Doctoral theses at NTNU, 2024:80

Anette H.Skjervold

Established and novel methods and biomarkers in breast cancer

Doctoral thesis

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Norwegian University of Science and Technology
Thesis for the Degree of
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ISBN 978-82-326-7754-2 (printed ver.)
ISBN 978-82-326-7753-5 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

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Printed by NTNU Grafisk senter

Etablerte og nye metoder og biomarkører innen brystkreft

Nytt blikk på metodikk rundt etablerte biomarkører, og vurdering av en mulig ny biomarkør

Biomarkører er målbare karakteristiske biologiske egenskaper som kan fortelle oss mye om vår helse generelt og ved ulike sykdommer. Velkjente eksempler er blodtrykk og blodprosent. Biomarkører spiller en viktig rolle i diagnostikk og behandling av brystkreft hvor vi måler egenskaper i selve svulsten. Blant disse er østrogen reseptor (ER) og Ki-67 (en markør som måler cellenes evne til dele seg). Kriteriene for hva som kjennetegner positivt uttrykk og deretter bestemmer videre behandling for pasienten er fortsatt en utfordring med disse biomarkørene. I denne avhandlingen ønsket vi å se nærmere på disse etablerte biomarkørene med nytt blikk og nye metoder. Ki-67 er en medvirkende faktor for å bestemme om pasienten skal få kjemoterapi eller ikke, og uttrykk av ER bestemmer om pasienten skal få hormon-blokkerende terapi. I tillegg studerte vi en mindre kjent biomarkør, genet *PAK1*.

I dag er det fortsatt diskusjon rundt de diagnostiske kriteriene for positivt Ki-67. Vurdering av Ki-67 påvirkes av mange faktorer, blant annet hvordan prøvene er behandlet ved ulike laboratorier i forkant av vurderingen, eller hvem som studerer og setter resultatet. Vi ønsket derfor å se om digital bildeanalyse kunne være en bedre metode for å standardisere tolkningen. Vi sammenlignet undersøkelse i mikroskop med digital bildeanalyse av de samme svulstene.

Vi fant at ved vurdering av Ki-67 ved hjelp av digital bildeanalyse kunne vi identifisere en større andel svulster med høye nivåer av Ki-67 sammenlignet med vurdering i mikroskop. Vi understreker også viktigheten av at diagnostiske grenseverdier bør kalibreres basert på hvilken metode som brukes.

For ER ligger nåværende diagnostisk grenseverdi ved 1% positive cellekjerner i svulsten, og dette avgjøres ved analyse i mikroskop uten nøyaktig telling, såkalt «eyeballing». Kriteriene for positiv ER ble i 2010 senket fra 10% til 1%. Var denne beslutningen bedre eller dårligere for gruppen med ER mellom 1 og 9% («ER Low Positive»)? Vi ønsket å se nærmere på de ulike nivåene av ER positivitet, og sammenlignet dem med andre kjennetegn i svulstene og fremtidsutsikter, med spesielt blikk på «ER Low Positive»-gruppen. Vi studerte 1955 svulster som vi delte i to grupper basert på pasientenes diagnosetidspunkt (før 1995, eller i 1995 eller senere). Rundt 1995 begynte kvinner med brystkreft å få medisinsk behandling i tillegg til operasjon. Vi fant at kvinner med «ER Low Positive» svulster

diagnostisert i 1995 eller senere hadde mindre aggressive svulster enn svulster tilhørende kvinner diagnostisert før 1995. Videre fant vi at kvinner diagnostisert i 1995 eller senere, hadde like fremtidsutsikter som de med høyt uttrykk av ER, sammenlignet med kvinner diagnostisert tidligere.

I en tid hvor søkelyset rettes mot individuell medisin og behandling, spiller biomarkører en viktig rolle. Det letes stadig etter nye som kan være avgjørende for den enkelte pasientens behandling. Genuttrykk av *PAK1* er økt i flere krefttyper, også brystkreft, og er lokalisert på en del av kromosom 11 som har flere gener assosiert med brystkreft. Økt antall *PAK1* gener har sammenheng med aggressive svulsttyper, resistens mot behandling og dårligere leveutsikter. Vi ville undersøke dette i brystkreft. I tillegg sammenlignet vi *PAK1* og *CCND1* genet, som også er lokalisert på kromosom 11 og er assosiert med brystkreft. Vi fant at økning i *PAK1* kopitall var assosiert med økning i kopitall av *CCND1*. Vi fant ingen signifikant forskjell mellom kopitallsøkning og risiko for å dø av brystkreft i tilfeller med økt kopitall av PAK1 alene, CCND1 alene, eller tilfeller med økt kopitall av begge genene.

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Finansieringskilde: Institutt for klinisk og molekylær medisin, Fakultet for medisin og helse

Ovennevnte avhandling er funnet verdig til å forsvares offentlig

for graden Doktorgrad innen Medisin og helse

Disputas finner sted i Kavli-senteret, auditorium MTA

Fredag 15.mars, kl. 12:15

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Acknowledgements

The projects presented in this thesis was carried out at the Department of Clinical and Molecular medicine at the Faculty of Medicine and Health Sciences, Norwegian university of Science and Technology (NTNU) from 2018 to 2023. The projects included in this thesis received funding from the Department of Clinical and Molecular medicine at the Faculty of Medicine and Health Sciences, NTNU.

I am humble and truly grateful for all the support and knowledge given by Professor Anna Mary Bofin, my main supervisor during these years. Thank you for all your encouragement, patience, help and feedback that kept me on track all the way to the finish line.

Furthermore, I am very grateful for all the insightful and constructive feedback and help given by my co-supervisors, Associate Professor/Consultant Pathologist Marit Valla and Associate Professor/Consultant Pathologist Henrik Sahlin Pedersen. Thank you for sharing your knowledge with me.

Many thanks to co-authors Associate Professor Signe Opdahl, Department of Public Health and Nursing, NTNU and Senior Biomedical Engineer Borgny Ytterhus, Histology Laboratory, Department of Molecular and Clinical Medicine, NTNU for their valuable contributions within their fields of expertise.

I would also like to give a special thank you to my co-workers at the office and in the laboratory, for all your emotional support and the numerous insightful talks and many good laughs.

Many, many thanks to friends and family for supporting me during these years. And last, but not least: To my dear child, Will (aka Yami), for cheering for me at every milestone, giving me all sorts of distractions (positive) and for making me talk so much English in our everyday life ♡

List of papers

This thesis is based on the following three papers:

Paper I:

Skjervold AH, Pettersen HS, Valla M, Opdahl S, Bofin AM. **Visual and digital assessment of Ki-67 in breast cancer tissue - a comparison of methods.** Diagn Pathol. 2022 May 6;17(1):45.

doi: 10.1186/s13000-022-01225-4. PMID: 35524221; PMCID: PMC9074355.

Paper II:

Skjervold AH, Valla M, Ytterhus B, Bofin AM. ***PAK1*** **copy number in breast cancer-Associations with proliferation and molecular subtypes.** PLoS One. 2023 Jun 27;18(6):e0287608.

doi: 10.1371/journal.pone.0287608. PMID: 37368917; PMCID: PMC10298784.

Paper III:

Skjervold AH, Valla M, Bofin AM. **Oestrogen receptor low positive breast cancer: associations with prognosis.** Breast Cancer Res Treat. 2023 Oct;201(3):535-545.

doi: 10.1007/s10549-023-07040-9. Epub 2023 Jul 18. PMID: 37462784; PMCID: PMC10460703.

Abbreviations

BC – Breast cancer

CN – Copy number

DIA – Digital image analysis

DP – Digital pathology

ER – Oestrogen receptor

FFPE – Formalin-fixed paraffin embedded

FISH – Fluorescence *in situ* hybridization

HER1 / EGFR – (Human) Epidermal growth factor receptor 1

HER2 – Human epidermal growth factor receptor 2

HIER – Heat-induced epitope retrieval

IHC – Immunohistochemistry

ISH – *In situ* hybridization

LN – Lymph node

ML – Machine learning

NST – No special type

PR – Progesterone receptor

TNM – Tumor-node-metastasis

TMA – Tissue microarray

VA – Visual assessment

WSI – Whole slide image

Sammendrag

Denne avhandlingen bygger på tre publiserte artikler. Arbeidet er utgår fra tre kohorter med kvinner fra Trøndelag fylke i Norge. Disse var kvinner født mellom 1886 og 1977 som ble diagnostisert med brystkreft. Etter diagnosen ble de fulgt frem til slutten av 2015, eller til tidspunktet for død av brystkreft, eller død av andre årsaker. Oppfølgingsdata ble gjort tilgjengelig fra nasjonale registre.

Hovedmålet med denne avhandlingen var å studere etablerte biomarkører for analyse av brystkreft med nye tilnærminger, og å studere gyldigheten av en ny biomarkør.

I den første studien vurderte vi uttrykk av proliferasjonsmarkøren Ki-67 i 248 invasive karsinomer (*of no special type* (NST)) ved hjelp av både den konvensjonelle metoden (visuelt i et lysmikroskop) og digital bildeanalyse (QuPath-programvare). Vi sammenlignet resultatene fra disse to metodene og fant at vurdering av Ki-67 i brysttumorer ved hjelp av digital bildeanalyse identifiserte en større andel tilfeller med høye nivåer av Ki-67 sammenlignet med visuell vurdering av de samme svulstene. Vi konkluderte med at diagnostiske grenseverdier bør kalibreres ved innføring av ny metodikk.

I den andre studien vurderte vi *PAK1* genkopitall i 512 brysttumorer ved hjelp av fluorescens *in situ* hybridisering (FISH) på snitt fra vevsmikromatrise (*tissue microarray* (TMA)). Kopitall ble estimert ved å telle antall fluorescerende signaler for *PAK1* og centromerproben *CEP11* i 20 tumorcellekjerner/tilfelle. Vi vurderte sammenhenger mellom *PAK1* kopitall og andre tumoregenskaper, samt *PAK1* og *CCND1* kopitall. *CCND1* er lokalisert nær *PAK1* på kromosom 11. Vi fant at økning i *PAK1* kopitall var assosiert med høy proliferasjon og høy histologisk grad, men ikke med prognose. Økning i *PAK1* kopitall var mest vanlig i HER2- og Luminal B (HER2-negativ) subtyper av brystkreft. Økning i *PAK1* kopitall var assosiert med økning i kopitall av *CCND1*. Vi fant ingen signifikant forskjell mellom kopitallsøkning og risiko for død av brystkreft i tilfeller med økt kopitall av *PAK1* alene*, CCND1* alene, eller tilfeller med økt kopitall av begge genene.

