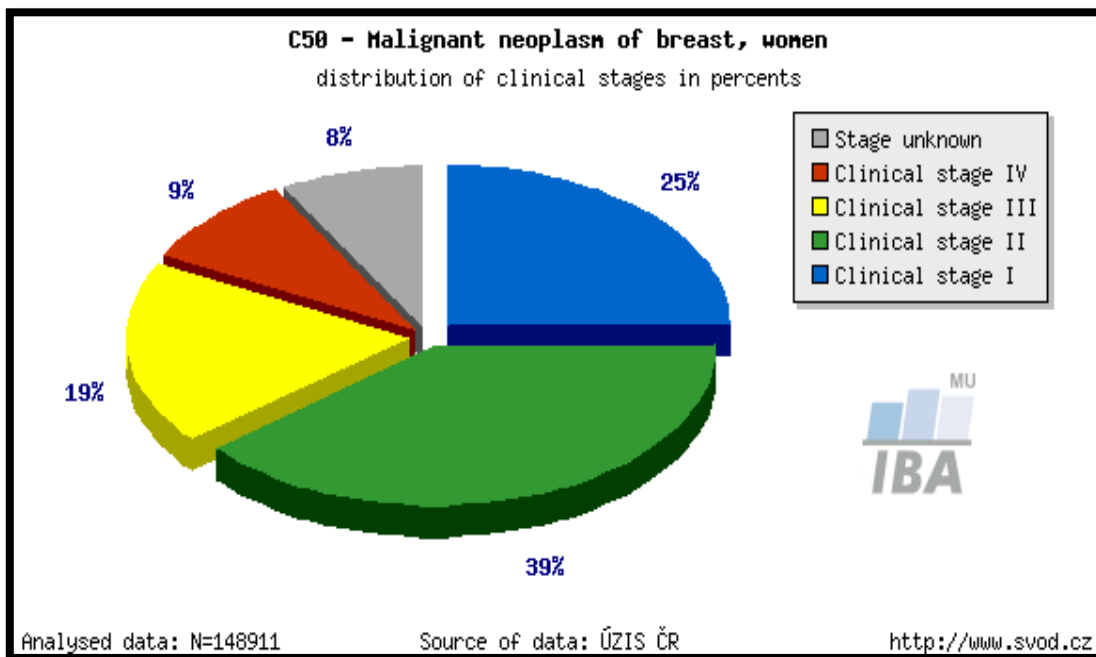
**Intrinsic Subtypes**

A landmark study by Perou *et al* in 2000 concluded that all breast tumours belonged to one of four intrinsic subtypes based on results of gene-expression profiling; Luminal/ER+, HER2, basal-like and normal breast like (34). These intrinsic subtypes were thought to represent distinct entities with diverse behaviour that could be identified using immunohistochemical surrogates (Fig. 6) (10, 35, 39). The differences in subtypes were thought to reflect differences in mutation profiles as well as differences in cell of origin (4). Of particular interest was the basal-like phenotype as it seemed to correspond with the immunohistochemically determined triple negative phenotype. At the 12th St Gallen International Breast Cancer conference the intrinsic subtypes were reclassified as luminal A, luminal B (HER2 negative), luminal B (HER2 positive), HER2 positive (non-luminal) and triple negative according to therapeutic options (35). At the 2013 St Gallen consensus conference a majority of the panel voted against multi-gene expression array profiling as a requirement for subtype definition (36).

Despite the fact that the conclusions drawn from the Perou study were premature and incomplete, it led to a revolution in breast cancer research resulting in the development of new cDNA gene-expression based tests that predict recurrence risk. Currently available on the market are MammaPrint (Agendia Inc., CA and Amsterdam), Oncotype DX (Genomic Health, Inc., CA), the Rotterdam signature and PAM50 (37). So far, only MammaPrint has United States Food and Drug Administration (FDA) approval however, Oncotype DX is the most widely used clinical gene expression assay in the United States (37). Two trials for determining the efficacy of both Oncotype DX and MammaPrint (Trial Assigning Individualized Options For Treatment (TAILORx) and Microarray in Lymph node-Negative Disease may Avoid Chemotherapy (MINDACT) respectively) are currently on-going and full results are unavailable (38). The first data from the TAILORx are expected in 2014 (37).

**Figure 6 Immunohistochemical phenotypes of intrinsic breast cancer subtypes (10, 35, 39)**
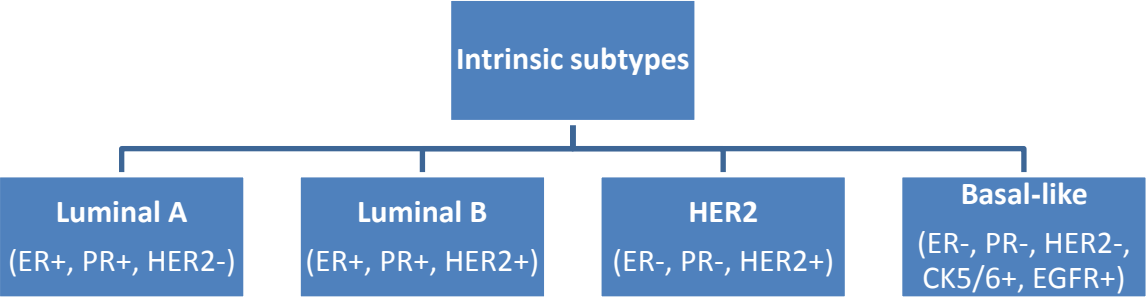
## 2.1.4. Predictive and Prognostic Markers

There is no single marker variable that adequately predicts outcome in breast cancer. A predictive marker predicts response to a particular type of therapy while a prognostic marker gives an estimation of clinical outcome in untreated patients (40, 41). Good prognosis would indicate that adjuvant systemic therapy may be unnecessary while poor prognosis is an indication for aggressive post-surgical systemic therapy (42).

**Nottingham Prognostic Index (NPI)**

NPI is a tool for stratifying prognosis in breast cancer patients (Table 5) (42, 43). In routine clinical practice, lymph node status, tumour size and histological grade are the strongest prognostic indicators in operable breast cancer (44). NPI combines these three prognostic factors, known to be independently associated with survival in order to produce a stronger predictor of outcome than any of the three alone (43, 45). NPI has been shown to predict long term survival in breast cancer patients and its reliability has been validated in several studies in its native Nottingham and around the world (40, 42, 44). In combination with predictive factors such as hormone receptor and HER2 status as well as individual patient needs and wishes, NPI can be used to select patients for adjuvant therapy (42).

Nottingham Prognostic Index = Lymph node stage (1-3) + Histological grade (1-3) + Tumour size (cm) x 0.2

**Table 5 Prognostic groups of Nottingham Prognostic Index (42)**

| NPI | Prognosis |
|---|---|
| 2.02-2.4 | Excellent |
| 2.41-3.4 | Good |
| 3.41-4.4 | Moderate 1 |
| 4.41-5.4 | Moderate 2 |
| 5.41-6.4 | Poor |
| 6.41-7.4 | Very poor |

**Oestrogen and progesterone (hormone) receptors (ER and PR)**

Approximately 75% of breast carcinomas express oestrogen receptors (45). Progesterone receptor expression is found in 55-65% of all breast carcinomas is thought to indicate a functioning ER pathway (45). ER+/PR+ tumours respond better to hormonal therapy than ER+/PR- tumours (45).

Hormone receptors (HR) are proven to be strong prognostic factors as patients with ER and PR expressing tumours are associated with the best outcomes with or without anti-hormonal therapy (46). According to the results of the SEER program, since 1990, women with ER+ tumours have experienced greater declines in breast cancer mortality than those with ER- tumours (47). Despite having lower growth rate than ER- tumours, ER+ tumours do not have lower metastatic potential (15). Mortality rate for HR+ tumours appears to remain constant over time from diagnosis while for HR- tumours the rate is high for the first 3-5 years and then it declines (45).

In our practice at the Fingerland Department of Pathology, we perform a semi-quantitative immunohistochemical assessment of hormone receptor status. An immunoreactive score (IRS) of 0-12 is assigned by assessing intensity of nuclear staining and percentage of positive cells (Table 6) (48).

IRS = intensity score x percentage score

**Table 6 Components of IRS system for immunohistochemical detection of oestrogen and progesterone receptors in breast cancer (48)**

| Intensity | Score | Percentage | Score |
|-----------|-------|------------|-------|
| Negative | 0 | 0 | 0 |
| Slight | 1 | ≤10 | 1 |
| Moderate | 2 | 11-50 | 2 |
| Strong | 3 | 51-80 | 3 |
| | | >80 | 4 |

**Human epidermal growth factor receptor (HER2)**

HER2 is a transmembrane receptor kinase that belongs to the same family of receptors as epidermal growth factor receptor (EGFR). There is no known ligand for HER2 however the receptor is the preferred molecule for dimerization with other members of the ERB family upon activation (49). Some authors report that up to 25% of all breast carcinomas overexpress HER2 (50-54). More recent, studies particularly in European populations, show that the incidence is lower than previously thought (55, 56). HER2 positive tumours are more aggressive than HER2 negative tumours and are more frequent in younger women (57). Overexpression of HER2 has been associated with poor clinical outcome in patients with axillary lymph node metastasis but not in patients with negative nodes (40). Overexpression also predicts positive response to HER2 targeted therapy (50). Despite the fact that HER2 positivity indicates poorer prognosis, treatment with trastuzumab, a recombinant humanized monoclonal antibody against HER2, appears to eliminate the differences in outcome (46, 58).

HER2 is routinely evaluated immunohistochemically and by in situ hybridization. Patients with tumours with positive HER2 staining or with HER2 gene amplification ($\geq$ six copies of HER2 gene per cell or HER2:CEP17 $\geq$2.2) are candidates for trastuzumab treatment (Table 7) (49).

**Table 7 HER2 testing in breast cancer (49)**

| Staining pattern | Score | HER2 staining assessment | Reflex HER2 in situ hybridization |
|---|---|---|---|
| No membrane staining observed | 0 | Negative | No |
| Faint partial staining of the membrane in any proportion of cancer cells | 1+ | Negative | No |
| Weak to moderate complete staining of the membrane in >10% cancer cells | 2+ | Equivocal | Yes |
| Strong, complete staining of the membrane in >10% cancer cells | 3+ | Positive | No |

**p53**

The p53 gene is recognized as a tumour suppressor gene and loss of function mutations are associated with development of varied malignancies (59, 60). P53 mutations are the most frequently observed mutations in carcinomas however the precise mechanism of wild-type p53 function is still not fully understood (59). Normal p53 may induce apoptosis or cell cycle arrest. Mutant p53 is more stable than wild-type p53 and it has a longer half-life (59, 61). It can therefore be more easily detected by immunohistochemistry. Mutations in p53 gene however may not be the only factors leading to p53 stability; it is possible that other genetic alterations in different loci may be responsible, in some cases, for prolonging p53 half-life (60). Immunohistochemical p53 overexpression is seen in neoplastic but not in normal breast tissue (59). Up to 54% of breast carcinomas are reported to show immunohistochemical p53 expression (59).

Aberrations of the p53 family of transcription factors have been reported to be predictive of response to chemotherapy (41, 62). Some studies show that mutations in the *TP53* gene are associated with chemoresistance however there are high inconsistencies across studies (62, 63). Bidard *et al* suggest that this is because molecular subclasses are not taken into account (64). They showed that p53 immunostaining is associated with a trend for higher rates of pCR in TNBC (64). For triple negative breast carcinomas Biganzoli *et al* suggest that p53 expression is associated with poor outcome (61). Pollner *et al* however failed to demonstrate a statistically significant association between p53 expression and survival (59).

**Ki-67**

Proliferation is one of the strongest prognosticators in node-negative breast cancer (65). Ki-67 is a non-histone nuclear protein that can be detected in nearly all phases of the cell cycle but not in resting cells; it is a well-established marker of cell proliferation (15, 29, 66). The number of immunohistochemically Ki-67 positive cells present in tumours may be used to classify patients as good or poor survivors (15, 27). Aleskandarany *et al* showed that using a cut-off point of 10% positively stained tumour cells, Ki-67 expression could be used to classify grade 2 breast carcinomas into two distinct subgroups with different outcome (27). A measurement of Ki-67 during/after neoadjuvant therapy also appears to be strongly predictive of long-term outcome (67). Aside from being a prognostic marker, Ki-67 also

predicts response to endocrine therapy and neoadjuvant chemotherapy (45, 68). Studies have shown that adjuvant chemotherapy is significantly beneficial to patients with rapidly proliferating tumours but not to patients with slowly proliferating tumours (65).

It has been suggested that Ki-67 as a marker of proliferation should be used to distinguish between luminal A and luminal B subtypes (36, 69).  However Ki-67 is not included in current clinical decision making because of a lack of clarity on how the measurements should influence clinical decisions (66). This is partially due to issues with quality control and assurance (65). Additionally various investigators have used different cut-off points for Ki-67 assessment (10%, 14%, 20%) (27, 66, 68). Also, some Ki-67 positive nuclei may not survive the cell cycle thus the prognostic value of the Ki-67 index may be blurred (65). Despite these limitations, immunohistochemical detection of Ki-67 is still considered to be a robust cost effective diagnostic tool that provides useful prognostic information and may be useful in planning therapy (27).

MIB1 is the most commonly used antibody for immunohistochemical detection of Ki-67 (27, 67).


**MammaPrint**

MammaPrint is an RNA based gene-expression prognostic tool that is used to guide adjuvant treatment decisions. It was developed based on the Amsterdam 70-gene expression profile that was shown to be a powerful predictor of outcome in young breast cancer patients (70). MammaPrint helps to identify patients that do not need adjuvant chemotherapy and can be spared the toxic side effects and financial burden (71). A 70-gene profile is analysed in fresh frozen tumour samples. The testing is performed by the manufacturers, Agendia Inc., CA and Amsterdam, (71). The assessment classifies tumours as having either poor prognostic signatures with high risk of recurrence or good prognostic signatures with low risk of recurrence. Straver *et al* preformed a study on 167 patients with stage I and II breast carcinoma (72). They showed that patients with poor prognostic signatures were more likely to achieve a pathologic complete response to chemotherapy than those with good prognostic signature. All the triple negative tumours analysed had poor prognostic signatures.

MammaPrint was not accepted by the St. Gallen 2011 international breast cancer consensus

panel for clinical decision-making (69). Only a minority of the 2013 St Gallen panellists (25%) thought that the 70 gene signature would predict chemotherapy response (36).

**Oncotype DX**

Oncotype DX is a 21-gene profile assay that uses RT PCR in formalin-fixed paraffin-embedded tissue. It is the most frequently used gene expression profile (GEP) in the United States (38). It predicts the likelihood of recurrence in node negative ER+ breast cancer. Though the assay works independently of nodal status, it appears to be useful only in patients with 3 or fewer positive nodes (73). It uses a recurrence score to stratify patients into 3 groups; those with high risk, intermediate risk and low risk. Patients categorized as low risk will be recommended for endocrine therapy alone (73). The patients with low risk recurrence can avoid adjuvant chemotherapy that they do not need.

The St. Gallen 2011 international breast cancer consensus panel considered Oncotype DX to be potentially useful for clinical decision making (69). A majority of the St Gallen panel in 2013 thought that the 21-gene recurrence score could predict chemotherapy response (36).

**Combinatorial approach**

All the factors discussed above form the basis for determining adjuvant therapy for patients with breast cancer. The predictive markers currently used are able to identify patients that will not respond to certain treatment (negative predictive value) but their ability to identify patients that will respond (positive predictive value) is suboptimal (45).

Isolated knowledge of the individual prognostic markers provides limited information on tumour biology. For this reason a combinatorial approach is employed nowadays for assessing prognosis and guiding patient management. Still, patients with similar clinico-pathologic features may show varied outcomes and may respond differently to therapy (71). This justifies the on-going intensive search for better predictive and prognostic markers for breast cancer and especially for the triple negative breast carcinomas.

### 2.1.5 Treatment options for breast cancer

**Locoregional treatment**

Surgical treatment for breast cancer is determined for each woman on an individual basis (74). For early stage breast cancer, breast conserving surgery followed by irradiation has replaced modified radical mastectomy as the preferred treatment option (74, 75). Mastectomy may be performed after neoadjuvant chemotherapy of stage III tumours, in cases of previous breast irradiation or when clear surgical margins cannot be secured (74). The standard technique for postoperative radiotherapy following breast conserving therapy is percutaneous irradiation of the entire breast (45-50 Gy) followed by a tumour bed boost (76). Routine post-mastectomy RT is endorsed for patients with more than 3 involved nodes (69). Post-mastectomy RT is not supported for large (T>2) node-negative tumours (69).

**Adjuvant and neoadjuvant (primary) chemotherapy**

Early studies showed increased proliferation in metastatic deposits after resection of the primary tumour. It was also observed that preoperative administration of cyclophosphamide resulted in maximum decrease in proliferation of cancer cells. These findings provided rationale for use of primary chemotherapy for non-metastatic breast cancer (77). Clinical evaluation of tumour size remains the gold standard assessing patient suitability for neoadjuvant chemotherapy (57). Neoadjuvant chemotherapy is not recommended for low-proliferating and in highly endocrine-responsive disease (69). Adjuvant systemic therapy greatly improves survival in women with both lymph node-positive and lymph node-negative breast cancer (Table 8). It is recommended for patients with stage IB-IIIB disease (78). Standard adjuvant chemotherapy regimens can be used in the neoadjuvant setting and anti-HER2 agents should be added for HER2 overexpressing tumours (69).

TNBCs have been reported to be more sensitive to anthracycline (adriamycine/doxorubicin) based neoadjuvant chemotherapy than hormone receptor positive tumours (79). Basal-like breast carcinomas (BLBCs) have also been shown to have higher sensitivity to anthracycline-based chemotherapy than luminal subtypes (80).

**Table 8 Preferred agents for adjuvant chemotherapy in different breast cancer subtypes (69)**

| Subtype | Recommendation |
|---|---|
| **Luminal A** | No preference |
| **Luminal B** | Anthracyclines and taxanes |
| **TNBC** | Cyclophosphamide |
| **Basal-like** | Anthracyclines and taxanes |

**Targeted therapy**

Knowledge of the biology of breast cancer has expanded greatly in the last few decades. A better understanding of the uniqueness of each tumour leads to a shift from a 'one size fits all' approach to a more elegant and personalized approach to oncological treatment. The lessons learnt about some key pathways involved in breast cancer development and progression have enabled the development of agents that specifically target these pathways. Some of the agents have already become part of standard care while others are not yet approved for use in clinical practice though they have shown some efficacy. Tyrosine kinase inhibitors, angiogenesis inhibitors and agents that interfere with DNA repair are examples of such novel therapeutics. Oestrogen focused and anti-HER2 therapies are now standard components of breast cancer care (81).

Endocrine therapy is the first and oldest form of targeted therapy for breast carcinoma (69). Indeed it is the first type of targeted therapy used for any type of cancer. Tamoxifen is a non-steroidal anti-oestrogen that started out as a failed contraceptive (82). It belongs to the group of selective oestrogen receptor modulators (SERMs) (82). It targets ER+ tumours by blocking oestrogen-stimulated growth at the oestrogen receptor (82). Aromatase inhibitors such as letrozol suppress oestrogen synthesis, reducing systemic oestrogen levels. For premenopausal patients tamoxifen alone is considered to be standard treatment. For cases in which tamoxifen is contraindicated, ovarian function suppression with aromatase inhibitors is the preferred therapeutic option. Tamoxifen use has been linked to endometrial cancer, osteoporosis and thromboembolism (40).

Trastuzumab is a humanized murine IgG monoclonal antibody that targets the extracellular domain of HER2 (49, 57, 58, 83). The mechanism of its action is not fully understood however it appears to inhibit DNA repair and induce apoptosis (83). Trastuzumab has been shown to reduce signalling through the P13K/Akt and Ras/Raf/MEK/MAPK pathways; it has also been suggested that it may promote antibody-dependent cellular cytotoxicity (83). In 1998 it was approved by the FDA as first part of line treatment for metastatic HER2+ breast carcinoma (58, 84).

In a study on women with metastatic breast cancer, Dawood *et al* showed that trastuzumab improves the prognostic outcome of women with HER2+ breast cancer beyond that of HER2- disease (58). Lapatinib, which gained FDA approval in 2007, is a small molecule tyrosine kinase (TK) inhibitor that has been shown to cause remissions in patients with HER2+ tumours that are resistant to trastuzumab (58). It selectively binds to both HER2 and EGFR intracellularly to prevent phosphorylation of pathways which activate cell proliferation survival (84). Diarrhoea, neutropenia and liver dysfunction are the common adverse effects of lapatinib therapy (84). Dual HER2-targeting therapy (trastuzumab plus TK inhibitor) is not currently accepted as a therapeutic option for HER2+ disease (69). Pertuzumab is a recombinant humanized monoclonal antibody that prevents HER2 dimerization thus it inhibits multiple HER signalling pathways (85). Pertuzumab, in combination with docetaxel and trastuzumab, is recognized as part of the preferred first-line treatment for patients with metastatic HER2+ breast cancer (85).

