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Molecular Subtypes and Survival in a Historic Cohort of Women with Breast Cancer

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Molekylære subtyper og overlevelse i en historisk kohort av kvinner med brystkreft

Målsetningen med studiene bak denne avhandlingen var å bidra til bedre klassifisering av brystkreft i prognostiske grupper. Arbeidet er basert på en historisk kohort av kvinner med brystkreft med lang oppfølging. Fra 1956-1959 ble alle kvinner fra Nord-Trøndelag som var født mellom 1886 og 1928, invitert med i et brystkreftscreeningprogram i regi av Kreftregisteret. Av de nesten 26 000 inviterte kvinnene, fikk 1393 brystkreft i perioden fra 1961-2008. De fleste av disse kvinnene ble operert, men hadde ellers ingen eller lite tilleggsbehandling. Alle tilgjengelige vevsblokker fra disse svulstene ble hentet fra arkivet til avdeling for patologi ved St Olavs Hospital.

Blokker med svulstvev fra 909 av disse kvinnene hadde god nok kvalitet, og disse er inkludert i prosjektet. Svulstene ble klassifisert etter histopatologiske typer og differensieringsgrad. Det ble brukt immunhistokjemi og in situ hybridisering, som er vanlige metoder innenfor molekylærpatologi, for å klassifisere i seks molekylære subtyper (Luminal A, Luminal B (HER2-), Luminal B (HER2+), HER2 subtype, Basal Phenotype og 5 Negative Phenotype). Videre ble det gjort studier av kjente markører hvor prognostisk nytte ikke er avklart.

Avhandlingen bygger på 3 delprosjekter som er publisert i 3 artikler. I den første studien ble prognosen for de ulike molekylære subtypene sammenlignet. Det ble funnet forskjeller mellom subtypene der Luminal A hadde best prognose, mens HER2 subtypen og 5 Negative Phenotype kom dårligst ut. Det mest interessante var at disse forskjellene var bare tilstedet for svulster med middels differensieringsgrad (histopatologisk grad 2) og bare de første 5 årene etter diagnose.

I den andre studien var målsetning å finne ut om *TOP2A* kan være til hjelp for å vurdere prognosen av brystkreft. *TOP2A* er et gen på kromosom 17 som koder for proteinet topoisomerase II α. Dette er enzymer som regulerer cellulære prosesser som replikasjon og transkripsjon, og som er målprotein for en type cellegift (Antracyklin) som er i bruk ved behandling av brystkreft. I denne studien var *TOP2A* sterkt assosiert med hormonreseptor og HER2 som er viktige prognostiske og prediktive markører i klinikken i dag, men denne markøren hadde ikke en selvstendig prognostisk betydning.

I den tredje studien ble prognose for de 2 vanligste histopatologiske typene brystkreft sammenlignet. Det ble funnet at lobulær brystkreft grad 2 hadde dårligere prognose enn duktal brystkreft grad 2, men prognosen var sammenlignbar med duktal brystkreft av mer aggressiv grad (histopatologisk grad 3). De fleste lobulær brystkreftsvulster er negative for bindingsproteinet E-cadherin. E-cadherin brukes i klinikken som et diagnostisk hjelpemiddel når det er tvil om en svulst er lobulær eller ikke. I den tredje studien ble det funnet at E-cadherin kan være nyttig som en prognostisk markør for lobulær brystkreft fordi E-cadherin negativ lobulær brystkreft hadde dårligere prognose enn E-cadherin positiv. For duktal brystkreft hadde E-cadherin ingen prognostisk verdi.

Studiene bak denne avhandlingen kan gi viktige bidrag i forståelsen av brystkreft. Først og fremst kan dette bidra til bedre klassifisering av brystkreft, og da spesielt den heterogene gruppen av grad 2 svulster. Videre er det vist at histopatologiske type kan ha selvstendig verdi som en prognostisk markør, og dette kan få betydning for valg av behandling. E-cadherin kan lett implementeres i klinikken som en prognostisk markør for lobulær brystkreft. Funnene må bekreftes i videre studier.

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Table of contents

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Finally; thanks to my husband Morten for support and faith and thanks to our three children Magnus, Helene and Vegard for showing me every day what is important in life.

2. List of papers

I. Engstrøm Monica J., Opdahl Signe, Hagen Anne I., Romundstad Pål R., Akslen Lars A., Haugen Olav A., Vatten Lars J., Bofin Anna M.
Molecular subtypes, histopathological grad and survival in a historic cohort of breast cancer patients. Breast Cancer Research and Treatment. 2013 Aug; 140(3):463-73.

II. Engstrøm Monica J., Ytterhus Borgny, Vatten Lars J., Opdahl Signe, Bofin Anna M.
TOP2A gene copy number change in breast cancer. Journal of clinical pathology. 2014 May; 67(5):420-5.

III. Engstrøm Monica J., Opdahl Signe, Vatten Lars J., Haugen Olav A., Bofin Anna M.
Invasive lobular breast cancer: The prognostic impact of histopathological grade, E-cadherin and molecular subtypes. Histopathology. 2015 Feb; 66(3):409-19

3. Abbreviations

ASCO	American Society of Clinical Oncology
BCSS	Breast cancer-specific survival
BP	Basal phenotype
CAP	College of American Pathologists
CI	Confidence intervals
CISH	Chromogenic *in situ* hybridization
cDNA	complementary DNA
CK5	Cytokeratin 5
E-cad	E-cadherin
EGF	Epidermal growth factor
EGFR	Epithelial growth factor receptor 1
ER	Oestrogen receptor
FDA	Food and Drug Administration
FFPE	Formalin-fixed, paraffin-embedded
FISH	Fluorescence *in situ* hybridization
GGI	Gene expression grade index
HER2	Human epidermal growth factor receptor 2
HES	Haematoxylin–erythrosin–saffron
HPF	High power field
HR	Hazard ratio
ISH	*In situ* hybridization
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry/immunohistochemical
ILC	Invasive lobular carcinoma
KM-plot	Kaplan-Meier plot
MHT	Menopausal hormonal therapy
NGS	Nottingham Grading System
5NP	Five negative phenotype
NHSBSP	National Health Services Breast Screening Programme
NST	No special type

PCR	Polymerase Chain Reaction
PR	Progesterone receptor
SI	Staining index
SMA	Smooth muscle actin
SN	Sentinel node
TMA	Tissue microarray
WHO	World Health Organisation

4. Background

4.1 Introduction

Breast cancer is the leading cause of death from cancer among women worldwide. Breast cancer accounts for one in every four cases of cancer and, in 2012, a total 1.7 million cases of breast cancer were diagnosed [1]. Incidence is highest in developed countries, but is increasing in developing countries where breast cancer mortality is relatively higher compared to more affluent parts of the world. This may, in part, be explained by late diagnosis and lack of optimal therapy [2]. Figures 1 and 2 show breast cancer incidence and mortality worldwide [1].

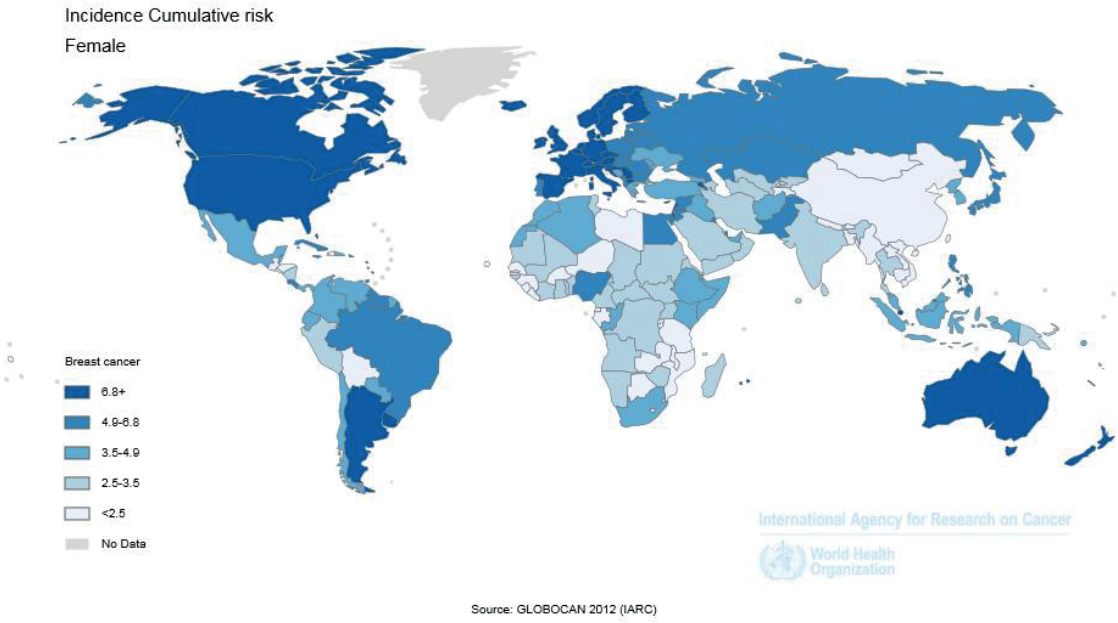


Figure 1 Incidence of breast cancer worldwide [1].

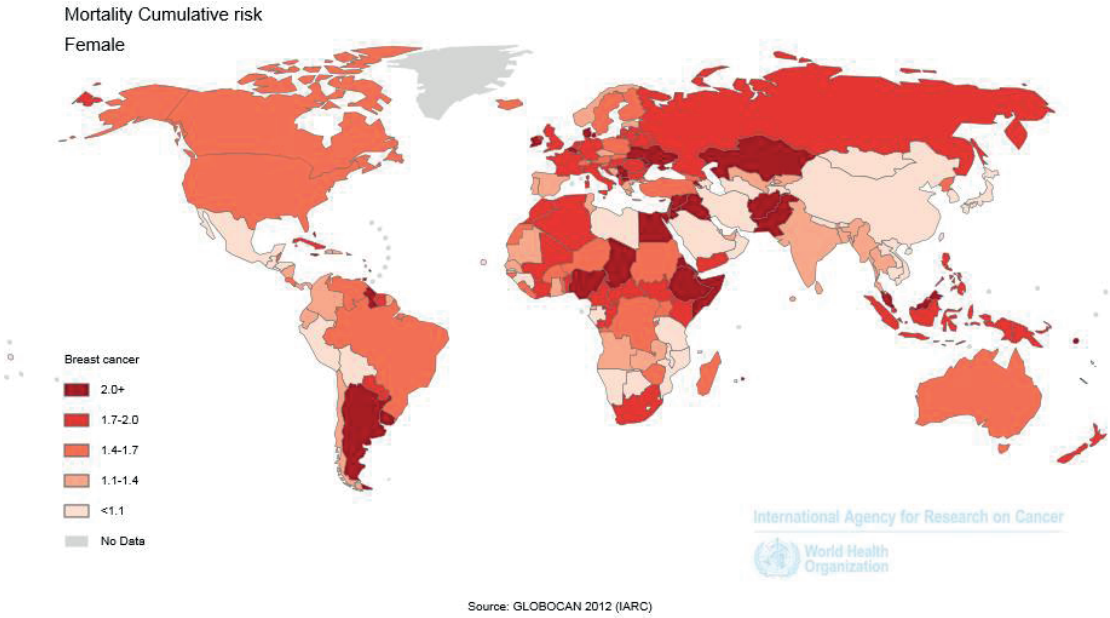


Figure 2. Mortality of breast cancer worldwide [1].

In 2012, 2 956 women were diagnosed with breast cancer in Norway and 645 deaths from the disease were registered. Breast cancer is the most common cancer and the second most common cause of death from cancer among women in Norway [3]. Figure 3 shows incidence for the ten most common cancers and Figure 4 shows the ten most common causes of cancer death among women in Norway in 2011 [4].

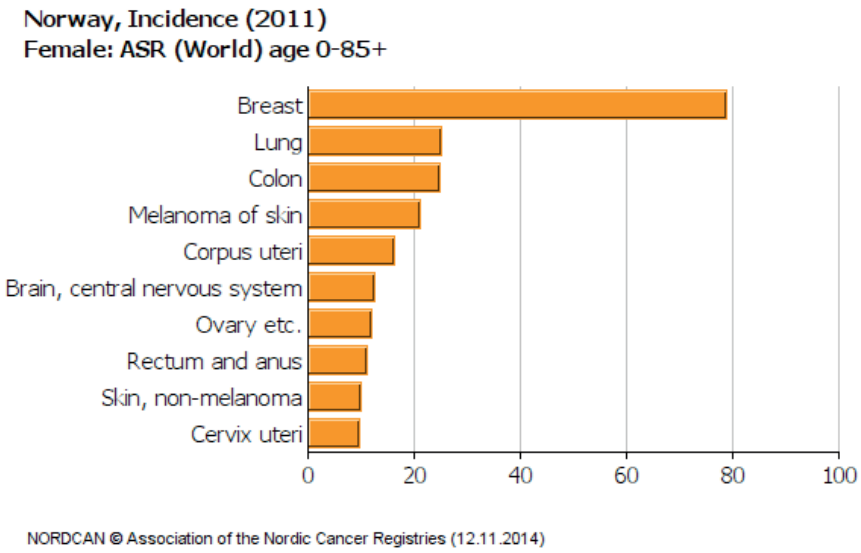


Figure 3. The ten most common cancers among women in Norway in 2011.

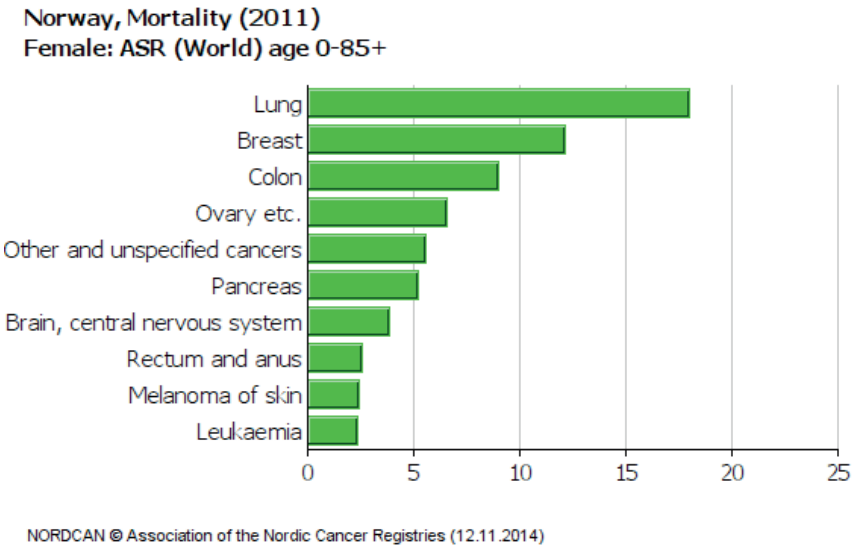


Figure 4. The ten most common causes of cancer death among women in Norway in 2011.

The incidence of breast cancer in Norway has increased from 1960 to 2005. Around 2005 there was a slight fall in incidence followed by an increase reaching a peak of 3094 in 2011 (Figure 5). In 2012, 2956 cases of breast cancer were diagnosed [3]. Despite the increase in incidence, mortality has declined. A reduction in mortality was first observed in the middle of the 1990s, probably due to a combination of earlier diagnosis and the introduction of new treatment options [5-7]. In the same period, survival has improved considerably, and, in 2010, 9 out of 10 women were still living five years after diagnosis (Figure 5).

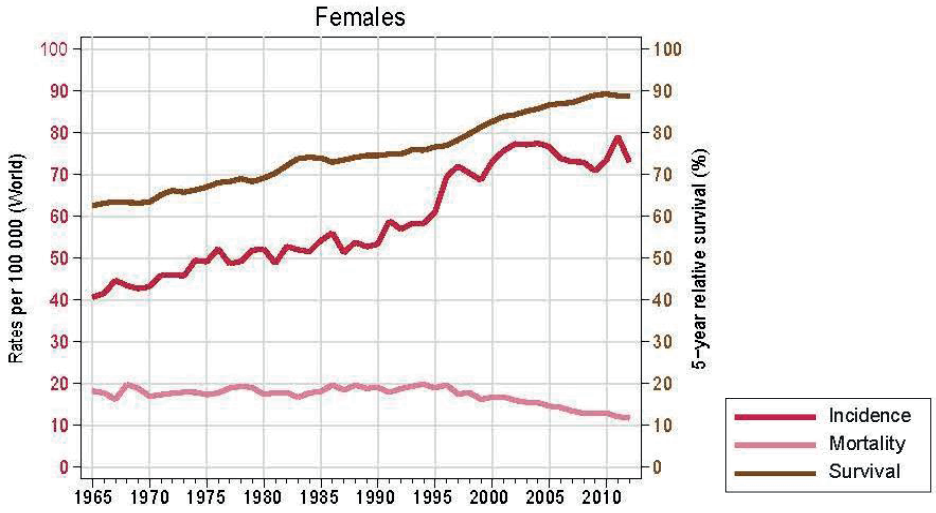


Figure 5. Breast cancer incidence, mortality and survival in Norway from 1965 to 2012 [3].

Survival is usually expressed as a 5-year rate which is the percentage of people still alive 5 years after diagnosis. Relative survival is the percentage of the patient population who are still alive after 5 years divided by the percentage of the general population alive at the end of the same time period. However, breast cancer patients have a higher risk of death compared to the general population more than 20 years after diagnosis [8] and 5-year survival is therefore a limited description of breast cancer prognosis.

4.2 Causes and risk factors of breast cancer

Epidemiological studies have identified a number of risk factors, but the causes of breast cancer are not known. The correlations between potential causes, risk factors and breast cancer are complex and not fully understood. Risk factors and possible causes are largely outside our influence. A relationship between various risk factors and breast cancer in general has been demonstrated, and more recently, more specific studies of associations between risk factors and different subtypes of breast cancer have been published [9-12].

Among breast cancer risk factors beyond the individual`s control are weight and length at birth, intrauterine oestrogen exposure, adult height and hereditary breast cancer risk. The same applies to mammographic breast density which is also associated with an increase in risk of developing breast cancer [13, 14]. The proportion of hereditary breast cancer is estimated to be between 5 and 10 % [15-17]. Mutations in the *BRCA1* and *BRCA2* genes are autosomal dominant by nature and account for 45 – 50 % of hereditary breast cancer. For a large proportion, the *BRCA1* mutations lead to triple negative breast cancer (hormone receptor negative and HER2 negative breast cancer). The risk of developing various breast cancer subtypes differs between ethnic groups. For example, African American women have a higher risk of basal phenotype or basal-like breast cancer [18, 19].

Alcohol consumption and body weight are established modifiable risk factors associated with life style. A dose-response association between alcohol consumption and risk of breast cancer has been shown, but the mechanisms are not known [20, 21]. Premenopausal obesity and postmenopausal obesity are associated differently with breast cancer risk. Premenopausal obesity has been shown to have a protective effect while postmenopausal obesity is associated with higher risk [22, 23]. Exposure to radiation could also be classified as a modifiable risk factor.

Reproductive factors such as early onset of menarche, high age at first birth, non-parity and late age at menopause are associated with higher risk of breast cancer [22, 24, 25]. These risk factors seem mainly to be linked to hormone receptor positive breast cancer [9, 22]. Breast feeding may lower the risk of some subtypes of breast cancer [26]. Pregnancy leads to a transient increased breast cancer risk followed by a prolonged protective effect [27-29]. These factors are linked to endogenous oestrogens although the mechanisms are not clarified [30].

Exogenous hormones have been shown to have an impact on breast cancer risk. Menopausal hormonal therapy (MHT) and hormonal contraception are associated with higher breast cancer risk [31-33]. The doses of both have been modified considerably since their introduction and this may have lead to some reduction of the increased risk. The combination of oestrogen and progestagen entails a higher risk than oestrogen alone which in itself is probably responsible for a very modest increase in risk [34, 35].

Several of the risk factors are linked to exposure to endogenous and exogenous oestrogen and the observed increased risk is mainly associated with hormone receptor positive breast cancer. For hormone receptor negative subtypes of breast cancer, associations with risk factors are much less clear [9, 36]. The complexity of this field and interactions between the different risk factors makes studies and interpretation of studies difficult.

4.3 Advances in breast cancer treatment

The treatment of breast cancer remained largely unchanged until the latter half of the 20th century. Surgical resection of breast cancer tumours was described as early as in the 1th century by Celsus [37]. In more recent times, William Halsted is credited for performing the first radical mastectomy in the 1890s. However, Charles Moore advocated mastectomy *en bloc* as early as 1867 [38]. In 1882 William Banks recommended routine axillary clearance when treating breast cancer [38].

In the 1950s, radical mastectomy versus simple mastectomy was discussed [39]. Still, radical mastectomy with removal of the pectoralis major muscle remained the treatment of choice until approximately 1970, followed by simple mastectomy which spared the muscle and reduced morbidity [40, 41]. As early as in the 1950s and 1960s and parallel to the discussions regarding the extent of mastectomy, clinical trials comparing mastectomy with breast conserving surgery followed by radiotherapy were carried out [42]. Breast conserving therapy for selected patients was gradually implemented from the 1970s, but was not accepted in Norway until the 1990s [43]. A number of studies have shown similar survival for breast conserving surgery and mastectomy [44-48]. More recently, even large tumours have been treated with breast conserving surgery following the development of more advanced oncoplastic techniques or after down-staging with neoadjuvant chemotherapy.

The sentinel node (SN) technique is another important advance in the surgical treatment of breast- and other cancers. The basis for this method is the hypothesis that the lymphatic drainage from the breast passes through one or more gate-keeper or sentinel lymph nodes in the axilla and to a lesser extent the retrosternal region [49, 50]. With lymphoscintigraphy and/or blue dye (methylene blue, patent blue v or isosulfan blue), the SN can be detected and removed for histopathological examination [51]. If no metastasis is found, further axillary surgery is not performed. For approximately 75 % of patients axillary clearance is thus avoided [52] and postoperative morbidity and risk of lymphoedema is reduced [53, 54]. For patients with clinical negative axilla and micrometastasis to the SN, axillary clearance can be omitted [55-57].

Advances leading to improvement of prognosis have been achieved with the advent of adjuvant therapy. Indications for and type of chemotherapy have changed over the years from perioperative chemotherapy recommended to all [58] to the more individually tailored treatment protocols of today [59]. For patients with positive hormone receptor status, endocrine therapy has been shown to improve survival [60]. From around 1970, endocrine therapy was indicated in cases of metastatic breast cancer [61]. In 1975, measurement of oestrogen receptor (ER) was introduced facilitating better selection of patients for treatment with tamoxifen thus increasing its use as an adjuvant therapy alternative [62]. Recent publications have documented better survival for ten years treatment versus five years for premenopausal women [63]. However, decreasing effect of treatment over time may be a disadvantage. Furthermore, poor compliance may also reduce benefit [64, 65].

Targeted therapy against HER2 is one of the major advances made in adjuvant breast cancer treatment in recent years. The monoclonal antibody trastuzumab was the first anti-HER2 agent approved for breast cancer treatment [66]. The treatment has improved prognosis for HER2 positive breast cancer when used in combination with chemotherapy. More recently, even more potent inhibitors of HER2 have been introduced. Anti-HER2 therapy has been shown to be useful in the adjuvant, neo-adjuvant and metastatic setting [66].

Radiation therapy is of importance in the control of local and regional breast cancer and thus impacts long-term survival [67, 68]. Radiotherapy is given based on breast cancer stage and as an integral part of breast conserving treatment. Prognostic biomarkers are not decisive in determining radiotherapy.

4.4 Morphology

The structural and functional tissue in the breast consists of epithelium arranged in lobules and ducts and their surrounding connective tissue. In normal breast tissue, the epithelium consists of two layers: the epithelial (luminal) layer lining the lumen and the myoepithelial (basal) layer between the epithelial layer and the basal lamina [69]. These two layers consist of three cell types [70]: luminal and basal cells in the epithelial layer and myoepithelial cells in the myoepithelial layer. In the non-lactating breast, the stromal elements (fat cells and fibrous connective tissue) comprise the majority of the breast volume. Both epithelium and stroma are hormonally responsive, and differentiation is dependent on the influence of oestrogen and on the stromal-epithelial interaction [71]. The proportion of fat and connective tissue varies among individuals, and changes throughout life under the influence of physiological and hormonal factors [72].

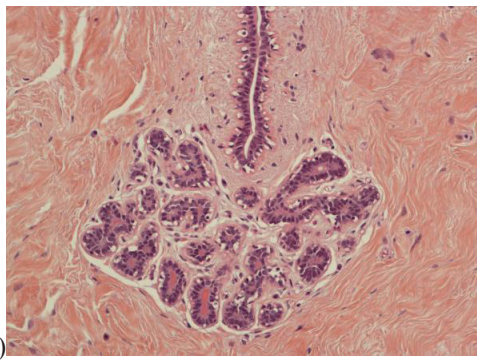
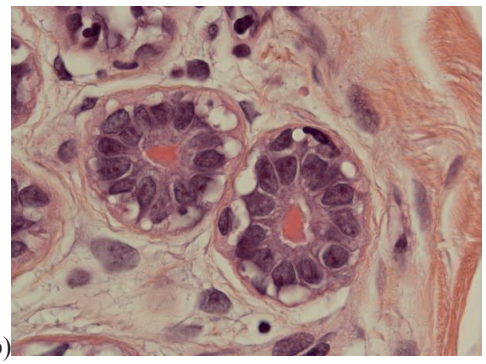
a)  b) 

Figure 6. Normal HES-stained breast tissue. a) A normal lobule (HES, 100x). b) Clear cell change in the myoepithelial layer (HES, 1000x). Photo: A M Bofin

Embryologic development from the milk lines is independent of steroid hormones early in fetal life. From 15 weeks, further differentiation is dependent on testosterone. Secretory activity is induced by maternal and placental steroid hormones and prolactin in the last weeks of fetal life. The maternal hormonal influence on the infant disappears during 1-2 months after birth, and further breast development is postponed until puberty [72]. Under the influence of oestrogen at the onset of puberty, growth and differentiation of both epithelium and stroma occurs. Prolactin, growth hormones and glucocorticoids also contribute. Further hormonal

changes cause differentiation of the breast tissue during pregnancy and lactation. With the onset of the menopause, glandular atrophy gradually occurs.

In addition to the luminal ductal cells and the myoepithelial cells, there is also evidence of the existence of breast stem cells dispersed in the glandular tissue [71, 73]. The function of normal stem cells is self-renewal and maintenance of homeostasis in tissue [74, 75]. The normal stem cells must be able to undergo frequent mitosis and migrate within the organ. These are also qualities characteristics of cancer cells.