I den tredje studien undersøkte vi sammenhenger mellom nivåer av østrogenreseptor (ER)-uttrykk og tumoregenskaper, og prognose hos 1955 tilfeller av brystkreft. Brystkreft-tilfellene ble delt i pasienter som sannsynligvis ikke hadde mottatt adjuvant terapi i henhold til behandlingsretningslinjer i bruk ved diagnosetidspunktet (før 1995), og de som kunne ha mottatt adjuvant terapi (diagnostisert i 1995 eller senere). Østrogenreseptor-status ble inndelt i tre kategorier: <1%, ≥1<10%, og ≥10% positive tumorcellekjerner. Histopatologisk grad, proliferasjonsstatus, og molekylære subtyper ble korrelert med ER-status innen hver tidsperiode separat og på tvers av tidsperioder. Den største andelen ER Lav positive tumorer (ER≥1<10%) fant vi i

Luminal B (HER2 +) subtype og grad 3 svulster. Risiko for død av BC var lavere i ER Lav Positiv og ER ≥ 10% sammenlignet med ER-negative svulster. Kvinner diagnostisert i 1995 eller senere hadde høyere andel ER Lav positiv brystkreft, og svulstene deres var mindre, hadde lavere grad, og lavere proliferasjon enn svulster tilhørende kvinner diagnostisert før 1995.

Summary

The present thesis is based on three published papers. The work arises from three cohorts of women from Trøndelag county in Norway. These were women born between 1886 and 1977, that were diagnosed with breast cancer (BC). After diagnosis they were followed until the end of 2015 or until time of death from BC or death by other causes. Follow-up data was made accessible from national registries.

The main aim of this thesis was to study established biomarkers in BC assessment with new approaches, and to study the validity of a new biomarker.

In the first study we assessed expression of the proliferation marker Ki-67 in 248 invasive carcinomas (NST) using both the conventional method (visually in a light microscope) and digital image analysis (QuPath software). We compared the results from these two methods and found that assessment of Ki-67 in breast tumours using digital image analysis identified a greater proportion of cases with high Ki-67 levels compared to visual assessment of the same tumours. We concluded that diagnostic cut-off levels should be recalibrated on the introduction of new methodology.

In the second study we assessed *PAK1* copy number (CN) in 512 breast tumours using fluorescence in situ hybridization (FISH) on tissue microarray (TMA) slides. Copy numbers were estimated by counting the number of fluorescent signals for *PAK1* and the chromosome enumeration probe for chromosome 11 (CEP11) in 20 tumour cell nuclei/case. We assessed associations between *PAK1* CN and tumour features, and *PAK1* and *CCND1* CNs. *CCND1* is located close to *PAK1* on chromosome 11. We found that *PAK1* CN increase was associated with high proliferation and high histopathological grade, but not with prognosis. *PAK1* CN increase was most frequent in the HER2- and Luminal B (HER2-) subtypes of BC. *PAK1* CN increase was associated with CN increase of *CCND1*. We found no significant difference in CN increase and risk of death from BC between cases with increased CN of *PAK1* alone, *CCND1* alone, or cases with increased CN for both genes.

In the third study, we assessed associations between levels of Oestrogen Receptor (ER) expression and tumour characteristics, and prognosis in 1955 cases of BC. All cases were stratified into patients unlikely to have received adjuvant therapy according to treatment guidelines at the time of diagnosis (before 1995), and those who could have received adjuvant therapy (diagnosed in 1995 or later). ER status was divided into three categories: <1%, ≥1<10%, and ≥10% positive tumour cell nuclei. Histopathological grade, proliferation status, and molecular subtypes were correlated with ER-status within each time period, and across time periods. The highest proportion of ER Low

Positive tumours (ER≥1<10%) were found in the Luminal B (HER2+) subtype and grade 3 tumours. Risk of death from BC was lower in ER Low Positive and ER≥10% compared to ER negative BCs. Women diagnosed in 1995 or later had a higher proportion of ER Low Positive BC, and the tumours were of smaller size, lower grade and lower proliferation than tumours in women diagnosed before 1995.

Introduction

Breast cancer (BC) is the most common cancer in women worldwide, causing nearly 700 000 deaths every year (1, 2). Breast cancer is a highly heterogenic disease with variable biology and patient outcomes (3). It is classified into histopathological type, histopathological grade and TNM stage, and into different molecular subtypes with different biological traits, prognosis and response to treatment (4). Thus, personalized treatment strategies are of great importance for BC patients.

Survival of BC has increased over the last decades, and this raises new perspectives and questions, such as the impact of overtreatment and the long-term side-effects after treatment. As prognosis and treatment strategies vary within different BC subtypes, which are partly decided by evaluation of biomarkers, there is a need to identify new prognostic biomarkers to further personalize prognostication and treatment. In addition, there is a need to re-evaluate established biomarker cut-offs for the same reasons.

Digital pathology (DP) is a rapidly growing sub-field within the field of pathology and entails the digitization of glass slides using a whole slide image (WSI) scanner. Digital image assessment of biomarkers has in the recent years increasingly become a method used to improve efficiency and reproducibility in cancer assessment and has already been implemented in some routine diagnostic laboratories (5-8). The field is growing with new knowledge, instrumentation, and software (9). Digital image analysis (DIA) has been shown to reduce inter- and intraobserver variability, which is a well-known issue within conventional pathology.

The main aim of this thesis was to study biomarkers with new and different approaches, and to investigate the properties of a new biomarker.

Background

Breast cancer epidemiology

Breast cancer is the most frequently occurring cancer (11.7% of all cancer cases in 2020) and is the most frequent cause of cancer-related death among women (1, 10). According to GLOBOCAN 2020, there were 2.26 million new cases of BC, and 685 000 BC deaths, globally (1) (See Figure 1).

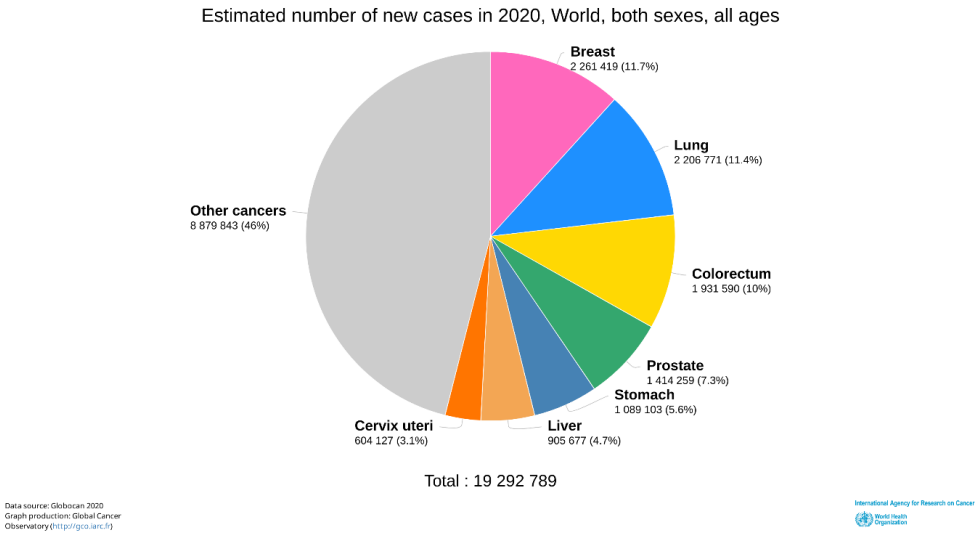

Figure 1: Pie chart showing estimated number of new cases of cancer cases worldwide, both sexes, all ages. Permission for reuse granted 2023. Globocan 2020, International Agency for Research on Cancer, WHO (1)

At the end of 2020, 7.8 million women worldwide had been diagnosed with BC during the previous 5 years, making it the world's most widespread cancer. Female gender is the strongest risk factor for BC. Breast cancers can occur in men as well, and accounts for approximately 0.5-1% of all BC cases diagnosed within a year (2). In Norway in 2022, 4247 new cases (23 males and 4224 females) were diagnosed (11).

Among women, BC accounts for 25% of all cancer cases, and 16% of deaths from cancer, and ranks first for incidence in most countries (Figure 2). In recent years BC mortality has decreased despite an apparent increase in new cases diagnosed in most countries, this is probably due to a combination of effective BC screening and improved treatment (12, 13).

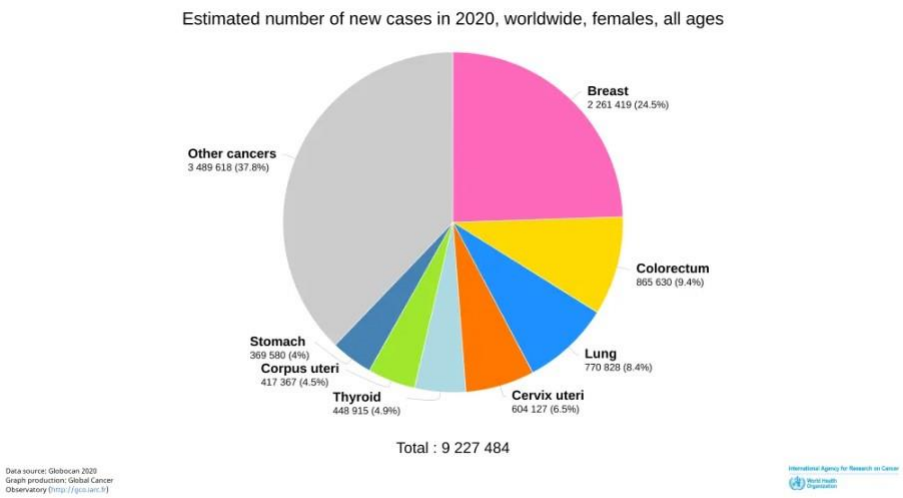

Figure 2: Pie chart showing the estimated number of new cancer cases worldwide, females of all ages. From Globocan 2020, International Agency for Research on Cancer, WHO (1)

According to World Health Organization (WHO) breast cancer mortality dropped by approximately 40% in high-income countries between 1980 and 2020. Countries that have succeeded in reducing BC mortality have been able to achieve a reduction of 2-4% per year (14). If an annual mortality reduction of 2.5% occurs globally, 2.5 million BC deaths could be avoided between 2020 and 2040 (1, 15). To be able to reach this kind of goal there is a need to better understand the biology of the cancer, its microenvironment, and molecular signaling in order to provide a personalized treatment regime to increase mortality.