Poly (ADP-ribose) polymerase (PARP) is an enzyme essential for base excision repair for single-strand DNA breaks (7). PARP inhibitors such as olaparib and iniparib target the base excision repair pathway of single-stranded DNA. The use of olaparib, an oral PARP inhibitor for treatment of breast cancer (locally advanced or metastatic) in women carrying a BRCA1 or BRCA2 mutation is associated with a high (41%) response rate (39).

Bevacizumab is a vascular endothelial growth factor (VEGF) inhibitor. VEGF is a key mediator of angiogenesis which is required for tumour growth, invasion and metastasis (86). Bevacizumab is reported to improve progression-free survival in patients with

metastatic TNBC when administered in combination with paclitaxel (39, 87). Despite this observation, there is no clear signal that bevacizumab has any special properties in TNBC compared with other subtypes of breast cancer (88).  Large trials investigating bevacizumab in TNBC are still on-going however it is currently not recommended by the FDA for treatment of metastatic breast cancer despite having previously been granted approval (89).

EGFR can be targeted using monoclonal antibodies (cetuximab, panitumumab) which target the extracellular domain of the EGFR receptor or with tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib or afatinib (78, 90, 91). A randomized phase II trial testing cetuximab in combination with carboplatin in stage IV TNBC showed low levels of response to platinum plus cetuximab and uncommon response to cetuximab alone (92). In this study, EGFR pathway analysis revealed that cetuximab blocked expression of the EGFR pathway in only a minority of cases (92).
Cetuximab is not yet approved for treatment of metastatic breast cancer (89).

**2.2 Triple negative breast cancer (TNBC)**

Triple negative breast cancer by definition is a diagnosis of exclusion. It includes all primary epithelial breast malignancies that do not overexpress HER2 and are negative for hormone (oestrogen and progesterone) receptors (87). They account for 10-17% of all breast carcinomas (34, 35, 93-96). Some authors report incidences of up to 24% (38). This discrepancy could be explained by the varying cut-offs for assessing ER, PR and HER2 status (97, 98). As of now, no clear evidence-based definition of triple negativity exists. Triple negativity is strongly associated with BRCA mutations. Approximately 70% of breast cancers in people carrying a germline BRCA1 mutation are triple negative (99). Women with triple negative (TN) tumours are shown to be younger than women with hormone receptor positive tumours (100). TNBCs are thought to be more common in young women (<50 years of age) and in women of African, Hispanic and South East Asian origin (94, 100). An American population based case-control study showed that women with TN tumours are also more likely to be obese, of lower socioeconomic status and likely to have experienced menarche at a younger age than women with hormone receptor positive tumours or controls (100). Young age at first full-term pregnancy and black race were also shown to be associated with TN phenotype (100).

This group of malignancies is morphologically, immunophenotypically and biologically heterogeneous (87, 101). In general triple negative breast carcinomas are thought to be more aggressive with worse outcome than other immunophenotypic subtypes (87). They are considered to have bad prognosis with a high risk of death in the first 5 years following therapy but reduced late recurrences/deaths (96). Relapse of TNBC usually occurs within the first three years of the breast cancer diagnosis (10, 93, 102). The liver, lungs and central nervous system are common metastatic sites (94). TNBCs are unlikely to respond to hormonal or anti-HER2 therapy thus the only standard systemic treatment option available is conventional chemotherapy (87, 101).

The morphological features associated with TN ductal NOS carcinoma are high nuclear grade, marked cellular pleomorphism, lack of tubule formation, scant stromal content, tumour necrosis, pushing borders of invasion, lymphoplasmacytic inflammatory infiltrate and, central acellularity (2, 35). There are however, certain rare morphological subtypes of

breast cancer with specific histological features and predictable clinical behaviour that are associated with triple negativity.

One example is adenoid cystic carcinoma (ACC). ACC has a distinct biphasic pattern consisting of true glandular spaces lined by epithelial cells and pseudocystic spaces lined by myoepithelial cells. Unlike ACC in the head and neck, primary breast ACC has excellent prognosis (26). Medullary carcinomas, which are also associated with triple negativity, whether typical or atypical, have better outcome than high grade ductal carcinomas NOS (103). Medullary carcinomas account for 1-7% of all breast carcinomas (15) and tend to occur in younger women (94, 104). 5 morphologic features define this subtype: syncytial architecture, absence of glandular structures, moderate to intensive lymphoplasmacytic infiltrate, high nuclear grade and complete microscopic circumscription (15, 105, 106). Tumours that do not fulfil all 5 of the histologic diagnostic criteria are sometimes referred to as atypical medullary carcinomas (103). Unlike with other morphological subtypes of breast cancer, ER positivity in medullary carcinomas is associated with poorer overall survival (94).

Secretory carcinoma of the breast, originally referred to as juvenile carcinoma, is a very rare histological subtype of breast cancer (0.15% of all breast cancers) (15, 107). It is the most common type of breast cancer seen in children however the disease is most frequent in young adults (108). It is a low grade, generally well circumscribed tumour composed of round to polygonal cells producing intra and extracellular PAS and alcian blue positive secretory material (15, 107, 108, 109).

Metaplastic carcinomas of the breast are epithelial malignancies with partial or complete transformation to a non-glandular epithelial or mesenchymal cell type (110). They are classified as matrix producing carcinomas, squamous cell carcinomas, spindle cell carcinomas, carcinosarcomas or metaplastic carcinomas with osteoclastic giant cells (110). Unlike other special histological types of triple negative breast cancer, metaplastic carcinomas appear to be inherently aggressive and associated with poorer prognosis than invasive ductal carcinoma NOS. Over 50 % of the patients develop local or distant metastasis within 5 years. Jung *et al* reported poorer 5-year DFS for metaplastic breast cancer than for any other subgroup of breast carcinoma (110).

Thanks to the discoveries from gene expression profile studies, there has been a great interest in identifying the so-called basal phenotype in TNBC and more recently, the claudin-low phenotype. The claudin-low phenotype was first identified by Herschkowitz *et al* in 2007 as a distinct molecular subtype of human breast cancer (111). Claudin-low tumours express mesenchymal genes with low gene expression of e-cadherin and tight junction proteins claudins 3, 4 and 7 (112, 113). They show inconsistent expression of basal cytokeratins, low expression of HER2 and luminal markers, and do not show high expression of proliferation genes (113).

Claudins belong to a family of tight junction proteins which are critical for epithelial cell polarity and maintaining the differentiated state of epithelial cells (114). Immunohistochemical studies on claudins in breast cancer have yielded results that are rather difficult to interpret. Claudins 3 and 4 appear to be overexpressed in breast cancer while claudins 1 and 7 appear to be down-regulated or completely absent (114). Claudin 1 positivity however has been reported to be more common in ER- than in ER+ tumours (114). Lanigan *et al* found that high claudin-4 expression was associated with poor prognosis and high tumour grade (115). Kulka *et al* demonstrated high levels of claudin-4 expression in basal-like breast carcinomas (116). The same research group observed loss of claudin-4 expression in well differentiated breast carcinomas (117).

The most widely accepted IHC definition for basal-like carcinoma is that proposed by Nielsen *et al*; any CK5/6 and/or EGFR positivity in TNBC (98). To the best of our knowledge there are no widely accepted immunohistochemical criteria for the identification of the claudin-low phenotype. Both basal and claudin-low subtypes are associated with poor outcomes (113) and a tendency to metastasize to the brain and lungs (118).

The basal-like phenotype is perhaps more attractive to study in TNBC because it may be reliably identified immunohistochemically and not only is EGFR a potential prognostic marker, it is also a very attractive molecule for biological therapy. About 60% of TNBCs are reported to express EGFR (93) however the role this plays in breast cancer development and progression is not fully understood.

## 2.3. Basal-like breast carcinoma

'Basal-like' is one of the four intrinsic subtypes of breast cancer initially proposed by Perou *et al* based on their cDNA microarray study of 43 different benign and malignant breast tissues (36 infiltrating ductal carcinomas, 2 lobular carcinomas, 1 DCIS, 1 fibroadenoma and 3 normal breast samples) (34).  In their sample, there was a group of 6 tumours that highly expressed genes characteristic for breast basal epithelial cells, including CK5, CK17, integrinβ4 and laminin (34). All six of these tumours showed positive immunohistochemical staining for CK5/6 and/or CK17. This was proof of the 'basal-ness' of this group of tumours. The basal-like tumours were ER- and appeared to be distinct from the HER2+ tumours (34).  It appeared that TNBC could be the immunohistochemical surrogate of BLBC.   Since the 2000 Perou publication, numerous authors have emphasized that TNBC and BLBC are not the same but that an overlap exists between the two groups of tumours. ER expression has been reported in 5-45% of BLBCs and HER2 expression in 14% (119). Foulkes *et al* define basal-like breast cancer as follows '*a subtype of breast cancer defined by unsupervised analysis of microarray gene-expression data… characterized by the absence of or low levels of expression of estrogen receptors, very low prevalence of HER2 overexpression, and expression of genes usually found in the basal or myoepithelial cells of the human breast*' (101). Thus like TNBC, BLBC appears to be a heterogeneous group of breast malignancies.

The significance of the basal-like subtype is controversial. While most studies have indicated that basal-like breast carcinomas have poor prognosis, not all of them have done so (120).  Jumppanen *et al.* suggest that there is no difference in survival between the basal and non-basal hormone receptor negative tumours (121).

BLBCs are usually ductal NOS carcinomas with solid architecture and high nuclear and histological grade (3, 120). Geographic necrosis, ribbon-like architecture and prominent lymphocytic infiltrate are common histological features of this group (6, 35, 120, 122). 95% of medullary carcinomas are basal-like (3). Use of oral contraceptives in women <40 years old, younger age at menarche, Hispanic ethnicity, abdominal obesity and metabolic syndrome are amongst the risk factors associated with BLBC (122).

The basal-like phenotype is common in patients with germline BRCA1 mutations (98).

GEP for identification of intrinsic subtypes is only performed in research settings. The tests are expensive and complex for routine diagnostic purposes however the information they yield may be useful in assessing prognosis. These issues are solved by finding immunohistochemical surrogates for identifying the prognostically significant subtypes of breast carcinoma. High molecular weight keratins CK5, CK5/6, CK14, and CK 17 have been used in various combinations by different investigators for identifying the basal-like phenotype in TNBC (119). Besides the 'gold standard' immunohistochemical definition of BLBC proposed by Nielsen *et al*, other investigators have suggested and used different criteria for identifying the basal-like phenotype. Rakha *et al* proposed using a cut off of 10% positively staining tumour cells for evaluating CK5/6 (123). Cakir *et al* suggest adding vimentin and CK14 to the panel in order to identify basal-like breast carcinomas (35). Cserni and Bori used expression of EGFR, CK5, CK14 or p63 to identify the basal phenotype in TNBC (124).

Poorly differentiated basal-like carcinomas have been shown to overexpress certain embryonic stem cell genes such as SOX2 and MYC (4). The stem cell has been presumed to be the cell of origin for basal-like carcinomas however studies have shown that the basal-like group bears a striking resemblance to luminal progenitor cells (4). This suggests that 'luminal progenitor' subtype is a more apt description for HR-/HER2-/CK5/6 and/or EGFR+ carcinomas than basal-like.

An immunohistochemical study of 6 secretory carcinomas carried out by Lae *at el* concluded that secretory carcinomas are low grade triple negative breast carcinomas belonging to the basal-like spectrum (107). Other reports have demonstrated expression of basal cytokeratins (CK5/6, CK14 and CK17) as well as EGFR in secretory carcinomas (125). Unlike for other basal-like breast tumours, prognosis for patients with secretory carcinoma is good (107, 109). It is somewhat paradoxical that these tumours with apparent features of luminal differentiation simultaneously express markers of basal differentiation. This finding suggests that 'basal' and 'luminal' differentiation may not be mutually exclusive.

## 2.4. Germ-line BRCA1 mutations and TNBC/BLBC

The BRCA1 gene located on chromosome 17 is known to be involved in a large proportion of people with inherited susceptibility to breast and ovarian malignancies (15). BRCA1-related tumours account for approximately 5% of all breast cancers (87). BRCA1 mutations may be suspected in breast cancer cases that are diagnosed at a young age (15).

It has been suggested that BRCA1 may be involved in breast epithelial cell differentiation. It appears to be required for transition from an ER- progenitor to an ER+ progenitor (7). As such, the inactivation of BRCA1 would give rise to tumours with stem cell-like features (122). BRCA1 related tumours are high grade malignancies with high mitotic activity, syncytial growth pattern, pushing margins, geographic necrosis and prominent lymphocytic infiltrates (3, 120). Also, when large, they are less likely to be lymph node positive than non-BRCA1 related tumours (126). The histological characteristics of BRCA1 related breast cancers are strikingly similar to those of basal-like or triple negative breast cancer, as described above. Indeed, 75% of breast cancers in women with germ-line BRCA1 mutations are basal-like and/or TN (39). Even in basal-like tumours without BRCA1 mutations, there is evidence of dysfunction in the BRCA1 pathway (39, 122).

Foulkes *et al* performed a study in order to determine whether BRCA1-related breast cancers were more likely to express a basal epithelial phenotype than non-BRCA1 related cancers (120). They found that BRCA1 related breast cancers are statistically significantly associated with expression of basal cytokeratin (CK5/6). It is reported that even partial suppression of BRCA1 function in human mammary epithelial cell cultures can induce EGFR expression and an increase in cancer stem-like cells (127).

The breast carcinomas arising in patients with BRCA1 mutations have defective DNA double strand break repair mechanisms thus therapeutic agents that target DNA repair pathways such as olaparib are effective in their treatment (39, 122).

High rates of pCR (up to 83%) have been observed in BRCA1 mutated tumours (39, 89, 101).

## 2.5. Basal Markers

### 2.5.1. P-Cadherin (*CDH3*)

P (placental)-cadherin belongs to the family of classical cadherins which are transmembrane glycoproteins that serve as calcium-dependent cell-cell adhesion molecules (128, 129). To this group also belong CDH1/e (epithelial)-cadherin, CDH2/n (neuronal)-cadherin and CDH4/r (retinal)-cadherin (129). The intracellular domains of cadherins are linked to actin cytoskeletons via cytoplasmic catenins. The cadherin-catenin complexes and the pathways they control play important roles in regulation of cell growth, differentiation, motility and survival. They are also essential for maintaining the structural integrity of epithelial tissues (130). In stratified and pseudo stratified epithelium, its expression is restricted to the basal layers (130, 131).

P-cadherin alterations are detected in many human tumours. Its exact role in carcinogenesis is uncertain as the consequences of its expression vary depending on tumour cell model and context (128, 129). In melanoma and colorectal carcinoma cell lines, p-cadherin seems to act as an anti-invasive and anti-migration molecule. In *in vitro* breast cancer models as well as in urothelial, pancreatic and cholangiocarcinoma cell lines however, p-cadherin seems to behave as an oncogene and its over-expression is associated with single cell motility and invasion capacity (129).

P-cadherin is expressed in normal breast myoepithelial cells and it shows no significant cross-reactivity with luminal epithelial cells, stromal myofibroblasts or vessels (129, 130). It also appears to be expressed by breast stem cells (130). It is associated with poorly differentiated carcinomas and with triple negativity (129, 130). P-cadherin is expressed in 20-40% of invasive carcinomas and in 25% of DCIS. Liu *et al* showed that p-cadherin expression is associated with decreased disease-free interval and overall survival (128). P-cadherin expression is strongly associated with BRCA1 mutations and is an important biomarker in the identification of BLBCs (129).

## 2.5.2. P63

Protein 63 (p63) is a member of the p53 family comprising p53, p63 and p73. The human gene encodes at least six different isoforms (132). The transactivating isoform has a similar function to p53 while the N-isoform inhibits the transcriptional activation of p53 (133). Its translation products are crucial for the maintenance of a stem cell population in human epithelium (132, 134). It is expressed by basal and intermediate cells of squamous epithelium and urothelium (132, 134). In normal breast tissue, p63 expression is usually limited to myoepithelial cells and mammary stem cells (135) (Table 9).  It is considered to be a highly specific marker for the nuclei of myoepithelial cells (132, 136) .Luminal cell expression however has been described in papillary lesions as well as in nuclei and cytoplasm of epithelial cells showing secretory differentiation (133). Secretory carcinomas which are usually triple-negative, have been shown to have either cytoplasmic or nuclear p63 positivity (133).

The role of p63 in breast carcinoma is unclear and the protein has been studied as both an oncogene and a tumour suppressor gene. In an RT PCR study of human bladder cancer cell lines and primary tumours, Choi *et al* showed that muscle invasive tumours expressing p63 had worse prognosis than those not expressing the marker (134).

In the breast, however the ΔN isoform of p63 has been shown to be a tumour suppressor (135).

Hanker *et al* suggest that p63 is a positive predictive marker for response to endocrine therapy and a marker of good prognosis (132).  A study by Rocca *et al* examined the significance of p63 expression in tumour response to chemotherapy. They reported a high rate of pCR in p63 positive tumours after primary chemotherapy with cisplatin only (without anthracyclins) (62).

They suggest that cisplatin-based regimens are more effective in p63 positive tumours than in p63 negative tumours.

## 2.5.3. CD10

CD10, also known as enkephalinase, is a zinc dependent membrane associated neutral endopeptidase that cleaves signalling peptides (4, 137). It is expressed on a variety of

normal cell types including fibroblasts and granulocytes, and it has been shown to be expressed during B-lymphocyte maturation at early-B and pre-B lymphoblastic stages (137). In the breast, it is strongly expressed in normal myoepithelial cells and those associated with benign proliferations (5, 138). While it stains stromal fibroblasts, vessels are negative for the marker (138).

Aside from being a myoepithelial marker, CD10 has been used to identify breast stem cells (4, 137, 139). CD10 is involved in mammary gland development, controlling cell growth and differentiation and it also maintains and regulates the mammary stem cell population (137, 139).

Expression of CD10 may be observed in the stroma of invasive breast carcinoma. It has been suggested that this feature is associated with poor prognosis (137, 140). CD10 expression is also seen in a subset of mammary sarcomas (138, 141).

CD10 staining has been observed in a variety of other tumours. Positive staining may be a marker of good prognosis in cervical and non-small cell lung carcinomas while in melanomas and colorectal carcinomas it is an indicator of poor prognosis (137).

## 2.5.4. CK5 and CK6 (detected using anti-cytokeratin 5/6 antibody)

Anti-cytokeratin 5/6 is a mouse monoclonal primary antibody that detects cytokeratins 5 and 6. CK5 is a basal cytokeratin expressed by mammary myoepithelial cells (131). It is also expressed by multipotent progenitor epithelial cells in the breast located between the basal and luminal layers in normal ducts (142). It has been suggested that tumours expressing CK5 originate from these progenitor cells. CK6 is expressed by proliferating squamous epithelial cells.

CK5/6 is the most commonly used CK marker for identifying basal-like breast carcinoma (98, 120, 143). Foulkes *et al* showed that expression of CK5/6 is statistically significantly associated with BRCA1-related breast cancers (143, 144). The same group showed that CK5/6 positive tumours are likely to be larger, high grade tumours occurring in younger women (143). In their study, they showed that CK5/6 expressing tumours were associated with worse disease-specific outcome than CK5/6 non-expressing tumours. In an immunohistochemical study of 105 cases of TNBC, Sutton *et al* showed that in CK5/6 positive TNBCs, intratumoral expression of CK5/6 was significantly higher in the node-

positive group than in the node-negative group (94). However, the difference in expression of CK5/6 between the node-negative and distant metastasis groups did not achieve statistical significance (p=0.057). Nielsen *et al* suggest that CK5/6 is a basal-like breast cancer specific marker (98).

## 2.5.5. Other high molecular weight cytokeratins (detected using 34βE12/HMW antibody)

34βE12 is a mouse monoclonal primary antibody that recognizes CKs 1, 5, 10 and 14. It is used for detection of basal epithelial cells such as basal cells in the prostate or of stratified squamous epithelium (145). In the breast, CK5 and CK14 are expressed by basal epithelial cells, including mammary myoepithelial cells (131). Both cytokeratins are widely used as markers of the basal phenotype (124, 146). The fact that HMW is able to detect multiple high molecular weight cytokeratins makes it a possible marker of BLBC. Bori and Cserni showed that absence of HMW staining had a 94% negative predictive value regarding the basal-like phenotype (124).