4.5 The development of cancer

The mechanisms of uncontrolled cellular proliferation are only partly understood. Cancer development is a multistep process reflected in intratumoural heterogeneity where the various cell types interact and the stroma and microenvironment participate actively [76]. Two important hypotheses are the stem cell theory of cancer and the theory of somatic evolution in cancer [77].

In the cancer stem cell theory, cancer stem cells have the ability to proliferate extensively and new cancer cells originate only from cancer stem cells [78]. Other non-stem cancer cells proliferate and contribute to the progression of the disease, but they cannot sustain the development of cancer independent of the stem cells. Liu et al demonstrated breast cancer stem cells in distinct mesenchymal-like and epithelial-like states [79]. Gene-expression profile studies of these states showed similar expression patterns for the different molecular subtypes suggesting the same cell of origin.

In the theory of somatic evolution in cancer, a neoplasm is the result of sequential mutations occurring because of genetic instability, environmental factors and/or other events that together lead to uncontrolled cell growth [77, 80]. In this theory, all cancer cells have the same potential for further growth under the same exposure [81].

Both theories are explanations of tumour heterogeneity and the one does not exclude the other [77].

Breast cancer arises from the epithelium, probably most frequently in the terminal duct lobular unit. Histopathologic examination of proliferative lesions may reveal a variety of

morphological entities such as usual hyperplasia, atypical hyperplasia, carcinoma *in situ* and invasive carcinoma. The different intraductal proliferative entities are associated with varying degrees of increased risk of developing invasive carcinoma [82]. The work in this project is restricted to invasive breast carcinomas.

4.6 Histopathological types

Breast cancer is a heterogeneous disease or group of diseases and this is reflected in the microscopical findings. Breast cancer is divided into morphological groups based on both the growth pattern and the appearance of the cells in the tumours. Pathologists have a long history of seeking to classify breast cancer into meaningful groups of similar pattern by assessment of HES-stained sections [83]. Classification in histopathological types is done according to World Health Organization (WHO) Classification of Tumours [82]. Updated versions of the WHO classification system have been published regularly since 1968 (Scarff, R. W. & Torloni, H. Histological Typing of Breast Tumours (WHO, Geneva, 1968)).The terminal duct lobular unit is the anatomical origin of the majority of breast carcinoma regardless of histopathological type. Although it is recognized that prognosis may differ for the various types, histopathological type is currently not decisive for treatment in Norway.

Tumours with distinct histopathological characteristics in HES-stained sections are classified as special types and approximately 30 different types and subtypes have been described [82, 84]. The special types account for 25 % of all breast carcinomas [85, 86]. Approximately 75 % of breast carcinomas fail to fit as special type [83] and are classified as invasive breast carcinomas of no special type (NST). This type or group of types is commonly referred to as invasive ductal carcinoma (IDC) and shows considerable variation in growth pattern and cell appearance. Figure 7 a and b show examples of invasive breast carcinomas of NST.

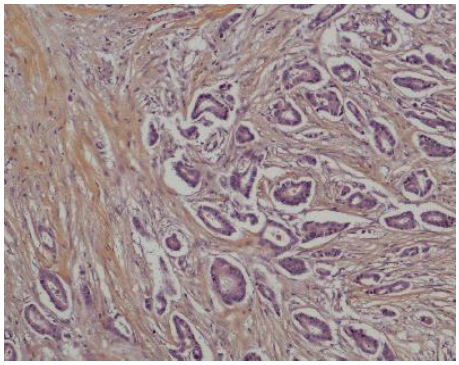
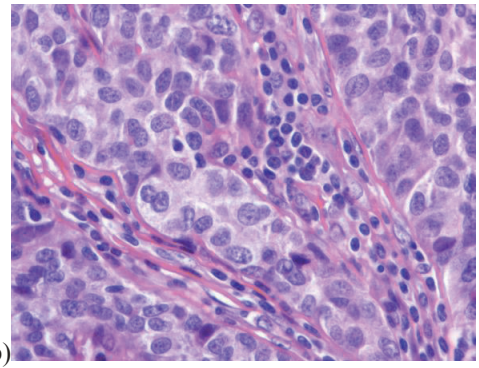
a)  b) 

Figure 7. a) Invasive carcinoma of no special type (HES) 100x b) 600x. Photo: A M Bofin

Invasive lobular carcinoma is the second most common type and most frequent of the special types comprising 5–15 % of all breast cancers. Invasive lobular carcinoma is defined as a tumour composed of non-cohesive cells growing in a dispersed fashion or in cell lines ("Indian file" pattern) in a fibrous stroma [82]. Classic microscopic features are small cells, uniform nuclei, infrequent mitoses and the absence of glandular structures. There are a number of variants making classification more difficult and some of these variant are infrequent. Mixed types, such as tubulolobular carcinoma also occur.

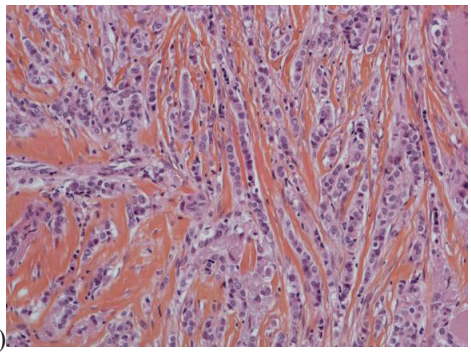
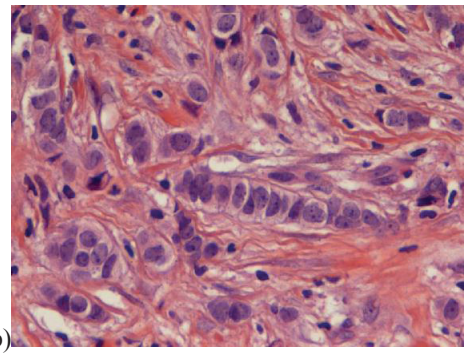
a)  b) 

Figure 8. Invasive lobular carcinoma with "Indian file" pattern (HES). a) 200x b) 600x
Photo: A M Bofin

Tubular carcinoma (2 %), mucinous carcinoma (2 %), medullary carcinoma (< 1 %), metaplastic carcinoma (0.2 – 5 %), papillary carcinoma (rare) and neuroendocrine carcinoma (< 1 %) are examples of other less frequent histopathological types [82]. Tubular carcinoma entails a favourable prognosis and is characterized by well-differentiated tubular structures in most of the tumour. In mucinous carcinomas, clusters of small uniform cells are seen floating in varying amounts of extracellular mucin. Capillary fragments are also seen. Medullary carcinoma is characterized by pushing borders, high-grade nuclei, lack of glandular structures and prominent lymphoid infiltration. Medullary breast cancer occurs more frequently among breast cancer patients with *BRCA1* mutation [87, 88]. In metaplastic carcinomas, the epithelial cells may differentiate into squamous cells or mesenchymal-like cells of various appearances. Some of the special types are infrequent or rare, and therefore studies of each of these types are difficult.

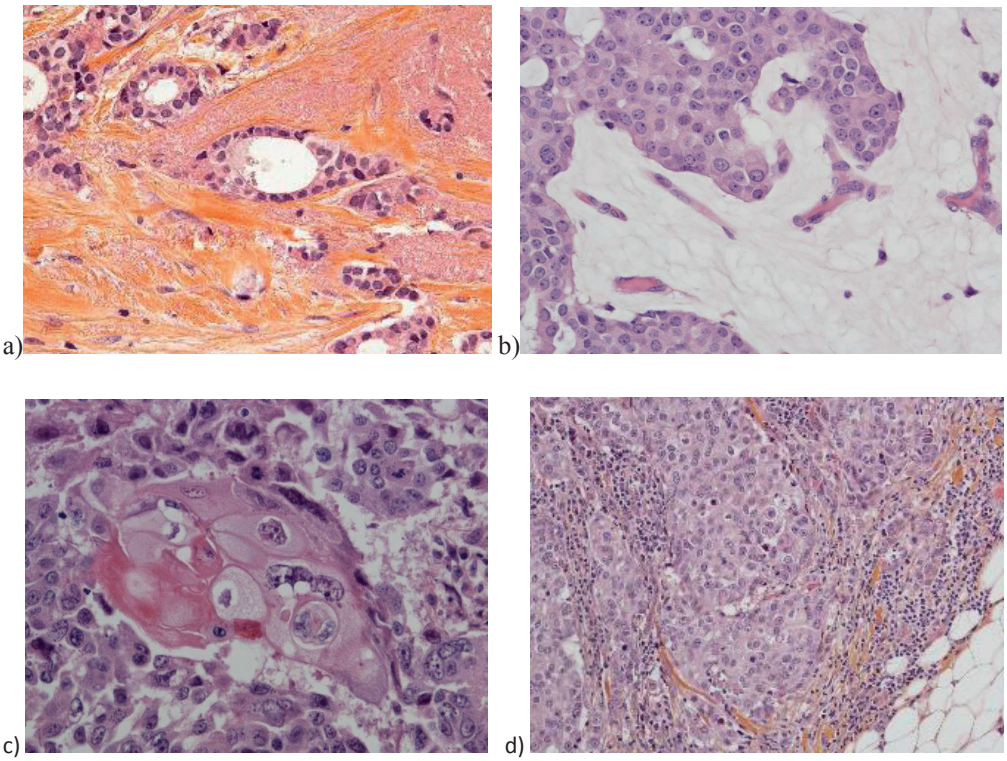


Figure 9. Invasive carcinoma, special types. a) Tubular carcinoma (HES, 400x), b) Mucinous carcinoma (HES,400x), c) Metaplastic carcinoma (HES, 600x) and d) Ductal carcinoma with medullary feature (HES, 200x). Photo: A M Bofin

4.7 Histopathological grading

Breast cancer is classified into histopathological grades which reflect the degree of differentiation of the tumour cells. Histopathological type and grade give complementary information regarding tumour features and prognosis [83]. Grading is usually done on full face HES-stained sections. The most commonly used method is the Nottingham Grading System (NGS) [89] which is a modification of the original Scarff-Bloom-Richardson grading system [90]. Histopathological grade provides prognostic information and is decisive for treatment. Even in the current genomic and molecular era, lymph node status, tumour size and histopathological grade are still considered to be the three strongest prognostic determinants in breast cancer [91, 92]. In experienced hands and under standardized conditions, the reproducibility is modest to good [89, 93, 94].

Figure 10 shows examples of invasive ductal carcinoma, grades 1 and 3. Three features are considered and scored in the NGS (see Table 1): The proportion of tumour with tubule formation, the number of mitoses and the degree of variation in nuclear size and shape are assessed and allotted a score. The sum of the scores for the three components generates the grade.

Tubular structures: The tumour is given a score of 1 if more than 75 % is composed of glandular structures and 3 if tubules are found in less than 10 % of the tumour. A pitfall in this assessment is shrinkage artefact due to poor fixation, and clearly visible lumina are important [89].

Nuclear pleomorphism: Nuclei are assessed for size, shape and variations of these features. The high-scoring nuclei are often vesicular with multiple nucleoli. Some histopathological types, such as lobular carcinoma, are characterized by small or relatively small nuclei and consequently result in a low score (1 or 2). Scoring of nuclear pleomorphism is to a certain extent subjective, and interindividual variation occurs [95].

Mitoses: The scoring criteria for the mitotic count are more well-defined [89, 95]. The number of mitoses is dependent on microscopic field diameter in the microscope used by the pathologist, and the guidelines in Figure 11 are followed.

Grade 1 (score 3-5) represents the most differentiated and least aggressive tumours, grade 2 (score 6-7) moderate and grade 3 (score 8-9) the most poorly differentiated, most aggressive tumours.

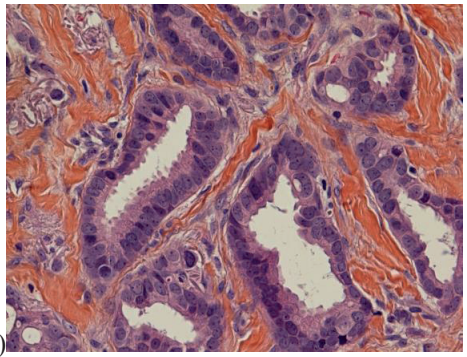
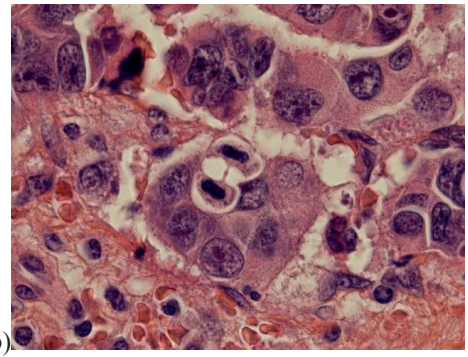
a)  b) 

Figure 10. Invasive ductal carcinoma. a) Tubular structures in grade 1 ductal carcinoma (HES, 400x). b) Profound nuclear pleomorphism in grade 3 invasive ductal carcinoma (HES, 600x). Photo: A M Bofin

Table 1. Feature considered when grading according to Nottingham Grading System, table slightly modified after Elston and Ellis [89].

Feature	Score
Tubule formation	
Majority of tumour (>75 %)	1
Moderate degree (10-75 %)	2
Little or none (<10 %)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Score 1-3 dependent on microscope field area.	1
Guidelines in Figure 11 [96]	2
	3

NHS
Cancer Screening Programmes

Breast Cancer Grading

Nottingham Criteria

Accurate grading of invasive breast cancer requires good fixation, processing, section cutting, staining and careful application of grading criteria. In the UK, about 20% of *symptomatic* breast cancers are grade 1, 30% grade 2, and 50% grade 3. These proportions may be different in asymptomatic cancers detected by mammographic screening. Special type cancers (lobular, etc) should also be graded. Three separate scores are given:

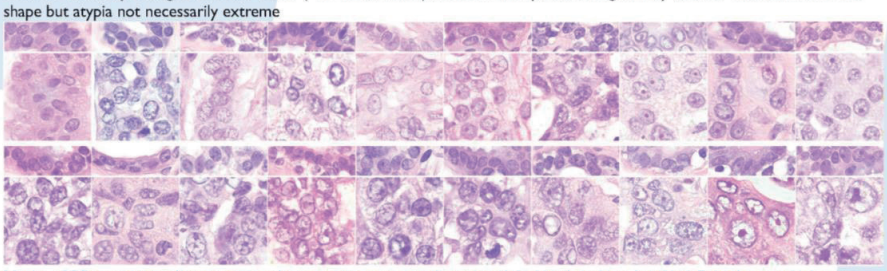
Gland (acinus) formation

Score 1: more than 75% of the whole carcinoma forms acini
Score 2: 10–75% of the whole carcinoma forms acini
Score 3: less than 10% of the whole carcinoma forms acini

Only score clearly formed glandular lumens surrounded by polarised cancer cells

Nuclear atypia/pleomorphism

Only about 5% of symptomatic cancers score 1 for nuclear atypia; about 50% score 3.
Score 1: nuclei only slightly larger than benign breast epithelium (< 1.5 × normal area); minor variation in size, shape and chromatin pattern
Score 2: nuclei distinctly enlarged (1.5–2 × normal area), often vesicular, nucleoli visible; may be distinctly variable in size and shape but not always
Score 3: markedly enlarged vesicular nuclei (> 2 × normal area), nucleoli often prominent; generally marked variation in size and shape but atypia not necessarily extreme



Nuclei of 20 consecutive breast cancers by increasing mean nuclear area (left to right, top to bottom). Paired non-neoplastic breast epithelium is shown above each case for comparison. Only one cancer (top left) has nuclei which score 1. The others in the top row score 2. All 10 in the bottom row score 3.

Mitosis counts

Measure diameter of high power field (hpf) on your microscope to the nearest 0.01 mm. Always use same objective *and* eyepieces; if either is changed, measure again. Read score thresholds from table below. Scan sections to find area with most mitotic activity (often at tumour edge). In this area count definite mitoses in 10 consecutive fields. Skip fields with few carcinoma cells or obvious necrosis. Convert to score (1–3).

Table of mitosis score thresholds

Field diameter (mm)	Mitotic frequency score 1	2	3	Field diameter (mm)	Mitotic frequency score 1	2	3	Field diameter (mm)	Mitotic frequency score 1	2	3
0.40	≤ 4	5–9	≥ 10	0.50	≤ 7	8–14	≥ 15	0.60	≤ 10	11–20	≥ 21
0.41	≤ 4	5–9	≥ 10	0.51	≤ 7	8–14	≥ 15	0.61	≤ 10	11–21	≥ 22
0.42	≤ 5	6–10	≥ 11	0.52	≤ 7	8–15	≥ 16	0.62	≤ 11	12–22	≥ 23
0.43	≤ 5	6–10	≥ 11	0.53	≤ 8	9–16	≥ 17	0.63	≤ 11	12–22	≥ 23
0.44	≤ 5	6–11	≥ 12	0.54	≤ 8	9–16	≥ 17	0.64	≤ 11	12–23	≥ 24
0.45	≤ 5	6–11	≥ 12	0.55	≤ 8	9–17	≥ 18	0.65	≤ 12	13–24	≥ 25
0.46	≤ 6	7–12	≥ 13	0.56	≤ 8	9–17	≥ 18	0.66	≤ 12	13–24	≥ 25
0.47	≤ 6	7–12	≥ 13	0.57	≤ 9	10–18	≥ 19	0.67	≤ 12	13–25	≥ 26
0.48	≤ 6	7–13	≥ 14	0.58	≤ 9	10–19	≥ 20	0.68	≤ 13	14–26	≥ 27
0.49	≤ 6	7–13	≥ 14	0.59	≤ 9	10–19	≥ 20	0.69	≤ 13	14–27	≥ 28

Final grading

Add scores for acinus formation, nuclear atypia and mitosis count. Total score must be in the range 3–9.

Total score 3, 4 or 5 = **grade 1** Total score 6 or 7 = **grade 2** Total score 8 or 9 = **grade 3**

Prepared by Dr James J Going, Department of Pathology, Western Infirmary, Glasgow, based on Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 1991; 19: 403–410, and reproduced with permission.

Additional copies are available from DH Publications Orderline (tel 08701 555 455 or email doh@prolog.uk.com) quoting BGRADPSTR.

Figure 11. Guidelines for grading of breast cancer tumours according to Nottingham Grading System [97].

4.8 Detection of biomarkers in breast cancer

Changes in cells can be studied at several points along the molecular biological pathway using appropriate methods. The Central Dogma of Molecular Biology was stated by Francis Crick in 1956 [98] and restated in 1970 [99]. This is a statement of sequential information transferred from DNA to RNA to proteins, but never in the opposite direction from protein to DNA. Figure 12 illustrates DNA, RNA and protein as three points on the molecular biological pathway. In this project, DNA is studied by *in situ* hybridisation (ISH) and proteins by immunohistochemistry (IHC).

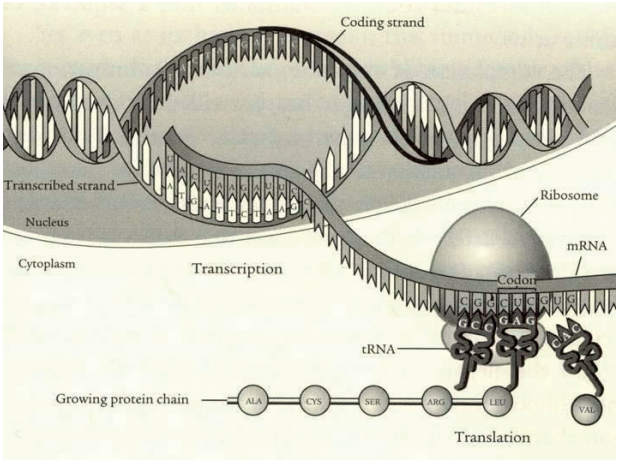


Fig 12. The molecular biological pathway. (K. Stern from D.G. Nathan Genes, Blood, and Courage: A Boy Called Immortal Sword 1995 Harvard University Press).

4.8.1 Immunohistochemistry

IHC is a method that unites histology, immunology and chemistry [100]. Immunological methods are used to visualise the distribution and localisation of specific antigens in histological sections. IHC is used in clinical diagnostics, for prognostication of disease and for prediction of treatment [70]. The method was introduced in 1941 by Albert Coons [101] on fresh frozen tissue. Fluorescence was initially used for visualization. The method was further

developed for use on Formalin-fixed, paraffin-embedded (FFPE) tissue and with peroxidase antibody conjugation enabling assessment under a light microscope [102, 103].

Specific antibodies are marked with a label visible by light or fluorescent microscopy and target antigens are detected by specific interaction between antibodies and antigens. The specific location on the antigen with affinity to the antibody is referred to as the epitop. To restore reactivity between antibody and epitop, reversing the effect of formalin fixation is necessary. This process is referred to as antigen retrieval or epitop retrieval, and is usually achieved by enzymatic reactions or by heating the sections [100, 104, 105]. After the introduction of retrieval techniques in the 1990s an increased number of antigens have become detectable [100].

IHC staining can be done as a one-step staining where the antibody with a visible label reacts with the epitop. An indirect staining method is more sensitive and more commonly in use, see Figure 13. The primary antibody is unlabelled and the target epitop is visualized by a secondary labelled antibody. Monoclonal or polyclonal antibodies can be used. Monoclonal antibodies require more resources in production than polyclonal, but are more specific because they recognize only one epitop within an antigen. Polyclonal antibodies can interact with several epitops within the same antigen and can be more robust than monoclonal antibodies when preanalytical conditions are unknown. However, non-specific staining may occur.

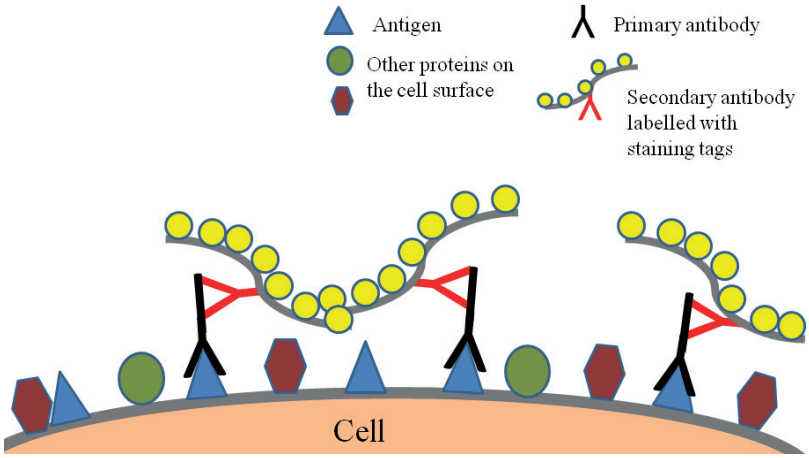


Figure 13. Immunohistochemistry: Indirect staining method. Illustration: MJ Engstrøm

Table 2 summarize steps and variables in the IHC-method. There are some limitations to be aware of. Preservation of the antigen is obviously an important factor for success. Formalin is the most used fixation solution. However, the pathologist or researcher has little control or influence over time from removal to fixation, the fixation time, solution volume relative to the tissue size or transport of the tissue. These issues may be of importance when interpreting immunohistochemical staining and represent potential pitfalls in the present project.

Table 2. Steps and Variables in an Immunohistochemical Test [100].

	Steps	Variables
Preanalytical phase	Sample procurement	Delayed fixation, prolonged ischemia, thickness of sample
	Fixation	Cross-linking vs coagulating fixatives, duration
	Decalcification	Type of decalcification solution and duration
	Tissue processing	Paraffin-embedded vs frozen tissues
	Tissue sectioning	Thickness of tissue section, drying temperature and duration, tissue section aging
Analytical phase	Deparaffination	Dewaxing agent
	Antigen retrieval	Detergents, enzymes, HIER
	Blocking nonspecific reactivities	Endogenous enzymes, hydrophobic binding, pigments
	Primary antibody	Monoclonal vs polyclonal, Ag recognition (native vs linear), specificity, species variability
	Detection system	Avidin-biotin vs polymer-based systems, ultrasensitive methods
	Enzyme-substrate-chromogen	Color detection
	Multiplex IHC	Enzyme-substrate combinations
	Counterstain	Contrast between chromogen and counterstain
Postanalytical phase	Control performance	Animal species compatibility, tissue processing
	Interpretation	Pathologist vs automated evaluation
	Report	Percentage of positive cells, positive vs negative threshold, stand-alone test vs ancillary test
		Diagnostic, prognostic, or theranostic test

HIER, heat-induced antigen retrieval; IHC, immunohistocehmistry.

In addition to the variables in the table, it is important to bear in mind that storage temperature for the sections after cutting may compromise antigen retrieval.

4.8.2 *In situ* hybridization

ISH is a method used to detect a specific DNA or RNA sequence in a section of tissue (*in situ*). The method makes it possible to see the localization of the sequence of interest in the tissue and to assess the amount or number of copies. This technology combines cytogenetics and molecular genetics [106]. Figure 14 shows the principles of the method. Double-stranded DNA, both probe and target sequences, are first denatured at high temperature. The probe, a

labelled complementary DNA or RNA, is added and hybridized to the target DNA or RNA sequence while the sections are annealed. The target sequences can be metaphase chromosomes or, as in ISH on tissue sections, interphase chromosomes and specific loci on chromosomes.

To visualize and locate the sequence of interest, the label is fluorescent (FISH), chromogenic (CISH) or silver (SISH). There is good correlation between these methods [107-109]. Using different coloured labels, multiple sequences may be localized simultaneously (multicolour ISH) [110].

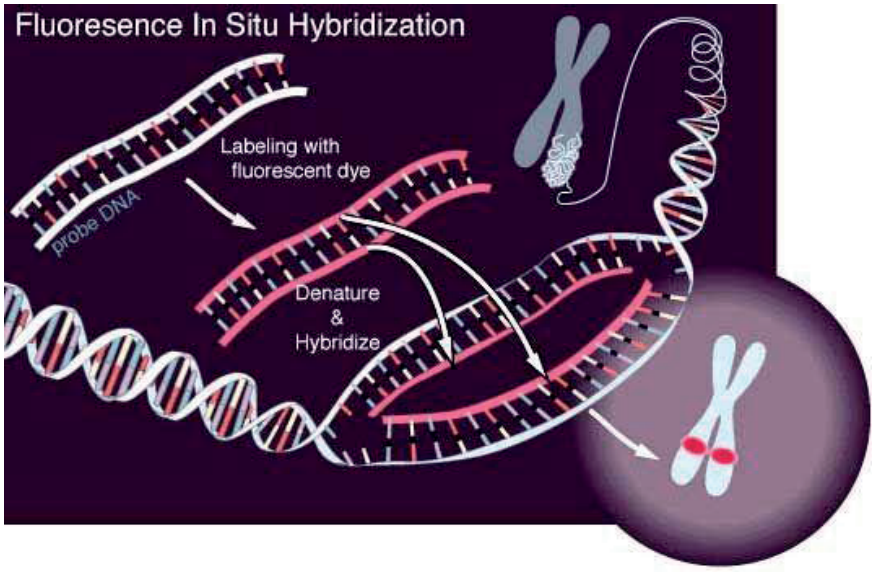


Figure 14 In situ hybridization. (National Human Genome Research Institute (NHGRI) http://www.genome.gov/12514471 by artist Darryl Leja).