Cancer is a highly variable disease in its genetics, cellular and tissue biology, and response to treatment. The original hallmarks of cancer were described and published by Hanahan and Weinberg in 2000 (16), and have since then been updated with additional hallmarks as research and knowledge has expanded (17, 18) (Figure 3). Figure 3 depicts the hallmarks of cancer, and shows the complexity, and thus the important areas to investigate for better understanding of cancer disease. Further knowledge of the molecular diversity of BC will enable us to understand the disease process and to explore molecular targets for improved treatment.

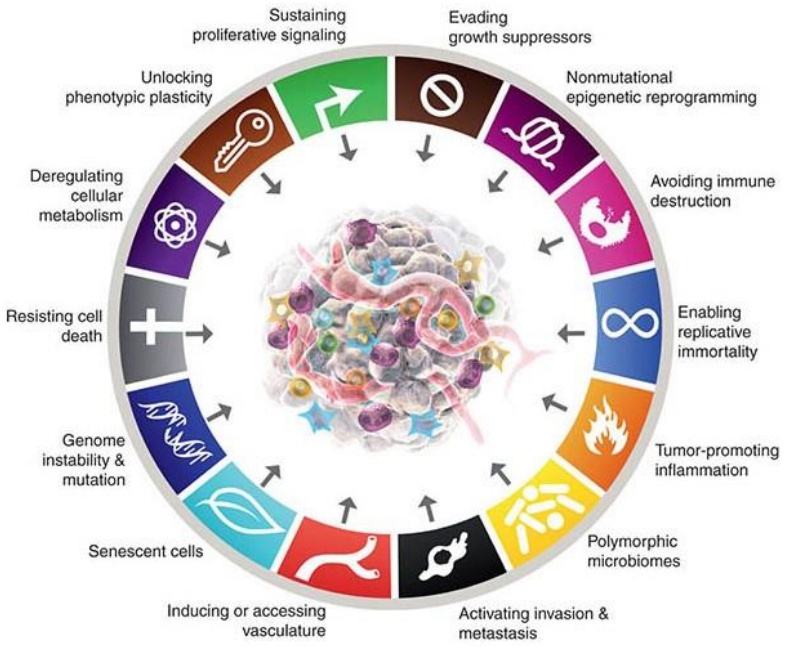

Figure 3: Hallmarks of cancer New Dimensions published in 2022 (18). Permission for reuse granted 2023. *Copyright © 2022, American Association for Cancer Research*

Breast cancer diagnosis

Breast cancer diagnosis usually involves clinical examination, medical imaging (mammography, ultrasound, magnetic resonance imaging) and examination of tissue samples from the lesion (fine-needle aspiration smears, biopsy). The conclusions drawn from these examinations form the baseline for further treatment.

Comprehensive pathology reports are of high importance for any cancer diagnosis. They are the foundation for correct diagnosis, customized optimal treatment, and the best possible prognosis and outcome for the individual patient. The pathology report comprises an assessment of histopathological type and grade, tumour size, resection edges, lymph node status and metastases, in addition to an assessment of a number of prognostic and predictive biomarkers.

Histopathological type refers to the growth pattern of a tumour. The most common histopathological type of BC is the invasive carcinoma of no special type (NST) (70-80%), followed by

lobular carcinoma (10-20%). There are also a number of special types (19, 20) (Figure 4 and 5). Histopathological types were described in terms of prognosis by Elston and Ellis in 1992 (21).

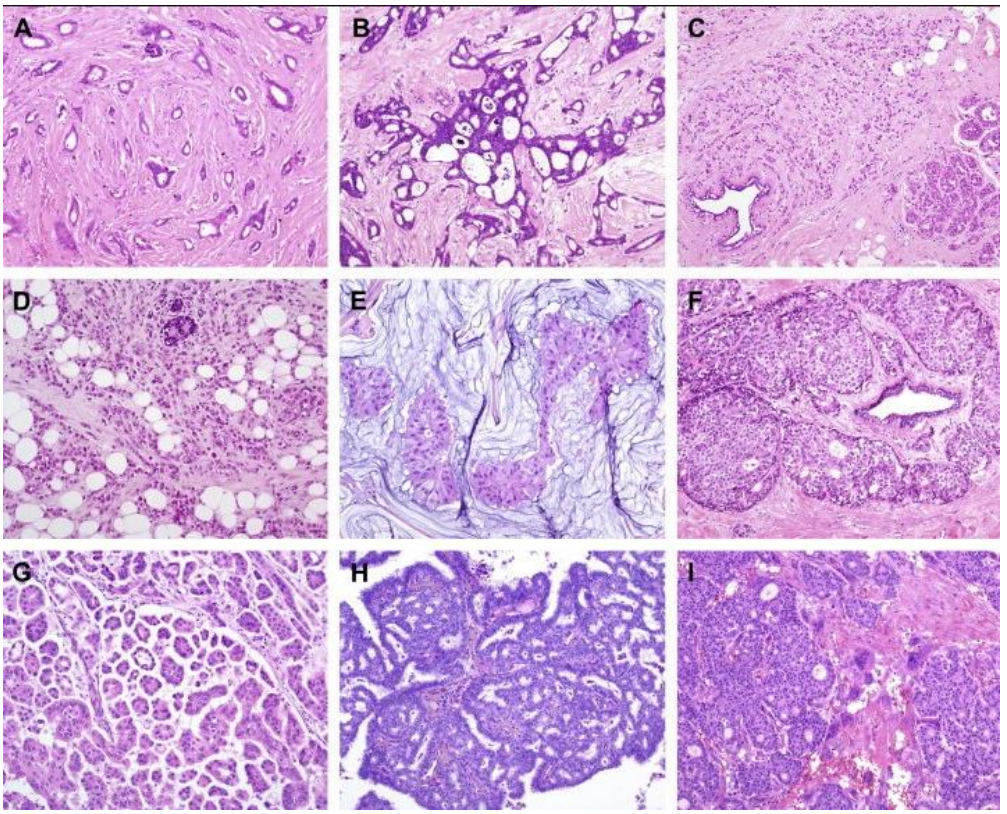

Figure 4: Histological special types of breast cancer preferentially oestrogen receptor positive. (A) Tubular carcinoma, (B) cribriform carcinoma, (C) classic invasive lobular carcinoma, (D) pleomorphic invasive lobular carcinoma, (E) mucinous carcinoma, (F) neuroendocrine carcinoma, (G) micropapillary carcinoma, (H) papillary carcinoma, (I) low grade invasive ductal carcinoma with osteoclast-like giant cells (20).

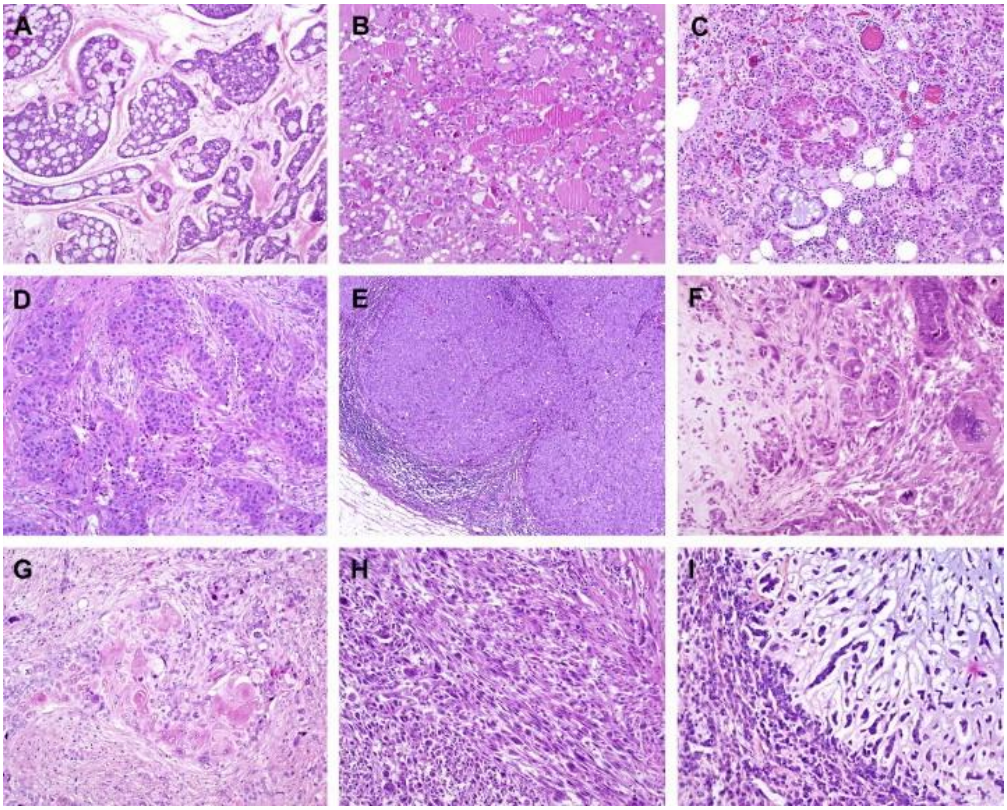

Figure 5. Histological special types of breast cancer preferentially oestrogen receptor negative. (A) Adenoid cystic carcinoma, (B) secretory carcinoma, (C) acinic-cell carcinoma, (D) apocrine carcinoma, (E) medullary carcinoma, (F) metaplastic carcinoma with heterologous elements, (G) metaplastic carcinoma with squamous metaplasia, (H) metaplastic spindle cell carcinoma, (I) metaplastic matrix-producing carcinoma (20). Permission for reuse granted 2023, © 2010 Federation of European Biochemical Societies

Histopathological grade refers to assessment of a tumour's degree of differentiation, which reflects the degree of resemblance the tumour cells bear to normal breast epithelial cells. The original protocol for histopathological tumour-grading was described and published by Bloom and Richardson in 1957 (22), and then revised by Elston and Ellis in 1991 (23). The Elston–Ellis modification of the Bloom-Richardson classification is commonly known as the Nottingham grading system (NGS). This grading system is still in use today when pathologists assess a BCs histopathological grade. The NGS is a semiquantitative assessment of three morphological characteristics: tubule/gland formation, nuclear atypia, and mitotic frequency (in 10 High Power Fields (HPF)). It can be performed on any BC tissue sample stained with Hematoxylin and Eosin (HE) (24). Grading itself is evaluated by a numerical scoring system of 1–3 per category (tubule formation; nuclear pleomorphism; mitotic count) (Table 1). The sum of the scores for each category indicates

the histopathological grade (3-5: Grade 1; 6-7: Grade 2; 8-9: Grade 3) (Figure 6). There is a highly significant association between histopathological grade and prognosis; the prognosis is poorer with increasing grade (23, 25).