An immunohistochemical microarray study with 58 TNBCs showed that expression of 34βE12 is an independent predictor of survival (146). The authors suggested that 34βE12 identifies a subset of TNBCs associated with better prognosis.

## 2.5.6. Epidermal growth factor receptor (EGFR)/HER1/ErbB1

The EGFR gene is located on chromosome 7 (147, 148). Its protein product EGFR belongs to the superfamily of transmembrane receptors with intrinsic protein tyrosine kinase activity (149). It is widely expressed on many cell types including all epithelial and stromal cells (149). In polarized epithelial cells, it is localized to the basolateral aspects, allowing for epithelial-stromal communication (149). EGFR is the sole or largely predominant receptor for several distinct ligands including EGF, TGF-alpha, amphiregulin and heparin-binging EGF (149). EGFR activation triggers numerous signalling pathways and the biological responses to activation include apoptosis, proliferation, migration and dedifferentiation depending on a number of extracellular factors (127, 149, 150). EGFR signalling is thought to play an important role in organ repair (149). In order to be functionally active, it must undergo dimerization, which is possible with all members of the

HER family.

EGFR and other members of the erbB family play important roles in tumour cell survival and proliferation (151). EGFR inhibition in both in vivo and in vitro models has been shown to induce apoptosis and inhibit angiogenesis and cell proliferation (152).

EGFR gene amplification and/or protein overexpression is seen in a wide variety of solid tumours and in some cases (e.g. non-small cell lung carcinoma, colorectal carcinoma), is associated with advanced stage and unfavourable prognosis (153). Amongst the therapeutic agents that target EGFR are monoclonal antibodies against the extracellular domain of the receptor (e.g. cetuximab, panitumumab and matuzumab) and low molecular weight tyrosine kinase inhibitors (e.g. erlotinib, gefitinib, lapatinib and afatinib) (127).

EGFR overexpression has been described in 8-36% (146) of breast carcinomas and is correlated with poor prognosis. In triple negative breast carcinomas, the proportion is even higher (in up to 60%) (148, 154, 155). High rates of EGFR positivity are also observed in metaplastic carcinomas and mammary NOS-type sarcomas (139).

While the consequences of EGFR expression in breast cancer are still largely unknown, EGFR remains an attractive candidate for targeted biological therapy, provided a suitable method for patient selection can be devised. As at now, EGFR inhibitors do not appear to be effective in breast cancer (146, 152).

## 2.6. Luminal marker - CK18

CK 18 is an acidic cytokeratin that is a major cytoskeletal component of cells of simple epithelia (156). In the breast it is expressed only by luminal/ductal epithelial cells (2, 7). Some studies report that CK18 expression is reduced in metastatic breast cancers (157). Additionally, CK18 expression is also shown to be lacking in a subset of micrometastatic tumour cells present in the bone marrow (157). In a 1458 case microarray study, CK18 loss was associated with high tumour grade, high mitotic index and large tumour size (157). Down-regulation of CK18 is more frequent in ductal than in lobular carcinomas (157). CK18 has been shown to be a prognostic indicator of both overall survival and cancer-specific survival. Buhler *et al* showed that high expression of CK18 in tumour cells is associated with reduced invasiveness *in vitro* and with lack of tumourigenicity in nude mice (158).

**Table 9 Molecular marker expression of various breast TDLU cell types (7, 142)**

|  | Luminal cells | Myoepithelial cells | Stem/progenitor cells |
|---|---|---|---|
| **CK5/6** | +/- | + | + |
| **CD10** | - | + | + |
| **P63** | - | + | + |
| **CK18** | + | - | +/- |
| **CK14** | +/- | + | + |
| **p-cadherin** | - | + | + |

## 3. Objectives

The main objectives of this work were twofold:

- To identify clear prognostically significant immunophenotypic and morphological subtypes within the triple negative group of breast carcinomas using immunohistochemical detection of p-cadherin, p63, CD10, CK5/6, EGFR, HMW, CK18 in tumour samples for classification as well as assessment of characteristic morphological features

and

- To study EGFR in triple negative breast carcinoma using in situ hybridization for detection of gene or chromosomal numerical abnormalities that may be of predictive and/or prognostic significance.

## 4. Materials and Methods

### Patients and tumour specimens

A retrospective search was performed in the archives of The Fingerland Department of Pathology for all cases of pre-treatment invasive breast carcinoma diagnosed between 2005 and 2008, inclusive.  From the pathology reports, the following information was obtained in each case: patient age at diagnosis, tumour type and grade as well as immunohistochemically detected expression of ER, PR, Ki-67 and p53. HER2 status was also noted.  Hormone receptor negativity was defined as immunoreactive score (IRS) = 0 for oestrogen and progesterone receptors – i. e. no immunohistochemical staining for ER and PR. HER score = 0, 1+ or 2+ non-amplified by FISH or dual-ISH (HER2 gene-chromosome 17 ratio < 2) was taken to be negative. Positive nuclear staining for Ki-67 in ≥20% of tumour cells was used to define high proliferative activity. p53 positivity was defined as nuclear staining in ≥ 50% tumour cells. Based on ER, PR and HER2 status we identified 4 immunophenotypic subtypes: ER and/or PR+ and HER2- (HR+/HER2-), ER and/or PR+ and HER2+ (HR+/HER2+), ER and PR- and HER2+ (HR-/HER2+) and ER and PR-, HER2- (HR-/HER2-).

For subsequent analysis we included only TN cases with sufficient archive material for further investigations and for which adequate follow up clinical data were available. The archived biopsy material was obtained in form of both core needle biopsies and resection specimens. For each case, one representative formalin-fixed paraffin-embedded tissue block was selected. Consultation cases as well as tumours in patients with a known history of prior breast carcinoma were all excluded. Also patients with distant metastases at the time of diagnosis were excluded from our final analysis.

A total of 52 formalin-fixed paraffin-embedded tissue blocks from 52 distinct patients fulfilled all the selection criteria and were included in the immunohistochemical and in situ hybridization study.

**Nottingham Prognostic Index (modified)**

As NPI is not routinely used in our institution, for each case NPI was calculated (NPI= lymph node stage + histological grade + tumour size x 0.2) and based on the results patients were assigned to one of five prognostic groups as described in section 2.1.4. For patients that did not receive neoadjuvant therapy, tumour size and lymph node stage were determined from diagnostic pathology reports. For those that did receive neoadjuvant therapy, tumour size and lymph node stage were taken from diagnostic mammography reports. In 2 cases a cT stage was assigned without noting exact tumour size. For these cases we estimated NPI based on the limits of tumour size given for the particular T stage. To simplify our classification, and because of our small sample size we combined the 'excellent' and 'good' groups, 'moderate 1' and 'moderate 2' groups and finally, we combined 'poor' and 'very poor' groups. Thus we classified our tumours as having good, moderate or poor prognosis.

**Morphologic Assessment**

The following parameters were assessed in the selected TN tumours from the original haematoxylin eosin slides used for diagnosis.

- nuclear atypia (degree of atypia scored on a scale of 1-4)
- tumour borders (pushing vs. infiltrating)
- tumour architecture (syncytial vs. non-syncytial)
- intra/peritumoural lymphoplasmacytic infiltrate
- presence/absence of central acellular zone (scar or necrosis)
- presence/absence of tumour necrosis
- presence/absence of in situ component
- stromal features
- presence/absence of angioinvasion

**Immunohistochemical Assay and Assessment**

Indirect immunohistochemical staining for p-cadherin, p63, CD10, HMW, CK5/6, EGFR and CK18 were performed in all cases. Characteristics of the monoclonal antibodies used are shown in table 10. Manual staining was performed for p-cadherin as described in table 11. For the rest of the antibodies, staining was performed using the fully automated BenchMark ULTRA platform (Ventana, Arizona, USA). Diaminobenzidine (DAB) was the chromogen used in all cases.

**Table 10 Details of antibodies used**

| Antibody | Manufacturer | Clone | Dilution |
|---|---|---|---|
| P-cadherin | Vector laboratories | 56C1 | 1:25 |
| TP63 | Ventana | 4A4 | original |
| CD10 | Novocastra | 56C6 | 1:10 |
| CK5/6 | DAKO | D5/16B4 | 1:100 |
| HMW | DAKO | 34βE12 | 1:25 |
| CK18 | DAKO | DC10 | 1:50 |
| EGFR | DAKO | pharmDx™ kit | --- |

**Table 11 Staining technique for p-cadherin**

| | | |
|---|---|---|
| 1. | Deparaffinization | Xylene 3x10min, 96% alcohol 2x5 min, 70% alcohol 5min |
| 2. | Rinse in distilled water | |
| 3. | Antigen retrieval | Citrate buffer pH6, peroxide 10min |
| 4. | Rinse in distilled water | |
| 5. | Application of primary antibody | 60 min |
| 6. | Application of EnVision ™+ Dual Link (Dako) | 30 min |
| 7. | DAB | (1ml DAB buffer + 1 drop DAB chromogen) 5 min |
| 8. | Rinse in distilled water | |
| 9. | Haematoxylin staining | 1 min |
| 10. | Dehydration, mounting | |

**Immunohistochemical staining evaluation**

All specimens were assessed by light microscopy without knowledge of the case histories. A semi-quantitative method was used to assess the immunohistochemical stains.

**P-cadherin**: Any cytoplasmic membrane staining was considered positive. A score from 0-3 was assigned in each case based on intensity of staining.

**p63**: Any nuclear or cytoplasmic staining was considered to be positive, percentage of positive tumour cells was recorded. Percentage of positive cells was scored as follows 1 ($<10\%$), 2 ($\geq10\%$, $<50\%$), and 3 ($\geq50$)

**CD10**: Any cytoplasmic staining was considered positive, percentage of positive tumour cells was recorded. Percentage of positive cells was scored as follows 1 ($<10\%$), 2 ($\geq10\%$, $<50\%$), and 3 ($\geq50$)

**CK5/6**: Any cytoplasmic staining was considered positive. A score from 0-3 was assigned in each case based on intensity of staining.  Additionally, percentage of positive tumour cells was recorded. Percentage of positive cells was scored as follows 1 ($<10\%$), 2 ($\geq10\%$, $<50\%$), and 3 ($\geq50$)

 **EGFR**: A semi-quantitative method for scoring EGFR expression was used employing a combination of staining intensity and percentage of positive tumour cells. Only cytoplasmic membrane staining was considered as positive. Intensity was graded on three levels – 1 (low), 2 (moderate) and 3 (high). Percentage of positive cells was scored as follows 1 ($<10\%$), 2 (11-50%), 3 (51-80%) and 4 ($>80\%$). A combination of these two parameters yielded a final EGFR score of 0-12.

**CK18**: Any cytoplasmic staining was considered positive. A score from 0-3 was assigned in each case based on intensity of staining.  Additionally, percentage of positive tumour cells was recorded. For classification we considered percentage of positively stained cells as described by Woelfe *et al* (157) i.e. normal expression: $\geq90\%$ stained tumour cells, partial loss of CK18 expression: $<90\%$ stained tumour cells, and complete loss of CK18.

**HMW**: Any cytoplasmic staining was considered positive. A score from 0-3 was assigned in each case based on intensity of staining.

**Immunophenotypic classification**

Based on expression of the above mentioned markers, we classified the tumours as having basal, luminal or luminal progenitor differentiation.

**Basal** differentiation was defined as expression of any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW, without expression of CK18.

**Luminal** differentiation was defined as expression of CK18 without expression of any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW

**Luminal progenitor** differentiation was defined as co-expression of CK18 with any of the following markers – p-cadherin, p63, CD10, EGFR, CK5/6 and HMW.

**Dual ISH Assay and Evaluation**

Dual in situ hybridization for detection of EGFR gene and chromosome 7 was performed in all cases. We used a dual colour staining technique for visualization of the EGFR gene and chromosome 7 centromere; with Ventana Silver *in situ* hybridization (SISH) detection kit for the EGFR gene and Ventana Alk Phos Red ISH detection kit for chromosome 7 centromere.

After deparaffinization of tissue sections (as shown in table 11), automated staining was performed using the BenchMark ULTRA platform (Ventana, Arizona, USA).

For each case, the numbers of copies of EGFR gene (black signals) and chromosome 7 (red signals) were counted and recorded in 40 different tumour cell nuclei. The average number of copies of the gene and chromosome for each case was recorded and the gene-chromosome copy (G:C) ratio was calculated and interpreted as follows;

$\quad$ **G:C** < 1.8 - no amplification of EGFR gene

$\quad$ 1.8 < **G:C** < 2.2 - borderline amplification of EGFR gene

$\quad$ 2.2 < **G:C** < 5.0 - EGFR gene amplification

$\quad$ **G:C** > 5.0 - high EGFR gene amplification

## Clinical Data

Clinical charts for all patients were reviewed. From these, we recorded type of treatment (neoadjuvant or adjuvant chemotherapy, radiotherapy, surgical therapy), progression free survival

(in months), overall survival (in months) and outcome at the end of the follow-up period. Five possible outcomes were recognized;

1.) Disease free (no sign of breast malignancy)

2.) Locoregional residual disease (presence of residual primary tumour or regional lymph node metastasis)

3.) Recurrence (metachronous breast cancer in ipsilateral or contralateral breast)

4.) Distant metastasis

5.) Death (death from breast/non-breast cancer related causes)


For our final analysis only those that were disease free at the end of the follow-up period were considered to have good outcome. All the others (locoregional residual disease, metachronous breast cancer, distant metastases and death) were collectively viewed as having bad outcome.


## Statistical Analysis

Statistical analysis was performed using Microsoft Excel (for basic descriptive statistics and graph construction). Fisher's exact test was employed for determining associations between our selected prognostic markers, tumour characteristics and survival data. P-values $<0.05$ were considered to be statistically significant. Results were assessed and interpreted with the help of a professional statistician.

## 5. Results

One thousand and forty nine (1049) primary invasive breast carcinomas (excluding consultation cases) were diagnosed at The Fingerland Department of Pathology between 2005 and 2008 (inclusive), including 3 carcinomas diagnosed in men. Most of the patients (809/1049; 77%) had HR+/HER2- tumours. The 2$^{nd}$ largest group was the TN group accounting for 12% (128/1049) of all carcinomas followed by the HR+/HER2+ group (64/1049; 6%). The smallest immunophentypic subgroup was the HR-/HER2+ group which comprised only 48 patients (5%).

The average age at diagnosis for all patients was 61 years (median: 61; range 26-96), for the TN patients it was 57 years (median: 58; range: 28-91) and for the non-TN patients 61 years. When the non-TN patients were further separated into the pre-defined immunophenotypic subtypes, it was observed that the HR+/HER2- patients were the oldest at diagnosis (average 62 years) followed by the HR-/HER2+ patients (average 58 years) (Table 12). The youngest patients (average 55 years) were those with HR+/HER2+ positive tumours.

Histological grade varied with immunophenotype (Fig. 7). None of the TN carcinomas were well differentiated. A majority of them (72%; 96/133) were poorly differentiated. Of the 4 immunophenotypic subtypes we identified, the TN group had the highest percentage of poorly differentiated (grade 3) tumours. All of the well differentiated carcinomas (grade 1) were HR positive. Only two of these also showed HER2 overexpression.

**Table 12 Age and immunophenotype of all patients diagnosed with breast cancer at the Fingerland Department of Pathology (2005-2008)**

|  | HR+/HER2- | HR+/HER2+ | HR-/HER2- (TN) | HR-/HER2+ |
|---|---|---|---|---|
| **Average** | 62 | 55 | 57 | 59 |
| **Median** | 62 | 57 | 58 | 58 |
| **Range** | 26-96 | 27-82 | 30-91 | 36-89 |

**Figure 7 Histological grade and immunophenotype of all breast carcinomas diagnosed in 2005-2008**
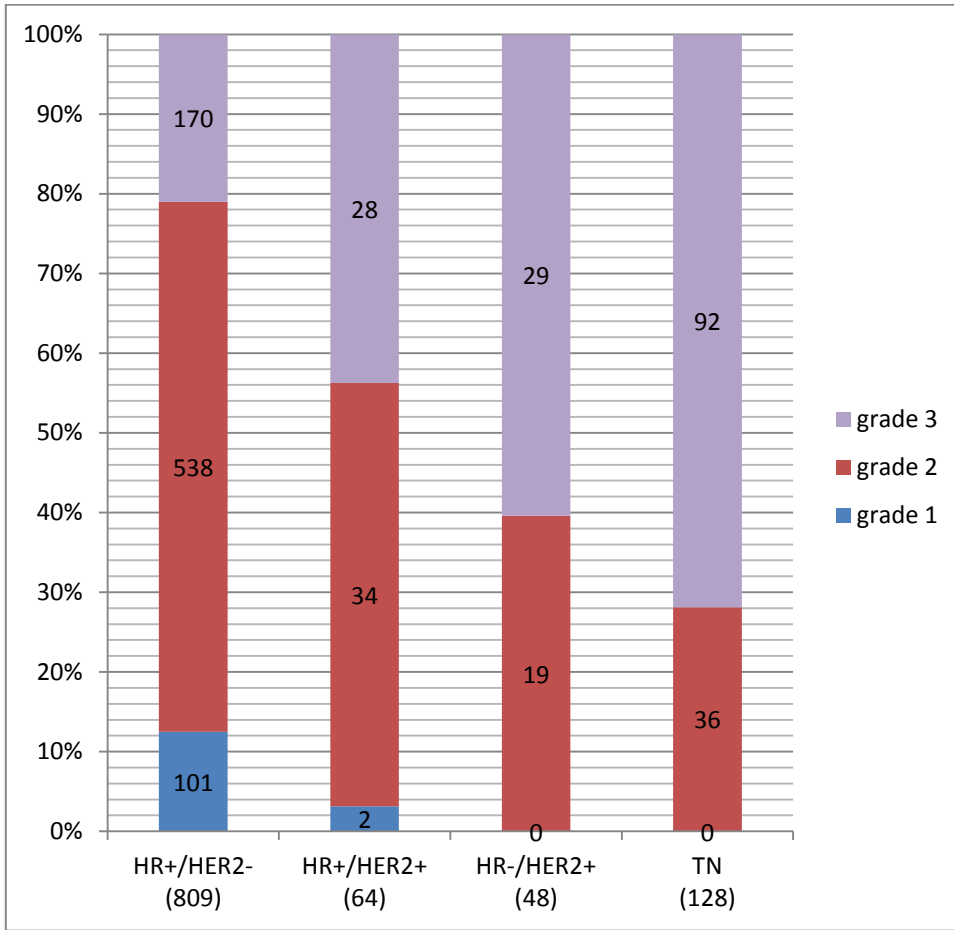
**Characteristics of Study Set**

Fifty-two patients with TNBC fulfilled all our selection criteria and were included in the study set for further investigation. Basic clinico-pathologic features of these cases are shown in table 13. The average age of our patients at diagnosis was 55 years (range: 28-84 years). Three of the patients were found to be carriers of BRCA mutations; BRCA status of all the other patients was unknown.

The group as a whole was characterized by high tumour grade, high proliferative activity, and p53 positivity. Ductal NOS was the most common histological subtype accounting for 88.5% of all cases. We also observed apocrine, atypical medullary and mucinous carcinomas. The average tumour size was 25.1mm (T2) and almost 60% (30/52) of the cases had regional lymph node metastases at the time of diagnosis.

**Table 13 Clinico-pathologic characteristics of cohort**

|  | Number | Percentage |
|---|---|---|
| **Patients (all female)** | 52 | 100 |
| **Age** | | |
| Range | 28-84 | |
| ≤40 | 7 | 13.5 |
| 40< | 45 | 86.5 |
| **Tumour type** | | |
| Ductal | 46 | 88.5 |
| Apocrine | 3 | 5.8 |
| Atypical medullary | 2 | 3.8 |
| Mucinous | 1 | 1.9 |
| **Histological grade** | | |
| 1 | 0 | 0 |
| 2 | 10 | 19.2 |
| 3 | 42 | 80.8 |
| **P53** | | |
| Positive | 33 | 63.5 |
| Negative | 19 | 36.5 |
| **Ki-67** | | |
| Low | 0 | 0 |
| High | 52 | 100 |

**Treatment**

All of the patients received varied combinations of locoregional and/or systemic therapy. A summary is shown in table 14. Treatment was selected based to individual patient and tumour characteristics. Partial mastectomy with sentinel lymph node biopsy was the most frequent surgical procedure performed and 75% of the patients received post-surgery/adjuvant radiotherapy. Anthracycline based regimens were the most frequently administered neo/adjuvant systemic treatments (Table 15). Forty-six (88.5%) patients received doxorubicin or epirubicin either as monotherapy or more frequently in combination with other agents. Two patients received adjuvant tamoxifen. Three patients did not receive any chemotherapy and 7 patients did not undergo any surgical procedure for varied reasons (i.e. contraindications due to comorbidities, patient refusal of surgical treatment, patients lost to follow-up during the course of neoadjuvant therapy). Nine (47.4%) of the 19 patients that underwent neoadjuvant chemotherapy had pathologic complete responses. These patients all were treated using AC-T regimen.