In breast cancer this technique is used to detect *HER2* amplification and FISH has been the method of choice for primary assessment of HER2-status or for confirmation of IHC [111, 112]. Other ISH methods have been shown to provide accurate and consistent results and are increasingly used [113, 114]. However, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) recommend validation of bright-field ISH by

comparing to a FISH-assay approved by the American Food and Drug Administration (FDA) before introducing these methods [115].

The possibility of visualising signals for several probes simultaneously or separately by changing filters is an advantage of FISH. Potential drawbacks of FISH are fading of the signals and difficulty in recognizing tumour tissue. CISH utilizing a chromogen rather than a fluorokrom opens for brightfield microscopy and tissue morphology is more easily appreciated. However, in some cases, signals may be difficult to enumerate due to close proximity or overlapping, especially in dual-colour CISH.

4.8.3 Gene expression analysis

To measure the amount of specific DNA or RNA, complementary DNA (cDNA) microarray technology can be used. In these methods, the complementarity of the two DNA strands is utilized. The DNA sequences from the specimen to be examined are denatured, and the cDNA is labelled with fluorescent dye (usually red). The reference cDNA is commonly from cell culture and is labelled with a different fluorescent dye (usually green). The mixture of these cDNA is hybridized to a microarray (Figure 15). Each spot in the microarray contains specific DNA sequences (probes) in known positions. The cDNA from the sample and from the reference are added to the microarray, and after hybridization to the probes, red spots express up-regulated genes in the sample and green spots express up-regulated genes in the reference.

The measurement of gene expression by cDNA is called gene expression analysis or gene expression profiling. The gene expression profile of a tumour represents the molecular signature unique for that tumour, and this signature may correspond to outcome for the patient [116]. Classification of breast cancer tumours based on gene expression profiling of a high number of genes has provided a deeper understanding of breast cancer [117, 118].

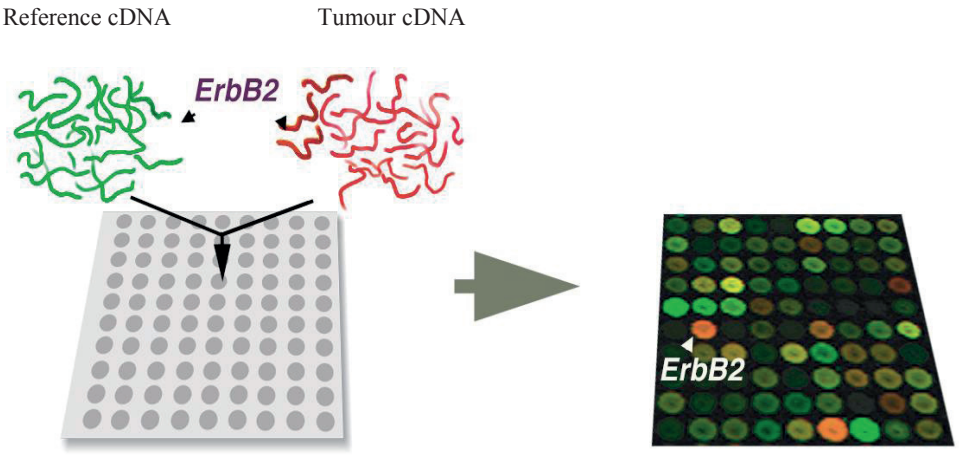


Fig. 15. Schematic description of cDNA microarray analysis of DNA copy-number changes [119].

4.9 Biomarkers in breast cancer

There are a number of prognostic and predictive biomarkers in clinical use in breast cancer. Treatment guidelines are based on hormone receptor-, HER2- and proliferation (Ki67) status in addition to histopathological grading, tumour size and lymph node status. These biomarkers are considered essential in prognostication of breast cancer. In addition, hormone receptor and HER2 are important predictive factors.

Great advances have been made in research on novel biomarkers and new classification systems in addition to improved methods and technology. However, research using well-known markers may provide useful knowledge in new areas contributing to better understanding of disease.

4.9.1 Hormone receptors

Hormone receptors are receptors that can bind to specific hormones and initiate multiple and complex signalling pathways in the cells. In breast cancer they play a major role as prognostic and predictive indicators [120, 121]. The function of these steroid receptors is to control

transcription of genes involved in cellular processes like growth and differentiation. The term hormone receptor in the context of breast cancer mainly refers to oestrogen receptor (ER) and progesterone receptor (PR). In addition, androgen receptor (AR) is frequently expressed in breast cancer [122] but its clinical impact is not clarified. ER and PR are localised to the parenchymal cell nuclei, mainly the luminal epithelial cell [123, 124].

Historically, measurement of ER in breast cancer tumours was done on fresh frozen tissue by an enzyme immune assay. In Norway, this method was used until late in the 1980s. After introduction of immunoperoxidase staining, ER could be assessed in FFPE and in smaller tumours. The Department of Pathology in Trondheim was among the first to introduce the method [125].

Currently, IHC are used to evaluate all breast carcinomas for ER and PR status. Most breast cancer tumours (70 – 80 %) are hormone receptor positive. According to guidelines from the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), tumours are considered ER or PR positive when ≥ 1 % of the tumour cells show positive nuclear staining [126]. At the biannual St. Gallen Breast Cancer Conference, recommendations regarding diagnostic and treatment of breast cancer are given based on consensus meetings. In 2009, it was recommended that a tumour is considered ER positive if there is any detectable ER [127]. There is no strict evidence for a cut-off of ≥ 1 % [128].

4.9.1.1 Oestrogen receptor

In normal breast tissue approximately 7 % of the nuclei show positive staining for ER and the ER positive cells are often singly distributed surrounded by ER negative cells [72, 124]. Signals from oestrogens are mediated through the nuclear receptors ERα and ERβ [129]. The two subtypes of ER regulate growth and development of mammary tissue through a fine balance of oestrogen signalling in cells [130]. Binding of oestrogen to ER leads to changes in gene transcription regulating cell growth, differentiation, apoptosis, and angiogenesis [121, 131].

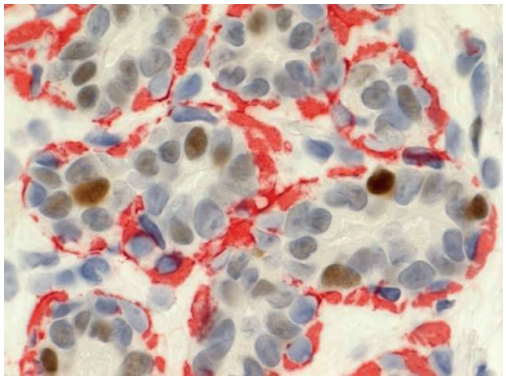


Figure 16. Normal breast tissue stained for ER and smooth muscle actin (SMA). ER positive nuclei surrounded by ER negative nuclei in ductal epithelium. SMA stained myoepithelial cells (1000x). Photo: A M Bofin

ERα is the classic ER receptor assessed by IHC in breast cancer tissue. The discovery of ERβ was published in 1996 [132, 133] but the clinical significance of ERβ has not yet been clarified. Expression of ERβ is shown to improve the prognosis of breast cancer patients receiving adjuvant tamoxifen [134] and in the future assessment of ERβ may be an option.

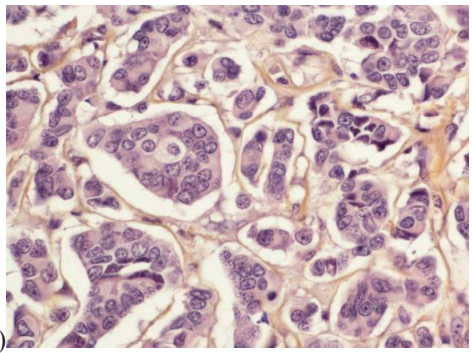
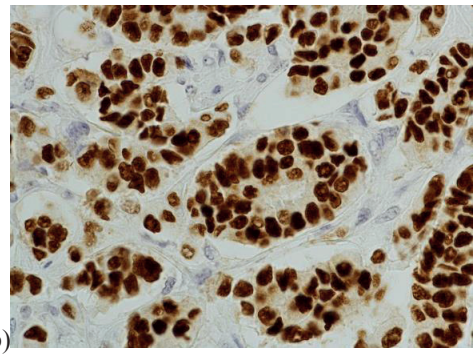
a)  b) 

Figure17. Invasive carcinoma NST. a) HES stained (600x). b) ER positive nuclei (600x). Photo: A M Bofin.

4.9.1.2 Progesterone receptor

ER and PR are usually co-expressed [123]. This co-expression is regarded as an indication of intact and functional ER since ER regulates the expression of PR [82]. There are two isoforms of PR (PRA and PRB) and for the biological effect of progesterone both isoforms are activated [135]. PR provides limited additional prognostic and predictive information. However, ER positive, PR negative breast cancer cases may have a poorer response to endocrine treatment compared to cases in which both receptors are expressed [136].

4.9.2 HER2

Human epidermal growth factor receptor 2 (HER2) is one of four receptors in the epidermal growth factor receptor (EGFR) family and the gene is located on chromosome 17q12-21 [137-139]. The other three receptors in the family are epidermal growth factor receptor (EGFR, HER1, erbB1), HER3 (erbB3), and HER4 (erbB4) [140, 141]. Epidermal growth factor (EGF) binds to EGFR on the cell surface and stimulates cell growth, proliferation and differentiation, and normal expression of EGFR is essential for normal cell growth and cell survival [142].

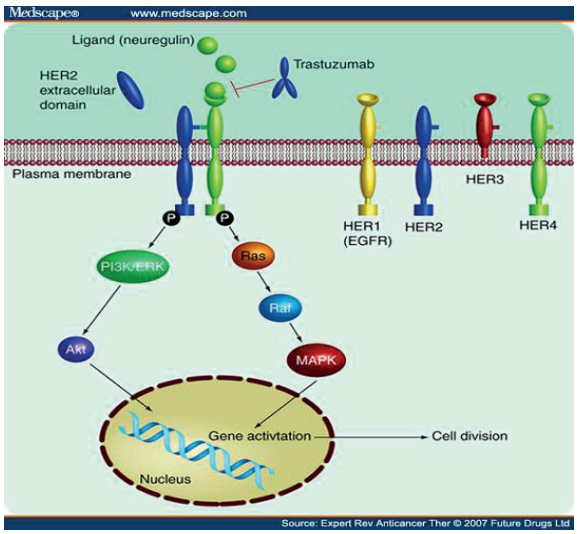


Figure 18. The four members of the EGFR family: EGFR, HER2, HER3 and HER4. HER2 is activated by heterodimerization with one of the other members of the family. The activation leads to initiation of cascade resulting in cell proliferation and survival [141].

HER2 overexpression in breast cancer is associated with poor prognosis. However, trastuzumab, a humanized monoclonal antibody targeting HER2 receptor, is an effective treatment for HER2 positive breast cancer in combination with chemotherapy [142]. Overexpression of the HER2 protein has been shown to be strongly correlated to *HER2* gene copy number [138, 143, 144] and HER2 status can be assessed by either IHC or ISH. There is an ongoing discussion regarding the optimal testing regime [113].

IHC is commonly used as the initial test for HER2 [145]. There are many commercially available antibodies and good agreement is shown for the most commonly used antibodies [145]. The membrane staining is semiquantitatively assessed according to strict criteria. Recommendations regarding assessment of IHC given by National Health Services Breast Screening Programme (NHSBSP) in United Kingdom are shown in Table 3 [146]. Tumours are considered HER2+ when IHC membrane staining score is 3+, negative when 0 or 1+ and equivocal when 2+.

Table 3. Recommended IHC scoring method from National Health Service Breast Screening Programme [146].

Score to report	**HER2 protein overexpression assessment**	**Staining pattern**
0	Negative	No staining is observed, or membrane staining in less than 10% of tumour cells.
1+	Negative	A faint/barely perceptible membrane staining is detected in more than 10% of tumour cells. The cells are only stained in part of the membrane.
2+	Borderline	A weak to moderate complete membrane staining is observed in more than 10% of tumour cells.
3+	Positive	A strong complete membrane staining is observed in more than 10% of the tumour cells.

In cases with equivocal IHC, *HER2* testing by ISH is recommended [113]. Interpretation of *HER2* after ISH is done by estimating gene to chromosome ratio after counting signals for both in a minimum of 20 nonoverlapping tumour cell nuclei [144]. A gene to chromosome ratio ≥ 2.0 is regarded as *HER2* amplification in most guidelines [113, 144]. ASCO/CAP

recommend recounting when ratio is between 1.8 and 2.2 [147]. The most recent revision of guidelines from ASCO/CAP (2013) differ slightly from those of NHSBSP regarding IHC and ISH and are shown in Table 4.

Table 4. Recommendations from ASCO/CAP regarding IHC and ISH for HER2. The table is slightly modified [115]

2013 HER2 Test Guidelines and Recommendations from ASCO/CAP
Must report **HER2 test result as positive for HER2 if:** ● IHC 3+ based on circumferential membrane staining that is complete, intense ● ISH positive based on: Single-probe average *HER2* copy number ≥ 6.0 signals/cell Dual-probe *HER2*/CEP17 ratio ≥ 2.0 with an average *HER2* copy number ≥ 4.0 signals per cell Dual-probe *HER2*/CEP17 ratio ≥ 2.0 with an average *HER2* copy number < 4.0 signals/cell Dual-probe *HER2*/CEP17 ratio < 2.0 with an average *HER2* copy number ≥ 6.0 signals/cell
Must report **HER2 test result as equivocal** and order reflex test (same specimen using the alternative test) or new test (new specimen, if available, using same or alternative test) if: ● IHC 2+ based on circumferential membrane staining that is incomplete and/or weak/moderate and within > 10% of the invasive tumor cells *or* complete and circumferential membrane staining that is intense and within ≤ 10% of the invasive tumor cell ● ISH equivocal based on: Single-probe ISH average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell Dual-probe *HER2*/CEP17 ratio < 2.0 with an average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell
Must report **HER2 test result as negative** if a single test (or both tests) performed show: ● IHC 1+ as defined by incomplete membrane staining that is faint/barely perceptible and within ≤ 10% of the invasive tumor cells ● IHC 0 as defined by no staining observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of the invasive tumor cells ● ISH negative based on: Single-probe average *HER2* copy number < 4.0 signals/cell Dual-probe *HER2*/CEP17 ratio < 2.0 with an average *HER2* copy number < 4.0 signals/cell
Must report **HER2 test result as indeterminate** if technical issues prevent one or both tests (IHC and ISH) from being reported as positive, negative, or equivocal.

Figure 19 show examples of HER2 CISH (a) and FISH (b).

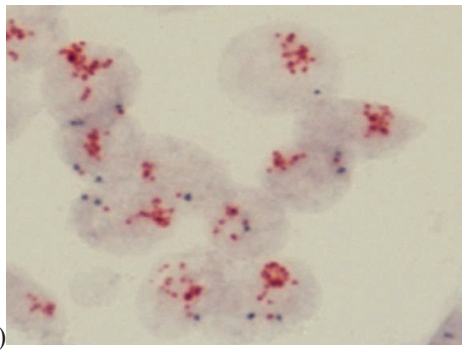
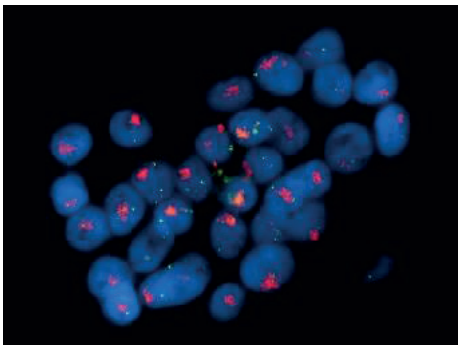
a)  b) 

Figure 19. High grade amplification of *HER2*. a) CISH in biopsy (1000x). Signals for HER2 are red, and signals for chromosome 17 centromere are blue. b) FISH in cytology (400x). Signals for HER2 are red, and signals for chromosome 17 centromere are green. Photo: A M Bofin.

4.9.3 Ki67

Ki67 is a nuclear protein present in the S, G2 and M phases of the cell cycle. In addition, Ki67 may be found in the G1 phase after mitosis [148]. Ki67 is regarded as a proliferation marker [148, 149]. The function of this protein has not been clarified, but a number of publications have shown prognostic value both in early stage [150, 151] and advanced [152] breast cancer. Ki67 is assessed by IHC. MIB1 is the most commonly used antibody [153].

Despite widespread use as a prognostic marker in a variety of different cancers types, estimation of Ki67 remains controversial and as yet, unresolved. To reduce intra- and interlaboratory variability there are given guidelines for analysing and reporting of Ki67 [154]. Still, direct comparison between laboratories and studies is difficult due to inconsistency in counting methods and results in assessment of Ki67 [153]. Area for counting and number of counted nuclei varies in different publications [154]. In the present project, Ki67 positive staining nuclei were counted in “hot spot” areas or best countable areas of the TMA cores. A minimum of 500 tumour cell nuclei were assessed, and the results were given as percentage positive cell nuclei.

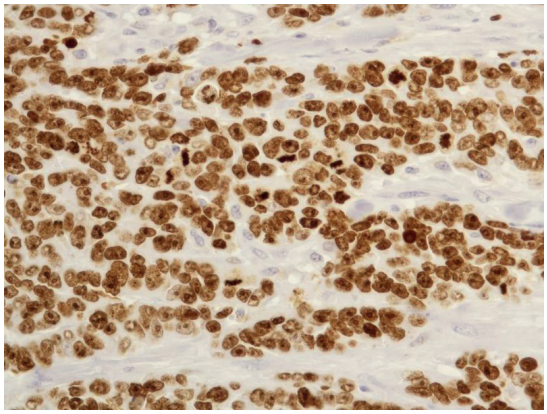


Figure 20. Invasive breast carcinoma with positive nuclear staining for Ki67 (MIB1) (600x). Photo: A M Bofin

4.9.4 Cytokeratin 5 (CK5)

Cytokeratins (CK) are cytoplasmic proteins important for the cytoskeleton of most eukaryote cells. CKs of different molecular weights can be used to distinguish between cell type and differentiation status [155]. In normal breast tissue, expression of different CKs can be used to distinguish between cell types [123]. Cytokeratin 5 (CK5) is expressed in myoepithelial and basal cells, and is one of the markers used to identify the basal phenotype/basal-like breast cancer. CK5 is assessed by IHC and is readily available although it is not in clinical use. A combination of CK5 and CK6 (CK5/6) is more commonly used but CK5 has a higher sensitivity for detection of the basal phenotype [156].

There are no accepted guidelines for the assessment of CK5. Some authors use a staining index (SI) where staining intensity and the proportion of cells with cytoplasmic staining are assessed separately and give a score. The scores are multiplied and the result is expressed as SI [157, 158]. Staining intensity is graded as 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong). The proportion of positive staining cells is scored as 1 (<10 %), 2 (10–50 %) and 3 (>50 %). There is no consensus on cut off for CK5. In some papers, SI 1-9 is considered positive [157-159]. In this project, the tumours were considered to be negative for CK5 when SI was 0–1 and positive when the SI was 2–9 [160].

The H-score is another method which may be used to assess IHC. In this method, the extent of immunoreactivity is evaluated by the following formula giving a score between 0 and 300: 3 x

proportion (in percent) of strongly stained cells + 2 x proportion (in percent) of moderately stained cells + proportion (in percent) of weakly stained cells [156, 161].

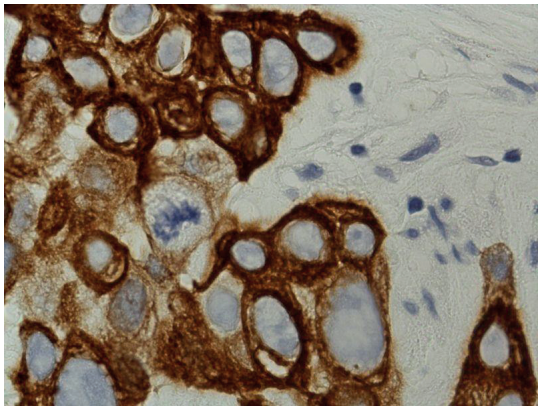


Figure 21. Invasive breast carcinoma stained for CK5. Positive cytoplasmic staining in tumour cells (1000x). Photo: A M Bofin

4.9.5 Epithelial growth factor receptor 1

Epithelial growth factor receptor 1(EGFR) is a transmembrane glycoprotein belonging to the HER-family of tyrosin kinase receptors [162], and is encoded by the *HER1*-gene. Activation of EGFR plays a central role in several important intracellular signalling pathways regulating cell proliferation, growth and survival. A simplified overview of the signalling pathway including inhibitory signals like PTEN and MKP1 is shown in Figure 22. Overexpression of EGFR in epithelial tumours may lead to more aggressive growth and invasion [162] and is associated with a poorer prognosis [163].

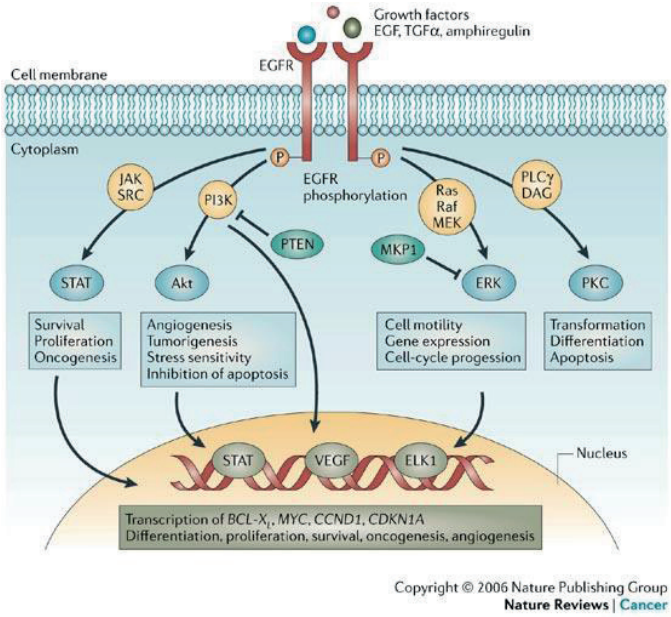


Figure 22. EGFR signalling pathway [164]

EGFR is not assessed routinely in breast cancer, but is of interest in research and has been shown to identify the tumours of basal phenotype among the triple negative tumours [165]. In Paper I, EGFR was assessed by IHC membrane staining, and scoring was done according to Table 5. SI was calculated with staining intensity multiplied by the proportion positive cells and a SI ≥ 2 was regarded as EGFR positive. Figure 23 shows a EGFR positive case.

Table 5. Assessment of EGFR. Staining index (SI) is score for staining intensity multiplied by score for proportion of positive cells.

Staining intensity	0 = no staining	1 = faint, incomplete membrane positivity	2 = moderate intensity; circumferential staining	3 = strong intensity; circumferential staining
Proportion positive cells	1a = < 1 %	1 = < 10 %	2 = 10 - 50 %	3 ≥ 50 %

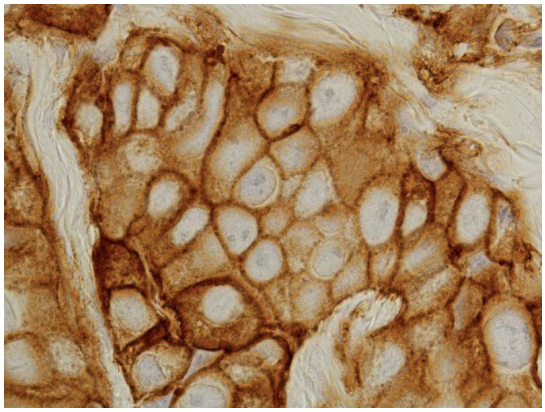


Figure 23. Invasive breast carcinoma stained for EGFR. Positive cytoplasmic staining in tumour cells (1000x). Photo: A M Bofin.

4.9.6 E-cadherin

E-cadherin (E-cad) is a protein in epithelial cells involved in cell-to-cell adhesion and suppression of invasion and metastasis [166]. During embryogenesis, E-cad play a role in the formation of tissue [167]. This transmembrane protein extends from the extracellular space through the cell membrane and into the cytoplasm [168]. The function of E-cad is dependent on a number of other proteins in the E-cadherin complex. The intracellular domain binds to and interacts with p120 catenin and b-catenin which in turn bind and interact with other intracellular proteins.

In current diagnostic breast pathology, IHC for E-cad may be used to distinguish lobular neoplasia, lobular carcinoma *in situ* and invasive lobular carcinoma from ductal carcinoma *in situ* and invasive carcinoma of no special type in difficult cases [168]. E-cad positive cases show a continuous membrane staining with moderate to strong intensity while focal or pearly expression is regarded E-cad negative [168]. Most lobular tumours are E-cad negative.

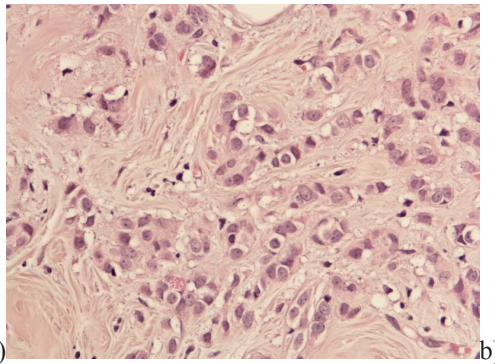
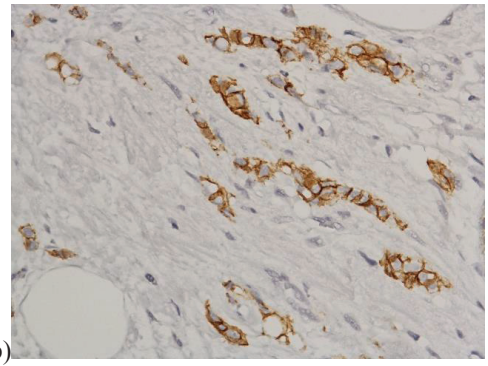
a)  b) 

Figure 24. Invasive lobular carcinoma at magnification 400x. a) HES staining. b) IHC showing E-cad positive status in the same tumour. A M Bofin

4.9.7 *TOP2A*

TOP2A is a gene close to *HER2* on chromosome 17. Topoisomerase II α is the protein product of *TOP2A* and the molecular target of anthracycline treatment. The function of the topoisomerase enzymes is the regulation of cellular processes like replication and transcription [169, 170]. A number of publications have shown frequent co-amplification of *TOP2A* and *HER2* [171, 172]. However, amplification of *TOP2A* has also been shown to occurs independent of *HER2* amplification [169]. The prognostic and predictive value of gene copy number changes in *TOP2A* has not been clarified and *TOP2A* is not included in the clinical biomarker repertoire.