Table 1: Features considered when grading by the Nottingham grading system. Table modified after Elston and Ellis (23).

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
	2
	3

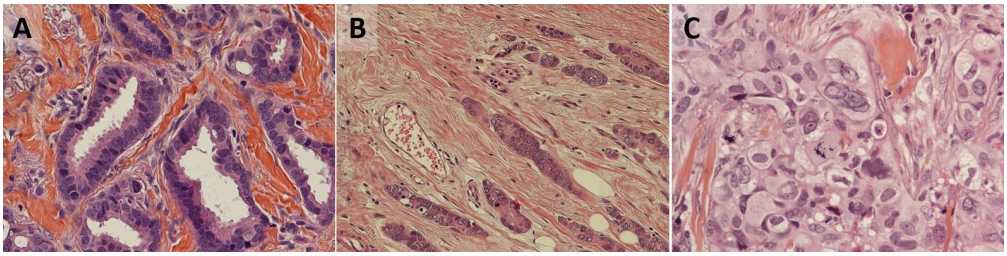

Figure 6: Hematoxylin-eosin-saffron (HES)-stained breast cancer tissue. A: Invasive carcinoma, no special type (NST) grade 1, 400X; B: Invasive carcinoma (NST) grade 2, 400X; C: Invasive carcinoma (NST) grade 3, 400X. Photo: Breast Cancer Subtypes research group, NTNU.

Breast cancer stage is a combined result of various information, including tumour size, lymph node status and metastasis (TNM staging system) (26). The TNM staging system of cancer was initially developed by Dr. Denoix during the years from 1943 to 1952 and was first published by The Union for International Cancer Control (IUCC) in 1968 (27). In 2017 The American Joint Committee on Cancer (AJCC) added biomarker status to a TNM breast cancer prognostic stage group (PSG) in the 8th version of the TNM Classification. The addition of biomarker as prognostic factor has later

been validated by several studies, and biomarker status is now a part of BC staging (28-30). TNM staging according to TNM Classification are shown in Table 2 (not included biomarker assessment).

Table 2: Staging of breast cancer according to the TNM-system (not included biomarker assessments) (26)

Stage	Tumour size	Nodes	Metastases
Stage IA	≤ 20mm	N0*	None
Stage IB	≤ 20mm	Nodal micrometastases (>0.2mm, <2.0mm)	None
Stage IIA	≤ 20mm >20mm ≤50mm	N1* N0	None None
Stage IIB	>20mm ≤50mm >50mm	N1 N0	None None
Stage IIIA	≤50mm >50mm	N2* N1 or N2	None None
Stage IIIB	Extension to chest wall and/or skin	N0, N1 or N2	None
Stage IIIC	Any T	N3	None
Stage IV	Any T	Any N	Detected

**N0 = no regional lymph node metastasis. N1= 1-3, N2= 4-9, N3 = ≥ 10 axillary lymph nodes involved.*

Breast cancer treatment

Breast cancer treatment can be highly effective, reaching 90% or higher chance for survival if treated, especially when the disease is identified early (31). Current treatment strategies generally include surgery and radiation therapy of the breast, lymph nodes and surrounding areas to control the disease, and systemic therapy to treat and/or reduce the risk of the cancer spreading (metastasis). Medical treatment includes endocrine (hormone) therapy, chemotherapy and targeted biological therapy (antibodies, immune receptors) (32-34).

In the past, all BCs were treated surgically by mastectomy (complete removal of the breast). Mastectomy may still be the best option for several reasons, such as large tumour size, known high-risk gene mutations, family history, patient preference, or lack of access to health care centres

offering radiation therapy (35). Radiation therapy can prevent a woman from having to undergo a mastectomy. The majority of BCs can now be treated with "lumpectomy", or breast-conserving treatment (BCT) (36), which is a procedure where only the tumour and surrounding tissue is removed from the breast. Radiation therapy to the breast is generally required after BCT to minimize the risk of recurrence. Furthermore, radiation therapy can reduce cancer recurrence risk after a mastectomy. For advanced stage of BC radiation therapy may reduce the likelihood of dying of the disease.

Regional lymph nodes are also removed during surgery for invasive cancers. Until the 1990s, surgical removal of the entire lymph node bed under the arm was considered necessary for prevention of BC metastasis. This often led to pain, swelling, numbness, and reduced mobility in the affected arm. A smaller lymph node procedure called "sentinel node biopsy" is now preferred as it has fewer complications (34, 37). For this procedure a radioactive tracer and/or dye is used to find the first lymph node(s) to which cancer could spread from the breast (38).

Medical (non-surgical) treatments for BCs may be given before (neoadjuvant) or after (adjuvant) surgery and are largely determined by assessment of size, grade and stage of the tumour, and biomarker expression. Cancers that express oestrogen receptor (ER) and/or progesterone receptor (PR) are most likely given endocrine therapy such as tamoxifen or other hormone blocking medication. Currently, endocrine treatment has a treatment period for up to 10 years(39, 40), and is given to patients with tumours expressing ≥1% ER, which is the current cut-off level (41), and the side-effects may affect the patient's quality of life considerably.

Some BCs may independently overexpress a receptor called HER2. The HER2 positive tumours may be treated by targeted biological agents such as the monoclonal antibody trastuzumab (42). When HER2-targeted therapy is given, it is often combined with chemotherapy (43). For patients with both HER2-positive and ER and/or PR-positive BC, clinicians may recommend either HER2-targeted therapy alone or, for selected patients, hormone-targeted therapy plus HER2-targeted therapy, or endocrine therapy alone (42).

The heterogeneous nature of BC and new treatment options demands a detailed assessment of the tumour's totality, including morphological features, biomarker assessment and gene-expression analysis (4). The effectiveness of BC therapies depends on a precise evaluation of predictive biomarkers and adherence to the prescribed course of treatment. Incomplete treatment is less likely to lead to a positive outcome (44-46). The ongoing investigation of new BC biomarkers could lead to new targets for treatment and improved personalized treatment to patients who may be over- or undertreated with current therapeutic strategies.

Biomarkers in medicine

Biomarkers used in medicine include measurements of blood pressure, and heart rate, x-ray findings, and complex molecular and genetic tests of blood and other tissues. Biomarkers provide measurable data and do not tell how a person feels or functions (47). Biomarkers can be characteristic biological properties or molecules that can be detected and measured in parts of the body like blood or tissue. They may indicate either normal or pathological processes in the body. Biomarkers can be specific cells, molecules, or genes, gene products, enzymes or hormones (48).

A biomarker is defined by the American National Cancer Institute as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease" (49). Cancer biomarkers are biological molecules that can predict the risk of developing cancer (risk biomarkers), detect or confirm the presence of cancer (diagnostic biomarkers), measure risk of cancer progression (prognostic biomarker), or potential response to therapy (predictive biomarkers). They are either produced by the tumour itself or by the body in response to the tumour.

Using immunohistochemistry (IHC) and *in situ* hybridization (ISH) to identify biomarkers in tissue sections, we can observe and assess the activity of the tumour.

Biomarkers in breast cancer

Breast cancer biomarkers play a central role when searching for the right diagnosis and treatment for BC patients.

Breast cancer is heterogeneous in both its morphological appearance and prognosis (50). Current diagnostic and treatment guidelines are based on pathologists' evaluation of tumour size, histopathological type, grade, lymph node- and resection margin status in addition to TNM stage, and assessment of a number of biomarkers: ER and PR, HER2 and the proliferation marker Ki-67 (34, 51). It is also possible to classify BCs into molecular subtypes with differing prognoses based on analysis of gene expression using cDNA microarray technology (52). More recently, commercially available gene expression panels are also in use to stratify BC cases into molecular subtypes, and thereby determine treatment (53, 54). These analyses are costly and may not be affordable for all laboratories worldwide, or for researchers who wish to study a few new biomarkers. However, IHC and ISH can be used as surrogates for gene expression analyses enabling us to reclassify formalin-fixed, paraffin-embedded (FFPE) BC tissue into molecular subtypes and study their associations with prognosis at a lower cost (55, 56).

Currently, biomarker assessment is largely done by visual assessment (VA) of the biomarkers by a pathologist under the light microscope. A main criticism of this approach is that it is highly variable and subject to intra- and interobserver variation. Digital image analysis (DIA) could enable us to adress the issue of inter- and intraobserver variability in the assessment of biomarkers (57-59).

Clinical biomarkers

Oestrogen Receptor

The discovery of endocrine therapy dates back to the late 1800s, early 1900s. Several studies, within the period 1886-1896, where bovine ovarian tissue was given as oral therapy to women experiencing menopausal symptoms showed remarkable symptomatic improvement for these women (60). Sir George Beatson proposed a connection between BC and the ovary in a publication from 1896 (61, 62). The hormone oestrogen was discovered in 1923 (63) and the ER was discovered in 1958 (64). The discovery of ER led a greater understanding of how hormones control the target gene expression through their receptors (65, 66). Now it is well-established that the presence of both ER and PR in a BC tumour indicates good response to endocrine therapy (67). Oestrogen receptor expression is associated with prognosis and is used to determine endocrine treatment. It is therefore considered to be both a prognostic and predictive biomarker.

Oestrogen receptor-signaling is the primary driver for ER-positive BCs, and inhibition of ER signaling has improved patient survival of ER-positive BC patients (68, 69). Expression of ER is seen in more than 70% of BC cases. Oestrogen receptor status is usually determined by IHC and ≥1% positive tumour cell nuclei is regarded as ER positive (70).