**Table 14 Treatment given to patients with TNBC**

| Type of treatment | N=52 | Percentage |
|---|---|---|
| **Neoadjuvant chemotherapy** | | |
| Yes | 19 | 36.5 |
| No | 33 | 63.5 |
| **Surgical treatment** | | |
| Partial ME with SN biopsy | 33 | 63.4 |
| Partial ME with AD | 5 | 9.6 |
| ME with AD | 7 | 13.5 |
| None | 7 | 13.5 |
| **Adjuvant chemotherapy** | | |
| Yes | 33 | 63.5 |
| No | 19 | 36.5 |
| **Radiotherapy** | | |
| Neoadjuvant | 4 | 7.7 |
| Adjuvant | 39 | 75.0 |
| None | 9 | 17.3 |

**Abbreviations: ME= mastectomy; AD= axillary dissection; SN= sentinel lymph node**

**Table 15 Chemotherapy agents and regimens administered to TNBC patients**

| | Neoadjuvant N=19 | Adjuvant N=34 |
|---|---|---|
| **Agent/Regimen** | | |
| AC-T | 13 | 14 |
| FEC | 2 | 4 |
| CMF | 0 | 1 |
| XeNA | 0 | 3 |
| AC | 0 | 8 |
| AT | 0 | 1 |
| A | 3 | 0 |
| T | 1 | 0 |
| T + carboplatin + bevacizumab | - | 1 |
| Tamoxifen | - | 2 |
| None | - | - |

**Abbreviations: A= Doxorubicin; AC= Doxorubicin Cyclophosphamide; AT= Doxorubicin Taxane; AC-T= Doxorubicin Cyclophosphamide plus Taxane; CMF= Cyclophosphamide Methotrexate Fluorouracil; FEC= Fluorouracil Epirubicine Cyclophosphamide; FEC= Fluorouracil Epirubicine Cyclophosphamide; T= Taxane; XeNA= Capecitabine plus Docetaxel**

**Outcome**

The average duration of follow-up was 58 months (range: 3-96 months, median: 60 months). At the end of the follow-up period 39/52 (75%) patients had no sign of residual breast carcinoma. 2 patients developed metachronous breast carcinoma in the contralateral breast 16 and 83 months after the primary diagnosis (Tables 16-17). The patients were aged 70 and 51 years respectively, at the time of the first breast cancer diagnosis. The younger patient, who was discovered to be a carrier of a BRCA mutation, was completely disease free at the end of the follow-up period.

Two of the 52 patients had locoregional residual breast disease at the end of the follow-up period. These women were lost to follow-up after 6 and 3 months respectively thus the true clinical behaviour of their disease remains unknown. Eight of the 52 (15.4%) patients developed distant metastasis, all within 37 months of initial diagnosis (average: 21 months, range: 12-37 months) (Table 18). All but one of the patients that developed distant metastases had lymph node involvement at the time of diagnosis. The only patient with pN0 cancer at diagnosis that developed distant metastasis had a pT2 grade 3 atypical medullary carcinoma.

The most frequent metastatic sites were the lung (in 3/8 patients), bone (in 3/8 patients), brain (in 3/8 patients), and lymph nodes (in 3/8 patients). Other locations for metastatic deposits were the liver (2/8), pleura (2/8), and meninges (1/8). One of the patients that developed lung and brain metastasis was alive and disease-free 67 months after diagnosis. Two patients died as a result of complications of metastatic disease 22 and 36 months after diagnosis. Clinico-pathologic characteristics of cases that developed distant metastasis are shown in table 16. Two other patients died, both 3 months after primary diagnosis, of possible complications of treatment. The last death that occurred in our cohort was as a result of generalization of a non-breast (pancreatic) malignancy 60 months after diagnosis of the breast cancer; she was breast cancer-free at the time of death.

**Table 16 Outcomes of all patients in cohort**

|  | N=52* | % |
|---|---|---|
| **Outcomes** | | |
| Disease free | 39 | 75.0 |
| Locoregional residual disease | 2 | 3.8 |
| Recurrence | 2 | 3.8 |
| Distant metastasis | 8 | 15.4 |
| Death | | |
| TNBC related | 4 | 7.7 |
| Non-TNBC related | 1 | 1.9 |

**Abbreviation: TNBC= triple negative breast cancer**

**\*There is an overlap in outcomes; one patient that developed distant metastases and another that had a recurrence were both disease-free at the end of the follow up period. Two patients that developed distant metastases died during the follow up period.**

**Table 17 Treatment and outcomes**

|  | Recurrence (%)<br>N=2 | Metastasis (%)<br>N=8 | Disease free (%)<br>N=39 |
|---|---|---|---|
| **Type of treatment** | | | |
| Neoadjuvant chemotherapy | | | |
|   Yes | 1 (50.0) | 4 (50.0) | 12 (30.8) |
|   No | 1 (50.0) | 4 (50.0) | 27 (69.2) |
| Surgical treatment | | | |
|   Partial ME with SN biopsy | 0 (0.0) | 0 (0.0) | 4 (10.3) |
|   Partial ME with AD | 2 (100.0) | 5 (62.5) | 28 (71.8) |
|   ME with AD | 0 (0.0) | 2 (25.0) | 5 (12.8) |
|   None | 0 (0.0) | 1 (12.5) | 2 (5.1) |
| Adjuvant chemotherapy | | | |
|   Yes | 2 (100.0) | 7 (87.5) | 25 (64.1) |
|   No | 0 (0.0) | 1 (12.5) | 14 (35.9) |
| Radiotherapy | | | |
|   Neoadjuvant | 0 (0.0) | 1 (12.5) | 2 (5.1) |
|   Adjuvant | 1 (50.0) | 5 (62.5) | 34 (87.2) |
|   None | 1 (50.0) | 2 (25.0) | 3 (7.7) |

**Abbreviations: ME= mastectomy; AD= axillary dissection; SN= sentinel lymph node**

**Table 18 Characteristics of metastasizing tumours**

| Age | Histologic subtype | Immuno. subtype | TNM stage | NPI (modified) | EGFR score | EGFR gene copy number | Location of metastases |
|---|---|---|---|---|---|---|---|
| 56 | Ductal | Luminal progenitor | IIB | Moderate | 3 | 2.75 | Bone, subclavian lymph nodes |
| 50 | Ductal | Luminal progenitor | IIB | Moderate | 4 | 3.90 | Lung, brain |
| 28 | Ductal | Luminal progenitor | IIIA | Poor | 0 | 2.20 | Lung, liver, bone, meninges, cervical lymph nodes |
| 70 | Ductal | Luminal progenitor | IIIC | Poor | 8 | 3.30 | Brain |
| 60 | Ductal | Luminal progenitor | IIA | Moderate | 4 | 3.60 | Liver, bone, pleura |
| 57 | Ductal | Luminal progenitor | IIB | Moderate | 6 | 2.20 | Lung, brain |
| 69 | Atypical medullary | Luminal progenitor | IIA | Moderate | 6 | 3.49 | Pleura |
| 67 | Ductal | Luminal progenitor | IIIA | Poor | 12 | 3.80 | Subclavian, cervical and mediastinal lymph nodes |

**Abbreviations: EGFR= epidermal growth factor receptor; Immuno.= immunophenotypic; NPI= Nottingham Prognostic Index; TNM= tumour node metastasis**

**Traditional prognostic markers**

Of the traditional prognostic markers (Table 19), we found that NPI (modified) was the best at identifying tumours that were unlikely to metastasize. The association between NPI and development of distant metastasis was statistically significant (p=0.036). TNM staging was better than lymph node stage or tumour size alone (p=0.183 and p=0.242 respectively) for identifying tumours likely to metastasize. Although none of the TNM stage I tumours metastasized during the follow up period, the finding was not statistically significant (p=0.124).

Interestingly p53 positivity appeared to be more frequent in non-metastasizing rather than in metastasizing tumours (68.2% vs. 37.5%). This finding however fell short of being statistically significant (p=0.097). There was no relationship between tumour grade and development of distant metastasis (p=0.642).

**Table 19 Traditional prognostic markers in metastasizing and non-metastasizing tumours**

| | Metastasis (%) N=8 | No Metastasis (%) N=44 | P-value |
|---|---|---|---|
| **Lymph node stage** | | | |
| 0 (22) | 1 (12.5) | 21 (47.7) | **0.183** |
| 1 (17) | 4 (50.0) | 13 (29.6) | |
| 2 (10) | 2 (25.5) | 8 (18.2) | |
| 3 (3) | 1 (12.5) | 2 (4.5) | |
| **Tumour stage** | | | |
| 1 (21) | 1 (12.5) | 20 (45.5) | **0.242** |
| 2 (20) | 5 (62.5) | 15 (34.1) | |
| 3 (7) | 1 (12.5) | 6 (13.6) | |
| 4 (4) | 1 (12.5) | 3 (6.8) | |
| **TNM stage** | | | |
| 1 (15) | 0 (0.0) | 15 (34.1) | **0.124** |
| 2 (25) | 5 (62.5) | 20 (45.5) | |
| 3 (12) | 3 (37.5) | 9 (20.4) | |
| **Histological grade** | | | |
| 2 (10) | 2 (25.0) | 8 (18.2) | **0.642** |
| 3 (42) | 6 (75.0) | 36 (81.8) | |
| **NPI (modified)** | | | |
| Good prognosis (18) | 0 (0.0) | 18 (40.9) | **0.036** |
| Moderate prognosis (24) | 5 (62.5) | 19 (43.2) | |
| Poor prognosis (10) | 3 (37.5) | 7 (15.9) | |
| **P53** | | | |
| Negative (19) | 5 (62.5) | 14 (31.8) | **0.097** |
| Positive (33) | 3 (37.5) | 30 (68.2) | |

**Abbreviations: NPI= Nottingham Prognostic Index; TNM= tumour node metastasis**

**Morphology**

We observed marked heterogeneity in the morphology of our sample. Most of the tumours (46/52) were classified as invasive ductal NOS carcinomas. There were 3/52 (5.8%) apocrine carcinomas (Fig. 8), 2/52 (3.8%) atypical medullary carcinomas (Fig. 9) and 1/52 (1.9%) mucinous carcinoma (Fig. 10). Even within the ductal carcinoma group morphology ranged from almost dedifferentiated tumours with bizarre nuclei and syncytial growth pattern to tumours with 100% tubule formation and only moderate nuclear atypia (Figs. 11-15).

One feature that was observed in a majority of our cases was intratumoural heterogeneity. This was a feature frequently observed in tumours with predominantly solid/syncytial architecture in which we detected a spindle cell sub-population. Intratumoural morphological heterogeneity was frequently accompanied by non-uniform expression of the molecular markers we investigated.

In our group of tumours we observed all tumour characteristics that have been described as being typical for TNBCs and/or BLBCs i.e. solid architecture, pushing borders, ribbon-like architecture, central acellular zone, geographic necrosis, high nuclear atypia and presence of lymphoplasmacytic infiltrate. Expression of basal markers was not limited to tumours with typical basal morphology.

None of the morphological characteristics we assessed showed a statistically significant association with development of distant metastasis (Table 20).  However, none of the 3 apocrine carcinomas metastasized; neither did the only mucinous carcinoma in our sample.

**Table 20 Morphological features of metastasizing and non-metastasizing tumours**

| | Metastasizing N=8 (%) | Non-metastasizing N=44 (%) | P-value |
|---|---|---|---|
| **Nuclear atypia** | | | |
| 1 (3) | 0 (0.0) | 3 (6.8) | **0.906** |
| 2 (8) | 1 (12.5) | 7 (15.9) | |
| 3 (33) | 5 (62.5) | 28 (63.6) | |
| 4 (8) | 2 (25.0) | 6 (13.7) | |
| **Borders** | | | |
| Pushing (10) | 1 (12.5) | 9 (20.5) | **1.00** |
| Infiltrating (42) | 7 (87.5) | 35 (79.5) | |
| **Architecture** | | | |
| Non-syncytial (35) | 6 (75.0) | 29 (65.9) | **1.00** |
| Syncytial (17) | 2 (25.0) | 15 (34.1) | |
| **DCIS** | | | |
| Absent (33) | 6 (75.0) | 27 (61.4) | **0.694** |
| Present (19) | 2 (25.0) | 17 (38.6) | |
| **Lymphoplasmacytic infiltrate** | | | |
| Minimal (8) | 2 (25.0) | 6 (13.6) | |
| Moderate (37) | 5 (62.5) | 32 (72.7) | **0.827** |
| Prominent (7) | 1 (12.5) | 6 (13.6) | |
| **Central acellular zone** | | | |
| Absent (35) | 5 (62.5) | 30 (68.2) | **1.00** |
| Present (17) | 3 (37.5) | 14 (31.8) | |
| **Necrosis** | | | |
| Absent (26) | 3 (37.5) | 23 (52.3) | **0.674** |
| Focal (9) | 2 (25.0) | 7 (15.9) | |
| Geographic (17) | 3 (37.5) | 14 (31.8) | |
| **Stroma** | | | |
| Minimal (14) | 5 (62.5) | 9 (20.5) | **0.128** |
| Moderate (31) | 3 (37.5) | 28 (63.6) | |
| Abundant (6) | 0 (0.0) | 6 (13.6) | |
| Mucinous (1) | 0 (0.0) | 1 (2.3) | |
| **Angioinvasion** | | | |
| Absent (41) | 5 (62.5) | 36 (81.8) | **0.343** |
| Present (11) | 3 (37.5) | 8 (18.2) | |

**Abbreviation: DCIS= ductal carcinoma in situ**

**Figure 8 Apocrine TNBC**
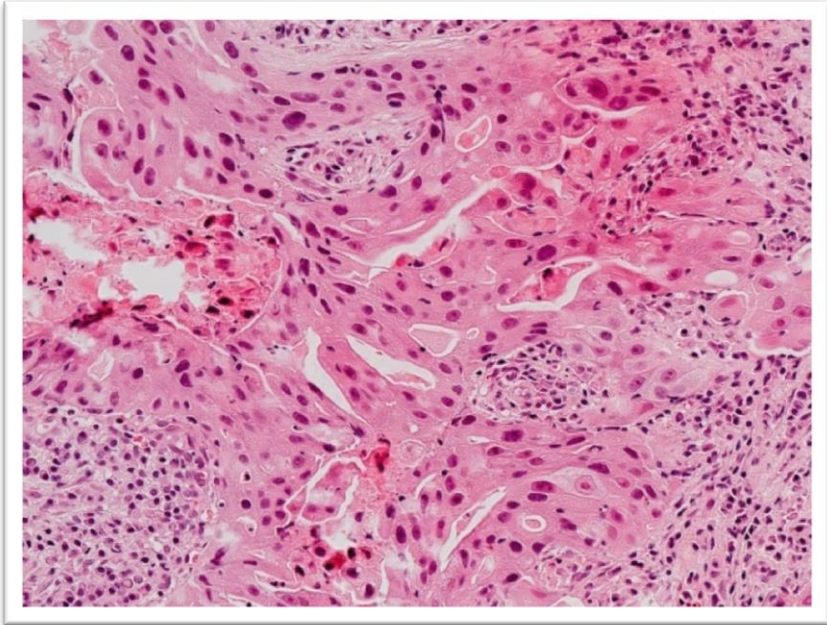


**Figure 9 Atypical medullary TNBC**
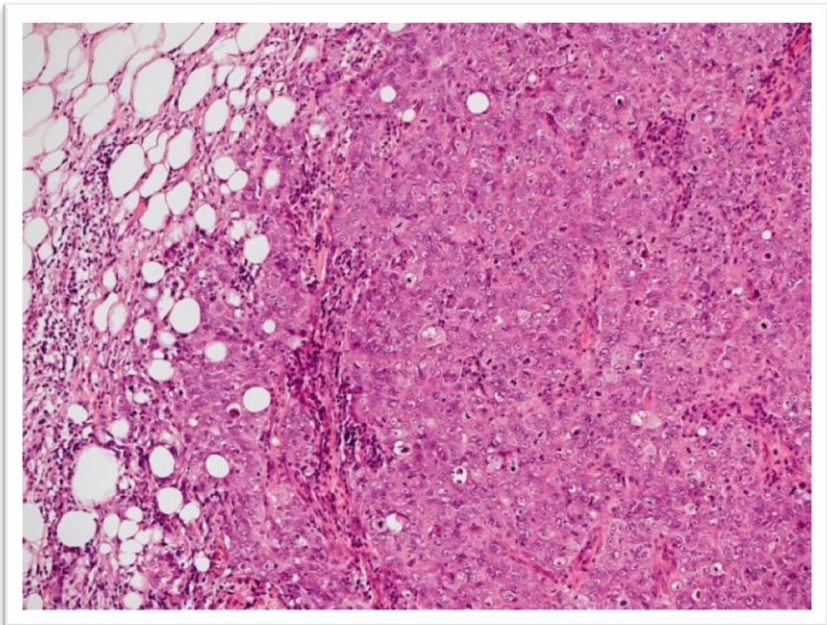
**Figure 10 Mucinous TNBC**
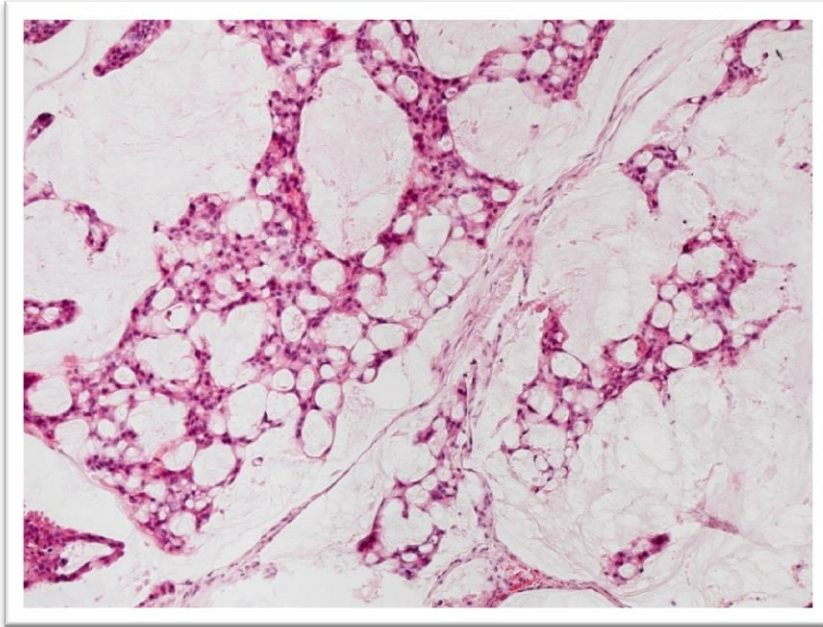
**Figure 11**
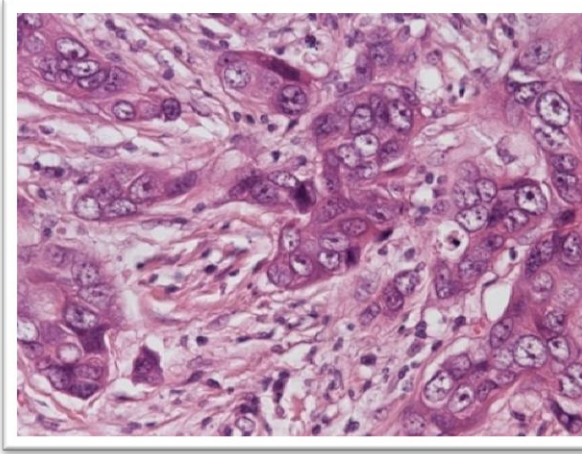