Detection of *TOP2A* gene copy number changes can be done by *in situ* hybridization. Gene to chromosome ratio is estimated based on signal counting in a minimum of 20 non-overlapping tumour cell nuclei. Cases with ratio ≥ 2 are usually considered *TOP2A* amplified, and *TOP2A* may be deleted when the gene to chromosome ratio ≤ 0.8 [173, 174]. Definition of monosomy is more controversial [137, 175]. In Paper II, only one signal for both gene and chromosome in more than 75 % of the recorded nuclei was defined as monosomy for *TOP2A*. Deletion and monosomy were grouped together and defined as *TOP2A* gene copy number loss.

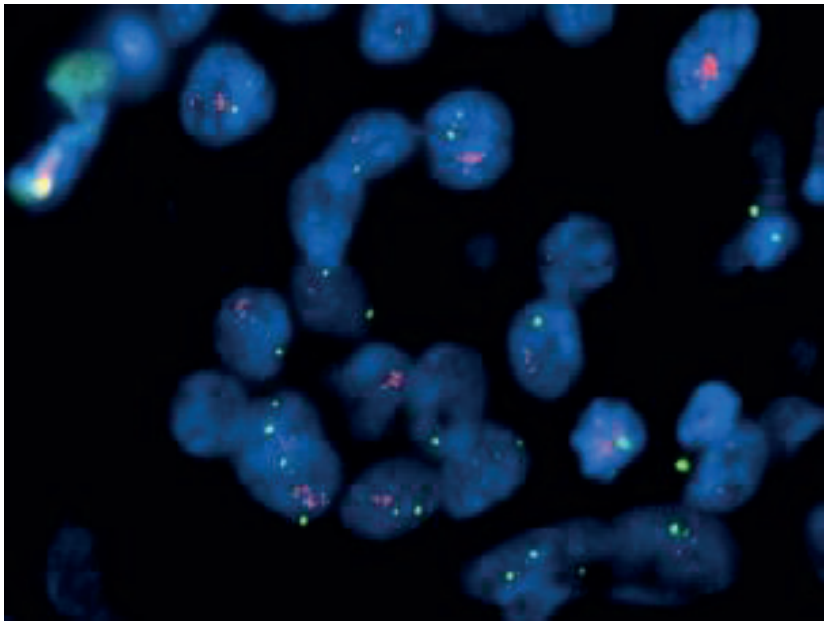


Figure 25. FISH with probes for chromosome 17 centromere and *TOP2A* showing increased copy number of *TOP2A* in invasive breast cancer (600x). Photo: A M Bofin

4.10 Tissue microarray

Tissue microarray (TMA) is a high through-put method that enables rapid analyses of a high number of specimens under the same conditions. The method was first described by Battifora in 1986 [176] and can be used to study genetic or molecular markers. The technique has since been modified and improved and a description of the procedure in current use was published in 1998 [177]. The use of the method has increased in recent years [178].

TMA is a collection of biopsies from FFPE tissue blocks and may comprise tumour tissue or normal tissue. TMA blocks are constructed by extracting tissue cores from the tissue of interest and inserting them into a recipient block. The most common core sizes are 0.6 – 1.0 mm, and each block may contain as many as 1000 cores [179]. From the TMA blocks, sections are cut and stained for routine staining, IHC and ISH. Construction of TMA is illustrated in Figure 26.

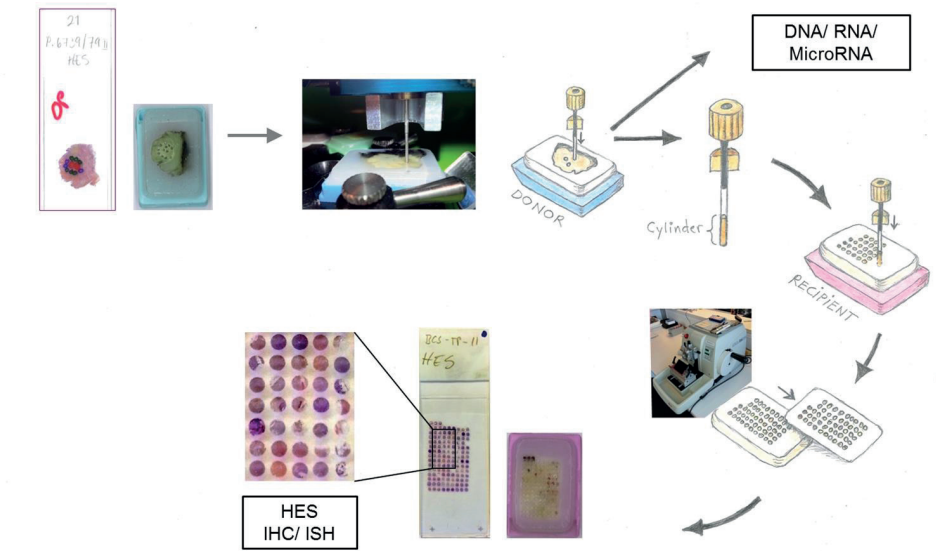


Figure 26. TMA construction in our laboratory. The area for TMA is selected by a pathologist on a full-face HES section. 1 mm cores are punched from the donor block and transferred to the recipient block. Sections are cut from the recipient blocks and staining is performed. Figure by Linda A. Dyrnes.

There are a number of benefits of this method. The opportunity to analyse multiple specimens at the same time is the main advantage. This applies primarily to research, but clinical laboratories with high numbers of specimens may benefit from this method. IHC and ISH techniques can be done on a high number of cases with small amounts of tissue and reagents facilitating economic use of both. Tissue utilization of large series can be maximized under uniform reaction conditions, and positive and negative controls can be included in the TMA.

There are some limitations to be aware of. TMA construction is labour intensive. Equipment is expensive and experienced users are required. Carcinomas are often heterogeneous and the cores in the TMA may not represent the whole tumour. This challenges the selection of areas in the donor tumour and the results of the analyses may be influenced. The users must be aware of the balance between use and conservation. However, donor paraffin blocks can still be used for full face sectioning after TMA-sampling (Figure 27). Loss of cores in the recipient blocks occurs. To ensure tumour representativity and avoid loss of cases, more than one core can be selected. In this project, three cores were selected from each case and spot loss was < 10 % and case loss < 4 % [180] .

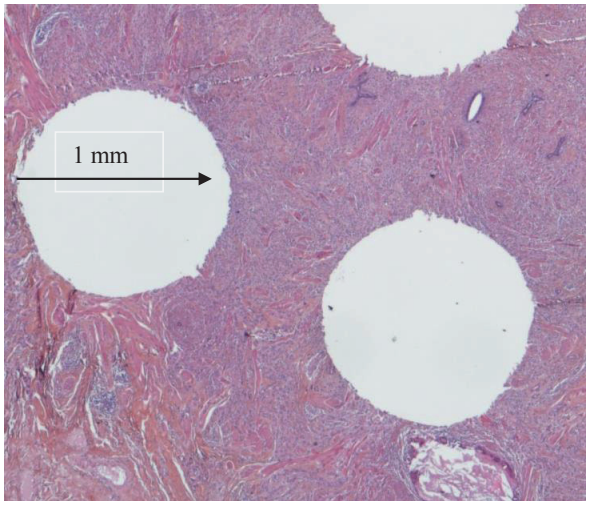


Figure 27. Full-face section from a donor block showing defects after core extraction. There is no damage to surrounding tissue (HES,20x). Photo: A M Bofin

4.11 Preanalytical conditions

Guidelines have been established to ensure standardized handling of tissue and cellular material prior to histopathological examination. The intention is standardization of all steps from surgical excision or biopsy to interpretation in order to avoid sources of errors. These guidelines encompass preanalytical, analytical and postanalytical conditions to minimize variability and tissue damage [126, 181]. However, in research on archival tissue information regarding preanalytical conditions is often lacking and all results must be evaluated in light of this limitation.

Histopathological examination is usually performed on FFPE tissue. The purpose of fixation is to limit or stop enzymatic degradation and preserve the tissue structure. The ability of formalin to fixate tissue has been known since the 1890s [182]. Interpretation of HES-sections is dependent on sufficient fixation. In IHC, inadequate fixation may result in false negative reactions and thereby false negative biomarker status [183].

Sample size, tissue type and the amount of fixative are important factors for adequate fixation. The time from removal to fixation should not exceed 1 hour, and the optimal tissue fixation time is 6 – 72 hours, depending on the size of the sample [126]. Too short fixation time is the most important limitation of the quality of the tissue. Some recommend a minimum of 24 hours [184]. Recommendations regarding formalin volume varies, but a formalin-volume to

tissue-volume ratio between 10:1 and 20:1 is the most commonly recommended volume [185]. Detailed procedures must be in place and their implementation is vital for optimal tissue handling.

There are variations in routines regarding the handling of biopsies and surgical specimens between institutions, within institutions and over time.

5. The aims of the study

The main aims of this study were to reclassify breast cancer tumours from a historic cohort of women into molecular subtypes based on surrogate markers for gene expression analysis, and to investigate whether this classification gives more precise prognostic information compared to histopathological grade. Further objectives were to explore the prognostic value of *TOP2A* in breast cancer and to study prognosis in invasive lobular carcinoma.

More specifically, the aims of each paper were as follows:

Paper I: To compare molecular subtyping by IHC and ISH with conventional histopathological grading in breast cancer in order to determine whether molecular subtyping provides more information regarding outcome.

Paper II: To study the frequency of *TOP2A* copy number change in breast cancer and to explore the prognostic value of these changes.

Paper III: To compare the prognosis for ILC and IDC and to evaluate the prognostic value of histopathological grading, molecular subtypes and E-cad in ILC.

6. Material and methods

6.1 Study population

6.1.1 Description of the cohort

The women in this cohort were invited to participate in a breast cancer survey organized by the predecessor of the Norwegian Cancer Society in the period 1956-1959 [186, 187]. The intention of the survey was to evaluate clinical breast examination as a screening method for early breast cancer detection. All women aged 20-69 years by January 1, 1956 (born in the period 1886-1928) living in three Norwegian Counties (Nord-Trøndelag, Aust-Agder and Vestfold) were invited. The long follow-up of this cohort has facilitated a number of studies of risk of breast cancer [9, 188-190], gynaecological cancer [189, 191] and thyroid cancer [192].

In addition to clinical examination conducted by a physician, the women were interviewed by a trained nurse or physician. The interview was carried out according to a structured questionnaire mainly focused on reproductive history. The youngest women were excluded due to incomplete reproductive history and difficulties in identification because of changes in family name after marriage. A total of 25 897 women from Nord-Trøndelag County were invited to participate in the survey.

In the period from January 1, 1961 to end of 2008, 1393 women in the Nord Trøndelag cohort were diagnosed with breast cancer. Of these, tissue was not available for 448 cases. For 276 patients, the operation specimens were sent for histopathological examination in other laboratories and were not available. In some cases tissue blocks were missing for unknown reasons. In the archives of the Department of Pathology and Medical Genetics, St. Olav's Hospital, Trondheim University Hospital, Norway, FFPE tissue was available for 945 cases. Some cases were excluded because of insufficient quality of the FFPE tissue, and 909 cases were included in the study. Of these, only biopsy was available for 79 cases. Figure 28 shows the study population.

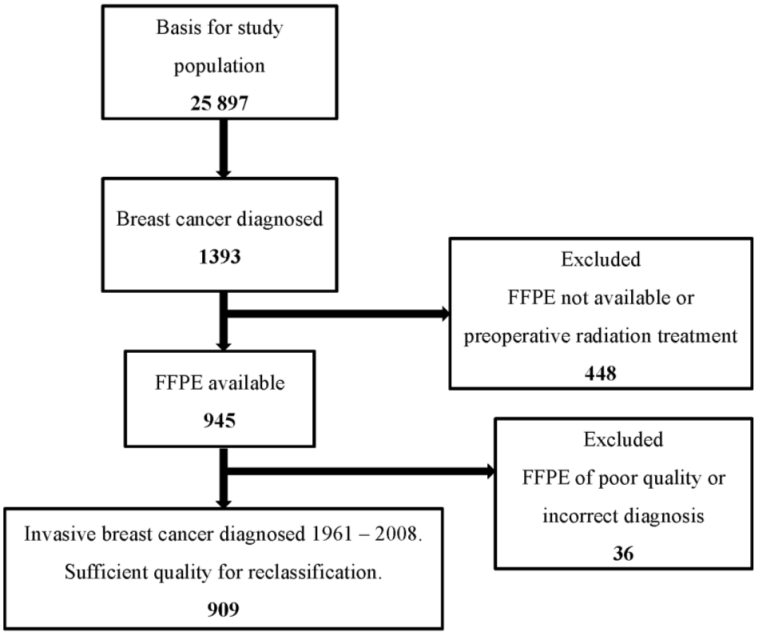


Figure 28. Flow chart showing number of cases in the study and reasons for exclusion (Paper I) [160].

6.1.2 Follow-up

All women diagnosed with breast cancer were followed from the date of diagnosis to the date of death from breast cancer, death from any other cause or to the end of follow-up (December 31, 2010), whichever came first.

6.1.3 Special characteristics of this historic cohort

Apart from the clinical examination offered during the survey, no organized screening for breast cancer was available to the women in this cohort in the follow-up period. In Norway, mammography screening was conducted as a pilot project in four counties from 1995-1996. The public screening program is governmentally funded and was gradually implemented in all counties from 1998 to 2004 [193]. In Nord-Trøndelag, all women in between 50-69 years of age have been invited to mammography every other year since 2001. The youngest women in

the cohort in the present study were 73 years of age in 2001. Hence, the breast cancer cases in this cohort are mainly clinical detected.

Menopausal hormonal therapy (MHT) has been available since the late 1950s. From 1990 to about 2000, MHT was in common use. There was a rapid increase in the use of oestrogen replacement therapy during and after menopause in Norway from 10 % in 1992 [194] to 30 % in 2002 [195]. Most of the women in this study population have probably not used MHT. The oldest women were 75 years of age in 1961 and the youngest were 50 years of age in 1978 when MHT were introduced in Norway [196]. Some of the youngest may have had hormone replacement therapy. However, in the 1980s there was a very modest use of MHT in Nord-Trøndelag [196]. Oral contraceptives were introduced in Norway in 1967 [197], however, their use in the study cohort must have been negligible due to age and time period.

There has been a great improvement in breast cancer treatment over the past 10 to 20 years. However, for a large proportion of the women in this project, current modern treatment for breast cancer was not an option due to the time period in which they were diagnosed. Patients diagnosed with breast cancer in the era of modern treatment, did not qualify for additional treatment regimes due to their age. Table 6 and 7 show an overview of treatment options. Most of the patients had surgery with mastectomy with or without axillary clearance or sentinel node diagnostics. Reliable information regarding hormone therapy is not available and the numbers in the table are based on the Norwegian guidelines from the relevant time period.

Table 6. Breast cancer therapy given to the patients in the cohort

	Of all cases (909)
Mastectomy	731 (80.4 %)
Breast conserving therapy	99 (10.9 %)
Only biopsy, no surgical treatment	79 (8.7 %)
Axillary surgery (clearance or sentinel node)	644 (70.8 %)
Hormone therapy*	192 (25.6 %)**
Trastuzumab	0
Chemotherapy	Unknown
Radiation	Unknown

* Estimated according to guidelines at diagnosis. ** % of the hormone receptor positive cases.

Table 7. Breast cancer therapy according to histopathological type. Table from paper III [198].

	Invasive ductal carcinoma n=611 (%)	**Invasive lobular carcinoma n=116 (%)**	**Total n=727 (%)**
Mastectomy	524 (85.8)	94 (81.0)	618 (85.0)
Breast conserving therapy	61 (10.0)	12 (10.4)	73 (10.0)
Only biopsy, no surgical treatment	26 (4.3)	10 (8.6)	36 (5.0)
Axillary surgery (clearance or sentinel node)	461 (75.5)	81 (69.9)	542 (74.6)
Hormone therapy*	134 (26.2**)	31 (30.4**)	165 (26.9**)
Trastuzumab	0	0	0
Chemotherapy	Unknown	Unknown	Unknown
Radiation	Unknown	Unknown	Unknown

* Estimated according to guidelines at diagnosis. ** % of the hormone receptor positive cases.

This population provides us with a unique opportunity to study a cohort of breast cancer patients almost exclusively treated with surgery alone.

6.2 Archives of the department of pathology

FFPE tissue allows for long-term storage in diagnostic or research biobanks. In Norway, FFPE tissue blocks archives have been maintained at the various departments of pathology since the 1930s [199]. FFPE tissue should be stored in the dark at room temperature, and thus can be preserved for an unlimited period of time [200]. The main challenges for further storing are of a logistical and practical character. These archives are exceptional and constitute a tremendous potential for medical research, especially when combined with public registries and population based surveys. Legal and ethical regulation of the use of these biobanks is practised in Norway [201].

6.3 TMA construction and slide scanning

TMA blocks were constructed using a Tissue Arrayer Mini-Core® 3 with TMA Designer2 software (Alphelys). Areas of interest in the HES-stained full-face sections were marked by an experienced pathologist. Three 1mm in diameter tissue cores were extracted from

peripheral regions of the tumour in the donor blocks and inserted into TMA recipient blocks. From the TMA blocks, 4 μm sections were cut and stained. IHC was carried out with antibodies for ER, PR, HER2 (CB11), CK5, Ki67, E-cad and EGFR in addition to HES staining. All HES- and IHC-stained slides were digitalized using the tissue scanner Ariol ™ SL-50 3.3 Scan system and analysis station (Genetix) at 5x and 20x magnification. IHC-stained sections were evaluated and scored by two observers independently using the Ariol review station.

6.4 Assay methods and classification

In all three studies, the REMARK recommendations for reporting in tumour marker studies were followed [202]. The assay methods are described in detail in Paper I and supplemented in Paper II and III. All immunostaining was done in a DakoCytomation Autostainer Plus (Dako). Table 8 shows the sources and dilutions of the primary antibodies used for visualization. In Paper I, CISH was used for detection of chromosome 17 centromere/ *HER2* copy number and in Paper II, FISH was used for detection of chromosome 17 centromere and *TOP2A*.

Table 8. Sources and dilutions of primary antibodies used in the studies.

Antibody	Clone	Manufacturer	Concentration of antibody	Dilution
ER	SP1	Cell marque	33 mg/ml	1:100
PR	16	Novocastra	360 mg/l	1:400
HER2	CB11	Novocastra	3.9 g/l	1:640
Ki67	MIB1	Dako	35 mg/l	1:100
CK5	XM26	Novocastra	50 mg/l	1:100
EGFR	2-18C9	Dako	Ready to use	No dilution
E-cad	NCH-38	Dako	55.2 mg/L	1:100

Review and classification of all cases into histopathological types and grades was done on full-face sections by two pathologists independently. Areas for TMA were selected and marked on the glass slide. Constructions of TMA, cutting and staining were performed by biomedical engineers. Assessment of all IHC and ISH markers were done by two researchers independently. In case of disagreement the sections were re-examined and consensus reached. Cut-off levels were set in accordance with clinical guidelines where possible after review of the literature.

Classification in molecular subtypes was done according to the algorithm in Figure 29.

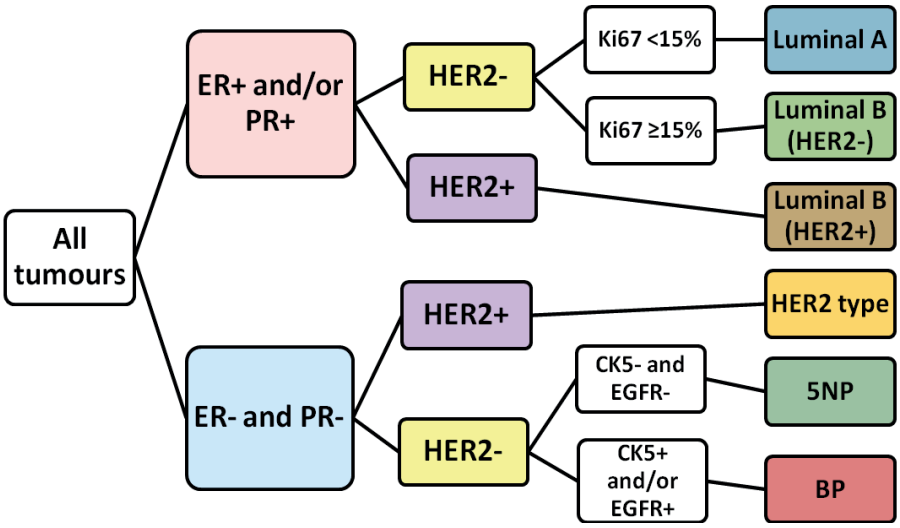


Figure 29. Classification system for molecular subtyping based on surrogate markers, based on Paper I [160].

6.5 Norwegian Public Registries and ethical committee

In Norway, there are a number of public registries that enable population based research. Reporting to these registries is mandatory by law and close to completeness is therefore ensured.

6.5.1 The Central Population Registry of Norway

The Central Population Registry of Norway was established in 1964 based on a census from 1960 and includes key information on all individuals who are or have been resident in Norway. Information on births, deaths, address, migrations, marital status and citizenship are recorded for all Norwegian citizens and all foreigners living in Norway for more than six months. Since 1964, all Norwegian citizens have an individual 11-digit birth number composed of the date of birth, a three-digit individual number and two check digits. This registry gives important population statistics. Data regarding birth, death and emigration were obtained from The Central Population Registry for this project [203].

6.5.2 The Cancer Registry of Norway

The Cancer Registry of Norway was established in 1951 after a proposal from WHO [204]. The purpose of the registry was to study the incidence of cancer. From its foundation in 1951 until 1979, the Cancer Registry of Norway was financed by The Norwegian Cancer Society which had income mainly from donations. In 1979, the Norwegian government took over financial responsibility [205]. The aim has gradually been expanded to include broader research and counselling on health care regarding cancer and prevention of cancer. Reporting has been mandatory by law since 1952 [206]. The Registry is one of the most complete registries in Europe [207]. Data from the study cohort were linked with the Cancer Registry of Norway and confirmation of breast cancer diagnosis and date of diagnosis was made available for the project [203].

6.5.3 The Cause of Death Registry of Norway

When Norwegians die in Norway or abroad, information is registered in the Cause of Death Registry of Norway. This registry is complete or close to completes and provides the opportunity to follow mortality trends and life expectancy. Data from the registry may be given to researchers on application including approval from the Regional Committee for Medical and Health Sciences Research Ethics.

6.5.4 The Regional Committee for Medical and Health Sciences Research Ethics

To conduct medical research in Norway, approval from The Regional Committee for Medical and Health Sciences Research Ethics for each project is mandatory [208, 209]. There are seven regional committees processing applications and when necessary The National Committee for Medical and Health Sciences Research Ethics gives advice and considers appeals. The basis for the work in these committees is founded in international conventions like the Declaration of Helsinki [210].

6.6 Statistical analyses

In all the studies, survival analyses were carried out using Kaplan-Meier (KM) methods and Cox proportional hazards models. All women in the studypopulation were followed from the date of breast cancer diagnosis to the date of death from breast cancer, death from any other cause or to the end December 2010, whichever came first. There were no cases with missing information due to emigration or other causes. Stata version 12.1 IC for Windows (Stata Corp.) were used for all the statistical analyses.

6.6.1 Kaplan-Meier

To compare Breast cancer-specific survival (BCSS) between groups, the KM-method was used. This method is widely used to estimate survival [211]. The KM survival curve reflects the probability for survival for a given length of time where time is considered in many small intervals. The survival is estimated by multiplying the survival probabilities of each time interval [212]. The Log-rank test was used to test the statistical significance of the differences between survival curves.

In KM estimates, it is assumed that life expectancy is the same for patients included early and late in the study [211]. This is probably not the case in this study where the women included were diagnosed with breast cancer from January 1961 to end of December 2008. In Norway, there was a notable increase in life expectancy in the first half of the 1990s. For women in Norway life expectancy increased from 73.2 years in 1950 to 83.2 years in 2010 [213]. A great proportion of this difference is due to changes in infant and child mortality and did not affect the women included in this project.

6.6.2 Cox Proportional Hazards Model

KM and log-rank-test are used to compare two groups for significant difference. However, it is not possible to include other independent factors in the analyses. In order to achieve this, Cox proportional hazards model was used to compare risk of death from breast cancer unadjusted and adjusted for age, stage and time period of diagnosis (10-year intervals). Hazard ratios (HR) were calculated with 95 % confidence intervals (CI). In each calculation, one group was defined as the reference group and was compared to groups with defined characteristics.

This method of studying the relationship between a known risk factor and the patient's time of death is commonly applied. A possible weakness in this model is the assumption that the HR for each explanatory variable is constant over time [214]. Thus, effects of time variation cannot be revealed.

6.7 Ethical approval

The studies were approved by the Regional Committee for Medical and Health Sciences Research Ethics (REK, Midt-Norge, ref. nr: 836/2009) and dispensation from the requirement of patient consent was granted.

7. Summary of results

7.1 Paper I: Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients

Paper I is the main work in this thesis and forms the basis for the other studies. A total of 909 cases were included and all were reviewed and classified according to histopathological grade and type. TMAs were constructed and using IHC and ISH as surrogates for genomic analyses, all cases were reclassified into the following molecular subtypes: Luminal A; Luminal B (HER2-); Luminal B (HER2+); HER2 subtype; Basal Phenotype (BP) and Five Negative Phenotype (5NP). The algorithm for molecular subtyping is shown in Figure 29.