Until the 1990s, ER protein expression was measured using a ligand-binding assay (LBA) with cut-off for ER-positivity at ≥10 fmol/mg cytosol protein, which relied on analyses of frozen tumour tissue. Initially, upon introduction of IHC staining, the binding of hormone receptor–specific antibodies were only successful on frozen tissue sections. As antigen retrieval methods developed during the early 1990s, and new antibodies allowed for application to routinely FFPE tissues, IHC became the best choice for ER and PR assessment and determining treatment options (71). To start with, the cut-off for ER positivity in IHC was set at 10%, since approximately 7% of normal breast epithelial cells express ER at any given time (72). The ER ≥1% cut-off level for endocrine treatment was first introduced in Norway in 2011 after recommendations in the ASCO/CAP guidelines published in 2010 (Figure 7) (41). However, pathologists should also report ER ≥10% and ≥50% (34, 73). Studies suggest that endocrine therapy may need to be further personalized (74, 75), as it is only logical to assume that there is a noteworthy difference between tumours with ER levels at, for example, 3% positivity, compared to tumours with ER at 85%. Studies have found that tumours with

ER 1-9%, accounting for approximately 3% of all ER-positive cancers, have a less favorable prognosis than tumours with ER ≥10%. The ER Low Positive tumours often have a basal-like genomic signature (76) and respond to neoadjuvant chemotherapy in a similar way as triple-negative BCs (77). Studies have also shown that outcomes for tumors with ER 1-9% lie between outcomes for ER-negative and ER≥10% (78).

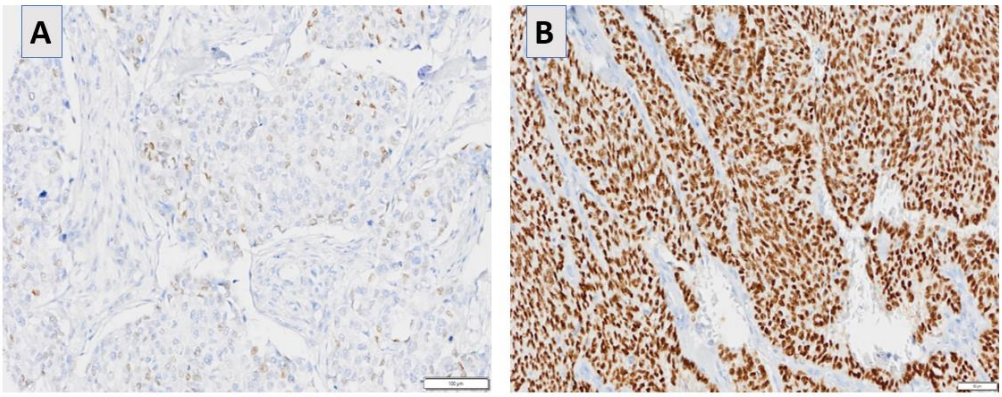

Figure 7: Positive nuclear oestrogen receptor expression. A: ER Low Positive (ER 1-9%); B: ER High Positive (ER ≥50%). Photo: Breast Cancer Subtypes research group, NTNU.

Progesterone Receptor

Progesterone is a hormone involved in the female menstrual cycle, maintenance of pregnancy and embryogenesis by binding to progesterone receptors. The name derives from latin *pro gestatiem* after these characteristics of involvement in processes preceding pregnancy or gestation (79).

The progesterone receptor regulates ER expression in BC; the *PGR* gene is an upregulated ER target gene, and PR expression depends on the presence of estrogen (80). Thus, PR expression may serve as an indicator of a functional ER-signaling pathway. Similar to ER, PR expression is observed in tumour cell nuclei on IHC. High expression of PR is more frequently observed in tumors with a good prognosis (Luminal A) than in tumors with a poor prognosis. In ER positive BC, negative PR is found to be associated with high risk of recurrence (81) and have a worse prognosis than ER and PR-positive tumours (82). Like ER, PR was initially measured by LBA, but is now assessed by IHC, and cut-off for positivity is ≥1% according to ASCO/CAP guidelines (73). If BCs express PR, but not ER, this may be an indication of residual function of ER or technical artifacts of IHC (81, 83, 84).

Human epidermal growth factor receptor 2

Human epidermal growth factor receptor 2 (HER2) is part of the epidermal growth factor receptor family, which comprises epidermal growth factor receptors (EGFR)/HER1, HER2, HER3, and HER4, and controls cell growth, survival, differentiation, and migration (85, 86). Breast cancer cells that overexpress HER2 generally have a higher proliferation than HER2 negative tumours (87). HER2 positive BC accounts for 20–25% of all BCs. They are aggressive and associated with poor prognosis (88, 89). HER2 is expressed on the tumour cell membrane and may be detected using IHC. Increased copy number of the *HER2* gene is associated with high protein expression and may be detected using FISH (42).

While HER2 overexpression in BC is associated with aggressive cancer, it responds well to BC treatment targeting the HER2-molecule (90). Multiple HER2-targeted therapies have been developed over the last few years, including the monoclonal antibody trastuzumab, which was approved in the early 1990s, (91) followed by tyrosine kinase inhibitors (TKI) lapatinib, neratinib, tucatinib, and pyrotinib (92-94). These drugs target and block HER2 or other receptors of the epidermal growth factor receptor family (95).

Ki-67, proliferation biomarker

Maintenance of continued proliferative signaling is one of the original hallmarks of cancer (17). The proliferative activity of a tumour cell provides important information about the growth of the tumour. Ki-67 is a nuclear antigen associated with cell proliferation (96). It is present in all active phases of the cell cycle (97, 98). Ki-67 was first discovered by Gerdes et al in 1983 (99). Ki-67 levels are low in the G1 and S phases, and peak during mitosis (100). To assess the proliferative activity of cells, Ki-67 antigens are usually stained by IHC using a Ki-67 antibody such as MIB1 (101). In assessment, the percentage positive tumour cell nuclei are reported (102). High values are associated with a poorer prognosis (103-107). As such, Ki-67 should be regarded as a prognostic biomarker.The proliferation marker Ki-67 is one of the biomarkers used to identify subpopulations of patients who are more likely to respond to chemotherapy (98). In this context, Ki-67 acts as a predictive biomarker.

According to recommendations from the International Ki-67 in Breast Cancer Working Group, only positive-staining nuclei and mitotic figures should be scored, regardless of staining intensity (102). Between 500 and 1000 tumour cell nuclei should be counted in hotspot areas. They underline that Ki-67 levels between 5% and 30% are subject to considerable interobserver and interlaboratory variability. They suggest that only very low (< 5%) or very high (≥ 30) levels should be considered clinically actionable (108). Despite Ki-67 being a well-established proliferation marker,

there is still controversy regarding the reproducibility of its assessment and cut-off levels, and its role as a predictive marker. A number of studies have been conducted in search for the optimal Ki-67 cut-off (58, 109-113). Some of these studies found that a cut-off at 20% for Ki-67 is appropriate in distinguishing between patients who should or should not receive adjuvant chemotherapy (109, 111). Inter- and intraobserver assessment of Ki-67 is poor (58, 114), inter- and intralaboratory cut-offs vary, and it has been suggested that each laboratory should calculate its own median Ki-67 value before estimating cut-offs for Ki-67-High, -Intermediate and -Low expression levels (115). More recently, some have recommended the use of automated digital analysis to improve assessment of Ki-67 (116-119). Further controversy has concerned the optimal number of cell nuclei to be assessed. Most guidelines recommend counting between 500 and 1000 tumour cell nuclei. However, counting a high number of nuclei in and around a hotspot may lead to hotspot dilution and a lower proliferation index potentially resulting in poorer prognostic value (113).

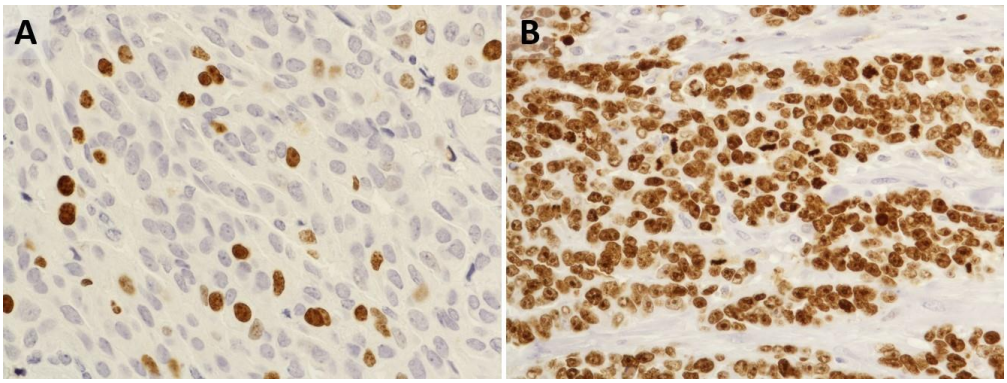

Figure 8: Ki-67 positive nuclear expression, A: Ki-67 <15%, B: Ki-67 >90%. Photo: Breast Cancer Subtypes research group, NTNU.

Ki-67 expression can be used to distinguish between the Luminal A and the Luminal B (HER2 negative) subgroups. Figure 9 shows a simplified algorithm for current clinical guidelines for treatment according to biomarker assessment.

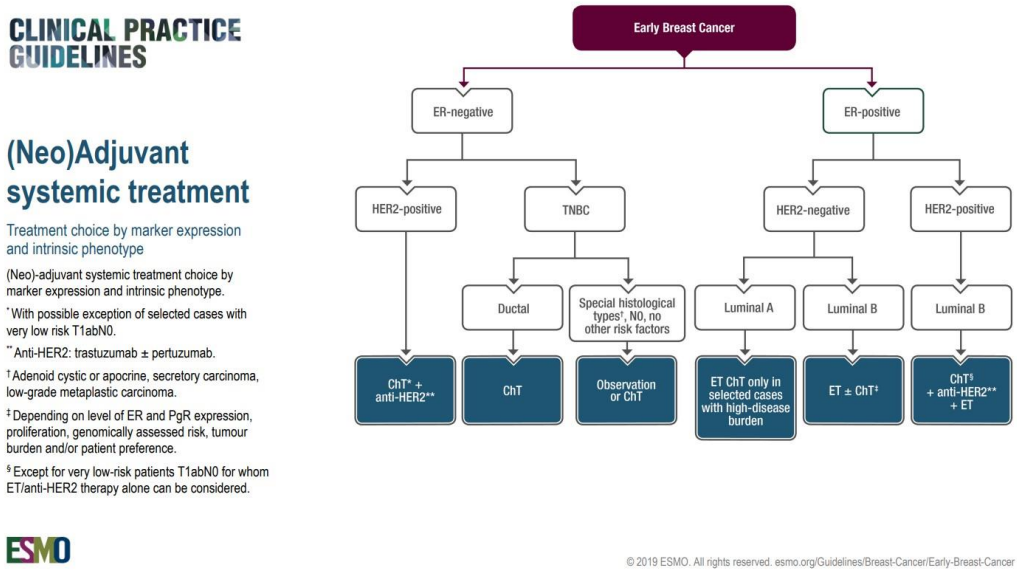

Figure 9: Algorithm for biomarker assessment in breast cancer and treatment according to European Society for Medical Oncology. © Copyright 2023 European Society for Medical Oncology. All rights reserved worldwide.