**Figure 12**
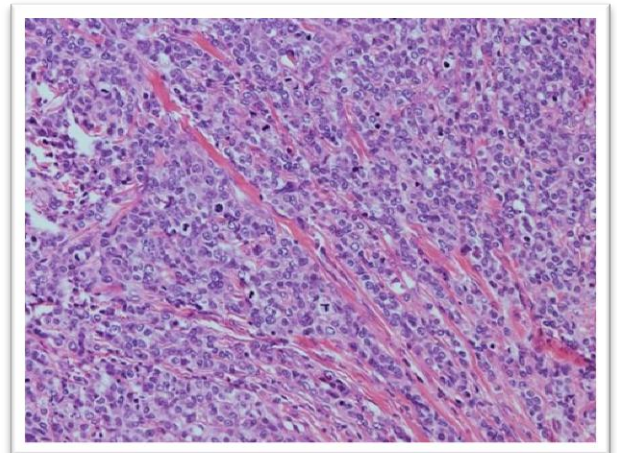


**Figure 13**
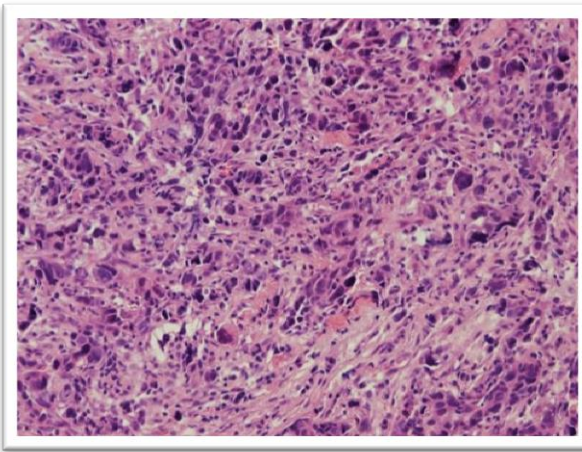


**Figure 14**
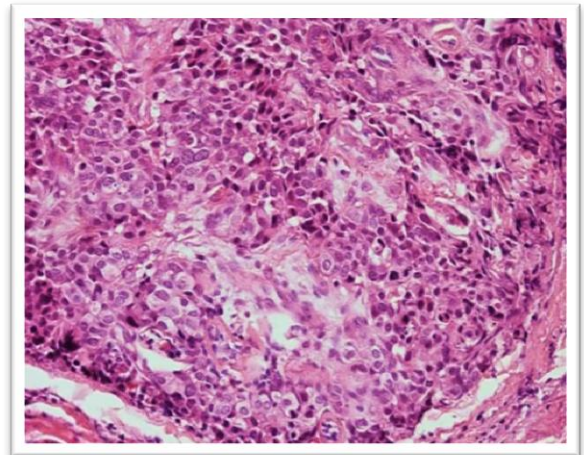


**Figure 11 Invasive ductal carcinoma NOS with vesicular nuclei and prominent nucleoli**
**Figure 12 Invasive ductal carcinoma NOS with minimal nuclear atypia**
**Figure 13 Invasive ductal carcinoma NOS with bizarre pleomorphic nuclei**
**Figure 14 Invasive ductal carcinoma NOS with spindle cell subpopulation**

**Figure 15 Tubule formation in TNBC**
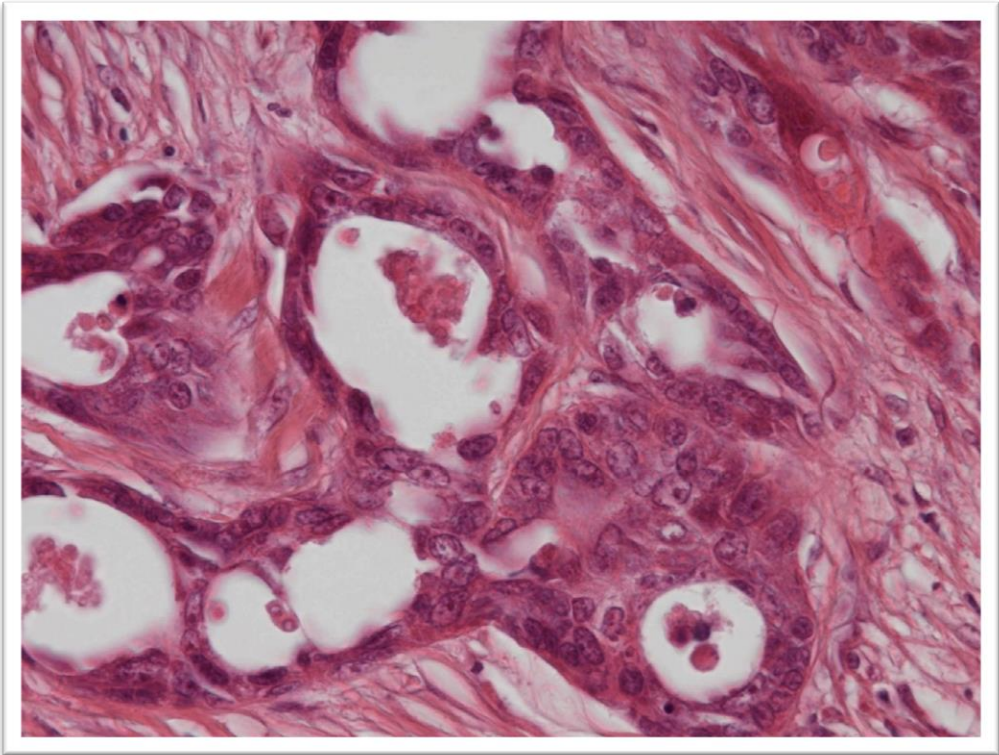


**Figure 16 Solid architecture in TNBC**
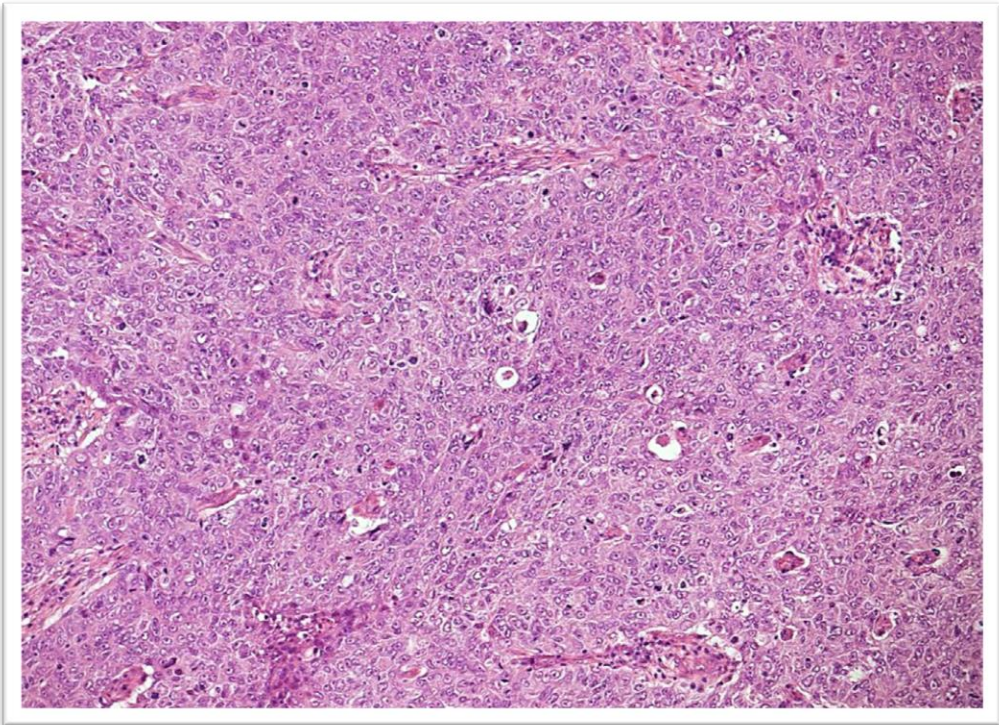
**Figure 17 High grade DCIS with microinvasion in TNBC**
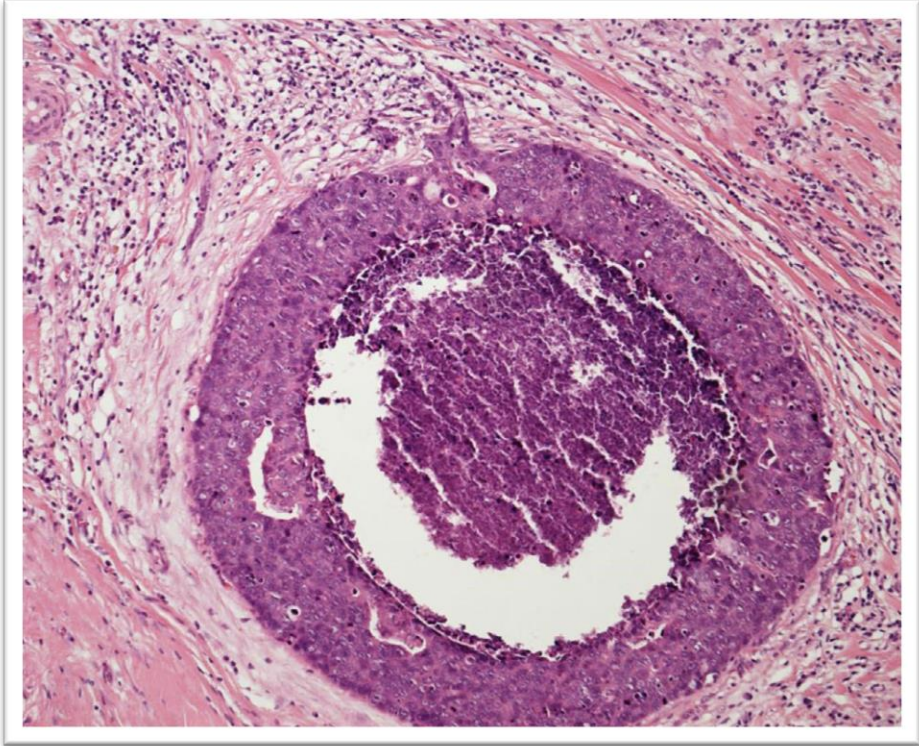


**Figure 18 Lymphangioinvasion in triple negative (apocrine) breast carcinoma**
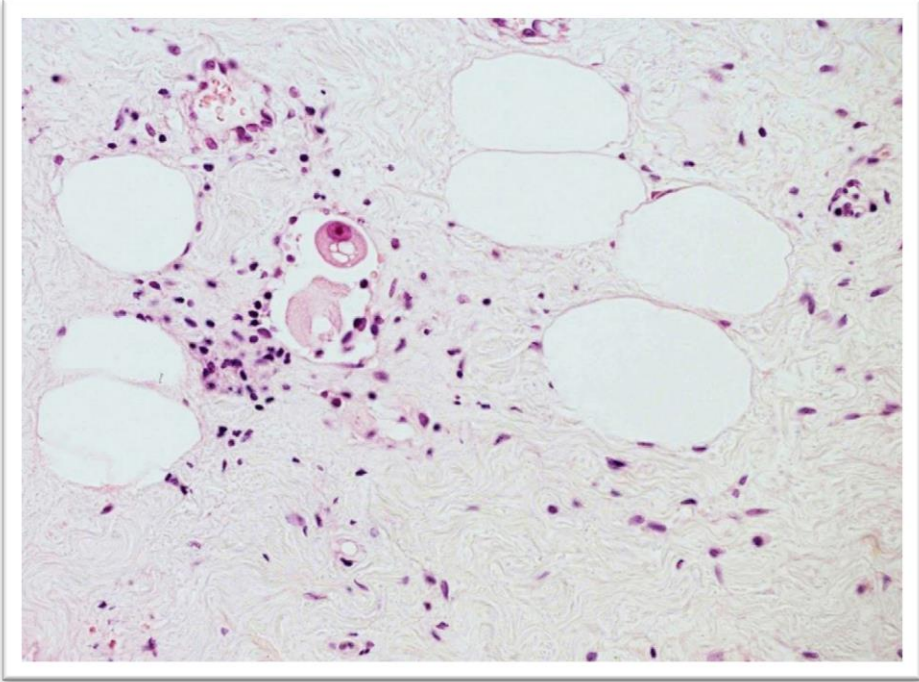
**P-cadherin**

Of all the molecular markers we investigated, p-cadherin was the most frequently expressed in our sample of TNBCs. All of the tumours showed some degree of p-cadherin positivity (Table 21). Intensity of staining was relatively uniform throughout the tumour tissue. Most of the tumours showed strong to moderate p-cadherin positivity. Twenty-six tumours (50.0%) were strongly positive for p-cadherin, 19/52 (36.5%) showed moderate staining intensity and only 7/52 (13.5%) cases were weakly positive (Figs. 19-21). We could not find any statistically significant association between intensity of p-cadherin staining and tumour grade (p=0.441), lymph node status (p=0.686), development of distant metastasis (p=0.230) or final outcome (p=0.245).

**Table 21 P-cadherin expression and selected tumour characteristics/clinical behaviour**

| Intensity of p-cadherin staining | | | |
|---|---|---|---|
| | Weak N=7 | Moderate N=19 | Strong N=26 | **P-value** |
| **Histological grade** | | | | |
| 2 (10) | 1 | 2 | 7 | **0.441** |
| 3 (42) | 6 | 17 | 19 | |
| **Lymph nodes** | | | | |
| Positive (30) | 3 | 11 | 16 | **0.686** |
| Negative (22) | 4 | 8 | 10 | |
| **Distant metastasis** | | | | |
| No (44) | 5 | 15 | 24 | **0.230** |
| Yes (8) | 2 | 4 | 2 | |
| **Final outcome** | | | | |
| Good (39) | 5 | 12 | 22 | **0.245** |
| Bad (13) | 2 | 7 | 4 | |

**Figure 19 Weak p-cadherin staining**
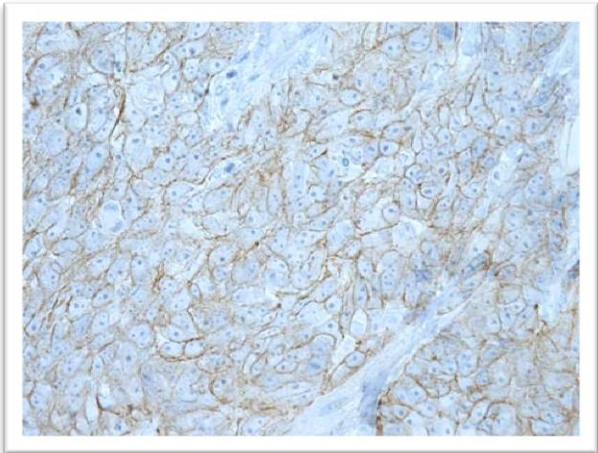


**Figure 20 Moderate p-cadherin staining**
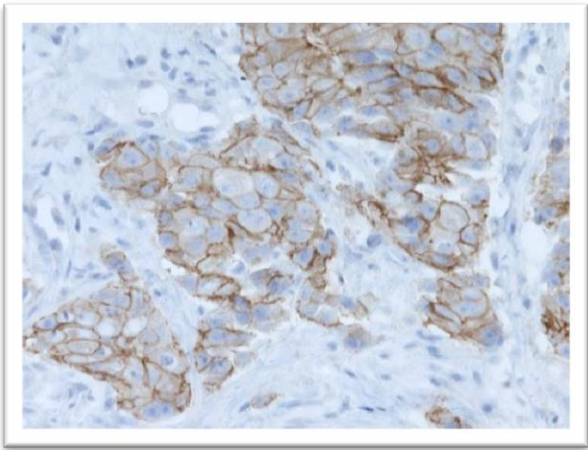


**Figure 21 Strong p-cadherin staining**
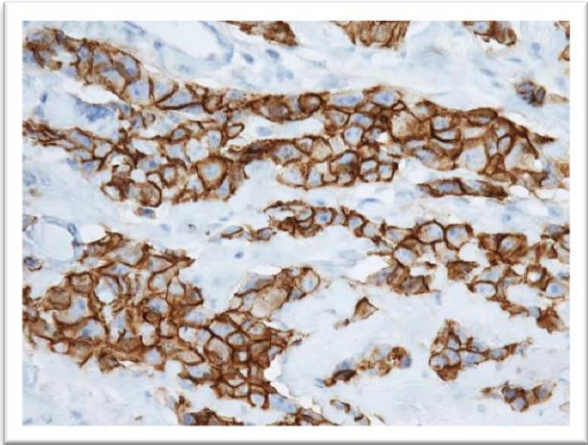
**P63**

P63 positivity was not a common feature of our samples (Table 22). Twenty-three (44.2%) were completely negative, 23/52 (44.2%) showed nuclear positivity in <10% tumour cells, 4/52 (7.7%) showed positive nuclear staining in ≥10% but less than 50% of tumour cells, and in only 2 cases (3.8%), over 50% of the tumour cells showed p63 positivity (Figs. 22-23). In the tumours with less than 10% positive tumour cells it was sometimes uncertain as to whether we were observing true tumour positivity or merely entrapped myoepithelial cells from residual breast tissue. One p63+ of the tumours was an apocrine carcinoma, the rest were ductal NOS carcinomas. Only 2 of the p63 positive tumours did not show concurrent p53 expression. Patients with <10% p63 positive tumour cells were less likely than those with ≥10% positively stained cells to have bad outcome (19.6% vs. 66.7% respectively). We found no statistically significant difference between p63+ and p63- tumours in terms of tumour grade (p=0.535), lymph node status (p=0.701) or development of distant metastasis (0.295).

**Table 22 p63 expression and selected tumour characteristics/clinical behaviour**

| | Percentage of p63 positive tumour cells | | | | |
|---|---|---|---|---|---|
| | 0%<br>N=23 | <10%<br>N=23 | ≥10%, <50%<br>N=4 | ≥50<br>N=2 | **p-value** |
| **Histological grade** | | | | | |
| 2 (10) | 5 | 4 | 0 | 1 | **0.535** |
| 3 (42) | 18 | 19 | 4 | 1 | |
| **Lymph node status** | | | | | |
| Positive (30) | 14 | 12 | 2 | 2 | **0.701** |
| Negative (22) | 9 | 11 | 2 | 0 | |
| **Distant metastasis** | | | | | |
| No (44) | 20 | 20 | 2 | 2 | **0.295** |
| Yes (8) | 3 | 3 | 2 | 0 | |
| **Final outcome** | | | | | |
| Good (39) | 19 | 18 | 1 | 1 | **0.085** |
| Bad (13) | 4 | 5 | 3 | 1 | |

**Figure 22 Weak focal p63 positivity**
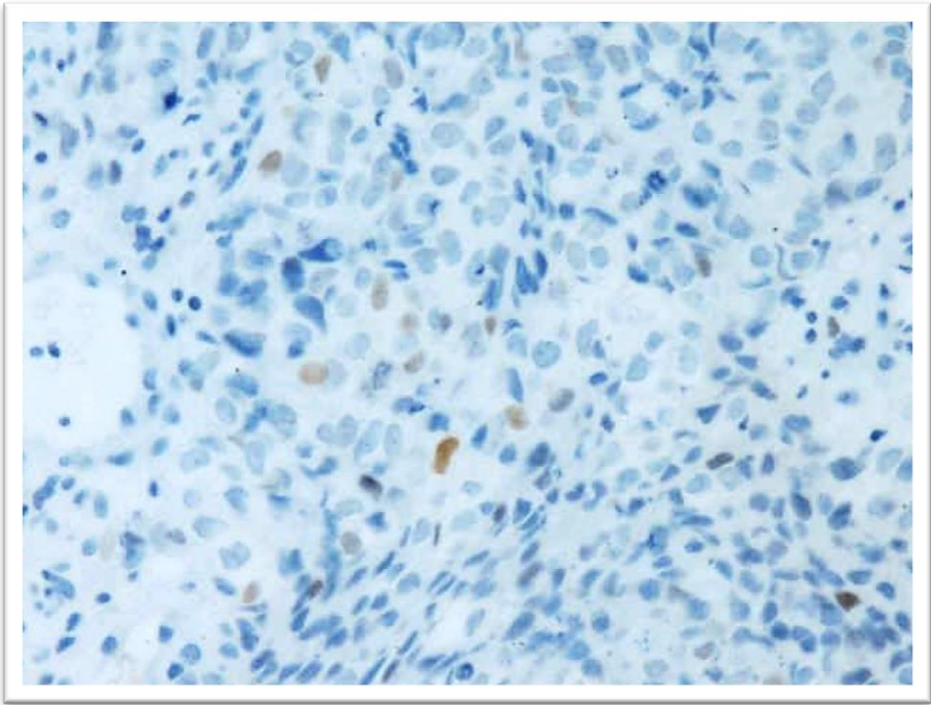


**Figure 23 Strong diffuse p63 positivity**
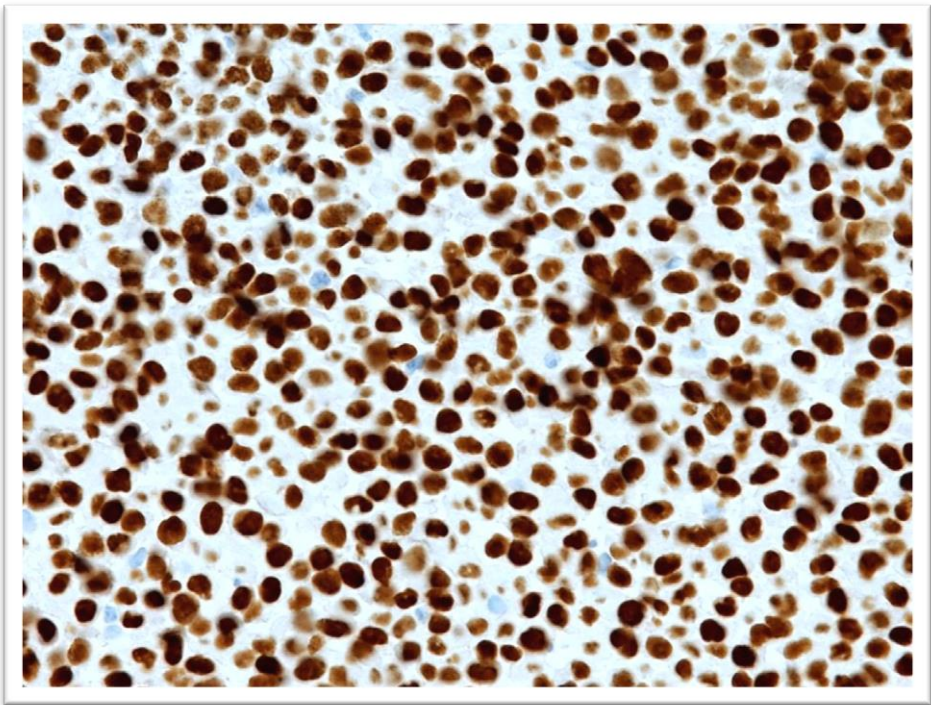
**CD10**

CD10 was the least frequently expressed marker in our group of TNBCs (Table 23). Although CD10 positivity was not a common feature of TNBC tumour cells, it was frequently seen in the tumour stroma (Fig. 24). Almost 70% (36/52) of our cases were completely negative for CD10. Staining in <10% tumour cells was observed in 15.4% (8/52), CD10 positivity in ≥10% tumour cell (but less than in 50%) was seen in 7.7% of cases. Only 4/52 (7.7%) tumours had ≥50% CD10 positive tumour cells (Fig. 25). Patients with CD10 negative tumours were less likely to have bad outcome than patients with tumours that showed any degree of CD10 positivity (16.7% vs. 43.7% respectively) however this finding did not achieve statistical significance (p=0.084). We also found no statistically significant link between CD10 expression and tumour grade (p=0.810), lymph node status (p=0.653) or development of distant metastasis (p=0.215).