The main findings in the study were significant differences in survival according to molecular subtypes with best survival for Luminal A and worst for HER2 and 5NP. However, the most

interesting result was that the significant difference in breast cancer specific survival was only found for grade 2 tumours and only the first five years after breast cancer diagnosis. For women surviving the first 5 years after diagnosis, there appears to be no difference in survival according to molecular subtype or histopathological grade.

7.2 Paper II: *TOP2A* gene copy number change in breast cancer

Changes in *TOP2A* copy number as a prognostic marker has been the subject of a number of studies, but its prognostic value has not been clarified. In the present study, the 670 cases suitable for TMA and ISH assessment of *TOP2A* and *HER2* copy number status were included. *TOP2A* status was assessed using FISH and *HER2* was assessed using CISH. Both *TOP2A* and *HER2* were classified as amplified when gene to chromosome ratios were ≥ 2. *TOP2A* deletion (gene to chromosome ratio≤ 0.8) or monosomy (only one signal for both gene and chromosome in more than 75% of nuclei) were defined as *TOP2A* gene loss.

In this study, *TOP2A* copy number changes were found in 66 cases (41 with amplification and 25 with gene loss) and *HER2* amplification in 110. Most of the *TOP2A* amplified cases were coamplified with *HER2* and the association between *TOP2A* copy number change and hormone receptor positive status was strong.

The risk of death from breast cancer was significantly higher for *HER2* amplified cases. This difference was present during the first five years after breast cancer diagnosis as shown in Paper I, and was not associated with *TOP2A* status. The main conclusion of the study was that *TOP2A* is of limited value as a prognostic marker in breast cancer.

7.3 Paper III: Invasive lobular breast cancer: The prognostic impact of histopathological grade, E-cadherin and molecular subtypes

The prognosis of ILC compared to IDC is still unclear. In addition, histopathological grading of ILC is controversial. E-cadherin is often used to confirm lobular type in difficult cases, but its potential as a prognostic marker has not been clarified.

In this paper the prognosis of ILC was compared with that of IDC. All 727 cases of ILC or IDC suitable for TMA were included. Of these, 611 were ductal and 116 were lobular.

The proportion of grade 2 tumours among the lobular tumours was much higher than for ductal tumours, respectively 85.3 % in ILC and 51.9 % in IDC. Only 6 % of ILCs were HER2+, whereas 16.9 % of IDCs were HER2+. As expected, the majority of ILCs had negative E-cad status and the majority of IDCs were E-cad positive.

Based on assessment of grade, ER and HER2, ILC would appear to have good prognosis. However, one of the main findings in this study was a poorer prognosis for grade 2 ILC compared to grade 2 IDC. BCSS of ILC grade 2 was comparable to that of IDC grade 3. E-cadherin negative ILC had a poorer prognosis compared to E-cadherin positive ILC and to IDC regardless of E-cadherin status. For IDC, E-cad had no prognostic value. E-cadherin in ILC may be more useful than histopathological grade in prognostication in ILC.

8. Discussion

Despite great advances in treatment in recent decades, there is still an urgent need for better and more precise prognostication of breast cancer. This thesis is based on a historic cohort of women with breast cancer with long follow-up. They were diagnosed with breast cancer in an era or at an age where modern treatment modalities were not available, thus providing a unique opportunity to study the near natural course of this disease. The main aim was to contribute to improved breast cancer prognostication. To achieve this, archival FFPE tumour tissue was utilized and IHC and ISH methods were used as surrogates for gene expression analyses in molecular subtyping and in studies of other characteristics of breast cancer.

8.1 Discussion of the main findings

The most important findings were significant differences in survival between the molecular subtypes with the best survival for the Luminal A subtype and poorest survival for the HER2 and 5NP subtypes. These findings are in accordance with the findings of others [215-218] . However, in this cohort survival was better for BP compared with 5NP. The high average age of the patients in this cohort may, in part, explain this phenomenon. The BP among post-menopausal women may differ from BP in younger patents.

Another interesting finding was the discovery that differences in BCSS between subtypes were present only during the first five years after breast cancer diagnosis and only for histopathological grade 2. To the best of our knowledge, this has not been described for molecular subtypes prior to this study. Risk of relapse and death from breast cancer is highest the first five years after diagnosis, and survival curves for patients with ER negative status fall rapidly during the first years after diagnosis [219]. Despite this, current five year survival for breast cancer is approximately 90 % [3]. However, there is still some risk of relapse after many years and an important question is why disease with apparently good prognosis may remain dormant for many years and then relapse. Negative prognostic factors appear to be of less significance after the first five years. The clinical impact of this is still unclear. Currently, diagnostic tools capable of identifying patients with apparently good prognosis but high long term risk of recurrence are missing.

Histopathological grading is an important prognostic tool in breast cancer. Several validation studies have been performed [220] and histopathological grade is highly decisive for adjuvant therapy. Interlaboratory and interpersonal inconsistence has caused discussions, but standardization of the method and experienced pathologists have improved the reproducibility [221]. However, grade 2 tumours constitute 30 % - 60 % of the breast carcinomas [222], and the heterogeneity of these tumours is well known. Gene profile studies have shown distinct gene profiles for histopathological grade 1 and 3, but a heterogeneous profile for grade 2 that represents a mixture of grade 1 and 3 [222]. Better prognostication of grade 2 tumours is required and molecular subtyping may be useful.

In this cohort of breast cancer patients with a near natural course of the disease, invasive lobular carcinoma has a poorer prognosis compared to invasive ductal carcinoma. The majority of ILC are grade 2 but their prognosis is significantly poorer that of IDC grade 2. In Norway, histopathological type is not included as a parameter in treatment guidelines. Patients with grade 2 ILC should probably be classified in same prognostic group as grade 3 IDC. However, at present, it is unclear whether the ILC patients would benefit from more adjuvant therapy and further research is needed to elucidate this point.

The histopathological types are distributed differently. Invasive breast carcinoma of no special type occurs far more often than the other types. This means that most studies are performed on invasive breast carcinoma of NST. The numbers of cases included in studies of

special types are often low. In clinical experience, some of the special types are recognized to have a good prognosis. However, the prognostic value of each histopathological type is not clarified. Lack of knowledge and documentation may lead to overestimation or underestimation of clinical value of prognostic markers. There is no doubt that histopathological grading is important in prognostication, but the findings in Paper III that ILC grade 2 has prognosis comparable to IDC grade 3 suggest that the significance of histopathological grade may differ in different histopathological types of breast cancer. The differing implications of histopathological grade in lobular and ductal carcinoma have also been demonstrated by others [223].

In ILC, histopathological typing may be difficult, and in clinical pathology E-cad is a useful marker to distinguish lobular neoplasms from ductal. Distinguishing between these types has value because of the differences in prognosis. E-cad positive ILC has a survival comparable to that of IDC of the same grade regardless of E-cad status. Identification of E-cad negative ILC is probably of greater prognostic significance than detection of ILC per se. Since E-cad is already widely in use to identify ILC, this will not entail introduction of new methods. However, further studies, preferably on full-face sections, are required before implementation of E-cad as a prognostic marker in routine pathology.

The study of *TOP2A* gave interesting and additional results. Previous research has been unable to clarify the prognostic and predictive value of *TOP2A* copy number change in breast cancer. Changes in *TOP2A* copy number are infrequent, and when found, these changes were strongly associated with positive status for hormone receptor and for HER2. The study revealed that apparent differences in prognosis regarding *TOP2A* positive and negative status were hormone receptor and/or HER2-dependent. Thus, *TOP2A* copy number change had no independent prognostic value in this cohort of breast cancer patients. This study contributes to dispel *TOP2A* as a prognostic marker. However, the predictive value of *TOP2A* copy number change remains unresolved.

8.2 Clinical benefit of molecular subtyping

Consensus for molecular subtyping based on surrogate markers is still not established, but in order to compare, recent publications were used when the algorithm for subtyping (Figure 29) was created. Breast cancer is a complex group of diseases and complete understanding of this

complexity is missing. Gene expression analyses have taken the understanding of breast cancer further [118], but still the clinical benefit remains to be documented. A number of studies of molecular subtyping using IHC and ISH techniques as surrogates for gene expression analyses have been published [215, 216]. However, the best way to classify breast cancer is not settled. Further studies of known and novel markers may provide better and more precise classification.

Gene profile studies are the background for molecular subtyping using surrogate markers. Some results from gene expression analyses are confirmed by studies based on IHC and ISH, but the different techniques do not necessarily provide the same information. In gene expression profiles, the number of mRNA molecules is counted as a measure of gene expression [92]. The proteins which are the functional units are not counted directly. Surrogate markers are easily available and can be applied to large cohorts. However, more studies are needed for both approaches and they are expected to be complementary in clinical practice [92]. Gene expression tests applicable on FFPE tissue are required. In new gene signature assays such as the PAM50, set of breast cancer related genes may be analysed in FFPE tissue thus enabling comparison between surrogate makers and gene signatures as predictors of survival [224, 225].

8.3 Cut-off controversies

Lack of consensus applies to some of the biomarkers in these studies. Even the threshold for the well-established hormone receptor status is still to a certain extent controversial and the optimal cut off is not clarified [226]. ASCO/CAP recommends 1 % as cut off [126] and from St. Gallen the recommendation is to regard any staining as positive [127]. Deyarmin et al argue for classification of ER status into three groups: ER negative (< 1 %), low ER-staining (1 % - 10 %) and ER positive (≥ 10 %) [121]. Most low ER-stained tumours show gene profile compatible with the Luminal B or a non-luminal subtype [226]. The proportion of tumours with positive ER-staining in 1 % - 9 % of the cells is small [226]. In the present cohort there were 24 cases with positive ER-staining in 1 % - ≤ 9 % of the cells (unpublished data). Of these, 16 were Luminal B which had a poorer prognosis compared to Luminal A. Patients with low ER-staining tumours may benefit from both adjuvant endocrine treatment and chemotherapy [121, 226].

Ki67 has been shown to have independent prognostic value in breast cancer [151]. St. Gallen recommended 14 % as cut off for Ki67 for distinguishing between Luminal A and Luminal B [227]. The evidence for this cut off is limited [228] and further studies are required. The agreement regarding Ki67 IHC measurement at both an interlaboratory and intralaboratory level is poor and assessment recommendations have been proposed [154]. Standardisation is still lacking and this is important to be aware of when comparing studies [228]. In this project, Ki67 < 15 % was defined as Ki67 low and Ki67 ≥ 15 % as Ki67 high. Ki67 was used to differentiate between Luminal A and Luminal B (HER2-) in accordance with St. Gallen [227]. All cases were assessed by two persons independently in order to improve the precision. However, we recognize the limitations of interpretation and the need for further research.

8.4 Limitations of material and methods

Fortunately, FFPE tumours have been archived and constitute valuable biobanks. The archives of Department of Pathology, St. Olav`s Hospital has made these studies possible. Still, attention to potential limitations is important. We have no control over the preanalytical conditions and the tissue may have been handled differently through time periods. However, in this project tissue quality was good irrespective of time period and IHC and ISH were successful in most cases. There was some loss of tissue, but the use of 3 cores per tumour in the TMAs minimized loss. Cases classified as 5NP may be false negative for one or more of the markers used in molecular subtyping. However, only seven of the 5NP cases showed no staining for any of the other markers used in these studies and only one case was not stained for any marker used in other studies in this project (unpublished data). This weighs in favour against false negative biomarker status.

TMA technology has advantages and limitations discussed in the section *4.7 Tissue microarray*. Transfer of the results from research using TMA to routine pathology cannot be done without further validation studies. In most institutions, assessment of IHC and ISH is done on full-face sections. It is necessary to ensure that the results from TMA studies are reproducible on full-face sections. This is important both when areas for TMA are selected according to specific criteria and when the areas are selected from random areas in the donor tissue. Cut-off levels for markers and their interpretation may differ and the results may, in the worst case, be invalid for full-face sections.

Two methods of visualization were used for *ISH* in this thesis. For HER2, a chromogen was used mainly because of the high number of cases on each slide (42 cases/slide) and the anticipation that the fluorescent signals might fade during analysis. Orientation in a TMA section when evaluating FISH is challenging. However, for an appreciable proportion of cases, the CISH signals were difficult to count and had to be classified as not possible to interpret. Consequently, for *TOP2A* in Paper II, FISH was chosen and assessment was done by two researchers one of whom counted the signals while the other recorded the results. In this way, signal fading was avoided.

A number of studies are performed regarding amplification and co-amplification of TOP2A and HER2 in breast cancer and proportion of gene copy number changes for *TOP2A* varies. The proportion of *TOP2A* in the study in Paper II was lower than in comparable studies. In this study, a short DNA probe for *TOP2A* was used. Due to high frequency of co-amplification with *HER2*, a long probe may because of overlap with *HER2* result in a false high number of *TOP2* [229].

All three studies in this thesis are performed on the same historic population from Nord-Trøndelag. The tumours are biopsied or removed over a wide span of time from the beginning of 1961 to the end of 2008 in 2 different hospitals. The TMAs are arranged according to time period and positive and negative controls have been implemented in all analyses to minimize misinterpretation. Some cases were excluded due to poor quality of the tissue. However, there was no clear association between time period and tissue quality.

Few studies have been performed on archival tissue dating several decades back in time. One recent study, however, has shown that IHC can be applied successfully to specimens from as far back in time as the 1940s [230]. In that study, a rise in ER expression over time was found while PR and HER2 were stable. The difference in ER expression may be due to changes in routines and procedures in laboratories regarding fixation time. However, this does not explain the stability of PR and HER2 compared to ER.

9. Conclusions

The conclusions in this thesis can be summarized as follows

1. **Histopathological grade:**

The main findings in this thesis are differences in survival for grade 2 tumours that can, to a great extent, be explained by differences in molecular subtypes. Statistically significant differences were demonstrated for grade 2 exclusively when comparing the molecular subtypes and when comparing ILC and IDC. The luminal subtypes had better prognosis compared to the non-luminal, and HER2 subtype and 5NP had poorest prognosis. Grade 2 ILC had poorer prognosis compared to grade 2 IDC. This is important contribution in prognostication of the heterogeneous grade 2 tumours.

2. **Histopathological types:**

In the present project, statistical difference in survival for ILC and IDC of same histopathological grade is shown. Histopathological type may have independent value as a prognostic marker and the various biomarkers used in prognostication may have different impact for the different histopathological types.

3. **Prognostic markers**

Two well-known biomarkers which are not used in clinical routine as prognostic markers were studied in this project. Gene copy number changes in *TOP2A* had no independent value as prognostic marker in this historic cohort of women with breast cancer. This result applies both amplification and loss of *TOP2A* gene. Gene copy number changes in *TOP2A* are strongly associated with positive hormone receptor and HER2 status.

E-cadherin is used as a diagnostic tool to distinguish lobular neoplasia, lobular carcinoma in situ and invasive lobular carcinoma from ductal carcinoma in situ and invasive carcinoma of no special type in difficult cases. In this cohort, E-cad negative ILC entailed a poorer prognosis compared to E-cad positive ILC. When not used in diagnostic pathology, E-cadherin may prove useful as a prognostic marker in ILC. For IDC, E-cadherin had no prognostic value.

10. Future perspectives

This thesis provides insight into the natural course of the disease in the various subtypes of breast cancer. This may have implications in further stratifying breast cancer patients for treatment. A substantial proportion of patients may be currently receiving adjuvant therapy unnecessarily. Some would probably not have experienced relapse even without adjuvant treatment and some fail to achieve sufficient effect. An important aim for future research is identification of the super survivors, patients who can avoid adjuvant treatment. This can be achieved through further development of the molecular subtyping based on surrogate markers. New application of well-known markers or implementation of novel markers is possible strategies. Furthermore, rapid developments in new technological approaches will enable us to carry out genetic analyses on archival tumour tissue. The results of the studies carried out in this project will be validated in further studies of other cohorts. E-cadherin may be useful in prognostication in ILC and validation studies on full-face sections are needed.

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12. Errata

Paper I: Two cases of mucinous carcinoma were interchanged regarding histopathological grade. One was histopathological grade 1 and the other was histopathological grade 3. This was corrected in Papers II and III.

Paper I and II: One case was wrongly registered as HER2 negative. This was corrected in Paper III.

Paper I: In Table 2 the numbers of cases with < 5 lymph nodes examined are displaced with the number of lymph nodes not examined. This is corrected in Paper III.

Paper II: In Table 3 the rows of numbers are displaced upwards in relation to the text in column 1.

Paper I

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PRECLINICAL STUDY

Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients

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Abstract Molecular subtyping of breast cancer may provide additional prognostic information regarding patient outcome. However, its clinical significance remains to be established. In this study, the main aims were to discover whether reclassification of breast cancer into molecular subtypes provides more precise information regarding outcome compared to conventional histopathological grading and to study breast cancer-specific survival in the different molecular subtypes. Cases of breast cancer occurring in a cohort of women born between 1886 and 1928 with long-term follow-up were included in the study. Tissue microarrays were constructed from archival formalin-fixed, paraffin-embedded tissue from 909 cases. Using immunohistochemistry and in situ hybridisation as surrogates for gene expression analyses, all cases were reclassified into the following molecular subtypes: Luminal A; Luminal B (HER2−); Luminal B (HER2+); HER2 subtype; Basal phenotype; and five negative phenotype. Kaplan–Meier survival curves and Cox proportional hazards models were used in the analyses. During the first 5 years after diagnosis, there were significant differences in prognosis according to molecular subtypes with the best survival for the Luminal A subtype and the worst for HER2 and five negative phenotype. In this historic cohort of women with breast cancer, differences in breast cancer-specific survival according to subtype occur almost exclusively amongst the histopathological grade 2 tumours. From 5 years after time of diagnosis until the end of follow-up, there appears to be no difference in survival according to molecular subtype or histopathological grade.

Keywords Breast cancer · Molecular subtype · Histopathological grade · Tissue microarray · Breast cancer-specific survival · Prognosis

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Abbreviations

BCSS	Breast cancer-specific survival
BP	Basal phenotype
CI	Confidence intervals
CISH	Chromogenic in situ hybridization
CK5	Cytokeratin 5
EGFR	Epithelial growth factor receptor 1
ER	Oestrogen receptor
FFPE	Formalin-fixed, paraffin-embedded
GGI	Gene expression grade index
HER2	Human epidermal growth factor receptor 2
HES	Haematoxylin–erythrosin–saffron
HR	Hazard ratio
IHC	Immunohistochemistry/immunohistochemical
PR	Progesterone receptor
5NP	Five negative phenotype
SI	Staining index
TMA	Tissue microarray

Introduction

Breast cancer is the most common cancer and leading cause of cancer-related death amongst women worldwide [13, 35]. The disease is heterogeneous in its histopathology, therapeutic response, metastatic patterns and outcome. Current treatment guidelines are based on histopathological grading, tumour size, lymph node-, hormone receptor-, human epidermal growth factor receptor 2 (HER2)- and proliferation (Ki67) status. More recently, gene expression analyses using c-DNA microarray technology have provided a deeper understanding of the complexity of breast cancer. Perou et al. [30] describe four molecular subtypes: Luminal-like, HER2 enriched, Basal-like and Normal-like. More recent publications have confirmed these subtypes with some modifications and it has been shown that molecular subtypes also differ in their response to treatment and outcome [4, 8]. Molecular subtyping with immunohistochemistry (IHC) and in situ hybridisation (ISH) as surrogates for gene expression analyses makes it possible to study large numbers of archival breast cancer cases with long-term follow-up.

Histopathological grade is a well-established prognostic factor [3, 12, 32]. Recent studies confirm the importance of grading in breast cancer prognostication, although grading systems based on gene expression, such as the Gene expression grade index (GGI), have recently emerged [7, 32, 37]. Molecular subtyping may provide additional information on patient outcome, but consensus has yet to be reached regarding IHC or ISH markers that could be used as surrogates for gene expression analyses [17]. Most surrogate markers used for subtyping are available in clinical practice today, but it remains to document the benefits of a new classification prior to implementation.

The aims of this study were to discover whether reclassification of breast tumours into molecular subtypes provides more information regarding outcome compared to conventional histopathological grading and to study breast cancer-specific survival (BCSS) for molecular subtypes over time. To achieve this, a cohort of breast cancer cases with long-term follow-up was reclassified into molecular subtypes. Most of the markers examined are widely used, such as oestrogen receptor (ER), progesterone receptor (PR), HER2 and Ki67. In addition, cytokeratin 5 (CK5) and epithelial growth factor receptor 1 (EGFR) were included [2, 6]. The patients in this population experienced breast cancer in a time period or at an age where adjuvant treatment after surgery was rarely an option and the disease thus had a near-natural course.

Materials and methods

Study population

Between 1956 and 1959, 25,897 women in the Norwegian county of Nord-Trøndelag, born between 1886 and 1928, were invited to participate in a screening programme for early diagnosis of breast cancer [22, 29]. The screening comprised a clinical examination and a questionnaire focussed on reproductive history. Data were linked with the Norwegian Cancer Registry and the Cause of Death Registry of Norway. In all, 1,393 new cases of breast cancer occurred between 1961 and 2008. Most of these were analysed at the Department of Pathology, St. Olav's Hospital, Trondheim University Hospital, Norway. A total of 448 cases were excluded from the study. For the remaining 945 cases, formalin-fixed, paraffin-embedded (FFPE) tissue was available and 909 were of sufficient quality for reclassification into molecular subtypes (see Fig. 1).

Specimen characteristics

Pathology reports and FFPE tissue from all cases were retrieved from the archives of the department of pathology. In cases with recurrent disease or second or multiple primary breast cancer, only the first primary tumour was included. New 4-μm-thick full-face sections were cut from representative paraffin blocks from tumours and lymph node metastases and stained with haematoxylin–erythrosine–saffron (HES). Forty cases comprised only core biopsies or small tissue fragments unsuitable for tissue microarray (TMA). From these, serial sections were made. The HES-stained sections were reviewed under a

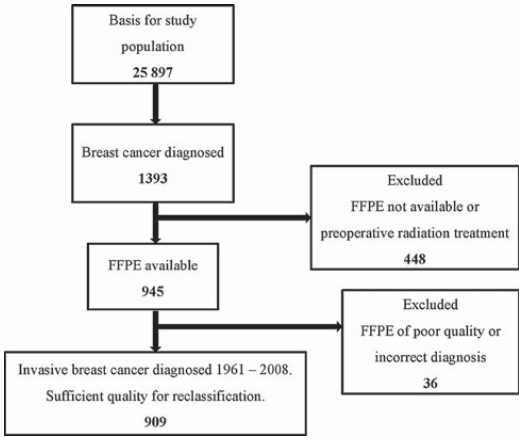

Fig. 1 Study population

microscope independently by two experienced pathologists (OAH, AMB) and classified according to histopathological type and grade according to the World Health Organization Classification of Tumours [23] and the Nottingham grading system [12, 33]. Any discrepancies in grade or type were discussed and consensus reached. In cases where tumour size was missing in the pathology report, size was measured in millimetres on the glass slide. Only cases with a measurement of the whole tumour in the pathology report and/or measurement of the full diameter on the glass slide were registered. All other cases were classified as size uncertain [$n = 268$ (29.5 %)].

TMA construction

TMA blocks were made using the Tissue Arrayer MiniCore® 3 with TMA Designer2 software (Alphelys). Areas of interest in the HES sections were marked by a pathologist. Three 1-mm-diameter tissue cores were extracted from peripheral regions of the tumour in the FFPE blocks and inserted into TMA recipient blocks. From the TMA blocks, 4-μm sections were cut and stained. IHC was done with antibodies for ER, PR, HER2(CB11), CK5, Ki67 and EGFR in addition to HES staining. In addition, HER2 status was also examined by chromogenic in situ hybridization (CISH).

Assay methods

Sections were mounted on Superfrost+glass slides, dried at 37 °C overnight and stored at −20 °C. All sections were stained within 12 weeks of sectioning. The slides were heated to 60 °C for 2 h. Pre-treatment was performed in a PT Link, Pre-Treatment Module for Tissue Specimens (Dako)

Table 1 Sources and dilutions of primary antibodies

Antibody	Clone	Manufacturer	Concentration of antibody	Dilution
ER	SP1	Cell marque	33 mg/ml	1:100
PR	16	Novocastra	360 mg/l	1:400
HER2	CB11	Novocastra	3.9 g/l	1:640
Ki67	MIB1	Dako	35 mg/l	1:100
CK5	XM26	Novocastra	50 mg/l	1:100
EGFR	2-18C9	Dako	Ready to use	No dilution

with buffer (High pH Target Retrieval Solution K8004) at 97 °C for 20 min. All sections were immunostained for ER, PR, HER2 (CB11), CK5 and Ki67 in a DakoCytomation Autostainer Plus (Dako). For visualization, the Dako REAL™ EnVision™ Detection System was used with Peroxidase/DAB+, Rabbit/Mouse, code K5007. EGFR was immunostained using EGFR pharmDx™ for autostainer, code K1494. See Table 1 for sources and dilutions of primary antibodies. Negative controls were included in each staining run. CISH was used to visualize the *HER2* gene (red chromagen) and chromosome 17 (blue chromagen) using the dual colour probe kit HER2 CISH pharmDx™ Kit, code 109 (Dako). Two of the steps in the CISH procedure were modified slightly. The incubation time for red chromogen solution was increased from 10 to 15 min, and the dilution of haematoxylin was increased from 1:5 to 1:7.

Scoring and reporting

All HES- and IHC-stained slides were digitalized using the tissue scanner Ariol™ SL-50 3.3 Scan system and analysis station (Genetix) at 5× and 20× magnification. Expression of ER, PR, HER2 (CB11), CK5, Ki67 and EGFR was evaluated using the Ariol review station. The images were viewed and subjectively scored by two persons independently. *HER2* gene amplification status was annotated under a bright field microscope. All cases were evaluated by at least one pathologist. Any discrepancies were discussed and consensus reached.

Classification of each marker

ER and PR were positive when ≥1 % of the tumour cells showed positive nuclear staining [19]. For Ki67, a total of 500 tumour nuclei were examined. Cases with ≥15 % positive nuclei were classified as Ki67 high and <15 % as Ki67 low [16].

HER2 was assessed using both IHC and CISH [11]. For HER2 IHC, the CB11 clone [31, 43] was used and the Herceptest (Dako) guidelines for interpretation were used with a membrane-staining score ranging from 0 to +3.

HER2 IHC was considered negative when the score was 0 or +1, positive when +3 and borderline when +2. Since the preanalytical treatment of the samples was unknown, the results of HER2 IHC were only used in cases where CISH was unsuccessful. In IHC (+2) and unsuccessful CISH (18 cases), the corresponding IHC was revised by two authors (AMB and MJE) and reclassified as either +1(14 cases) or +3(4 cases).