PAK1

P21-activated kinases (PAKs) are a group of serine/threonine protein kinases which consists of six isoforms (*PAK1*–6). They are overexpressed in BC, colon cancer and lung cancer, and in neurofibromatosis (120), as well as in other human tumours. PAKs play an important role in proliferation, cytoskeletal dynamics, and cell survival (120, 121). Their roles in these cell processes make them potential therapeutic targets. More is known of the functions of PAK1 and PAK4, than of the other isoforms (122, 123).

PAK1 is located on chromosome 11 (q13.5-q14.1). Increase in CN of *PAK1* and high PAK1 protein levels are found in BC, and several other human cancers (124-126). Copy number increase and high protein levels of PAK1 are linked to aggressive tumour types, chemotherapy resistance and poor prognosis (121, 127-131). In 2000, Mira *et al.* found that *PAK1* had an important role in BC proliferation (132). Since then, *PAK1* has been found to regulate several signaling pathways in BC (121, 133-138). *PAK1* amplification has recently been found to be significantly associated with reduced relapse-free survival of ER-positive BC patients (136). Cyclin D1 (CCND1) is found to be overexpressed in breast cancer, and studies have shown that *PAK1* regulates CCND1 expression in BC (125, 139). *PAK1* and *CCND1* are both located on the chromosomal band 11q13, which are

amplified in 15-20% of BCs (140). Co-amplification of genes located here has been found to be associated with poor prognosis in breast cancer (141). Increased *PAK1* CN could be a predictive marker for the effect of endocrine treatment (131, 142, 143).

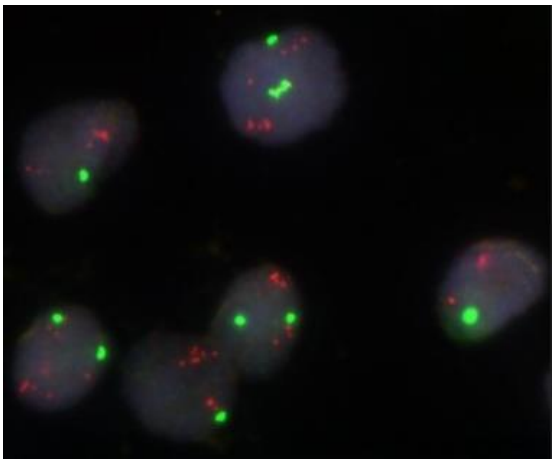

Figure 10: Increased copy number of P21-activated kinase 1 gene (*PAK1)* in breast cancer cell nuclei. Red fluorescent signals show PAK1 gene loci, green signals show centromere probe CEP11 loci in the nuclei. Cell nuclei are stained with DAPI. Photo: Breast Cancer Subtypes research group, NTNU.

Laboratory methods

Immunohistochemistry

Immunohistochemistry is a laboratory method for visualizing localization and distribution of cellular components such as proteins or other macromolecules (antigens) in tissue sections or cells. The method is based on antibody-antigen interactions to detect and visualize selected antigens. This is known as immunostaining or immunodetection and is an important tool to identify abnormal cells in diseases such as cancer or to stratify patients into optimized treatment regimes. The IHC technique was first introduced in 1941 by Albert Coons (144) and is now widely used in health care and pathology. Fluorescence was initially used for visualization, but later the method was developed for FFPE tissue using chromogens and assessment under a light microscope (145, 146).

The most important feature of the primary antibody is its specificity for the target antigen. The specific antigen location with affinity to the antibody is referred to as the epitope. For FFPE tissue, the reactivity between the antigen and the epitope must be restored through a process called antigen- or epitope-retrieval, which is a reversing of the effect of formalin fixation in the tissue for access to the antigen. This is usually done by enzymatic reactions or by heating of the sections (147-

149). The target epitope can be stained either directly, through a label that is directly conjugated to the primary antibody, or indirectly, using a labeled secondary antibody bound to the primary antibody. The indirect staining method is more commonly used, since two or more labeled secondary antibodies are able to bind to a single primary antibody, the result is increased signal and an increase in the analytical sensitivity (easier to detect areas with less antigen receptors) (Figure 11). Both monoclonal and polyclonal antibodies are available and can be used for antigen visualization. The monoclonal antibodies bind to one specific epitope/ antigen, while the polyclonal antibodies will bind to several epitopes. Thus, monoclonal antibodies will provide a more specific binding and visualization of a specific antigen, and polyclonal antibodies can give more non-specific staining, but will be more robust. The antibody is detected using either a fluorescent label, or an enzyme that converts a soluble substrate into a visible chromogenic product (most commonly 3,3'-Diaminobenzidine (DAB)).

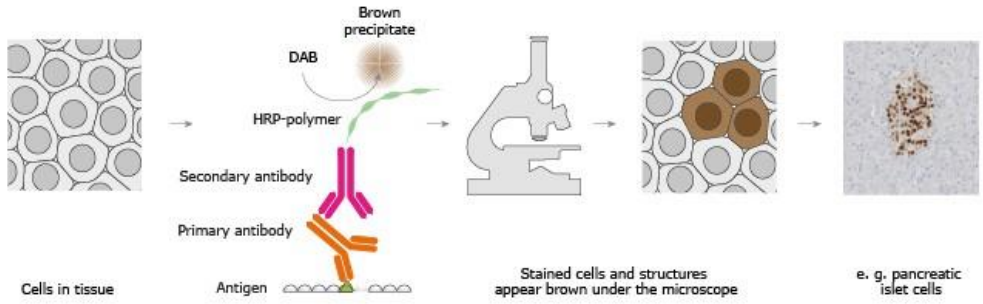

Figure 11. Indirect immunohistochemistry (IHC). Image credit: The Humane Protein Atlas (https://v15.proteinatlas.org/learn/method/immunohistochemistry)

There are some limitations to be aware of using IHC, as the method involves many steps in the laboratory, and any complications from any of these steps will in many cases impact the results. Variables impacting the IHC method can be assigned to the preanalytical, analytical or postanalytical phases (shown in Table 3). Some pre-analytical variables are often beyond the laboratory's control, such as the time from surgery until the specimen is placed in formalin (150)

Table 3: Steps and variables in the immunohistochemical process (147, 150):

	Steps	**Variables**
Preanalytical	Tissue sampling	Delayed fixation, prolonged ischemia, thickness of sample etc
	Fixation	Type of fixative and duration of fixation
	Decalcification	Type of solution and duration
	Tissue processing	Frozen tissues or FFPE
	Tissue sectioning	Section thickness, drying temperatures and duration, storage times and temperatures
Analytical	Deparaffination	Dewaxing solution
	Epitope retrieval	Detergents, enzymes, HIER
	Blocking non-specific reactants	Endogenous enzymes, hydrophobic binding, pigments
	Primary antibody	Monoclonal or polyclonal, specificity, species
	Detection system	Avidin-biotin or polymer-based, ultrasensitive methods
	Enzyme-substrate	Color-detection
	Chromogen	Enzyme-substrate combinations
	Multiplex IHC	Contrast
	Counterstain	
Postanalytical	Positive and negative controls	Species compatibility, tissue processing
	Interpretation	Pathologist or automated assessment
	Report	Percentage, cut-offs for pos/neg, type of scoring system, morphological context, misinterpretation, inadequate statistical analyses etc

Fluorescence *in situ* hybridization

In situ hybridization is a laboratory technique which allows for detection of specific RNA or DNA molecules in tissue- or cell samples (151). *In situ* means “in its original place”. Since its introduction in 1969 by Gall and Pardue (152) the method has a wide variety of uses due to its capability of

visualizing nucleic acid sequences without altering the cell's cytological, chromosomal or histological integrity. In 1980 Bauman et al introduced FISH (153). Fluorescence in situ hybridization has become an established method in pathology laboratories. The probes are labeled by fluorescent dyes that produce bright clear signals upon excitation in a fluorescence microscope (151). Fluorescence *in situ* hybridization is used for many purposes, for example analyses of chromosomal damage or gene mutations, gene mapping, in clinical diagnostics, and also in molecular toxicology and cross-species chromosome investigations. The method allows for identification and localization of regions of DNA or RNA within samples such as fixed cells or tissue sections (154). This technique is used not only in research laboratories, but also in diagnostics, prognostics and disease-monitoring in health care (155).

Figure 12 illustrates the FISH principle. In this technique, the double-stranded DNA are first denatured at high temperature and then hybridized with a fluorescent dye. The target in ISH on tissue sections may be whole interphase chromosomes and/or specific loci on chromosomes. This technique is commonly used to assess *HER2* CN in BCs (156, 157).