**Table 23 CD10 expression and selected tumour characteristics/clinical behaviour**

| | Percentage of CD10 positive tumour cells | | | | |
|---|---|---|---|---|---|
| | **0%** | **<10%** | **≥10%, <50%** | **≥50%** | **p-value** |
| | N=36 | N=8 | N=4 | N=4 | |
| **Histological grade** | | | | | |
| 2 (10) | 7 | 2 | 1 | 0 | **0.810** |
| 3 (42) | 29 | 6 | 3 | 4 | |
| **Lymph node status** | | | | | |
| Positive (30) | 22 | 3 | 3 | 2 | **0.653** |
| Negative (22) | 14 | 5 | 1 | 2 | |
| **Distant metastasis** | | | | | |
| No (44) | 32 | 5 | 3 | 4 | **0.215** |
| Yes (8) | 4 | 3 | 1 | 0 | |
| **Final outcome** | | | | | |
| Good (39) | 30 | 4 | 2 | 3 | **0.084** |
| Bad (13) | 6 | 4 | 2 | 1 | |

**Figure 24 Strong stromal CD10 positivity, tumour cells completely CD10 negative**
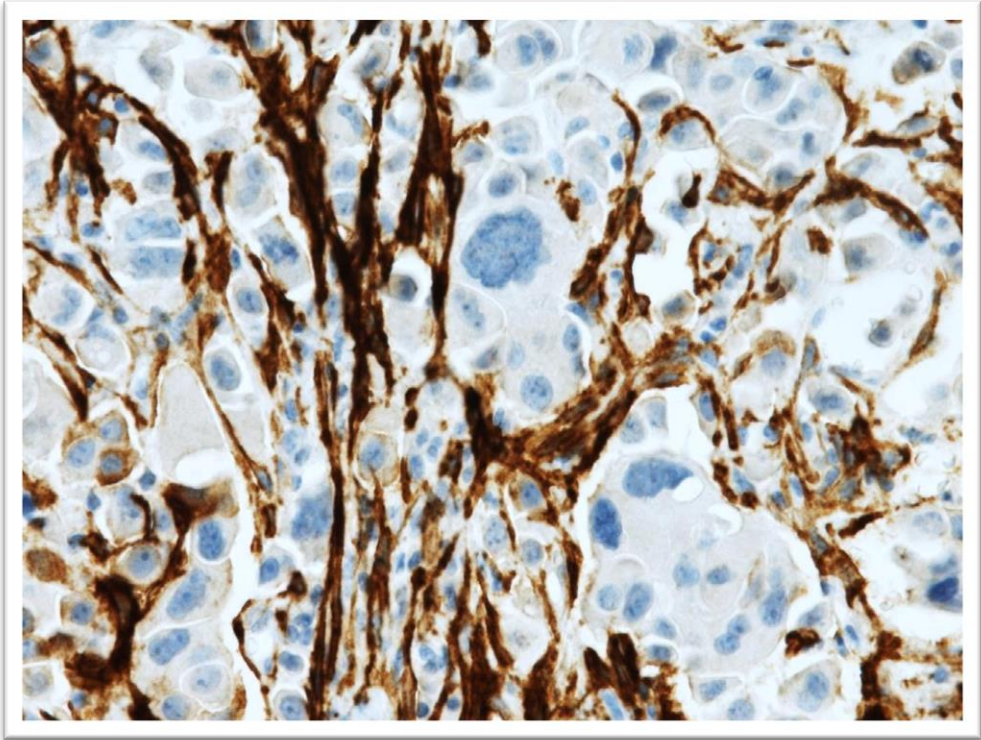


**Figure 25 CD10 positivity of >50% of tumour cells**
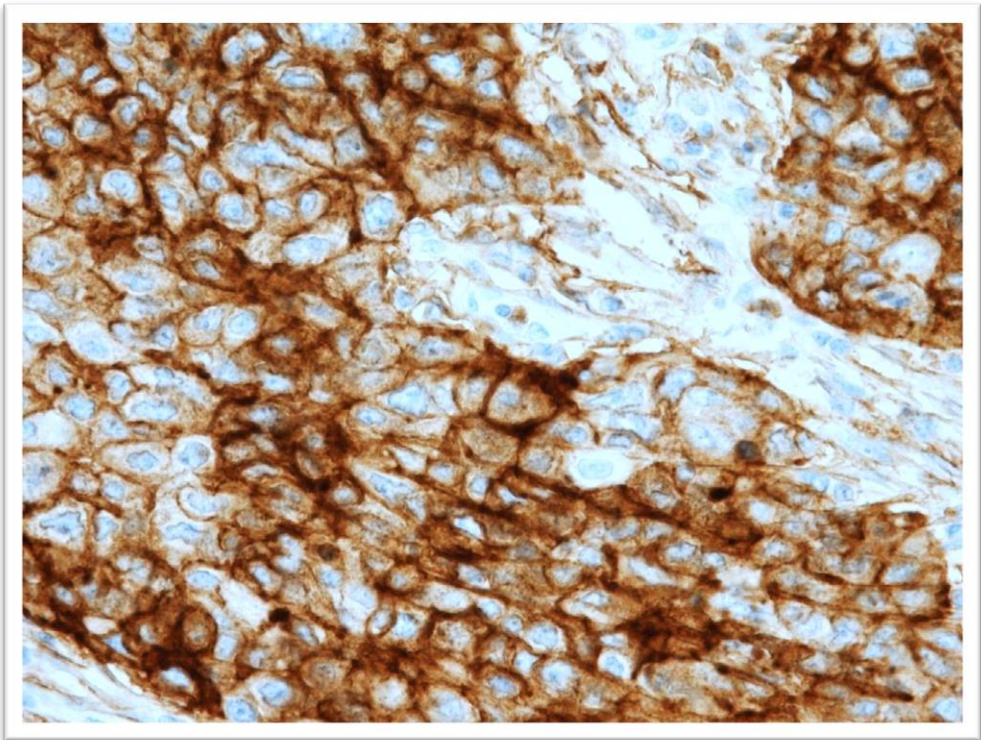
**CK5/6**

CK5/6 expression was observed in 78.8% (41/52) of our cases (Table 24) (Fig. 26). Uniform, diffuse staining however was a feature of only 13.5% (7/52) of cases (Fig. 27). We could not demonstrate a statistically significant association between degree of intratumoural CK5/6 expression and tumour grade (p=0.308), lymph node status (p=0.425), development of distant metastasis (p=0.759) or final outcome (0.557).

**Table 24 CK5/6 expression and selected tumour characteristics/clinical behaviour**

| | Percentage of CK5/6 positive cells | | | | |
|---|---|---|---|---|---|
| | 0%<br>N=11 | <10%<br>N=9 | ≥10%, <50%<br>N=11 | ≥50<br>N=21 | p-value |
| **Histological grade** | | | | | |
| 2 (10) | 2 | 0 | 2 | 6 | **0.308** |
| 3 (42) | 9 | 9 | 9 | 15 | |
| **Lymph node status** | | | | | |
| Positive (30) | 4 | 6 | 6 | 14 | **0.425** |
| Negative (22) | 7 | 3 | 5 | 7 | |
| **Distant metastasis** | | | | | |
| No (44) | 9 | 7 | 10 | 18 | **0.759** |
| Yes (8) | 2 | 2 | 1 | 3 | |
| **Final outcome** | | | | | |
| Good (39) | 9 | 5 | 9 | 16 | **0.557** |
| Bad (13) | 2 | 4 | 2 | 5 | |

**Figure 26 Partial CK5/6 staining**
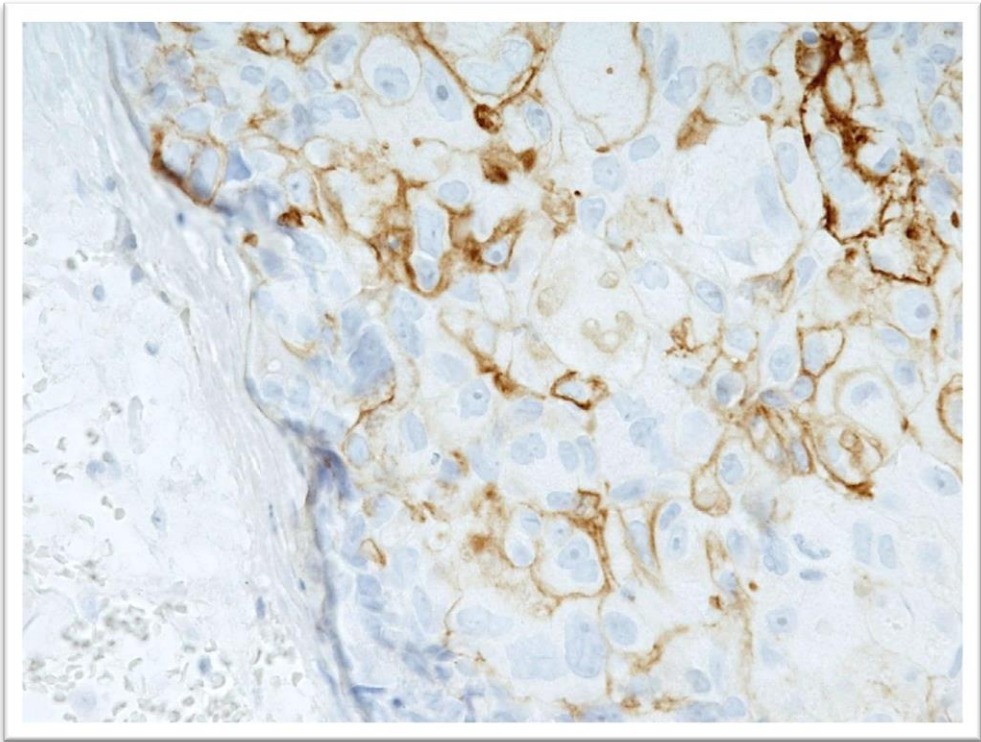


**Figure 27 Strong complete CK5/6 staining**
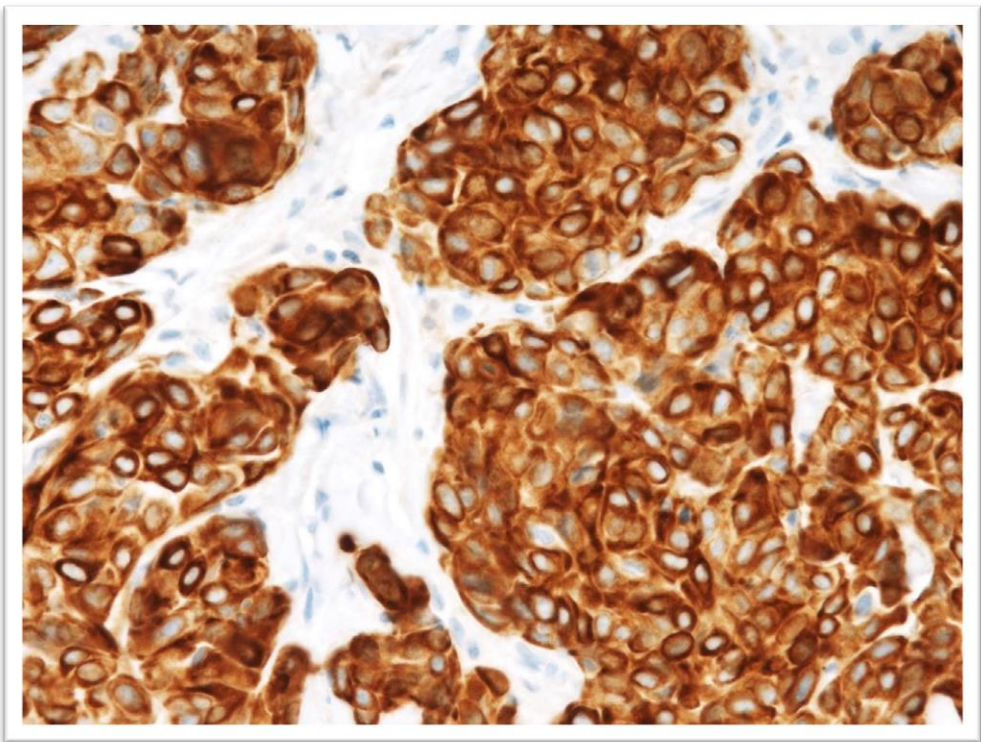
**HMW**

HMW staining was a common feature in our sample (Figs. 28-30). Intratumoural heterogeneity of expression was not uncommon. Only 3/52 (5.8%) tumours were negative for this marker (Table 25). These tumours were all grade 3 ductal NOS carcinomas. None of the patients with HMW negative carcinomas developed distant metastasis; they all had good outcomes at the end of the follow up period however these findings were not statistically significant (p=0.715, p=0.762 respectively). We also observed no statistically significant association between HWM staining and tumour grade (p=0.806) or lymph node status (p=0.659)

**Table 25 HMW expression and selected tumour characteristics/clinical behaviour**

| | Intensity of HMW staining | | | | |
|---|---|---|---|---|---|
| | **0** | **1** | **2** | **3** | **p-value** |
| | N=3 | N=12 | N=11 | N=26 | |
| **Histological grade** | | | | | |
| 2 (10) | 0 | 3 | 1 | 6 | **0.806** |
| 3 (42) | 3 | 9 | 10 | 20 | |
| **Lymph node status** | | | | | |
| Positive (30) | 1 | 6 | 6 | 17 | **0.659** |
| Negative (22) | 2 | 6 | 5 | 9 | |
| **Distant metastasis** | | | | | |
| No (44) | 3 | 9 | 9 | 23 | **0.715** |
| Yes (8) | 0 | 3 | 2 | 3 | |
| **Final outcome** | | | | | |
| Good (39) | 3 | 8 | 9 | 19 | **0.762** |
| Bad (13) | 0 | 4 | 2 | 7 | |

**Figure 28 Weak HMW staining**
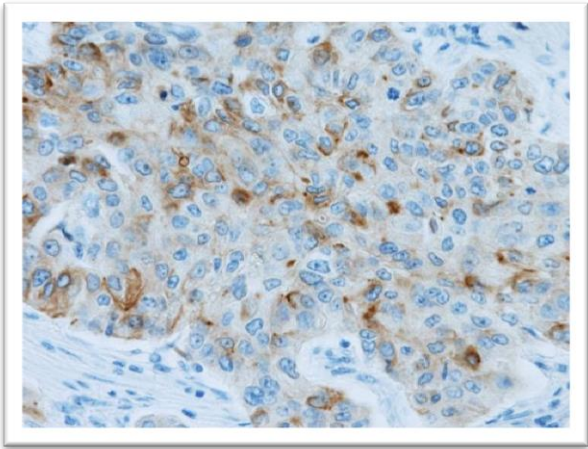


**Figure 29 Heterogeneous HMW staining**
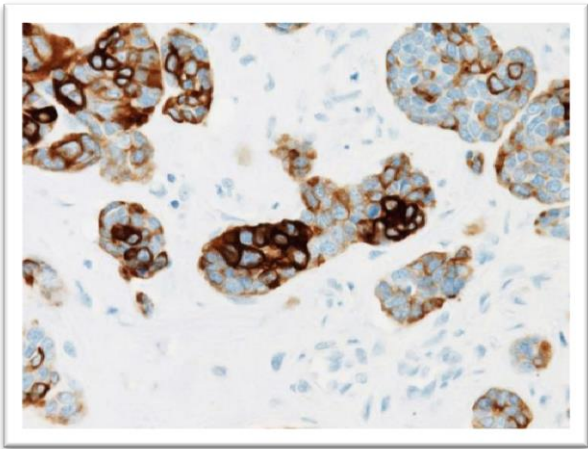


**Figure 30 Strong HMW staining**
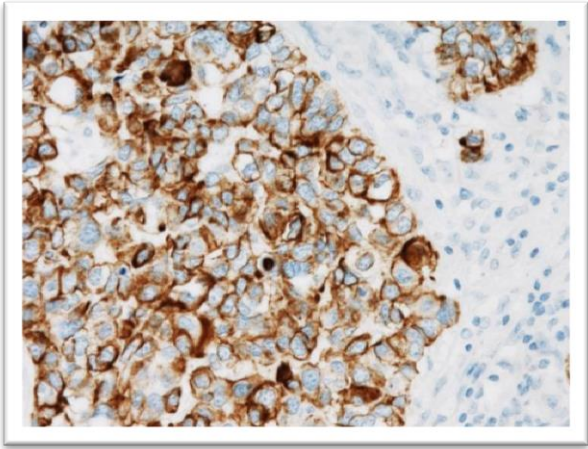
**EGFR (IHC)**

Forty-six (88.5%) of the 52 tumours in our sample showed some degree of EGFR expression (Table 26) (Figs. 31 and 32). We found no statistically significant relationship between EGFR score and tumour grade (p=0.379), lymph node status (p=0.531), development of distant metastasis (p=0.899) or final outcome (p=0.680).