The *HER2* gene was considered amplified if the gene to chromosome ratio was ≥2.0 [1, 34]. A minimum of 20 non-overlapping nuclei with signals for both chromosome and gene were assessed.

For CK5, a staining index (SI) was estimated. Staining intensity was graded as 0 (no staining), 1 (weak), 2 (moderate) and 3 (strong). The proportion of positive staining cells was scored as 1 (<10 %), 2 (10–50 %) and 3 (>50 %). The score for intensity multiplied by proportion is the SI [14, 26]. In this study, the results were considered to be negative when SI was 0–1 and positive when the SI was 2–9. For EGFR, membranous staining was scored according to the guidelines in the Dako PharmDx kit and a SI was calculated when this was combined with the proportion of cells showing positive staining resulting in a SI as described above.

Classification of molecular subtypes

Using the six biomarkers, the tumours were then classified in molecular subtypes: Luminal A, Luminal B (HER2−), Luminal B (HER2+), HER2 subtype five negative phenotype (5NP) and Basal-like phenotype (BP) (Fig. 2).

Statistical analyses

All women were followed from the date of breast cancer diagnosis to the date of death from breast cancer, death from any other cause or to the end of follow-up (December 31, 2010), whichever came first. BCSS according to molecular subtypes and histopathological grade was estimated using Kaplan–Meier methods and compared by log-rank tests. Cox proportional hazards models were used to estimate risk of death from breast cancer adjusted for age (5-year intervals), stage (in five categories: stage I–IV and unknown) at diagnosis according the data from the Cancer Registry [21] and time period of diagnosis (10-year intervals). Hazard ratios (HR) were calculated with 95 % confidence intervals (CI) for two time periods: first 5 years after diagnosis and from 5 years after diagnosis and onwards (conditional on surviving the first 5 years). Cox analyses of the first 5 years were stratified by histopathological grade. Statistical analyses were carried out using Stata version 12.1 IC for Windows (Stata Corp.). This study complies with the REMARK reporting recommendations for tumour marker studies [25].

Ethics

The study was approved by the Regional Committee for Medical and Health Sciences Research Ethics (REK, Midt-Norge, ref. nr: 836/2009) and dispensation from the requirement of patient consent was granted.

Results

Description of the population

In all, 909 cases were included. Mean age at diagnosis was 72.5 years (SD 10.7; range 41–102). Only 12.5 % were <60 years and 58.9 % were 60–79 years. Most tumours were 2–5 cm in diameter (43.2 %), but for 29.5 %, tumour size was unknown or uncertain. At the end of the observation period, 359 (39.5 %) had died of breast cancer, 390 (42.9 %) of other causes and 160 (17.6 %)

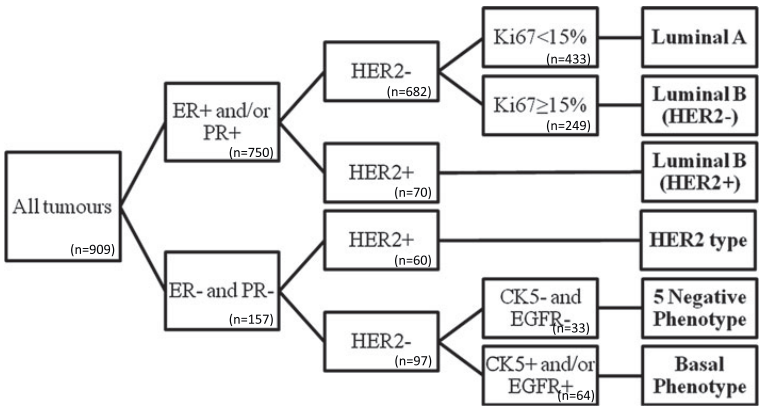

Fig. 2 Classification algorithm for molecular subtyping

were still alive. Median follow-up was 6.4 years [interquartile range (IQR) 10.0 years]. See Table 2 for patient and tumour data.

Histopathological characteristics

Of the 909 tumours, 12.9 % were grade 1, 53.7 % grade 2 and 33.4 % grade 3. The histopathological types were as follows: ductal: 70.0 %; lobular: 13.6 %; and other special types: 16.4 %. All cases were reclassified into molecular subtypes based on assessment of ER, PR, HER2, Ki67, CK5 and EGFR. Table 2 shows distribution of histopathological types and grades for each molecular subtype. Table 3 shows the number of positive cases of each marker.

Distribution of molecular subtypes

The distribution of subtypes was as follows: Luminal A: 47.6 %; Luminal B (HER2−): 27.4 %; Luminal B (HER2+): 7.7 %; HER2 subtype: 6.6 %; 5NP: 3.6 %; and

Table 2 Descriptive statistics for the 909 breast cancer cases

	Luminal A	Luminal B (HER2−)	Luminal B (HER2+)	HER2 type	5 Negative phenotype	Basal phenotype	Total
Number (%)	433 (47.6)	249 (27.4)	70 (7.7)	60 (6.6)	33 (3.6)	64 (7)	909
Mean age at diagnosis (SD)	73.9 (9.9)	71.9 (10.9)	69 (11.4)	67.3 (11.6)	75.9 (11.1)	71.7 (11.3)	72.5 (10.7)
Median years of follow-up after diagnosis (IQR)	7.4 (9.3)	7.0 (11.1)	5.2 (12.5)	3.2 (8.1)	3.4 (8.5)	5.1 (9.1)	6.4 (10.0)
Tumour grade (%)							
1	91 (21.0)	20 (8.0)	2 (2.9)	0	0	4 (6.3)	117 (12.9)
2	297 (68.6)	120 (48.2)	33 (47.1)	10 (16.7)	21 (63.6)	7 (10.9)	488 (53.7)
3	45 (10.4)	109 (43.8)	35 (50.0)	50 (83.3)	12 (36.4)	53 (82.8)	304 (33.4)
Histopathological type (%)							
Ductal	299 (69.1)	182 (73.1)	57 (81.4)	47 (78.3)	14 (42.4)	37 (57.8)	636 (70.0)
Lobular	68 (15.7)	35 (14.1)	6 (8.6)	1 (1.7)	12 (36.4)	2 (3.1)	124 (13.6)
Tubular	4 (0.9)	0	0	0	0	0	4 (0,4)
Mucinous	31 (7.2)	8 (3.2)	3 (4.3)	0	0	1 (1.6)	43 (4.7)
Papillary	19 (4.4)	7 (2.8)	3 (4.3)	1 (1.7)	0	2 (3.1)	32 (3.5)
Medullary	0	6 (2.4)	0	6 (10.0)	2 (6.1)	7 (10.9)	21 (2.3)
Metaplastic	0	1 (0.4)	0	2 (3.3)	1 (3.0)	9 (14.1)	13 (1.4)
Other	12 (2.8)	10 (4.0)	1 (1.4)	3 (5.0)	4 (12.1)	6 (9.4)	36 (4.0)
Tumour size[a] (%)							
<2	94 (21.7)	50 (20.1)	12 (17.1)	4 (6.7)	3 (9.1)	6 (9.4)	169 (18.6)
2–5	193 (44.6)	97 (39.0)	27 (38.6)	21 (35.0)	18 (54.5)	37 (57.8)	393 (43.2)
>5	35 (8.1)	19 (7.6)	7 (10.0)	13 (21.7)	2 (6.1)	3 (4.7)	79 (8.7)
Uncertain	111 (25.6)	83 (33.3)	24 (34.3)	22 (36.7)	10 (30.3)	18 (28.1)	268 (29.5)
Lymph node invasion[a]							
Yes	129 (29.8)	82 (32.9)	25 (35.7)	32 (53.3)	15 (45.5)	27 (42.2)	310 (34.1)
No (≥5 nodes or SNB[b])	142 (32.8)	66 (26.5)	24 (34.3)	15 (25.0)	5 (15.2)	21 (32.8)	273 (30.0)
No (<5 nodes examined)	123 (28.4)	84 (33.7)	20 (28.6)	9 (15.0)	10 (30.3)	11 (17.2)	257 (28.3)
Uncertain	39 (9.0)	17 (6.8)	1 (1.4)	4 (6.7)	3 (9.1)	5 (7.8)	69 (7.6)
Stage[c]							
I	238 (55.0)	123 (49.4)	29 (41.4)	23 (38.3)	15 (45.5)	27 (42.2)	455 (50.1)
II	157 (36.3)	90 (36.1)	28 (40.0)	27 (45.0)	14 (42.4)	30 (46.9)	346 (38.1)
III	23 (5.3)	17 (6.8)	3 (4.3)	7 (11.7)	3 (9.1)	4 (6.3)	57 (6.3)
IV	13 (3.0)	17 (6.8)	8 (11.4)	3 (5.0)	1 (3.0)	3 (4.7)	45 (5.0)
Unknown	2 (0.5)	2 (0.8)	2 (2.9)	0	0	0	6 (0.7)

[a] Histologically confirmed

[b] Sentinel node biopsy

[c] Cancer Registry of Norway, combined clinical and histological stage

Table 3 The number of positive cases for each marker

Marker	No. of positive (%)	Not possible to interpret
ER	749 (82.4)	2 (0.2 %)
PR	521 (57.3)	1 (0.1 %)
HER2	130 (14.3)	0
Ki67	406 (44.7)	1 (0.1 %)
CK5	164 (18.0)	0
EGFR	64 (7.0)	0

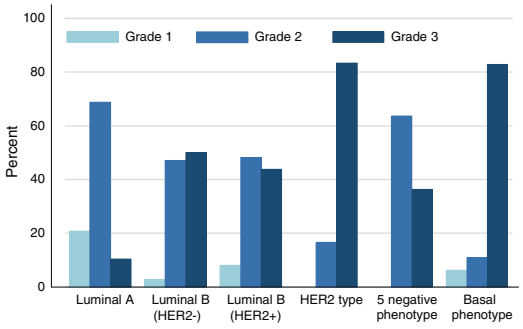

Fig. 3 Distribution of grade in percent according to subtype

BP: 7.0 %. See Table 2. Mean age at diagnosis was 72.8 (SD 10.5) for women with luminal tumours and 70.9 (SD 11.8) for non-luminal tumours. Luminal A had the highest proportion of grades 1 and 2 (Fig. 3). Only HER2 subtype and BP comprised a higher proportion of grade 3 than grade 2. Grade 1 was not found in HER2 and 5NP subtypes. The Luminal B subtypes had very similar distribution of grades despite differences in other characteristics.

Breast cancer-specific survival, molecular subtypes and histopathological grade

Luminal A subtype had the best survival, closely followed by Luminal B (HER2−) with 5-year BCSS higher than 75 %. The HER2 and 5NP subtypes had the poorest prognosis, with 5-year survival around 50 %. Of the triple-negative cases, BP had a better prognosis than 5NP. BP and Luminal B (HER2+) were similar in terms of 5-year survival (Fig. 4).

Figure 5 shows BCSS according to histopathological grade for up to 20 years of follow-up. Adjustment for age did not substantially influence the curves, but after adjustment for stage, survival for grade 1 tumours was improved (data not shown).

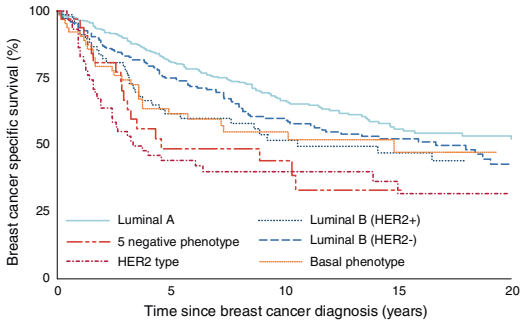

Fig. 4 Kaplan–Meier plot. Breast cancer-specific survival according to molecular subtypes. *P*-value from log-rank test of differences in BCSS was <0.0001

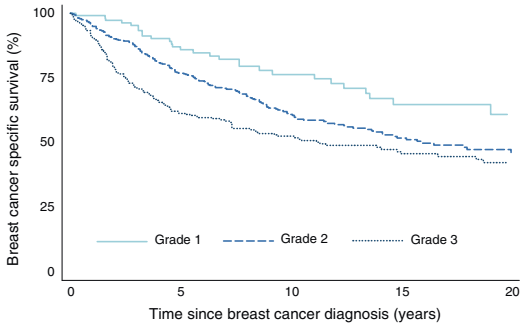

Fig. 5 Kaplan–Meier plot. Breast cancer-specific survival according to grade. *P*-value from log-rank test of differences in BCSS was 0.0001

Risk of death from breast cancer

Table 4 shows risk of death from breast cancer according to molecular subtype and histopathological grade. During the first 5 years, grades 2 and 3 had a poorer prognosis compared to grade 1 with HR 3.8 (95 % CI 2.14–6.75) for grade 3 and HR 1.97 (95 % CI 1.11–3.51) for grade 2. In the same time period, the hormone receptor-negative and/or HER2-positive subtypes had the poorest prognoses compared to Luminal A. Particularly poor prognoses were shown for the HER2 subtype [HR 4.24 (95 % CI 2.79–6.42)] and 5NP [HR 3.34, (95 % CI 1.91–5.82)]. After 5 years, neither grade nor molecular subtype showed any clear association with survival. Adjustment for age had no impact on the results, and adjustment for stage only slightly attenuated risk estimates.

Table 5 shows risk of death from breast cancer the first 5 years after diagnosis according to molecular subtype for grade 2 and 3. For grade 2, the HR for HER2 subtype compared to Luminal A was 6.62 (95 % CI 2.82–15.57), and adjustment for age and stage did not substantially

Table 4 Risk of death from breast cancer according to molecular subtype and histopathological grade

	No. of cases	Deaths from breast cancer	HR 95 % CI unadjusted		HR 95 % CI adjusted for age		HR 95 % CI adjusted for stage	
Histopathological grade, follow-up first 5 years after diagnosis								
1	117	13	1.00		1.00		1.00	
2	488	101	1.97	1.11–3.51	1.95	1.09–3.48	1.47	0.82–2.64
3	304	110	3.80	2.14–6.75	3.74	2.10–6.66	3.12	1.75–5.55
	909	224						
Histopathological grade, follow-up from 5 years after diagnosis[a]								
1	78	18	1.00		1.00		1.00	
2	291	83	1.37	0.82–2.29	1.29	0.77–2.16	1.21	0.72–2.02
3	153	34	0.98	0.55–1.74	0.94	0.53–1.67	0.90	0.51–1.60
	522	135						
Molecular subtype, follow-up first 5 years after diagnosis								
Luminal A	433	73	1.00		1.00		1.00	
Luminal B (HER2−)	249	56	1.42	1.01–2.02	1.42	1.00–2.02	1.29	0.92–1.84
Luminal B (HER2+)	70	25	2.33	1.48–3.67	2.36	1.48–3.74	2.11	1.33–3.33
HER2	60	32	4.24	2.79–6.42	4.39	2.86–6.72	3.72	2.44–5.65
5 Negative phenotype	33	15	3.34	1.91–5.82	3.18	1.81–5.61	3.17	1.81–5.53
Basal phenotype	64	23	2.43	1.52–3.89	2.38	1.48–3.82	2.39	1.48–3.82
	909	224						
Molecular subtype, follow-up from 5 years after diagnosis[a]								
Luminal A	271	69	1.00		1.00		1.00	
Luminal B (HER2−)	148	44	1.15	0.79–1.68	1.21	0.82–1.77	1.15	0.80–1.69
Luminal B (HER2+)	36	10	0.81	0.41–1.57	0.88	0.44–1.73	0.92	0.46–1.83
HER2	23	4	0.66	0.24–1.80	0.71	0.26–1.96	0.66	0.24–1.82
5 Negative phenotype	12	3	0.84	0.27–2.68	0.89	0.27–2.90	0.94	0.30–3.01
Basal phenotype	32	5	0.58	0.23–1.43	0.62	0.25–1.55	0.58	0.23–1.46
	522	135						

HR hazard ratio, *CI* confidence interval

[a] Conditional on surviving the first 5 years

influence the estimate. In grade 3, there was no clear difference in risk of death from breast cancer according to molecular subtype. Since 12 of the 13 patients who died of grade 1 tumours had Luminal A tumours, HRs were not calculated. Adjustment for time period of diagnosis did not change the results (not shown).

Amongst HER2-positive cases, the hazard ratio for the HER2 subtype compared to Luminal B (HER2+) was 1.8 (95 % CI 1.07–3.05) (not shown in table).

Discussion

In this long-term follow-up of breast cancer patients, the HER2 and 5NP subtypes showed the poorest prognosis during the first 5 years after diagnosis. After 5 years, BCSS did not significantly differ amongst the six molecular subtypes. However, the numbers of 5-year survivors in these two groups are low. The patients came from a cohort of women with breast cancer who lived through a time period with limited access to adjuvant treatment. However, 192 women would have qualified for antihormonal treatment according to the treatment guidelines operative at the time of diagnosis. None were qualified for treatment with trastuzumab. Kaplan–Meier BCSS estimates for patients with ER-positive tumours who may have received treatment and those who did not qualify for treatment do not differ significantly (data not shown).

During the first 5 years of follow-up, differences in survival according to subtype occurred almost exclusively amongst patients with grade 2 tumours. Grade 2 was significantly associated with poorer survival for all subtypes except Luminal B (HER2−).

These results support the findings of others that hormone receptor status defines two groups within HER2-positive breast cancer with differing BCSS [42]. The HER2 subtype had the poorest 5-year survival of all subtypes, whereas the Luminal B (HER2+) subgroup had a substantially better

Table 5 Risk of death from breast cancer according to molecular subtype for each histopathological grade

	Number of cases	Deaths from breast cancer	HR 95 % CI Unadjusted		HR 95 % CI Adjusted for age		HR 95 % CI Adjusted for stage	
Molecular subtype, follow-up first 5 years after diagnosis: grade 2								
Luminal A	297	45	1.00		1.00		1.00	
Luminal B (HER2−)	120	24	1.45	0.88–2.38	1.50	0.91–2.48	1.33	0.81–2.19
Luminal B (HER2+)	33	12	2.67	1.41–5.04	2.97	1.54–5.70	2.29	1.20–4.38
HER2	10	6	6.62	2.82–15.57	7.81	3.18–19.18	5.64	2.36–13.51
5 Negative Phenotype	21	11	4.68	2.42–9.06	3.91	1.97–7.76	4.42	2.26–8.64
Basal Phenotype	7	3	3.39	1.05–10.92	2.56	0.75–8.69	3.35	1.03–10.85
	488	101						
Molecular subtype, follow-up first 5 years after diagnosis: grade 3								
Luminal A	45	16	1.00		1.00		1.00	
Luminal B (HER2–)	109	31	0.73	0.40–1.34	0.73	0.39–1.36	0.57	0.31–1.04
Luminal B (HER2+)	35	13	1.00	0.48–2.09	0.96	0.45–2.05	0.85	0.41–1.79
HER2	50	26	1.60	0.86–2.99	1.60	0.84–3.05	1.21	0.64–2.29
5 Negative Phenotype	12	4	0.90	0.30–2.70	0.87	0.28–2.64	0.94	0.31–2.82
Basal Phenotype	53	20	1.07	0.55–2.06	1.07	0.54–2.11	0.86	0.44–1.68
	304	110						

Follow-up first 5 years after diagnosis. HRs were not calculated for histopathological grade 1 because 12 of the 13 patients who died of grade 1 tumour had Luminal A tumour

5-year survival, supporting the significance of ER status in determining survival. It has been shown that, despite problems associated with crosstalk between ER and HER2, Luminal B (HER2+) benefits from antihormonal treatment [20]. The hazard ratio for the HER2 subtype compared to Luminal B (HER2+) would appear to confirm this.

To predict response to endocrine therapy, the cutoff for ER was previously set at 10 % positive staining nuclei [28]. In accordance with current guidelines, the cutoff is now set at $\geq$1 % [19]. In this study, 24 cases showed ER-positive staining in $\geq 1 < 10$ % of tumour cell nuclei and were classified as Luminal. A majority (16 cases) were Luminal B, and in the Luminal B (HER2+) subtype, they accounted for 9 % of cases. Deyarmin et al. [10] have suggested that the classification of ER-low tumours as Luminal may be inappropriate. These cases exert little or no influence on the results of the Kaplan–Meier and Cox analyses in the present study.

Classification of breast cancer into molecular subtypes with surrogate markers for gene expression is widely used. In 2010, Blows et al. [4] published a large collaborative analysis that showed survival for different subtypes, where the subtyping in all the 12 included studies was done by IHC. These methods are more accessible and affordable than gene profile studies and can be applied to archival FFPE tissue. The St. Gallen Consensus Discussion in 2011 opened for molecular subtyping of breast cancer using ER, PR, HER2 and Ki67/grade, all factors already in clinical use, though the cutoff for Ki67 is still controversial [18]. The panel did not support the incorporation of EGFR or CK 5/6, thus the basal phenotype and the five negative phenotype were classified as 'triple negative' [15, 17]. Discussion is ongoing regarding which markers are best suited for the classification of molecular subtypes.

In the present study, 5-year survival was better for BP compared to 5NP. This is in contrast with the findings of others [4, 6, 40]. The 5NP subtype had poorer prognosis despite the fact that it comprised a higher proportion of histological grade 2 tumours. Validation studies will reveal whether or not this finding is consistent. This may be a group that would have benefited from adjuvant treatment as offered today.

Histopathological grade, tumour size and lymph node status are strong prognostic factors and are well established in clinical practice. Reduced long-term survival is associated with higher grade [4, 36, 44]. In the present study, high grade was associated with non-luminal subtypes. However, the prognostic value of the different factors may vary with time after diagnosis [24]. Since the risk of relapse and death is the highest during the first 5 years, particularly for ER-negative disease [27, 41], two periods of time were analysed separately in this study: the first 5 years after diagnosis and the subsequent years. Even after many years, there is some risk of breast cancer recurrence. Interestingly, in this cohort, there are no differences in survival according to subtypes for those who have survived the first 5 years. Further research may reveal whether adjuvant treatment modifies this tendency.

Histopathological grade 1 tumours are associated with the best prognosis, whereas grade 3 tumours are associated with the poorest prognosis. Grade 2 tumours comprise a more heterogeneous group where the majority has an intermediate prognosis, but some cases may exhibit similarity with grades 1 and 3 [7, 32]. The same applies in this study. It is possible to classify grade 2 tumours into low risk and high risk of recurrence using the GGI which is based on analysis of 97 genes [37]. A 3-gene proliferation score using PCR assay to identify *TOP2A, FOXM1* and *MKI67* has similar prognostic value as GGI and might be easier to implement [39]. However, the present study shows that it is possible to obtain significant additional information of prognostic value by using already implemented or readily accessible tests, and this may be of value in prognostication of grade 2 tumours.

This study contributes to the understanding of breast cancer heterogeneity partly because of the unique nature of the study population. These women lived in a time before birth control pills and hormone replacement therapy at menopause were available, and they had not undergone organized mammography screening. Furthermore, due to age and time period, they had limited postoperative treatment and thus we come as close to the natural course of the disease as possible. One drawback in this study is the relatively high age of the cohort and the results must be considered in light of this fact. This may explain the relatively high proportion of grade 2 tumours and the slightly lower proportion of HER2-positive tumours [38]. Another weakness may be the IHC estimation of HER2 where standardized preanalytical conditions were unattainable, thus precluding a semiquantitative estimation of protein expression. Despite this, there was full correlation between IHC and CISH in 587 cases. 13 cases were IHC + 3, but showed chromosome 17 polysomy with ratios <2.0. Two cases scored +3, but no changes in chromosome or gene copy number. For the same reason, false-positive and -negative results may have occurred for the other biomarkers. However, the distribution of subtypes is comparable to that of other studies [4, 5, 9]. All laboratory tests were carried out under standardized conditions and their interpretation together with complete revision of the histopathological diagnoses, type and grade was done within the context of this study according to present-day guidelines. By adding two markers to identify the basal phenotype to the set of markers in clinical use, it was possible to subdivide triple-negative cases into BP and the 5NP. In this study, these two subtypes had significantly differing BCSS. Molecular tests such as GGI are promising in terms of clinical benefit, but so far the documented benefit is complementary to histopathological methods [32]. Similarly, molecular subtyping using surrogate markers may provide important additional information for selected subgroups of breast cancer patients.

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Conflict of interest The authors declare that they have no competing interests.

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Paper II

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TOP2A gene copy number change in breast cancer

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ABSTRACT

Aims The clinical significance of *TOP2A* as a prognostic marker has not been clarified. The aims of this study were to investigate the frequency of *TOP2A* copy number change; to correlate *TOP2A* with *HER2* status, hormone receptor (HR) status and molecular subtype, and further to explore differences in breast cancer-specific survival according to *TOP2A* and *HER2*.

Methods In this study, *TOP2A, HER2* and chromosome 17 copy number were assessed in 670 cases of breast cancer using in situ hybridisation techniques. Gene to chromosome ratios ≥2 were classified as amplification. *TOP2A* deletion (gene to chromosome ratio ≤0.8) or monosomy (only one signal for both gene and chromosome in more than 75% of nuclei) were classified as gene loss.

Results A strong association between *TOP2A* change and HR and *HER2* status was found. During the first 5 years after diagnosis, the risk of death from breast cancer was significantly higher for cases with *HER2* amplification irrespective of *TOP2A* status.

Conclusions *TOP2A* copy number change was strongly associated with HR and *HER2* status and as a prognostic marker *TOP2A* is probably of limited value.

INTRODUCTION

The *HER2* gene has a well-established biological and clinical role in breast cancer, and the HER2 amplicon on chromosome 17 harbours a number of genes involved in breast cancer pathophysiology. Copy number change among these genes is frequently observed though their significance remains to be clarified.[1]

TOP2A is one of the genes close to *HER2* and its protein product, topoisomerase II α, is the molecular target of anthracycline treatment. *TOP2A* amplification status has been thought to be linked to response to treatment. However, data are conflicting and, as yet, unresolved.[2] *HER2* and *TOP2A* are associated with high histopathological grade[3] and high proliferation,[4] but the clinical significance of *TOP2A* and its relationship to *HER2* have not been clarified.

The aims of this study were to investigate the frequency of *TOP2A* copy number change in a well-characterised cohort of women with breast cancer[5] and to correlate *TOP2A* with *HER2* status, hormone receptor (HR) status and molecular subtype. A further objective was to explore differences in breast cancer-specific survival (BCSS) according to *TOP2A* and *HER2*.