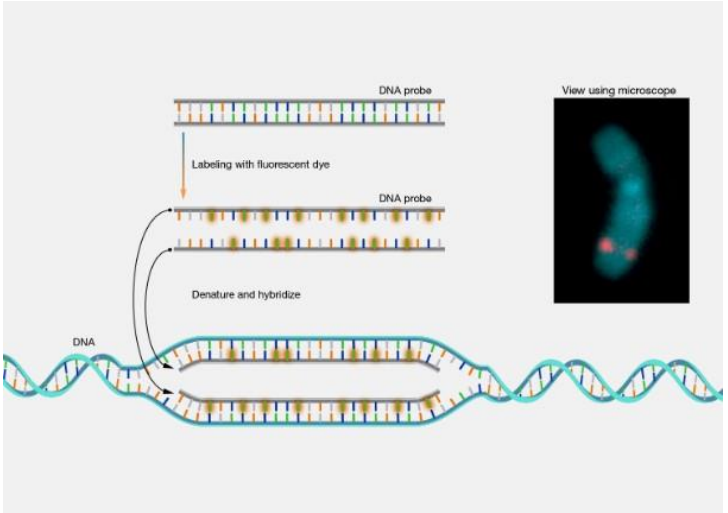

Figure 12: Fluorescence in situ hybridization. Figure Courtesy: National Human Genome Research Institute (https://www.genome.gov/genetics-glossary/Fluorescence-In-Situ-Hybridization)

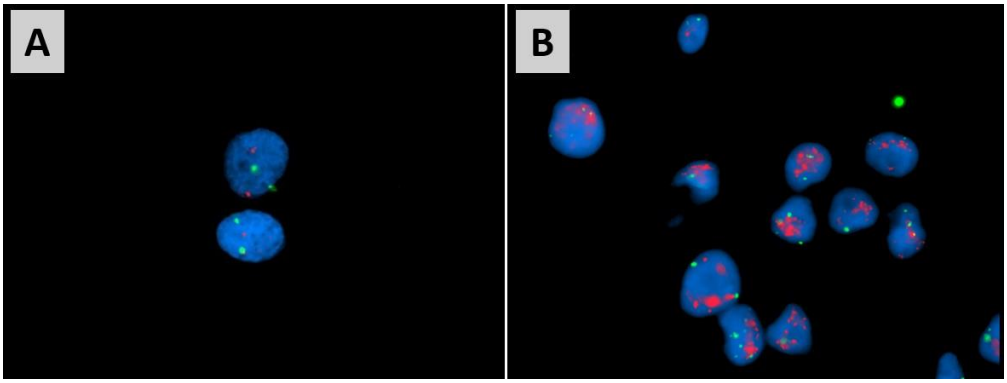

Figure 13: Fluorescence signals of A: Normal copy number of topoisomerase 2A (*TOP2A)* in breast cancer nuclei; B: Increased copy number of *TOP2A*; Red fluorescent signals show *TOP2A*, and green signals show the centromere probe *CEP17.* Photo: Breast Cancer Subtypes research group, NTNU.

An advantage of FISH is the opportunity to visualize signals for multiple probes at the same time or separately by simply changing filters in the fluorescence microscope. Other advantages are the high sensitivity with little to no background disturbance, as well as the advantage of assessing gene signals in specific tissue areas (e.g., tumour tissue). One drawbacks of this technique are the short-lived nature of the fluorescent signals. Unlike the insoluble products of some enzymatic reactions used in IHC, fluorescent dyes fade over time, and bleach out rapidly while illuminated during observation under the microscope, and if stored in artificial light or daylight.

Tissue microarray

Construction of tissue microarrays (TMA) allows for combinations of tens to hundreds of paraffin-embedded tissue specimens in one single paraffin block. This makes it possible to assemble a great number of different tissues, or pathological structures from the same organ, on the same slide for the same analyses (158, 159). Small tissue cylinders (usually 0.6-2.0mm in diameter) are punched out from selected regions of a donor blocks using a thin stainless-steel tube. Hematoxylin-eosin-saffron (HES)-stained sections laid over the donor block help guide sampling from representative areas of the FFPE tissue sample. The small tissue cylinder from the donor block are then transferred to a predefined and arrayed coordinated position in the recipient TMA-paraffin block (Figure 14) (159). Tissue microarray paraffin blocks may then be cut and stained for IHC and ISH in the same way as other FFPE tissue blocks.

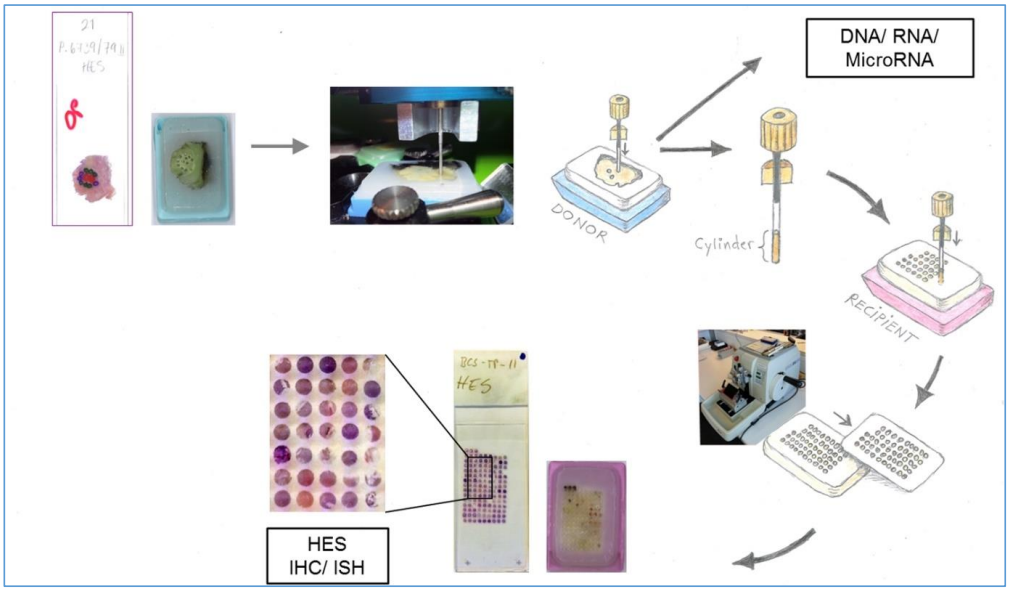

Figure 14: The process of tissue microarray (TMA) construction at our laboratory. The representative areas were selected and marked by a pathologist on a full-face tissue HES-stained tissue sections. 1mm cylinder cores were punched out from the area in the donor block and transferred to a recipient/TMA-block. Sections were cut from recipient block and stained with HES and selected biomarkers. Figure by LA. Dyrnes, Breast Cancer Subtypes research group, NTNU

Preparation and construction of TMA blocks require a considerable amount of work prior to use, compared to routine tissue blocks. However, the output of the work is rewarded as TMA-sections use less reagents per sample, assessment takes less time, and less tissue from the donor block is used (158). TMA is particularly beneficial for studying large historic cohorts, or tissue samples of limited size, or from rare conditions (160). Another advantage of TMA is that since all samples are gathered in one tissue slide, they will undergo the exact same procedure at the same time. Should it not be possible to reach a conclusion based on the information in the spot in the TMA block, it is possible to go back and make a full-face section of that particular tumour later. In most cases loss of TMA spots is a challenge, and it may be useful to choose more than one tissue cylinder from one sample, to make sure that at least one representative spot from the original tissue block is available for interpretation (161).

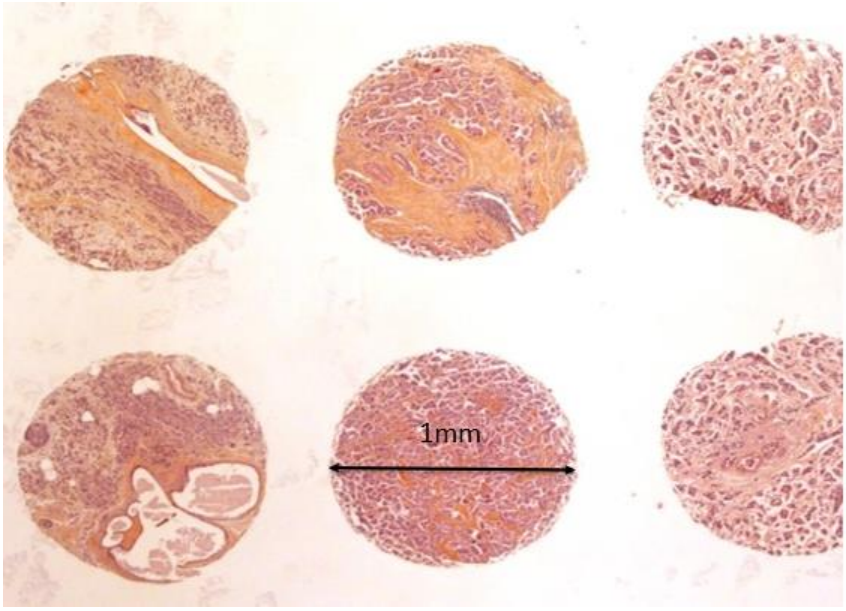

Figure 15: HES-stained sections from a tissue microarray block with 1mm in diameter cores/spots. Photo: Breast Cancer Subtypes research group, NTNU

When staining TMAs, preanalytical conditions may result in varying staining intensity. Examples of such preanalytical conditions are type of fixative used, duration of fixation, processing procedure and storage conditions of donor blocks prior to TMA construction, in addition to the inherent tissue quality (Table 3) (160, 162, 163). In research based on historical material, we often have little or no control over these preanalytical conditions.

During TMA construction, or during the staining procedure some of the core spots may go missing or get damaged. Some of the cylinders may contain areas of tissue that holds no valuable information, due to inaccuracy during construction. Typically, the spots may fold (Figure 16), making the tissue uninterpretable, and if the spot is lost, the area initially marked in the donor block as most interesting, will be irretrievable. Missing tissue in a TMA block can also be caused by cylinders falling deep into the well in the recipient block or if they get stuck on the way down into the recipient well. The core cylinder is approximately 3 mm long, though this depends on the thickness of the donor block and the content of tumour tissue. Thus, the core not necessarily contain tumour tissue throughout the entire cylinder.

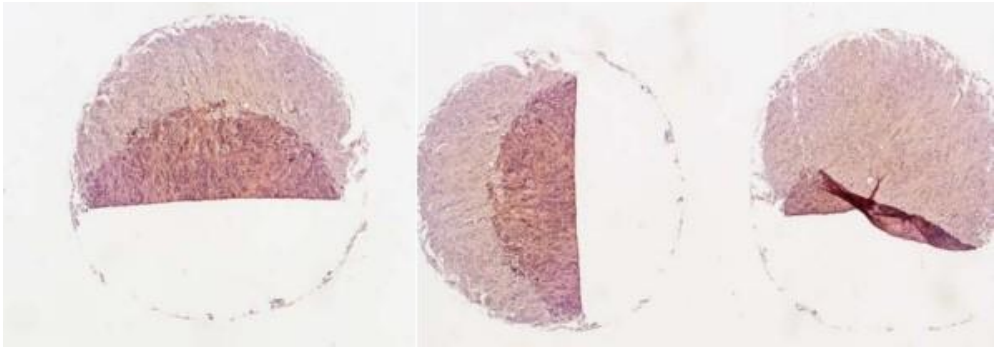

Figure 16: Examples of folded spots on TMA sections. Folding may affect large or small areas. Photo from "The Efficacy of Tissue Microarray in a Large, Historic Breast Cancer Cohort Study" (unpublished), SH Isaksen, 2014, Breast Cancer Subtypes research group, NTNU

When the cylinders are mounted, the tissue in the cylinder may have a concave or convex surface relative to the surface of the TMA block. This phenomenon will create a spot that looks like a donut when the TMA block is cut (Figure 17), or only a small circle in the middle.