**Table 26 EGFR score and selected tumour characteristics/clinical behaviour**

| EGFR Score | | | | |
|---|---|---|---|---|
| **0** | **1-4** | **5-8** | **9-12** | **p-value** |
| N=6 | N=21 | N=15 | N=10 | |
| **Histological grade** | | | | |
| 2 (10)  0 | 3 | 5 | 2 | **0.379** |
| 3 (42)  6 | 18 | 10 | 8 | |
| **Lymph node status** | | | | |
| Positive (30)  2 | 14 | 8 | 6 | **0.531** |
| Negative (22)  4 | 7 | 7 | 4 | |
| **Distant metastasis** | | | | |
| No (44)  5 | 18 | 12 | 9 | **0.899** |
| Yes (8)  1 | 3 | 3 | 1 | |
| **Final outcome** | | | | |
| Good (39)  5 | 17 | 11 | 6 | **0.680** |
| Bad (13)  1 | 4 | 4 | 4 | |

**Figure 31 Weak to moderate incomplete EGFR staining (EGFR score 4)**
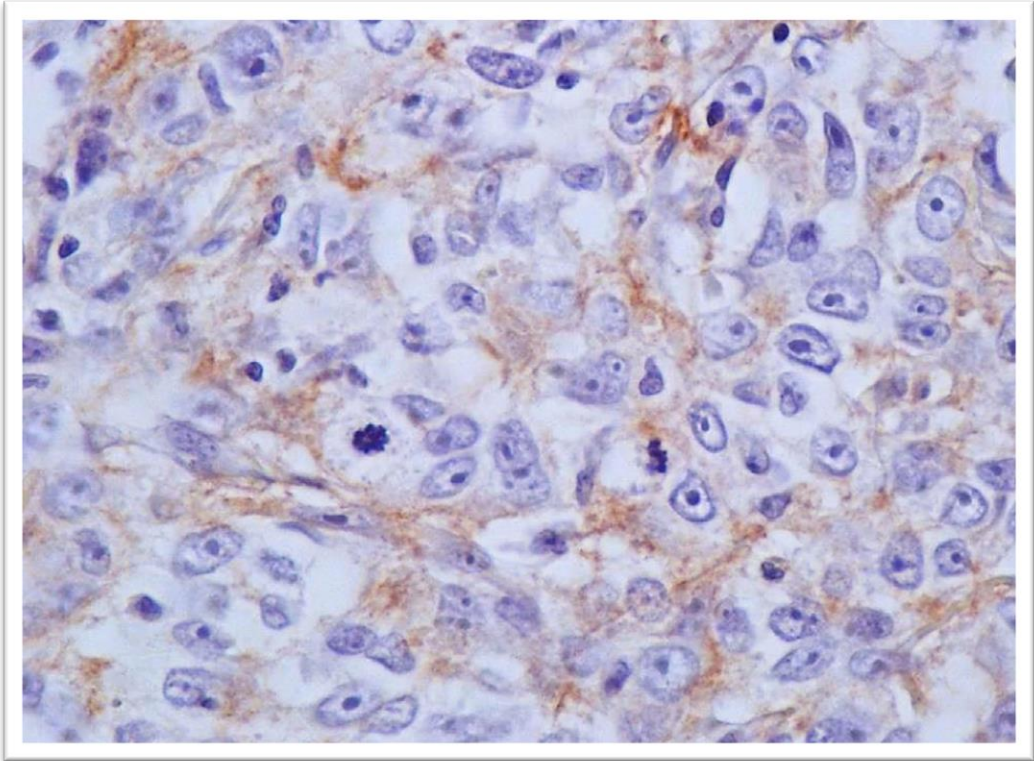


**Figure 32 Strong diffuse EGFR staining (EGFR score 12)**
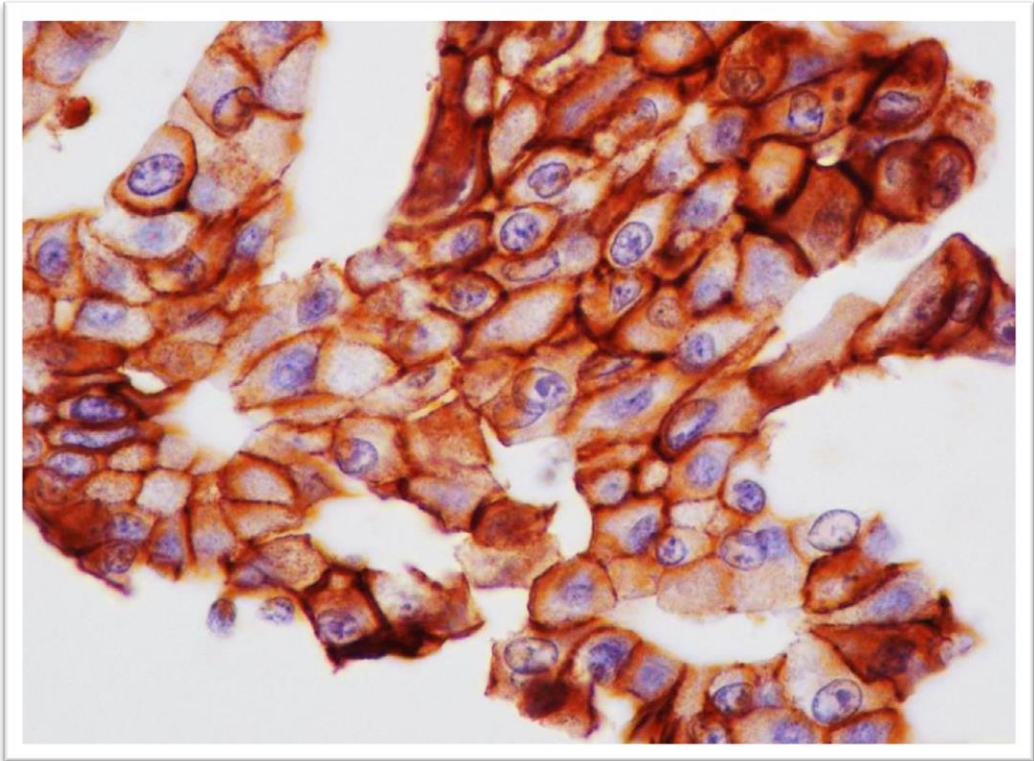
**EGFR (dual ISH)**

Details of gene copy number changes are shown in table 27. Almost all of the tumours had normal G:C ratios (Figs. 33-34), only one tumour showed EGFR gene amplification (G:C=4.05) (Fig. 35). The EGFR amplified tumour was a grade 3 ductal carcinoma in a 42 year old woman.  Eight tumours had ≥4 EGFR gene copies per cell (table 28). None of the tumours with ≥4 EGFR gene copies per cell metastasized during the course of the follow-up period. Three of them were grade 3 ductal carcinomas NOS, 2/8 grade 3 apocrine carcinomas, 1/8 medullary carcinoma, 1/8 mucinous carcinoma and 1/8 was a grade 2 ductal carcinoma NOS. Gene copy number showed a statistically significant association with final outcome (p=0.036); high gene copy number (≥4 copies per cell) was associated with positive outcome. There were no statistically significant relationships observed between gene copy number and tumour grade (p=0.898), lymph node status (p=0.863) or development of distant metastasis (p=0.211)

**Table 27 EGFR gene copy number per cell and selected tumour characteristics/clinical behaviour**

| EGFR gene copy number per cell | | | |
|---|---|---|---|
| | **2-2.99** N=26 | **3-3.99** N=18 | **≥4** N=8 | **p-value** |
| **Histological grade** | | | | |
| 2 (10) | 5 | 3 | 2 | **0.898** |
| 3 (42) | 21 | 15 | 6 | |
| **Lymph node status** | | | | |
| Positive (30) | 16 | 10 | 4 | **0.863** |
| Negative (22) | 10 | 8 | 4 | |
| **Distant metastasis** | | | | |
| No (44) | 23 | 13 | 8 | **0.211** |
| Yes (8) | 3 | 5 | 0 | |
| **Final outcome** | | | | |
| Good (39) | 21 | 10 | 8 | **0.036** |
| Bad (13) | 5 | 8 | 0 | |

**Table 28 Clinico-pathologic characteristics of tumours with high (≥4) EGFR gene copy numbers**

| Age | Tumour type | Tumour grade | TNM stage | NPI | EGFR score | EGFR gene copy number | G:C |
|---|---|---|---|---|---|---|---|
| 46 | Ductal | 3 | IIA | Moderate | 6 | 5.00 | 1.51 |
| 83 | Apocrine | 3 | IIIA | Poor | 2 | 4.14 | 1.01 |
| 48 | Atypical medullary | 3 | I | Good | 4 | 4.26 | 1.65 |
| 45 | Ductal | 3 | I | Good | 9 | 4.19 | 1.06 |
| 55 | Apocrine | 3 | IIIA | Poor | 12 | 4.09 | 1.03 |
| 84 | Mucinous | 2 | IIA | Good | 6 | 4.30 | 1.30 |
| 42 | Ductal | 3 | IIA | Moderate | 12 | 12.77 | 4.05 |
| 73 | Ductal | 2 | IIA | Good | 12 | 4.04 | 0.91 |

**Abbreviations: G:C= EGFR gene-chromosome 7 ratio; NPI= Nottingham Prognostic Index; TNM= tumour node metastasis**

**Figure 33 No numerical chromosome 7 or EGFR gene abnormalities (G:C=1.03)**
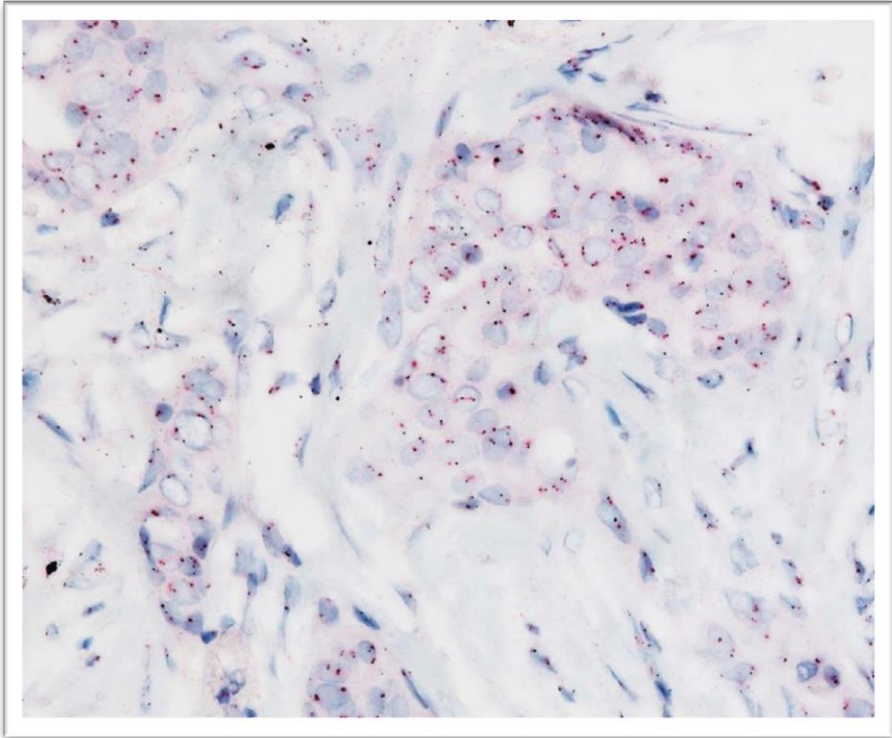


**Figure 34 Polysomy of chromosome 7 without EGFR gene amplification (G:C=1.01)**
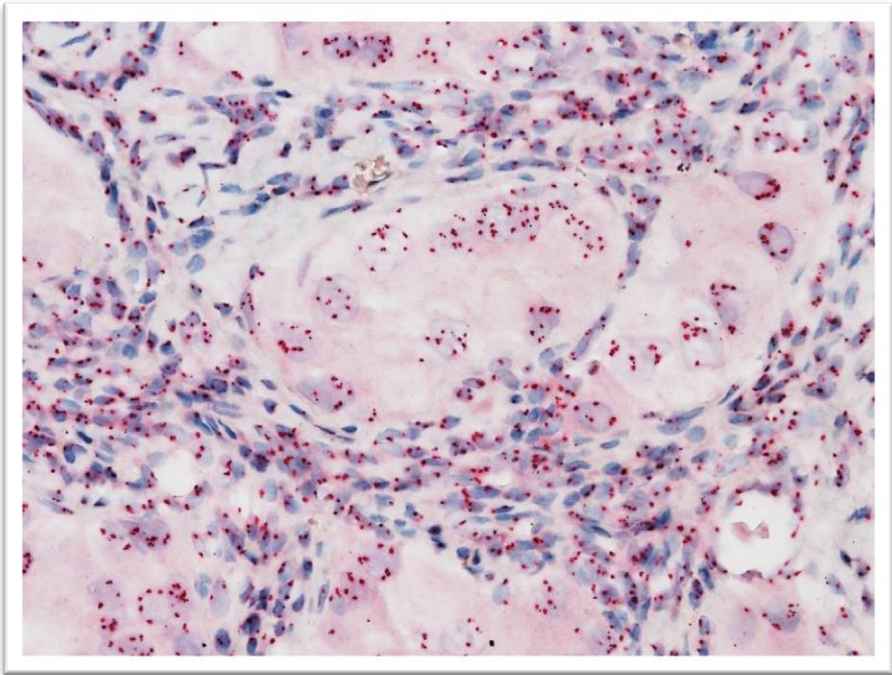
**Figure 35 EGFR gene amplification (G:C=4.09)**
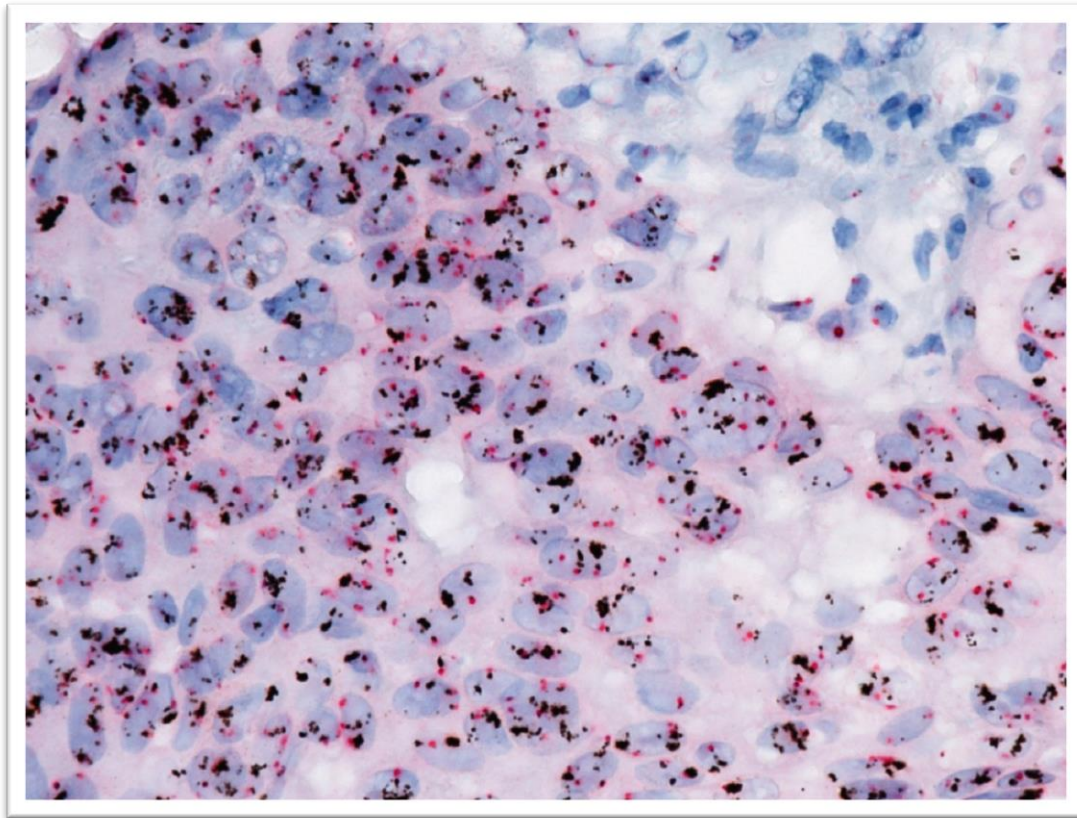
**CK18**

Only 2 (3.8%) tumours showed complete loss of CK18 (Table 29). Both tumours were grade 3 ductal NOS tumours. Staining intensity and extent varied from weak partial to strong complete positivity in the rest of the tumours (Figs. 36-37). Neither of the patients with CK18 negative tumours developed distant metastasis however this finding was not statistically significant. We also found no statistically significant association between CK18 staining and tumour grade (p=0.359), lymph node status (p=0.781) or final outcome (p=1.00).

**Table 29 CK18 expression and selected tumour characteristics/clinical behaviour**

| | CK18 staining | | | |
|---|---|---|---|---|
| | Normal staining N=30 | Partial loss N=20 | Complete loss N=2 | p-value |
| **Histological grade** | | | | |
| 2 (10) | 8 | 2 | 0 | **0.359** |
| 3 (42) | 22 | 18 | 2 | |
| **Lymph node status** | | | | |
| Positive (30) | 16 | 13 | 1 | **0.781** |
| Negative (22) | 14 | 7 | 1 | |
| **Distant metastasis** | | | | |
| No (44) | 25 | 17 | 2 | **1.00** |
| Yes (8) | 5 | 3 | 0 | |
| **Final outcome** | | | | |
| Good (39) | 22 | 15 | 2 | **1.00** |
| Bad (13) | 8 | 5 | 0 | |

**Figure 36 Partial loss of CK18 staining**
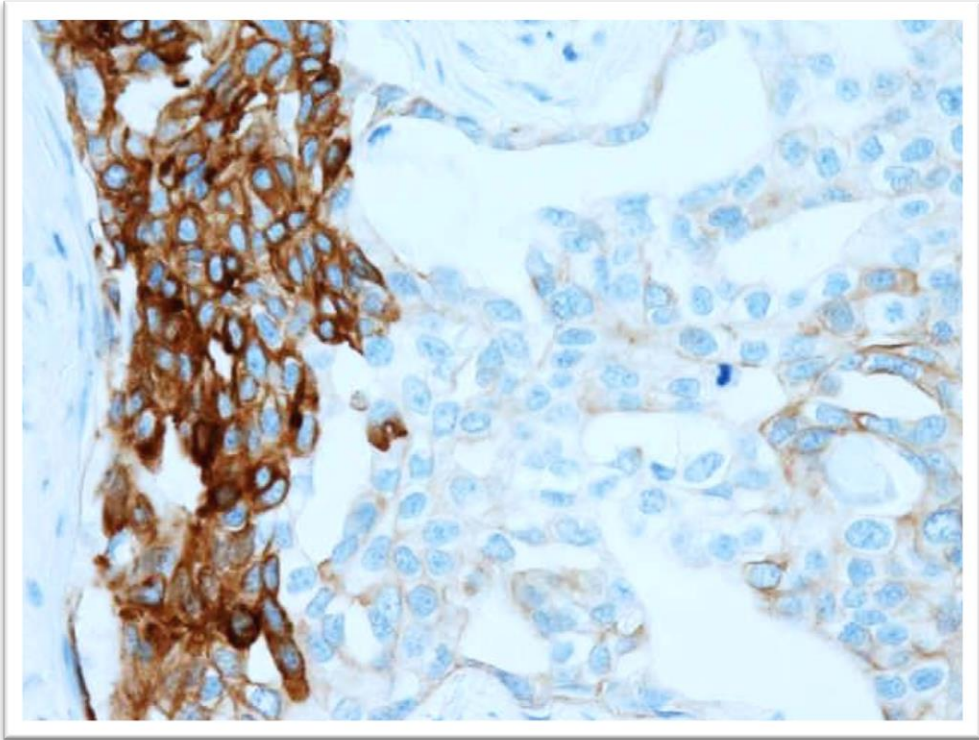


**Figure 37 Normal (complete) CK18 staining**
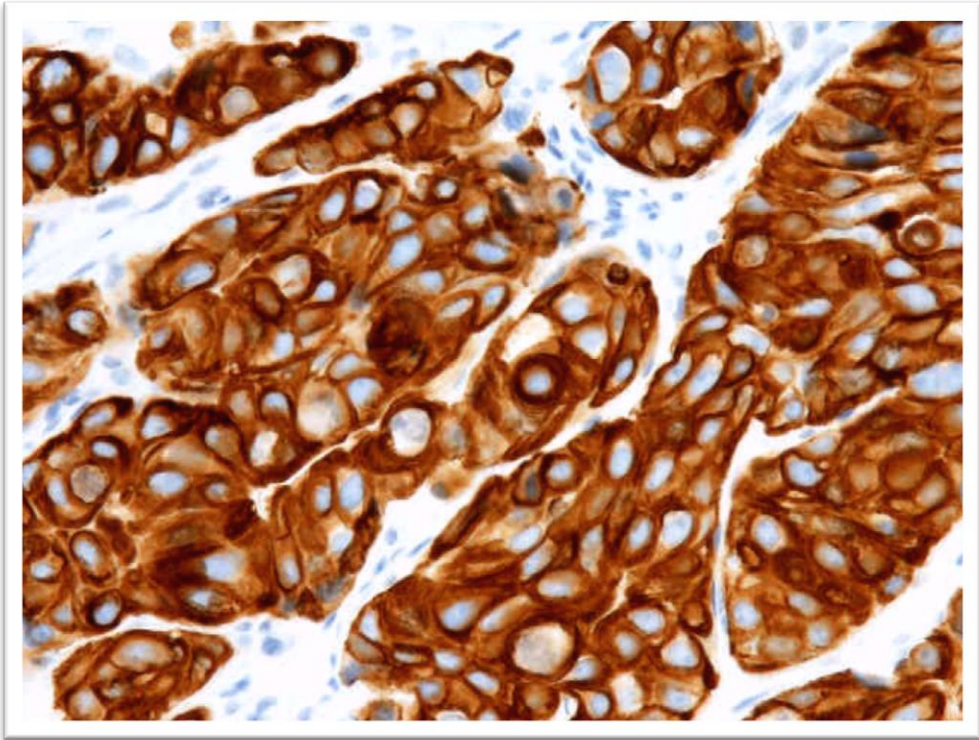
**Immunophenotypic subtypes**

All of the tumours expressed at least one of the basal markers. Based on our predefined criteria, 50/52 tumours showed luminal progenitor (luminal and basal) differentiation (Table 30). The remaining 2 tumours showed pure/'true' basal differentiation with complete loss of CK18 expression. According to Nielsen's criteria (TNBC with CK5/6 and/or EGFR+), 50/52 of our TNBC were basal-like.