MATERIALS AND METHODS

Study population

A screening programme for early diagnosis of breast cancer was conducted by the Norwegian Cancer Registry between 1956 and 1959. The patients developed breast cancer in a time period with limited access to adjuvant treatment. None were treated with anthracyclines or trastuzumab. According to the guidelines at the time of diagnosis, 30.7% patients may have qualified for treatment with tamoxifen. The population has been described in detail previously.[5–7] A total of 1393 women in the underlying population developed breast cancer in the follow-up period from 1961 to the end of 2008. Of these, 945 had tissue samples available at the Department of Pathology and Medical Genetics, St. Olav's Hospital, Trondheim, Norway, and 670 were suitable for assessment of *TOP2A* and *HER2* copy number. Survival data were generated after linkage between the Cause of Death Registry of Norway and the Norwegian Cancer Registry.

Specimen characteristics

All cases in this study have previously been classified according to histopathological type and grade and reclassified in molecular subtypes according to figure 1[5] using oestrogen receptor (ER),

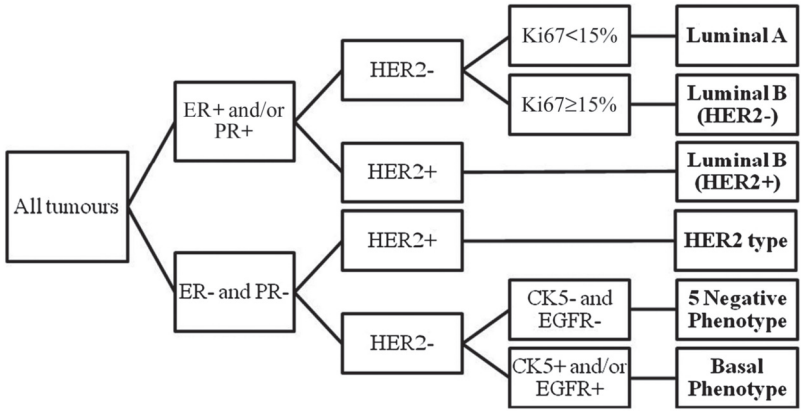

Figure 1 Classification algorithm for molecular subtyping.

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Table 1 Descriptive statistics of the 670 breast cancer cases

	TOP2A normal	*TOP2A* amplified	*TOP2A* loss	*HER2* normal	*HER2* amplified	Co-amplified	*TOP2A* loss, *HER2* amplified	*TOP2A* amplified, *HER2* normal	*TOP2A* loss, *HER2* normal	Normal *TOP2A* and *HER2*	Total
Number (%)	604 (90.2)	41 (6.1)	25 (3.7)	560 (83.6)	110 (16.4)	32 (4.8)	6 (0.9)	9 (1.3)	19 (2.8)	532 (79.4)	670
Mean age at diagnosis (SD)	73.4 (9.7)	69.5 (9.6)	72.0 (12.5)	74.0 (9.3)	68.3 (11.0)	68.2 (10.1)	69.2 (13.6)	74.3 (5.6)	72.8 (12.3)	74.0 (9.2)	73.1 (9.8)
Median years of follow-up after diagnosis (IQR))	6.7 (9.4)	5.8 (11.9)	6.4 (5.9)	7.1 (9.1)	4.5 (10.6)	5.1 (12.6)	5.0 (7.9)	6.0 (10.0)	6.7 (8.1)	7.1 (9.2)	6.6 (9.4)
Tumour grade (%)											
1	71 (11.8)	1 (2.4)	0	71 (12.7)	1 (0.9)	0	0	1 (11.1)	0	70 (13.2)	72 (10.8)
2	319 (52.8)	16 (39.0)	16 (64.0)	318 (56.8)	33 (30.0)	11 (34.4)	2 (33.3)	5 (55.6)	14 (73.7)	299 (56.2)	351 (52.4)
3	214 (35.4)	24 (58.5)	9 (36.0)	171 (30.5)	76 (69.1)	21 (65.6)	4 (66.7)	3 (33.3)	5 (26.3)	163 (30.6)	247 (36.9)
Tumour size (%)											
<2	136 (22.5)	8 (19.5)	5 (20.0)	135 (24.1)	14 (12.7)	5 (15.6)	0	3 (33.3)	5 (26.3)	127 (23.9)	149 (22.2)
2–5	292 (48.3)	15 (36.6)	7 (28.0)	270 (48.2)	44 (40.0)	14 (43.8)	3 (50.0)	1 (11.1)	4 (21.1)	265 (49.8)	314 (46.9)
>5	41 (6.8)	3 (7.3)	5 (20.0)	33 (5.9)	16 (14.6)	2 (6.3)	1 (16.7)	1 (11.1)	4 (21.1)	28 (5.3)	49 (7.3)
Uncertain	135 (22.4)	15 (36.6)	8 (32.0)	122 (21.8)	36 (32.7)	11 (34.4)	2 (33.3)	4 (44.4)	6 (31.6)	112 (21.1)	158 (23.6)
Molecular subtypes (%)											
Luminal A	300 (49.7)	7 (17.1)	10 (40.0)	317 (56.6)	0	0	0	7 (77.8)	10 (52.6)	300 (56.4)	317 (47.3)
Luminal B (HER2−)	166 (27.5)	1 (2.4)	6 (24.0)	173 (30.9)	0	0	0	1 (11.1)	6 (31.6)	166 (31.2)	173 (25.8)
Luminal B (HER2+)	37 (6.1)	23 (56.1)	1 (4.0)	0	61 (55.5)	23 (71.9)	1 (16.7)	0	0	0	61 (9.1)
HER2 type	35 (5.8)	8 (19.5)	5 (20.0)	0	49 (44.6)	9 (28.1)	5 (83.3)	0	0	0	49 (7.3)
Five negative phenotype	22 (3.6)	0	0	22 (3.9)	0	0	0	0	0	22 (4.1)	22 (3.3)
Basal phenotype	44 (7.3)	2 (4.9)	3 (12.0)	48 (8.6)	0	0	0	1 (11.1)	3 (15.8)	44 (8.3)	48 (7.2)
Hormone receptor											
Positive	503 (83.3)	31 (75.6)	17 (68.0)	490 (87.5)	61 (55.5)	23 (71.9)	1 (16.7)	8 (88.9)	16 (84.2)	466 (87.6)	551 (82.2)
Negative	101 (16.7)	10 (24.4)	8 (32.0)	70 (12.5)	49 (44.5)	9 (28.1)	5 (83.3)	1 (11.1)	3 (15.8)	66 (12.4)	119 (17.8)

progesterone receptor (PR), Ki67, cytokeratin 5 and epithelial growth factor receptor (EGFR) 1 as surrogate markers for gene expression. *HER2* status was assessed using chromogenic in situ hybridisation (CISH).

Assay methods

For the present study, fluorescence in situ hybridisation (FISH) was employed for detection of *TOP2A* and chromosome 17 according to the manufacturer`s guidelines. Pretreatment was done using Histology FISH Accessory Kit, code K5799 (Dako). The probe mix (VYSIS TOP2A/CEP 17 FISH Probe Kit, code 03N89-020 Abbott Molecular Inc) was applied and denatured at 73°C for 5 min before hybridisation at 37°C overnight. For *HER2* and chromosome 17, the *HER2* CISH pharmDx Kit, code 109 (Dako), was used and immunostaining for ER (ER SP1 Cell Marqque 33 mg/mL 1:100) and PR (PR 16 Novocastra 360 mg/mL 1:400) was done in a DakoCytomation Autostainer Plus (Dako) using Dako REAL EnVision Detection System with Peroxidase/DAB+, Rabbit/Mouse, code K5007, as previously described.[5]

Scoring and reporting

TOP2A gene copy number was evaluated under a fluorescence microscope (Nikon Eclipse 90*i*) and *HER2* gene under a bright field microscope (Nikon Eclipse 80*i*) by three of the authors (AMB, BY and MJE). A minimum of 20 non-overlapping tumour cell nuclei with signals for both chromosome and gene were counted in each case. Gene to chromosome ratios ≥2 were classified as amplification.[8–11] *TOP2A* was considered to be deleted when the gene to chromosome ratio was ≤0.8.[9 12] Cases with only one signal for both gene and chromosome in more than 75% of all nuclei were recorded as monosomy. In the analyses, deletion and monosomy were grouped together. ER and PR were classified as positive when ≥1% of the tumour cells showed positive nuclear staining.

Statistical analyses

Follow-up was from breast cancer diagnosis to death from breast cancer, death from any other cause or to December 31, 2010, whichever occurred first. BCSS was estimated using the Kaplan–Meier method, and Cox proportional hazards models were used to estimate risk of death from breast cancer. HRs were calculated with 95% CIs using Stata V.12.1 IC for Windows (Stata Corp).

RESULTS

Description of breast cancer cases

Of the 670 cases, 251 (37.5%) died of breast cancer, 314 (46.9%) died of other causes, and at the end of the observation period, 105 (15.6%) were still alive. Mean age at diagnosis was 73.1 years (SD 9.8; range 41–96 years), and median follow-up was 6.6 years (IQR 9.42 years). Histopathological grade, tumour size and molecular subtypes are given in table 1.

Amplification and deletion

Table 2 shows amplification of *TOP2A* was found in 41 cases (6.1%) and monosomy or deletion in 25 (3.7%). *HER2* was amplified in 110 cases (16.4%) and co-amplified with *TOP2A* in 32 cases (4.8%). Of the 25 cases with *TOP2A* loss, 6 were amplified for *HER2*. The majority with *TOP2A* amplification (78.1%) were co-amplified with *HER2*, whereas 34.5% of the *HER2* amplified tumours were either *TOP2A* amplified or showed *TOP2A* loss. The proportion of HR+ tumours was higher among cases with *TOP2A* amplification (75.6%) and *TOP2A* loss (68.0%) compared with *HER2* amplification (55.5%).

Table 2 Number of positive and negative cases for each marker

IHC (%)	*TOP2A* normal	*TOP2A* amplified	*TOP2A* loss	Total
HER2+	72 (11.9)	32 (78.1)	6 (24.0)	110 (16.4)
HER2−	532 (88.1)	9 (21.9)	19 (76.0)	560 (83.6)
ER+	500 (82.8)	31 (75.6)	17 (68.0)	548 (81.8)
ER−	102 (16.9)	10 (24.4)	8 (32.0)	120 (17.9)
PR+	361 (59.8)	19 (46.3)	5 (20.0)	385 (57.5)
PR−	243 (40.2)	22 (53.7)	20 (80.0)	285 (42.5)
Ki67 >15%	270 (44.7)	24 (58.5)	13 (52.0)	307 (45.8)
Ki67 >15%	333 (55.1)	17 (41.5)	12 (48.0)	362 (54.0)
CK5+	115 (19.0)	9 (21.9)	5 (20.0)	129 (19.3)
CK5−	489 (81.0)	32 (78.1)	20 (80.0)	541 (80.8)
EGFR+	46 (7.6)	1 (2.4)	3 (12.0)	50 (7.5)
EGFR−	558 (92.4)	40 (97.6)	22 (88.0)	620 (92.5)
Total	604 (90.2)	41 (6.1)	25 (3.7)	670 (100.0)

ER, oestrogen receptor; PR, progesterone receptor.

Amplification and loss according to molecular subtypes

With the exception of 5NP, *TOP2A* copy number aberrations were found in all subtypes and were associated with both HR and *HER2* status. A majority of 56.1% of *TOP2A* amplified cases were Luminal B (HER2+). Loss of *TOP2A* was found among the HR+ and *HER2* negative subtypes (Luminal A and Luminal B (HER2−)) (64.0%) or HER2 subtype (20.0%). One of four *TOP2A* deleted case was Luminal B (HER2+).

BCSS, *TOP2A* and *HER2*

The Kaplan–Meier plots in figures 2 and 3 show BCSS according to *TOP2A* and *HER2*, respectively, and in figure 4 the BCSS according to the status of both genes. Loss of *TOP2A* in the absence of *HER2* amplification did not affect BCSS. The Kaplan–Meier plots show poorest survival in *HER2*-amplified cases and *TOP2A* aberrations did not affect this.

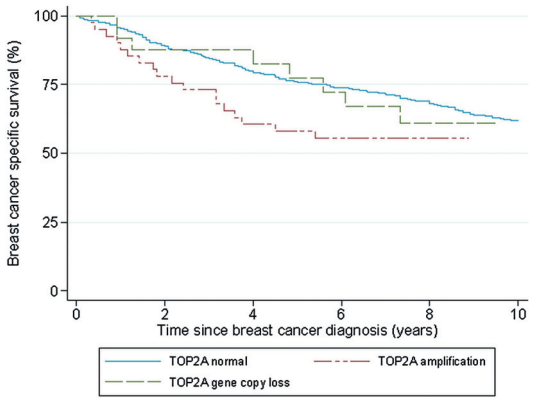

Figure 2 Kaplan–Meier plot. Breast cancer-specific survival (BCSS) according to *TOP2A*. p Value from log-rank test of differences in BCSS first 5 years after diagnosis was 0.02. After 5 years, the p value was 0.4.

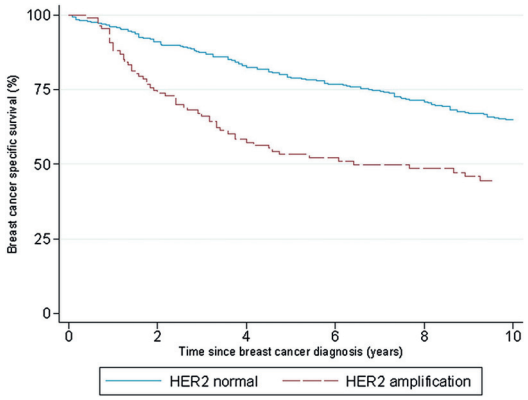

Figure 3 Kaplan–Meier plot. Breast cancer-specific survival (BCSS) according to *HER2*. p Value from log-rank test of differences in BCSS first 5 years after diagnosis was <0.0001. After 5 years, the p value was 0.9.

Risk of death from breast cancer, *TOP2A, HER2* and HR status

During the first 5 years, risk of death from breast cancer appears to be significantly higher in cases with amplification of *TOP2A* and *HER2* when analysed separately. When compared with no amplification for *TOP2A* and *HER2*, respectively, the HR for *TOP2A* amplification was 2.03 (95% CI 1.22 to 3.360) and for *HER2* was 2.77 (95% CI 1.97 to 3.89). Adjusting for age and stage did not change the results. For those who survived the first 5 years after diagnosis, there were no statistically significant differences in survival according to gene amplification status.

However, as shown in table 3 and figure 4, *TOP2A* did not exert an independent effect on prognosis. Adjusting for HR status in the Cox proportional hazards model did not change the results (data not shown). During the first 5 years after diagnosis, the risk of death from breast cancer was significantly higher for HR+ cases with *HER2* amplification irrespective of *TOP2A* status. Among the HR− cases, the numbers in each category were low and the results must be interpreted with caution.

DISCUSSION

TOP2A gene copy number change in breast cancer is an infrequent finding and its significance has been difficult to establish. In this study of 670 cases of breast cancer with long-term follow-up, the number of cases with *TOP2A* amplification or loss was far lower than the number of *HER2*-positive cases. However, there was a large proportion of co-amplification. In contrast to others who have found that amplification of one or both genes entails a poorer prognosis compared with cases with no amplification,[11 13 14] this study demonstrates that

Table 3 Risk of death from breast cancer according to *TOP2A* and *HER2* amplification

	Number of cases	Deaths from breast cancer	Hazard ratio 95% CI unadjusted		Hazard ratio 95% CI adjusted for age		Hazard ratio 95% CI adjusted for stage	
TOP2A								
Follow-up first 5 years after diagnosis	604	132	1.00		1.00		1.00	
Not amplified	41	17	2.03	1.22 to 3.36	2.07	1.24 to 3.47	2.11	1.27 to 3.50
Amplified	25	5	0.91	0.37 to 2.21	0.82	0.33 to 2.01	0.70	0.29 to 1.73
Loss	670	154						
TOP2A								
Follow-up from 5 years after diagnosis*	359	87	1.00		1.00		1.00	
Not amplified	22	5	0.75	0.30 to 1.85	0.74	0.30 to 1.86	1.02	0.41 to 2.54
Amplified	15	5	1.63	0.66 to 4.03	1.93	0.77 to 4.84	1.41	0.56 to 3.52
Loss	396	97						
HER2								
Follow-up first 5 years after diagnosis	560	105	1.00		1.00		1.00	
Not amplified	110	49	2.77	1.97 to 3.89	2.81	1.95 to 4.04	2.66	1.89 to 3.75
Amplified	670	154						
HER2								
Follow-up from 5 years after diagnosis*	346	83	1.00		1.00		1.00	
Not amplified	50	14	0.95	0.54 to 1.67	0.95	0.52 to 1.73	1.04	0.60 to 1.86
Amplified	396	97						
HER2 and TOP2A								
Follow-up first 5 years after diagnosis	532	100	1.00		1.00		1.00	
Normal *TOP2A* and *HER2*	38	17	2.61	1.56 to 4.36	2.76	1.63 to 4.69	2.68	1.60 to 4.51
TOP2A change and *HER2* amplification	28	5	0.96	0.39 to 2.37	0.89	0.36 to 2.21	0.77	0.31 to 1.90
TOP2A change and *HER2* normal	72	32	2.86	1.92 to 4.26	2.81	1.84 to 4.29	2.59	1.74 to 3.87
Amplified *HER2, TOP2A* normal	670	154						
HER2 and TOP2A								
Follow-up from 5 years after diagnosis*	328	79	1.00		1.00		1.00	
Normal *TOP2A* and *HER2*	19	6	0.99	0.43 to 2.28	0.97	0.41 to 2.28	1.44	0.62 to 3.37
TOP2A change and *HER2* amplification	18	4	1.07	0.39 to 2.94	1.26	0.45 to 3.50	0.91	0.33 to 2.51
TOP2A change and *HER2* normal	31	8	0.92	0.45 to 1.91	0.95	0.44 to 2.04	0.84	0.40 to 1.79
Amplified *HER2, TOP2A* normal	396	97						

*Conditional on surviving the first 5 years CI.

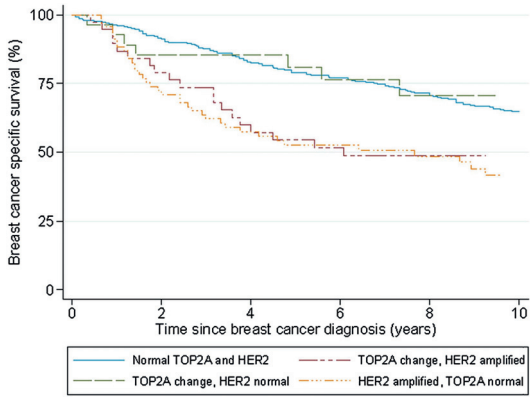

Figure 4 Kaplan–Meier plot. Breast cancer-specific survival (BCSS) according to *TOP2A* and *HER2*. p Value from log-rank test of differences in BCSS first 5 years after diagnosis was <0.0001. After 5 years, the p value was 1.0.

associations between BCSS and *TOP2A* copy number change are not independent of *HER2* and HR status.

The most important finding in this study is the strong association between *TOP2A* copy number change and HR and *HER2* status. These markers are well established as prognostic and predictive factors, and are to a high degree decisive for treatment after surgery. To the best of our knowledge, few studies have been designed to examine the prognostic value of *TOP2A*, though it has been shown that *TOP2A* amplification affects BCSS and risk of death from breast cancer[15] and that *TOP2A* may be a prognostic marker in ER+ breast cancer.[14 16] However, when the analyses include HR and *HER2* status, the present study shows that *TOP2A* has no independent prognostic impact. *TOP2A* may still have some modulating effects on prognostication, but this is probably of limited benefit in clinical practice.

Twenty of twenty-five cases with *TOP2A* loss were PR−, and of these, 12 were ER+. PR negativeness is a predictor of poor prognosis and appears to be associated with *TOP2A* loss. However, in this study, survival tended to be better in PR− cases with loss of *TOP2A* compared with cases with normal or amplified *TOP2A* (data not shown).

The proportion of amplification and co-amplification of *TOP2A* and *HER2* in breast cancer varies between studies. *HER2* amplification is reported to be around 15%.[2] For *TOP2A*, amplification varies from 5% to 19%.[3 17 18] In *HER2*-positive breast cancer, amplification of *TOP2A* varies from 25% to 42%.[1 19] Both amplification and deletion of *TOP2A* in the absence of *HER2* amplification have been demonstrated.[3 20] In the present study, 29.1% of the *HER2*-amplified cases were co-amplified with *TOP2A*. The proportion of *TOP2A* positive tumours in this study was lower than in other studies.[2] However, the frequency of *HER2* amplification is comparable with others, and this weighs against methodological problems. Furthermore, a short DNA probe for *TOP2A* was used to avoid overlap with *HER2*.[21] This may in part account for the low number of *TOP2A*-amplified cases in this study compared with previous studies and may reflect the true frequency of this finding.

Assessment of loss should be carried out with caution in histopathological sections because nuclear truncation may lead to a falsely low estimation of copy number. The cut-off for amplification is usually set at a gene/chromosome ratio of ≥2.0, and for deletion the cut-off level ranges from 0.5 to 1.0.[21] It is possible that monosomy may have an impact similar to loss of individual genes, but this is uncertain. In this study, only four cases showed deletion and monosomy and deletion were grouped together.

HER2-positive breast cancer has been shown to be more aggressive than *HER2*–negative breast cancer. Co-amplification with other genes, such as *STARD3* and *GRB7*, may contribute to and possibly strengthen this aggressive behaviour.[2] The proportion of amplification and co-amplification of *TOP2A* and *HER2* in breast cancer is low, and even in a series of 670 patients, the numbers are too low to draw reliable conclusions. As a prognostic marker, *TOP2A* is probably of limited value. *TOP2A* aberrations are strongly associated with HR and *HER2* status, and the importance of these markers in prognostication is still unchallenged.

Take-home messages

- *TOP2A* gene copy number change is an infrequent finding in breast cancer.
- There is a strong association between *TOP2A* copy number change and hormone receptor and *HER2* status.
- As a prognostic marker, *TOP2A* is probably of limited value, and hormone receptor and *HER2* status remain unchallenged.

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Competing interests None.

Ethics approval Approval of the study and dispensation from the requirement of patient consent was granted by the Regional Committee for Medical and Health Sciences Research Ethics (REK, Midt-Norge, ref. nr: 836/2009).

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Paper III

Engstrøm Monica J., Opdahl Signe, Vatten Lars J., Haugen Olav A., Bofin Anna M.. Invasive lobular breast cancer: The prognostic impact of histopathological grade, E-cadherin and molecular subtypes. Histopathology. 2015 Feb; 66(3):409-19

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Invasive lobular breast cancer: the prognostic impact of histopathological grade, E-cadherin and molecular subtypes

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Invasive lobular breast cancer: the prognostic impact of histopathological grade, E-cadherin and molecular subtypes

Aims: The aim of this study was to compare breast cancer specific survival (BCSS) for invasive lobular carcinoma (ILC) and invasive ductal carcinoma (IDC) and, further, to evaluate critically the prognostic value of histopathological grading of ILC and examine E-cadherin as a prognostic marker in ILC.

Methods and results: The study comprised 116 lobular and 611 ductal breast carcinomas occurring between 1961 and 2008. All cases had been classified previously according to histopathological type and grade, stained for oestrogen receptor (ER), progesterone receptor (PR), antigen Ki67 (Ki67), epithelial growth factor receptor (EGFR), cytokeratin 5 (CK5) and human epidermal growth factor receptor 2 (HER2) and classified into molecular subtypes. For the present study, immunohistochemical staining for E-cadherin was performed. The Kaplan–Meier method and Cox proportional hazards models were used in the analyses. Grade 2 tumours comprised 85.3% of the lobular tumours and 51.9% of the ductal tumours. BCSS in ILC grade 2 was comparable to that of IDC grade 3. E-cadherin-negative ILC had a poorer prognosis compared to E-cadherin positive ILC and to IDC regardless of E-cadherin status.

Conclusions: The implication of histopathological grading may differ in ILC compared to IDC. E-cadherin may be useful in prognostication in ILC and thereby influence the determination of treatment strategies for this group of women.

Keywords: breast cancer, breast cancer-specific survival, E-cadherin, histopathological grade, invasive lobular carcinoma, prognosis

Introduction

Invasive lobular carcinoma (ILC) is defined as an invasive carcinoma comprising non-cohesive cells dispersed individually in a single-file linear pattern in a fibrous stroma and accounts for 5–15% of breast cancers.[1–3] A number of variants of ILC do not show the classical morphological pattern, but loss of cell-to-cell cohesion is a common feature.[3]

Histopathological grade is an important prognostic tool.[4–6] The Nottingham grading system classifies patients into groups with different prognoses.[7] However, in ILC the suitability of grading is uncertain.[8,9] Glandular structures are absent, mitoses are infrequent and the nuclei uniform. Thus, most ILCs are grade 2 and the prognostic value of grading is unclear.

Breast cancer treatment guidelines are based on hormone receptor, human epidermal growth factor receptor 2 (HER2) and proliferation (Ki67) status, in

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addition to histopathological grade, tumour size and lymph node status.[10] Histopathological type is not always included as a parameter in treatment guidelines, although favourable types may influence the choice of treatment.

E-cadherin (E-cad) is a transmembrane protein involved in cell-to-cell adhesion, and its loss promotes invasion and metastasis.[11] Loss of E-cad is common in ILC,[11,12] and supports the diagnosis of ILC.[13] Although it has been suggested that low levels of E-cad are associated with poorer prognosis,[14–16] its potential as a prognostic marker in ILC has not been clarified.

The aims of this study were to compare breast cancer-specific survival (BCSS) in ILC with invasive ductal carcinoma (IDC) in a cohort of breast cancer patients with a long follow-up, to assess the prognostic value of histopathological grading of ILC and to examine the potential of E-cad as a prognostic marker in ILC.

Material and methods

STUDY POPULATION

Between 1956 and 1959, women from Nord Trøndelag County in Norway were invited by the Norwegian Cancer Registry to participate in a breast cancer survey. The population has been described previously.[17,18] Briefly, 25 897 women, born between 1886 and 1928, were invited. From 1961 to 2008, 1393 women developed breast cancer. Cases occurring prior to 1961 were excluded. A total of 945 tissue samples were available at the Department of Pathology and Medical Genetics, St Olav's Hospital, Trondheim, Norway, and 867 were suitable for inclusion in tissue microarrays (TMA). After linkage with the Cause of Death Registry of Norway and the Norwegian Cancer Registry, survival data were generated. Only cases of IDC of no special type and ILC (727 cases) were included in the present study.