**Table 30 Morphological and immunophenotypic subtypes**

| | Immunophenotypic subtype | | |
|---|---|---|---|
| | Luminal<br>N=0 | Luminal progenitor<br>N=50 (%) | 'True' basal<br>N=2 (%) |
| **Ductal NOS** | 0 | 44 (88.0) | 2 (100.0) |
| **Apocrine** | 0 | 3 (6.0) | 0 (0.0) |
| **Atypical medullary** | 0 | 2 (4.0) | 0 (0.0) |
| **Mucinous** | 0 | 1 (2.0) | 0 (0.0) |

## 6. Discussion

Taken as a whole, patients with triple negative breast carcinomas have worse prognosis than those with hormone receptor positive tumours or appropriately selected HER2-positive tumours that receive HER2 targeted therapy. The group however is clearly biologically heterogeneous as the patients have divergent outcomes regardless of treatment or known prognostic factors including stage. Some patients with triple negative breast carcinomas develop generalized disease resulting in death within two years of diagnosis while others with advanced carcinomas remain disease free for over 6 years after standard oncological therapy.

Clearly, there are important underlying differences in the nature of the entities comprising the group demonstrated by the observation of different outcomes in patients with age, stage and grade matched tumours that received identical treatment. Identifying the patients with truly aggressive disease would help to modify and optimize their management. In addition, providing patients with more accurate information on their prognosis earlier on in the course of the disease would likely improve their ability to cope with their illness and its treatment.

Results from studies on triple negative carcinomas with emphasis on basal-like carcinomas are difficult to compound and compare because of the lack of a standard definition of triple negativity. Some investigators have defined triple negativity as positive ER and PR nuclear staining in <10% of tumour cells some use 5% as the threshold while other more stringent investigators use 1%. We excluded from our definition of triple negative all tumours that showed any staining for hormone receptors as studies show that even tumours with minimal hormone receptor expression could respond to hormonal therapy (159). By using such tight boundaries in our definition, we ensured that only the tumours that are most likely to be truly biologically distinct from hormone receptor positive carcinomas were included in the study.

Our TNBC patients differed from the non-TNBC patients in terms of age and tumour features. The TNBC patients were younger than the HR+ patients and the same age as

HER2+ patients. The TN group had the highest proportion of poorly differentiated tumours. Our TNBCs were not associated with higher stage when compared with figures for breast carcinoma in general taken from the Czech Cancer Registry.

**TNBC and lymph node metastasis**

Lymph node metastasis is the most important independent prognostic factor for patients with breast carcinoma (15). Approximately 40% of women with breast cancer have regional lymph node involvement at the time of diagnosis. Despite the fact that lymph node status alone is the most important independent prognostic factor in women with breast cancer, approximately 25% of patients with node-negative disease die from metastatic disease and a similar proportion of patients with node-positive disease do not develop distant metastases. Thus negative lymph node status alone does not automatically suggest good prognosis (73). Node-positive disease is associated with an overall mortality rate of approximately 20% (73). The greater the number of nodes involved, the worse the prognosis (42). Despite the fact that relationship between TNBC and prevalence of lymph node metastasis is unclear, the accepted theory is that TNBC seems to spread to axillary lymph nodes less frequently than non-TNBC (43). Almost 60% of our TNBC cases had lymph node metastasis at the time of diagnosis. Of our 30 node-positive patients, 7 (23.3%) went on to develop distant metastasis during the follow up period. On the other hand, only one of the 22 node-negative patients (4.5%) developed distant metastasis. Negative lymph node status was thus a useful parameter for identifying tumours less likely to metastasize. However, we found no significant association between lymph node stage and metastasis amongst the lymph node positive cases.

**TNBC and NPI**

NPI combines time dependent (tumour size and lymph node metastases) and tumour dependent (histological grade) characteristics (42). Using NPI, 34.6% (18/52) of our TNBCs had good prognosis, 46.2% (24/52) had moderate prognosis and 19.2% (10/52) had poor prognosis. None of the patients with good prognosis developed distant metastases, 20.8% of the patients with moderate prognosis developed metastases and 30% of those with poor prognosis developed metastases. NPI was thus a useful tool in stratifying risk of

metastasis. The association between NPI and outcome was statistically significant (p=0.036). The finding suggests that tumour burden and histological grade may play a more important role in outcome of TNBC than other tumour characteristics.


**Morphology of TNBC**

It seems that TNBCs as a group of carcinomas are as morphologically heterogeneous as breast carcinomas in general. We observed a wide range of morphologic pictures even within the group of ductal NOS carcinomas. One common morphologic feature of the ductal NOS carcinomas was the presence of a population of spindled cells.

None of the morphological tumour characteristics we studied was significantly associated with development of distant metastasis. As all of our tumours expressed basal markers, we could not compare morphological characteristics in basal versus non-basal TNBCs.

Half (4/8) of the tumours that showed high EGFR copy numbers were 'special' histological types of breast cancer (apocrine, mucinous and atypical medullary breast carcinomas). This suggests that morphology is an important manifestation of breast tumour biology and thus could play a significant role in patient selection for various types of targeted therapy. Reis-Filho *et al* also showed a link between breast tumour morphology and EGFR changes. They observed frequent overexpression of EGFR and EGFR gene amplification in metaplastic breast carcinomas and suggested that some patients with metaplastic breast carcinomas might benefit from EGFR targeted therapy (160).


**TNBC, basal-like breast cancer and other immunophenotypic subtypes**

Jumppanen *et al* wrote '*Apart from hypothesis-generating scientific research, a breast cancer classification should correlate with clinical outcome of patients or predict efficacy to therapy*' (121). We were interested in seeing whether or not there was any clinical value in sub-classifying TNBCs as basal or non-basal like with the aim of possibly expanding our panel of standard prognostic and predictive markers in order to provide more precise information for clinicians and patients alike.

We did not find identification of the basal-like phenotype useful in prognostic stratification of TNBC. This was because according to the 'gold standard' Nielsen definition (TNBC

with CK5/6 and/or EGFR+), 96.2% of our cases were basal-like, with only 2 tumours being non-basal. Even these two tumours showed expression of other markers of basal differentiation (i.e. p-cadherin/p63 and p-cadherin/HMW, respectively).

Many authors have repeatedly emphasized that not all TNBCs are basal-like (7, 101, 161); our findings rather suggest the opposite, perhaps as a result of our strict immunohistochemical definition of triple negativity.

Silver *et al* also reported that using stricter criteria for defining triple negativity (less than 1% nuclear staining for oestrogen and progesterone receptors and HER2 score 0 or 1+ or HER nonamplified by FISH) reliably predicted classification of breast carcinomas into the basal-like subtype by hierarchal cluster analysis of the intrinsic genes (99).

One problem with the term 'basal-like' is that it implies myoepithelial-like as well as progenitor cell-like. By definition, it includes tumours that resemble basally-located myoepithelial cells and the breast progenitor cells which are not limited to any anatomical location in the TDLU (2). Rakha *et al* classified breast carcinomas as non-basal carcinomas, tumours with basal phenotype (CK5/6 and/or CK14+) and tumours with myoepithelial phenotype (p63 and/or SMA+) (162). They found that breast cancers with basal and myoepithelial phenotype are distinct groups that share some common morphological features and an association with poor prognosis.

In our opinion the term basal-like is too ambiguous for use in daily practice and provides less clinical information than the triple negative designation. While both TNBC and BLBC are known to be heterogeneous groups generally associated with poor prognosis, unlike BLBC, the TNBC designation provides predictive information useful for directing patient management.

Figure 38 shows our proposal for immunophenotypic classification of TNBC. Not surprisingly, none of our tumours showed exclusive luminal marker expression. All of them fell into either the 'true' basal (2/52) category or the dual/luminal progenitor (50/52) category.

Our finding of co-expression of luminal marker CK18 with basal markers (and minimal expression of p63 and CD10) in the majority of our cases is in support of the hypothesis

that the so called basal-like tumours, which form the vast majority of TNBCs, may arise from luminal progenitor cells rather than stem cells or myoepithelial progenitor cells (4).

The clinical significance of subtypization in TNBC based on cell of origin is questionable. In our sample, identification of immunophenotypic subtypes with focus on identification of the 'basal' phenotype was not clinically useful.

**Figure 38 Immunophenotypic classification of TNBC**

```
                    ┌─────────────────────┐
                    │ Triple negative      │
                    │ breast carcinoma     │
                    └─────────────────────┘
           ┌────────────────┼────────────────┐
  ┌──────────────┐  ┌──────────────┐  ┌──────────────────────┐
  │ Luminal      │  │ True basal   │  │ Dual/luminal progentior│
  │ CK18+        │  │ Basal markers+│ │ Basal markers+         │
  │ Basal markers-│ │ CK18-        │  │ CK18+                  │
  └──────────────┘  └──────────────┘  └──────────────────────┘
```
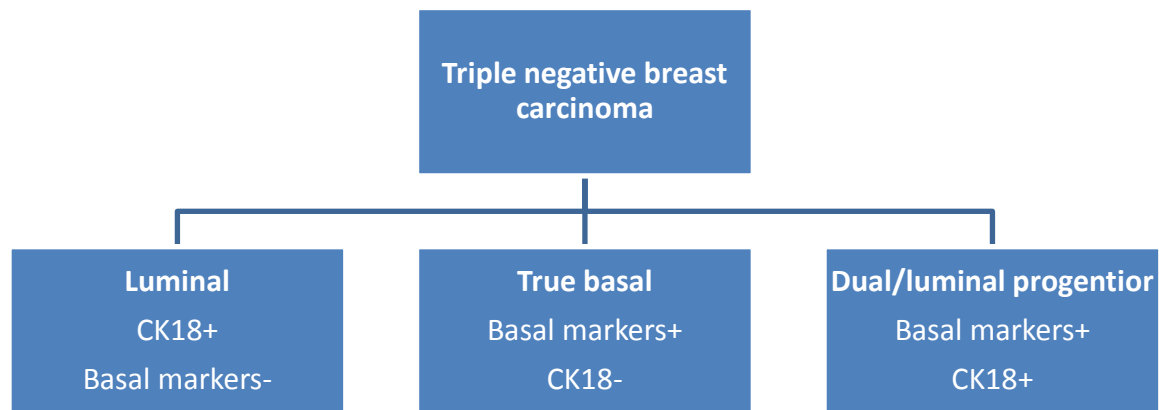
**TNBC and BRCA mutations**

Three of the patients in our sample were discovered to be carriers of BRCA mutations (the BRCA statuses of the rest of the cohort were unknown). All of them had stage IIA grade 3 ductal carcinomas with varied expression patterns of all the investigated molecular markers. All of them had surgical treatment and underwent AC-T chemotherapeutic regimen. Two of them had adjuvant radiotherapy. Although one patient developed metachronous breast cancer in her contralateral breast, all three of them were disease free at the end of the follow-up period (average: 75 months; range: 56-74 months).

**TNBC and response to therapy**

Nine of the 19 patients that received neoadjuvant chemotherapy achieved pCR. AC-T chemotherapeutic regimen was administered in all the 9 cases. All nine of them underwent partial mastectomy with axillary dissection and all but one had adjuvant radiotherapy. All of the patients that achieved pCR remained disease free at the end of the follow up period (average: 83 months; range: 54-98 months). These patients showed varied clinico-pathologic characteristics and varied expression patterns of the molecular markers we investigated. We found no single unifying characteristic amongst all the patients that achieved pCR to distinguish them from the other patients that underwent neoadjuvant chemotherapy but did not have pCR.

We note that 2 of the patients that underwent neoadjuvant chemotherapy and ended up not having surgical treatment were without signs of disease progression at the end of the follow up period (79 and 46 months, respectively). Both patients had stage IIIA disease and both underwent radiotherapy.

Choice of treatment appeared not to play a big role in development of distant metastasis or patient outcome.


**Predicting outcome in TNBC**

Rakha *et al* reported that time dependent pathological factors are the most useful in outcome prediction (123). In general, our findings are in accordance with theirs as in our sample, NPI was statistically significantly associated with development of distant metastasis. It is important to note that NPI, which also takes into account tumour histological grade, was a better predictor that the purely time dependent TNM stage (Fig. 39). P53 expression was higher in patients that did not develop distant metastasis (though not statistically significant; $p=0.097$). This could be a reflection of better response of p53 expressing TNBCs to chemotherapy as suggested by Bidard *et al* (64).

EGFR gene copy number was the only molecular characteristic we observed that showed a statistically significant, however limited, ability to predict outcome. All the TNBCs with 4 and higher EGFR gene copies per cell showed no sign of progression at the end of the follow up period. None of the other molecular markers we investigated were able to stratify TNBCs in a clinically significant way, neither were the morphological features we assessed.

**Figure 39 Factors leading to metastasis development**
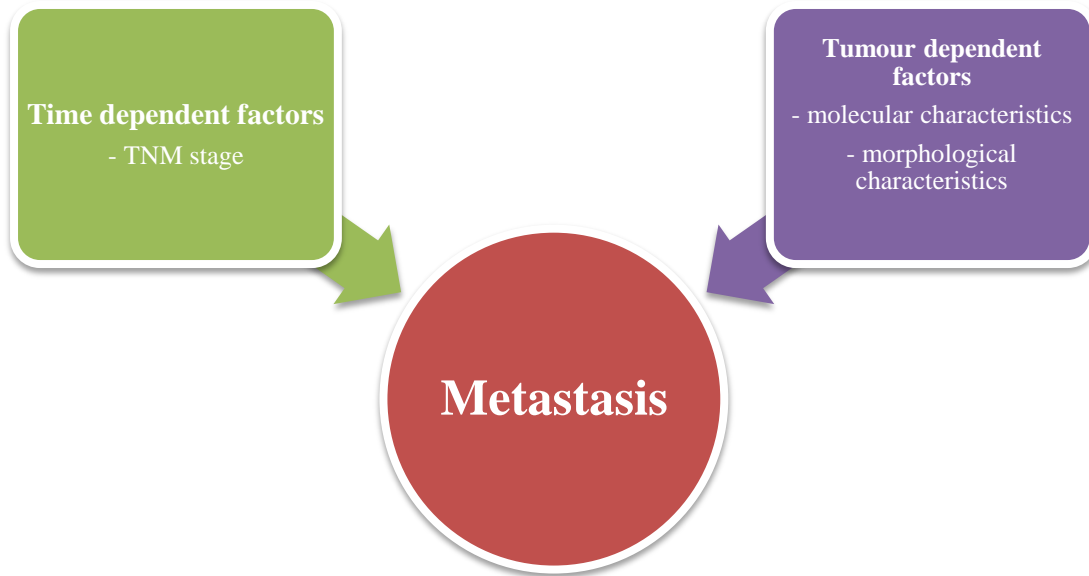


**Anti-EGFR therapy for TNBC**

Though 88.5% of our sample showed EGFR expression, amplification of the gene was seen in only one case. Our finding of rare EGFR gene amplification in TNBC was also reported by Nakajima *et al* in their study of 84 TNBCs (148). None of their tumours showed EGFR amplification. In addition to IHC and in dual in situ hybridization, they also performed EGFR gene mutation analysis. No EGFR gene activating mutations were found. Jacot *et al* also found no EGFR-activating mutations in their group of 229 TNBCs (163); neither did Uramoto *et al* in their PCR-based study on 84 breast carcinomas, 45% of which were ER negative. They concluded that EGFR tyrosine kinase inhibitors are unlikely to provide any benefit for Japanese breast cancer patients (164).

Lv *et al* on the other hand observed a higher incidence of EGFR gene amplification in their set of 139 unselected breast carcinomas; positivity in 33.1% of all cases (146). They did, however, also report a low rate of EGFR mutations (in 1.4% of all cases) and concluded

that EGFR mutations should not be used in trials testing anti-EGFR therapy in breast cancer.

Based on a study comparing 40 patients with TNBC to 158 patients with non-TNBC, Tang *et al* proposed that EGFR overexpression predicts better response to neoadjuvant chemotherapy in TNBC as EGFR overexpression was significantly associated with pCR in these patients (165).


Pitchard noted that an ideal target for targeted therapy should have the following characteristics;

- It should be a critical driver of the malignancy when it is abnormal
- It is associated with poor outcomes
- It can be successfully targeted by an agent without significant toxicity that acts though a well understood mechanism (166)


We were not able to demonstrate that EGFR changes are associated with poor outcome, neither are we certain that EGFR abnormalities are critical drivers of TNBC. This makes us question the suitability of EGFR as a therapeutic target for TNBC, certainly not for all subtypes of TNBC. The observation of a statistically significant link between high EGFR copy number and good outcome makes us further doubt that blocking the action of EGFR in TNBCs will produce favourable results.

After a phase II trial testing cetuximab in patients with metastatic TNBC Carey *et al* concluded that '*therapy targeting growth factor pathways in this subtype* (TNBC) *may require a far better understanding of the pathway*s *maintaining EGFR activity'* (92). Our findings put us in agreement with this statement. The role of EGFR in breast cancer appears to be highly complex and using anti-EGFR agents for treatment of TNBC at this point seems premature.

## 7. Conclusions

- Axillary lymph node involvement is a relatively common feature of TNBC
- Time dependent factors are significant predictors of prognosis in TNBC
- All TNBCs express at least 1 basal marker and could thus be considered 'basal-like'
- Complete loss of CK18 is a rare feature of TNBC
- EGFR gene amplification is a rare event in TNBC
- High EGFR gene copy numbers ($\geq$4 copies per cell) may be associated with favourable outcome in TNBC.


We observed a wide variation in the clinical behaviour of different cases of TNBC showing that assigning a blanket 'poor' prognosis in all cases is misleading and could result in overtreatment of patients that may not need aggressive adjuvant therapy. Based on our findings, we recommend a combinatorial approach to prognostication in TNBC using simple tools such as NPI, which proved to be useful in the stratification of patients into prognostic groups.

Identification of the basal-like phenotype using varied IHC definitions, in TNBC, had no clinical impact. Also, as the vast majority of our cohort showed co-expression of luminal marker CK18 and markers of basal differentiation, we are in favour of adopting the term luminal progenitor-like to better describe this group of TNBCs in order to differentiate them from carcinomas with (basally located) myoepithelial differentiation.

The clinical significance of molecular classification based on cell of origin is doubtful and limited, partially because of the significant overlap in protein expression amongst the cell types comprising the TDLU. The findings of this study put us in agreement with Rahka *et al* who observed that the concept of tumour differentiation rather than histiogenesis is more appropriate in the era of tailored therapies and predictive classification systems (119). It is likely that molecular profiling will play an increasingly important role in breast cancer diagnosis and management in the future, however, like Hanby (167) we believe the importance of morphology in tumour assessment should not be underestimated.

The unexpected finding of good outcome in patients with carcinomas showing high EGFR copy number calls to question the suitability of anti-EGFR treatment in patients with TNBC. Though our study was limited by its size and its retrospective nature, we found nothing to indicate that EGFR was a driver of aggressive behaviour. We were thus unable to provide evidence supporting the use of anti-EGFR therapy in unselected cases of TNBC. We did, however, discover that EGFR gene copy number may be of use in determining prognosis in TNBC. High EGFR gene copy number could be an independent marker of good outcome in TNBC.

The molecular markers and morphological characteristics we investigated were not useful in providing a basis for clinically relevant classification of TNBC. Instead, we confirmed the importance of simple combinatorial prognostic tools like the Nottingham Prognostic Index.

We propose that high EGFR copy number should be further investigated as a potential marker of good outcome in TNBC.

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## 9. Figure legends

**Tables**

**Figures**

## 10. Acknowledgements

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