SPECIMEN CHARACTERISTICS

All cases were classified into histopathological type and grade and reclassified into molecular subtypes using surrogate markers for gene expression analyses (Figure 1).[17] Histopathological typing and grading was performed independently on full-face sections by two experienced pathologists (O.A.H., A.M.B.).[3,5,19] Three 1-mm tissue cores from the periphery of each tumour were selected and assembled in TMAs. Immunohistochemical (IHC) staining was performed for

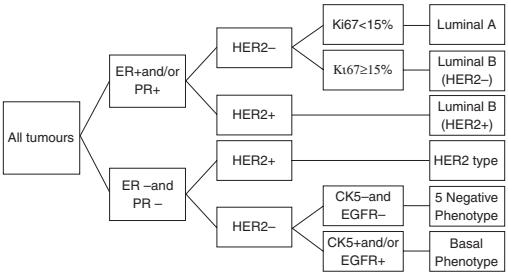

Figure 1. Classification algorithm for molecular subtyping.[17]

oestrogen receptor (ER), progesterone receptor (PR), antigen Ki67 (Ki67), HER2, cytokeratin 5 (CK5) and epithelial growth factor receptor 1 (EGFR). *HER2* gene amplification status was estimated using chromogenic *in-situ* hybridization (CISH). For the present study, IHC staining was performed for E-cad.

ASSAY METHODS

Assay methods for all markers except E-cad have been described in detail previously.[17] For the present study, IHC for detection of E-cad was performed according to the manufacturer's guidelines (Dako, Glostrup, Denmark). The sections were mounted on Superfrost+ glass slides, dried at 37°C overnight and stored at −20°C. Before staining, the slides were heated to 60°C for 2 h and pretreated in a PT Link pretreatment module for tissue specimens (Dako) with buffer (high pH target retrieval solution K8004) at 97°C for 20 min. Monoclonal mouse antibody (clone NCH-38), 55.2 mg/l dilution 1:100, was applied. For visualization, the Dako REAL™ EnVision™ detection system was used with peroxidase/diaminobenzidine (DAB)+, rabbit/mouse, code K5007.

SCORING AND REPORTING

The REMARK reporting recommendations for tumour marker studies were followed.[20] All IHC evaluations were performed independently by two researchers. ER and PR were positive if ≥1% of the tumour cells showed positive nuclear staining. For Ki67, ≥15% stained nuclei was classified as $Ki67^{high}$ and <15% as $Ki67^{low}$. A staining index (SI) (intensity × proportion) was calculated for CK5 and EGFR; SI of 0–1 was considered to be negative and 2–9 was considered to be positive, as described previously. *HER2* gene amplification was defined as gene to chromosome ratio ≥2. In cases where CISH failed, +3 IHC staining for HER2 was recorded as positive.[17] In the

present study, only moderate or strong continuous membrane staining for E-cad in >50% of tumour cells were classified as positive. There were very few cases with aberrant staining (cytoplasmic staining or intermittent membranous staining), and these were classified as negative.

STATISTICAL ANALYSES

Follow-up was from date of diagnosis until death or 31 December 2010. Kaplan–Meier methods were used to estimate BCSS for ILC grade 2 compared to IDC grades 1, 2 and 3, and for comparing survival of ILC and IDC grade 2, E-cad$^+$ and E-cad$^-$ tumours. Grade 2 ILC and IDC were compared for each of the following biomarker categories separately: ER+, Ki67^{low} and HER2$^-$. Comparison was made between ILC and IDC grade 2 tumours with the favourable biomarker profile (ER$^+$ and HER2$^-$ and Ki67^{low}). BCSS for luminal A and luminal B (HER2$^-$) subtypes were compared for ILC and IDC separately. The log-rank test was used to compare survival curves, $P < 0.05$ was considered statistically significant. Cox proportional hazards models were used to estimate relative risks of death from breast cancer adjusted for age (5-year intervals), stage at diagnosis (I, II, III, IV, unknown) and time-period of diagnosis. Hazard ratios (HR) for ILC compared to IDC were calculated with 95% confidence intervals (CI). The numbers of cases of ILC grades 1 and 3 were too low for reliable analyses of grade and BCSS in ILC. The number of cases with an unfavourable biomarker profile (ER$^-$, HER2$^+$ and Ki67^{high}) was too small for separate analysis ($n = 39$). Statistical analyses were performed using Stata version 12.1 (Stata Corp., College Station, TX, USA).

ETHICS

Approval was granted by the Regional Committee for Medical and Health Sciences Research Ethics, including dispensation from the requirement of patient consent (REK, Midt-Norge, ref. no. 836/2009).

Results

DESCRIPTION OF THE POPULATION

Of the 727 cases, 16% were ILC and 84% were IDC (Table 1). During follow-up, 297 (40.9%) died from breast cancer and 304 (41.8%) died of other causes. At the end of the period, 126 (17.3%) were still alive. Mean age at diagnosis was 71.3 years for IDC and 73.3 years for ILC. Table 2 shows the treatments given.

TUMOUR CHARACTERISTICS

Histopathological grade, tumour size, lymph node status, stage and molecular subtypes are given in Table 1. Table 3 shows the results of IHC and CISH. The proportion of histopathological grade 2 tumours was higher in ILC (85.3%) compared to IDC (51.9%). In ILC 87.9% were ER$^+$ and 6.0% were HER2$^+$, compared to 83.6% ER$^+$ and 16.9% HER2$^+$ in IDC. A higher proportion of ILC (16.4%) than IDC (7.5%) were >5 cm. However, the proportions of tumours between 2 and 5 cm were similar (42.2 versus 45.5%).

GRADE, TYPE AND PROGNOSIS

Figure 2 shows BCSS for ILC grade 2 compared to IDC grades 1, 2 and 3. ILC grade 2 had poorer BCSS compared to IDC grade 2 ($P = 0.01$, log-rank test).- There was no significant difference in BCSS between ILC grade 2 and IDC grade 3 ($P = 0.48$, log-rank test). Table 4 shows the risk of death from breast cancer according to type. ILC grade 2 was compared to IDC grades 1, 2 and 3 separately. HRs were similar for ILC grade 2 and IDC grade 3, whereas IDC grade 2 had a significantly better survival than ILC grade 2 (HR: 0.66, 95% CI: 0.46–0.94). Adjustment for age, stage and time of diagnosis did not influence the results.

PROGNOSTIC VALUE OF TYPE IN ER$^+$, HER2$^-$ AND KI67^{LOW} TUMOURS

Table 5 shows risk of death from breast cancer according to type among patients with grade 2 tumours and clinically favourable biomarker profiles. For each marker status (ER$^+$, HER2$^-$, Ki67^{low}), respectively, there was a significantly higher risk of death from ILC compared to IDC. Similarly, risk of death from breast cancer for patients with grade 2 tumours expressing a complete favourable biomarker profile (ER$^+$, HER2$^-$ and Ki67^{low}) was higher for ILC than for IDC (HR: 2.16, 95% CI: 1.34–3.49). Analysis of all grades did not alter the results (data not shown).

PROGNOSTIC VALUE OF MOLECULAR SUBTYPES

The proportions of HER2$^+$ and/or ER$^-$ ILC were low compared to IDC, as reflected in the distribution

Table 1. Summary of patient and tumour characteristics

Patient and tumour characteristics	Ductal	Lobular	Total
Number (%)	611 (84.0)	116 (16.0)	727 (100.0)
Number of breast cancer deaths (%)	246 (40.3)	51 (44.0)	297 (40.9)
Mean age at diagnosis (SD)	71.3 (10.7)	73.3 (9.1)	71.7 (10.5)
Median years of follow-up after diagnosis (IQR)	7.2 (10.6)	4.8 (7.9)	6.8 (10.4)
Tumour grade (%)			
1	61 (10.0)	9 (7.8)	70 (9.6)
2	317 (51.9)	99 (85.3)	416 (57.2)
3	233 (38.1)	8 (6.9)	241 (33.2)
Tumour size (%)			
≤2 cm	182 (29.8)	20 (17.2)	202 (27.8)
>2 cm, ≤5 cm	221 (36.2)	43 (37.1)	264 (36.3)
>5 cm	46 (7.5)	19 (16.4)	65 (8.9)
Uncertain	162 (26.1)	34 (29.3)	196 (27.0)
Lymph node status			
No metastasis	234 (38.3)	45 (38.8)	279 (38.4)
Metastasis detected	236 (38.6)	38 (32.8)	274 (37.7)
Not examined for metastasis	141 (23.1)	33 (28.4)	174 (23.9)
Stage at diagnosis			
Stage I	294 (48.1)	52 (44.8)	346 (47.6)
Stage II	246 (40.3)	49 (42.2)	295 (40.6)
Stage III	37 (6.1)	11 (9.5)	48 (6.6)
Stage IV	29 (4.8)	4 (3.5)	33 (4.5)
Stage uncertain	5 (0.8)	0	5 (0.7)
Molecular subtypes (%)			
Luminal A	290 (47.5)	63 (54.3)	353 (48.6)
Luminal B ($HER2^-$)	170 (27.8)	33 (28.5)	203 (27.9)
Luminal B ($HER2^+$)	54 (8.8)	6 (5.2)	60 (8.3)
HER2 type	49 (8.0)	1 (0.9)	50 (6.9)
Five negative phenotype	13 (2.1)	11 (9.5)	24 (3.3)
Basal phenotype	35 (5.7)	2 (1.7)	37 (5.1)

SD, standard deviation; IQR, interquartile range; HER2, human epidermal growth factor receptor 2.

of molecular subtypes (Table 1). Among 353 luminal A cases, 290 (82.2%) were ductal and 63 (17.8%) were lobular. Figure 3 shows that luminal A ILC had a poorer prognosis than luminal A IDC ($P = 0.02$, log-rank test). Luminal B ($HER2^-$) IDC had a slightly better prognosis than luminal A and luminal B ($HER2^-$) ILC ($P = 0.39$, log-rank test). Table 6 shows that risk of death from grade 2

Table 2. Summary of breast cancer therapies for all cases

	Invasive ductal carcinoma *n* = 611 (%)	Invasive lobular carcinoma *n* = 116 (%)	Total *n* = 727 (%)
Mastectomy	524 (85.8)	94 (81.0)	618 (85.0)
Breast conserving therapy	61 (10.0)	12 (10.4)	73 (10.0)
Only biopsy, no surgical treatment	26 (4.3)	10 (8.6)	36 (5.0)
Axillary surgery (clearance or sentinel node)	461 (75.5)	81 (69.9)	542 (74.6)
Hormone therapy*	134 (26.2**)	31 (30.4**)	165 (26.9**)
Trastuzumab	0	0	0
Chemotherapy	Unknown	Unknown	Unknown
Radiation	Unknown	Unknown	Unknown

*Estimated according to guidelines at diagnosis; **% of the hormone receptor-positive cases.

Table 3. Results of immunohistochemical and *in-situ* hybridization markers

	Ductal (*n* = 611)	Lobular (*n* = 116)	Total (*n* = 727)
ER^+	511 (83.6)	102 (87.9)	613 (84.3)
ER^-	98 (16.0)	14 (12.1)	112 (15.4)
Not possible to interpret	2 (0.3)	0	2 (0.3)
PR^+	364 (59.6)	58 (50.0)	422 (58.1)
PR^-	246 (40.3)	58 (50.0)	304 (41.8)
Not possible to interpret	1 (0.2)	0	1 (0.1)
$HER2^+$	103 (16.9)	7 (6.0)	110 (15.1)
$HER2^-$	508 (83.1)	109 (94.0)	617 (84.9)
$Ki67^{high}$	280 (45.8)	39 (33.6)	319 (43.9)
$Ki67^{low}$	330 (54.0)	77 (66.4)	407 (56.0)
Not possible to interpret	1 (0.2)	0	1 (0.1)
$CK5^+$	120 (19.6)	4 (3.5)	124 (17.1)
$CK5^-$	491 (80.4)	112 (96.6)	603 (82.9)
$EGFR^+$	41 (6.7)	3 (2.6)	44 (6.1)
$EGFR^-$	570 (93.3)	113 (97.4)	683 (93.9)
$E\text{-}cad^+$	523 (85.6)	27 (23.3)	550 (75.7)
$E\text{-}cad^-$	69 (11.3)	86 (74.1)	155 (21.3)
Not possible to interpret	19 (3.1)	3 (2.6)	22 (3.0)

EGFR, epithelial growth factor receptor; ER, oestrogen receptor; PR, progesterone receptor.

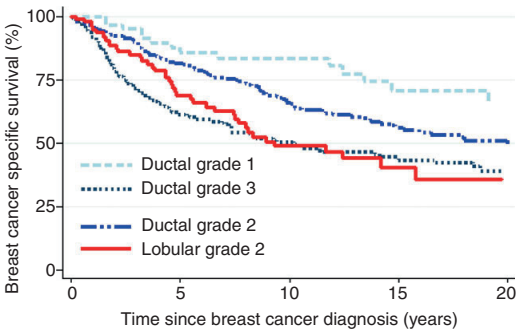

Figure 2. Breast cancer specific survival for invasive lobular carcinoma grade 2 compared to ductal carcinoma grades 1, 2 and 3. *P*-value from log-rank test of differences in breast cancer specific survival (BCSS) was 0.01.

breast cancer was higher for luminal A ILC, luminal B (HER2$^-$) ILC and luminal B (HER2$^-$) IDC compared to luminal A IDC. The difference between luminal A IDC and ILC was statistically significant. The numbers in the other subtypes were too low for analysis.

PROGNOSTIC VALUE OF E-CADHERIN

Table 3 shows that 23.3% of ILC were E-cad$^+$. Figure 4 shows BCSS for grade 2 E-cad$^+$ and E-cad$^-$ ILC and IDC. E-cad$^-$ ILC had poorer prognosis than E-cad$^+$ ILC ($P = 0.005$, log-rank test). Figure 5 shows examples of E-cad IHC staining. Table 7 shows that risk of death from breast cancer for ILC E-cad$^-$ was nearly twofold (HR: 1.96, 95% CI: 1.32–2.89) compared to IDC E-cad$^+$. There was no clear difference in prognosis between IDC E-cad$^+$, IDC E-cad$^-$ and ILC E-cad$^+$. Adjustment for age, stage and time-period did not influence the results.

Discussion

The main finding in this study of a cohort of breast cancer patients with long-term follow-up was a significantly poorer prognosis for grade 2 ILC compared to grade 2 IDC. The prognosis for grade 2 ILC was comparable to that of grade 3 IDC. A similar pattern was observed when the analyses were restricted to tumours with positive prognostic marker profiles (ER$^+$, HER2$^-$ and Ki67^{low}). Furthermore, E-cad expression appeared to be a favourable prognostic marker in ILC.

In the Nottingham grading system gland formation, nuclear atypia/pleomorphism and mitosis counts are considered.[5] However, because the morphological features of ILC differ from IDC, grade may have a different prognostic significance.[8,21] This is an important discussion, because histopathological grade is one of several factors determining adjuvant therapy, whereas type is disregarded.

In agreement with others,[1,21,22] there were few ILCs of grade 1 (7.8%) and grade 3 (6.9%) in this study, and the low numbers preclude survival analyses. Histopathological grading has been shown to be of independent prognostic value in ILC.[23] However, the implications of grading in ILC may differ from IDC and its value as a prognostic tool must be considered in this light, particularly when determining treatment strategies.

ER, HER2 and Ki67 are important prognostic and/or predictive markers. In this study, the proportion of ILCs with a favourable marker profile was higher

Table 4. Risk of death from breast cancer. Invasive lobular carcinoma grade 2 compared to invasive ductal carcinoma grades 1, 2 and 3

			HR	95%CI	HR	95%CI	HR	95%CI	HR	95%CI
Tumour characteristics	Number of cases	Deaths from breast cancer	Unadjusted		Adjusted for age		Adjusted for stage		Adjusted for time period of diagnosis (10-year intervals)	
Lobular grade 2	99	42	1.00		1.00		1.00		1.00	
Ductal grade 1	61	17	0.43	0.24–0.75	0.47	0.27–0.84	0.49	0.28–0.87	0.40	0.23–0.71
Ductal grade 2	317	114	0.66	0.46–0.94	0.67	0.47–0.95	0.59	0.41–0.85	0.66	0.46–0.94
Ductal grade 3	233	115	1.10	0.77–1.56	1.13	0.79–1.61	1.10	0.77–1.57	1.03	0.72–1.47
	710	297								

HR, hazard ratio; CI, confidence interval.

Table 5. Risk of death from invasive lobular grade 2 compared to invasive ductal carcinoma grade 2

Tumour characteristics	Number of cases	Deaths from breast cancer	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
			Unadjusted		Adjusted for age		Adjusted for stage		Adjusted for time period of diagnosis (10-year intervals)	
ER^+										
Ductal	297	100	1.00		1.00		1.00		1.00	
Lobular	88	37	1.71	1.17–2.50	1.68	1.14–2.47	1.97	1.33–2.91	1.82	1.24–2.68
	385	137								
$Ki67^{low}$										
Ductal	224	71	1.00		1.00		1.00		1.00	
Lobular	70	30	2.01	1.31–3.01	1.95	1.26–3.03	2.20	1.42–3.43	2.03	1.31–3.14
	294	101								
$HER2^-$										
Ductal	287	97	1.00		1.00		1.00		1.00	
Lobular	93	39	1.76	1.21–2.56	1.74	1.19–2.55	1.98 1.30–2.90		1.22–2.60	1.78 1.22–2.60
	380	136								
ER^+, $Ki67^{low}$ and $HER2^-$										
Ductal	201	61	1.00		1.00		1.00		1.00	
Lobular	56	24	2.16	1.34–3.49	2.04	1.25–3.34	2.45	1.50–4.01	2.31	1.42–3.76
	257	85								

HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2.

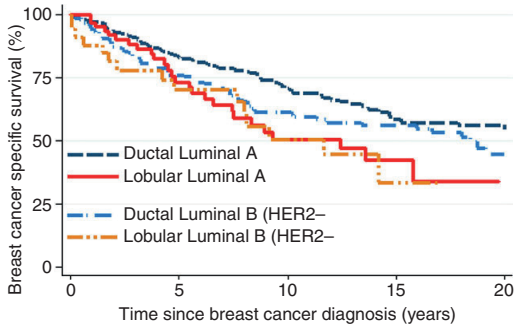

Figure 3. Breast cancer specific survival for invasive lobular and ductal carcinoma grade 2 according to luminal A and luminal B [human epidermal growth factor receptor 2 (HER2)] subtypes. *P*-value from log-rank test of differences in breast cancer specific survival (BCSS) was 0.02.

compared to IDC, implying a better prognosis for ILC. However, even when restricting analyses to cases with favourable marker profiles, a significantly poorer prognosis was found in ILC compared to IDC. $HER2^+$ cases in ILC were few (Table 2), thus limiting its utility as a prognostic marker in ILC. Better prognostic markers for ILC are required.

In this study, $E\text{-cad}^+$ grade 2 ILC was prognostically comparable to grade 2 IDC (both $E\text{-cad}^+$ and $E\text{-cad}^-$). E-cad- ILC had a poorer prognosis. The identification of patients with ILC of expected poor prognosis may have implications when determining adjuvant therapy. If the prognostic utility of E-cad for ILC is confirmed in future studies and robust guidelines for interpretation of E-cad IHC are developed,[14,15] this could extend the use of a well-known marker for the benefit of a substantial proportion of breast cancer patients.

The loss of E-cad expression is shown to promote invasion and metastasis of epithelial cancers, including breast cancer.[24] E-cad may be involved in other cellular processes of importance as a tumour suppressor gene.[25] Cell-to-cell adhesion involves cytoplasmic catenins and the actin cytoskeleton in addition to

Table 6. Risk of death from invasive lobular carcinoma grade 2 and invasive ductal carcinoma grade 2 according to luminal A and luminal B (HER2$^-$) subtypes

			HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
	Number of cases	Deaths from breast cancer	Unadjusted		Adjusted for age		Adjusted for stage		Adjusted for time-period of diagnosis (10-year intervals)	
Ductal luminal A	203	62	1.00		1.00		1.00		1.00	
Ductal luminal B (HER2$^-$)	74	29	1.48	0.95–2.31	1.55	0.99–2.42	1.70	1.09–2.67	1.36	0.87–2.12
Lobular luminal A	56	24	2.11	1.31–3.39	2.08	1.28–3.38	2.53	1.55–4.12	2.21	1.36–3.57
Lobular luminal B (HER2$^-$)	26	10	1.78	0.91–3.48	1.81	0.92–3.57	2.10	1.07–4.14	1.74	0.88–3.41
	359	125								

HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2.

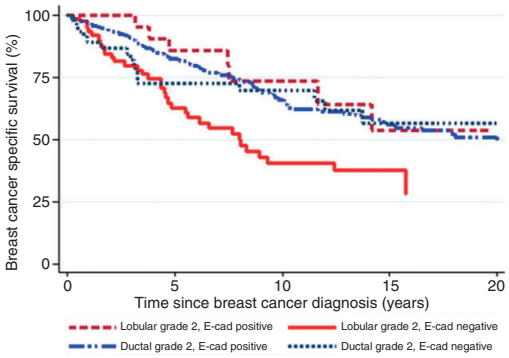

Figure 4. Breast cancer specific survival for for invasive lobular and ductal carcinoma grade 2 according to E-cadherin status. *P*-value from log-rank test of differences in breast cancer specific survival (BCSS) was 0.005.

E-cad, and these mechanisms are complex.[26] Loss of tumour suppressor function and impaired cell-to-cell adhesion, both of which are dependent in part on E-cad, underline the importance of this molecule in breast cancer.

The proportion of E-cad$^+$ ILC reported varies from 0 to 20%.[27–29] In this study, where histopathological typing was based on morphology only, 23.3% were E-cad$^+$. No cases were revised according to histopathological type in light of E-cad status. Mixed lobular and ductal carcinomas are not infrequent.[3] In this study, mixed tumours were classified as ductal.[27,30,31]

Molecular subtyping is based mainly on studies of IDC.[32] IDC is the most common histopathological type, although type is rarely mentioned.[33–35] For other types, the prognostic value of molecular subtyping remains uncertain. In this study, there were too few ILCs in the non-luminal and HER2 subtypes for reliable results. However, the differences in BCSS in the HER2$^-$ luminal subtypes between ILC and IDC are comparable to the results of the biomarker analyses. Considered together, the results confirm that histopathological type has an independent impact in the prognostication of ILC.

The main strength of this study is the historical nature of the patient cohort enabling complete long-term follow-up. The vast majority of women in this study developed breast cancer in an era prior to the use of hormonal contraception, menopausal hormonal therapy (MHT) and mammography screening, and did not qualify for new therapies as they were introduced, thus enabling insight into the near-natural course of this disease. A drawback is the relative high age of the women, and should be considered when interpreting the results. Others have shown better,[36] similar[2,37] or poorer[38,39] prognosis for ILC compared to IDC. Differences in patient populations, follow-up and adjuvant therapy may explain these inconsistencies. Some studies have shown an increased risk of ILC when using MHT.[40–42] It is unclear whether or not there are differences in prognosis between MHT-associated ILC and ILC in non-users.[43] The majority of cancers in the present study were diagnosed in a time-period or at an age when MHT was rarely used.

In this study, 99 of 116 ILCs were histopathological grade 2. The numbers of grades 1 and 3 were low, and this can be attributed to the morphological features of ILC. This impairs grading as a prognostic

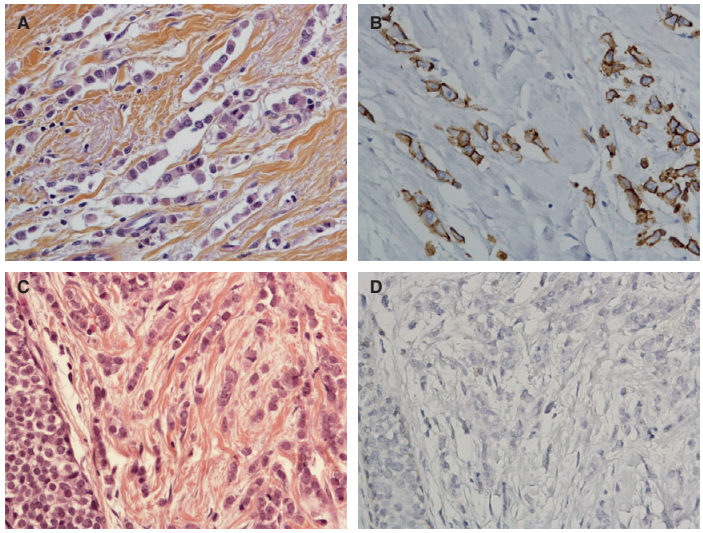

Figure 5. Invasive lobular carcinoma (ILC): **A**, ILC stained wih Haematoxylin–erythrosin–saffron (HES). **B**, Same case as **A**. Positive membrane staining for E-cadherin. **C**, ILC HES. **D**, Same case as **C**. No membrane staining for E-cadherin.

Table 7. Risk of death from invasive lobular carcinoma grade 2 and invasive ductal carcinoma grade 2 according to E-cadherin status

			HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI
	Number of cases	Deaths from breast cancer	Unadjusted		Adjusted for age		Adjusted for stage		Adjusted for time period of diagnosis (10-year intervals)	
Ductal, E-cad$^+$	260	94	1.00		1.00		1.00		1.00	
Ductal, E-cad$^-$	46	16	1.03	0.61–1.75	1.00	0.59–1.71	1.17	0.68–2.00	1.03	0.60–1.76
Lobular, E-cad$^+$	24	7	0.84	0.39–1.81	0.86	0.40–1.88	0.87	0.40–1.89	0.83	0.38–1.79
Lobular, E-cad$^-$	74	35	1.96	1.32–2.89	1.88	1.27–2.80	2.30	1.54–3.44	2.03	1.36–3.01
	404	152								

HR, hazard ratio; CI, confidence interval.

tool in ILC. Similarly, the prognostic value of HER2 in ILC may be limited due to the low number of ILCs expressing HER2. However, grade 2 ILC had a consistently poorer prognosis when compared to grade 2 IDC, and the differences were also apparent when the analyses included only tumours with presumed favourable biomarkers. Due to the low number of lobular tumours in our study, we did not have sufficient statistical power to investigate the prognostic value of an unfavourable biomarker profile within lobular cancers. The present study supports the claim that lobular lesions are a distinct family of neoplastic lesions in the breast.[12] The role of E-cad in ILC may not only be in the determination of histopathological type; it may also be more useful than grade in prognostication and in the determination of treatment.

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Conflicts of interest

The authors declare that they have no competing interests.

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