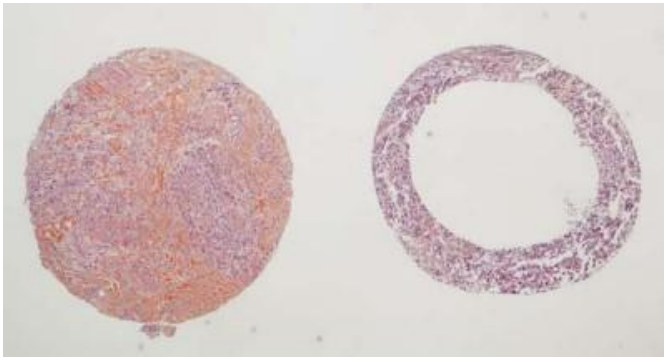

Figure 17: Examples of a successful spot on the left and spot with "donut"-effect on the right. Photo from "The Efficacy of Tissue Microarray in a Large, Historic Breast Cancer Cohort Study" (unpublished), SH Isaksen, 2014, Breast Cancer Subtypes research group, NTNU

Assessing biomarkers in TMAs from only one small biopsy or preselected area (spot) has been shown to have similar correlation with risk and survival compared to when the pathologist assesses an entire full-face section (164, 165). Several studies have shown good concordance between TMA and WS assessment of biomarkers (166-168), even though heterogeneity of tumours is considered an issue with TMA.

Digitization of TMA slides greatly reduces difficulties with orientation within the TMA block. Each spot is assigned an ID upon scanning, and it enables the reviewer to easily switch back and forth between images of spots. This enables comparison between the spots, while still maintaining a good overview.

Digital pathology

Digital Pathology (DP) involves converting an optical image captured from a microscopy glass slide into a digital image which can be stored, uploaded, shared, viewed and analysed on a computer. Digital pathology has its roots back in the 1960s, when telepathology was introduced as a means for pathologists to collaborate across distances (169). The term first became established in the late 1990s along with the introduction of the first whole slide image (WSI) scanners (170-172). Over the last decades, advances in software, processing power and cloud-based storage solutions have enabled the use of digital images for a wide variety of purposes in pathology. As a result, more and more pathology departments have implemented digital imaging for tasks such as image archiving, sharing digital slides and digital image analysis (173-176).

Whole slide image scanners have become more and more affordable and are slowly becoming the accepted solution for research laboratories and routine diagnostics laboratories (177, 178). Implementation of digital pathology in routine laboratories still has its challenges on technical, logistical, and financial levels. Whole slide image scanners are cleared for use in the European Union under directive 98/79/EC of the European Commission for in vitro diagnostics (in vitro diagnostic medical device directive (IVDD)) (179, 180). As of May 2022, developers of all in-vitro medical devices, including WSI scanners and digital pathology standalone software, such as WSI viewers or automated image analysis for specific tasks (e.g., immunohistochemical quantification), can also apply for and receive the CE mark IVD-MD for medical devices under the new in vitro diagnostic medical device regulation (IVDR) of the European Parliament. An approval requires a performance report which includes a scientific validity report and an analytical and clinical performance report (181).

Table 4: Pros and cons in digital pathology.

Digital Pathology Feature	**Possible Advantages**	**Possible Disadvantages**
In-house telepathology	Quick second opinion Social distancing (COVID-19 pandemic)	Second opinion overuse (interrupted workflows). Less face-to-face communication.
Remote telepathology	Service for remote areas. Specialization through DP in low volume labs. Home-office use. Healthcare cost reduction through global histopathology market.	Social isolation in remote telepathology. Loss of routine on-site expertise through home office. Wage competition through global histopathology market.
Consultation telepathology	Quick access possible. No physical slide transfer. Lower threshold for consultation due to shorter turnaround time.	No tissue blocks available for additional stains/molecular assays. Consulted pathologist unaccustomed to work-up (stains/scanner calibration) at the primary center. Compatibility issues due to diverse proprietary DP formats. Possible medico-legal implications due to restricted work-up.
WSI-general	No physical slide distribution. No fading of stored slides. No irretrievable/lost slides. Shorter sign-out time. Reduced misidentification of slides due to barcoded slides automatically allocated to the case. Easy dynamic workload allocation (e.g., management of backlogged work, redistribution in case of sick leave).	Time to evaluable-ready slide increased due to additional scan time. Integration into a laboratory information system (LIS) for full efficiency gains needed → possible costs for LIS update. Regular calibration required (scanners/displays). Small particles omitted by scan → manual checking for rescan. Artifacts (out-of-focus areas, digital stitching artifacts). Increased IT-dependence (IT-downtime) compared to optical microscopy.
WSI-reporting/user experience	Parallel (side-by-side) viewing, digital slide superposition. Shorter sign-out time. Quick access to prior slides → less immunohistochemistry. Facilitates slide presentation at multidisciplinary tumor board.	Slower evaluation compared to optical microscopes Mostly only single focus plane in routine DP → difficulties with interpretation Some structures harder to recognize on WSI → glass slide needed Polarization not possible on DP → glass slide

Digital Pathology Feature	Possible Advantages	Possible Disadvantages
	Easy image sharing in clinical communication. Computational pathology possible (see below). Occupational health: less neck strain, more flexible posture.	needed Extra training for safe practice required (perceived insecurity on digital sign-out) if not DP from career start Easy availability of prior digital slides might shift medico-legal onus towards more extensive re-examination → increased workload Dual infrastructure generally necessary (glass and digital) Occupational health: Computer Vision Syndrome (CVS)
WSI-Image Analysis, ML/AI	Faster/efficient and more accurate measurements/quantifications. Exact quantification of tumor cell content for molecular analyses. Digital enhancement of image features. AI for second-read safety net. Direct link morphology to clinical parameters “novel biomarker” beyond human recognition. Inspection/correction of suggestions from AI-apps in development on WSI-viewer: “human-in-the-loop” interaction.	Benefit of more accurate quantification not necessarily clinically relevant. Applications beyond human evaluation not yet approved/used for clinical management. AI intransparent (“black box”). Regulatory oversight challenges with self-modifying (adaptive) AI as algorithm/performance not constant over time.
WSI-education	Digital images for presentation and exams readily available. Remote teaching and self-study. Increased student motivation, modern appeal.	None.
Costs and efficiency gains	Work time saved through faster turnaround times. Decreased additional techniques (less immunohistochemistry). Decreased physical slide-transfer costs.	DP implementation and maintenance and storage costs add to current fixed costs if productivity gains remain unrealized (fixed work contracts). Dual infrastructure costs (workstations and microscopes if kept). Glass and digital storage still necessary. Technical expert knowledge for hardware acquisitions needed.

WSI: whole slide imaging, AI: artificial intelligence, ML: machine learning

Automated estimation of biomarkers using DIA offers a less subjective approach and is more reproducible and accurate than traditional VA under a microscope (177). A recent meta-analysis that included twenty-five biomarker-studies and a total of 10 410 histology samples indicated an equivalent performance of DP compared to the use of conventional light microscopy. Overall concordance showed an agreement percentage of 98.3% between the digital assessments compared to the clinicians decisions in light microscope (9).

Most of the WSI scanner systems available today use either line scanning or tile scanning, both of which generate multiple smaller images (in the form of lines or tiles) of high resolution. The lines or tiles are then aligned and stitched together to recreate the image of the original whole tissue section. Collecting image data is achieved using a carefully controlled motorized scanning stage or objective assembly. Most systems include scanning at 10X, 20X and 40X magnification. These magnifications can be adjusted as in the light microscope, though the system is motorized and controlled using a computer. Some systems can also scan under oil immersion at 60X. Whole slide image scanners can scan brightfield slides and fluorescent slides.

The primary challenges of DP are the initial investment in expensive equipment, the growing number of software available with very specific uses, and lack of standardized protocols and reporting systems (Table 4). Among advantages of DP is increased flexibility, enabling the pathologist and/or students to work out of office, easy transfer from one person to another for consultation and collaboration purposes, and advanced software available for tissue assessment and evaluation. In diagnostics, it is often necessary to re-evaluate previous biopsies from the same patient. This is more easily done digitally, compared to having to visit the physical slide-archive to search for the old tissue slide in question.

Global perspective

Breast cancer survival for up to 5 years after diagnosis ranges from more than 90% in high-income countries, to 66% in India and 40% in South Africa according to WHO (1) (Figures 18 and 19). Early detection and improved treatment has proven to be successful for preventing death from BC in high-income countries. Implementation of screening- and diagnostic programs is still limited in some countries. The great majority of drugs used for BC are already on the WHO Essential Medicines List (EML). Making this treatment available for all could result in major global improvements in BC survival.

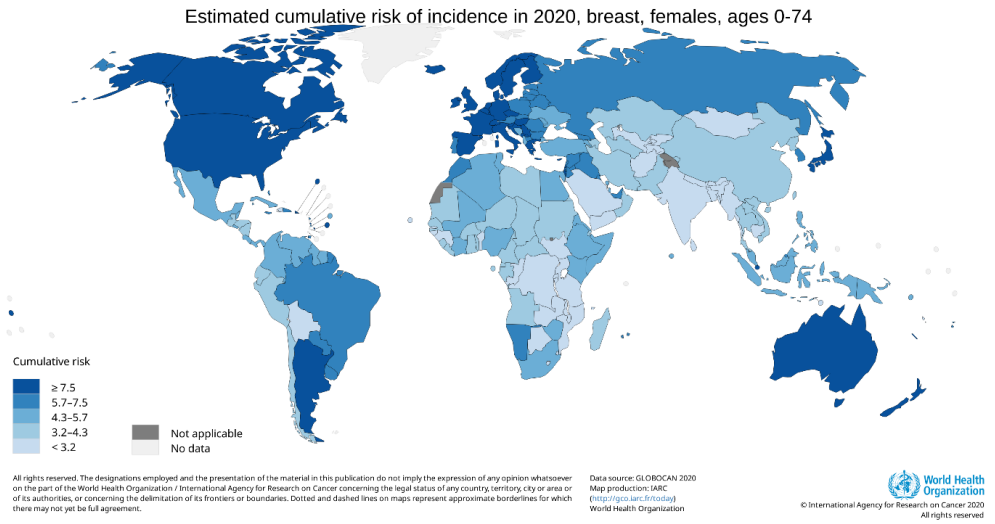

Figure 18: Incidence of breast cancer worldwide 2020 (1)

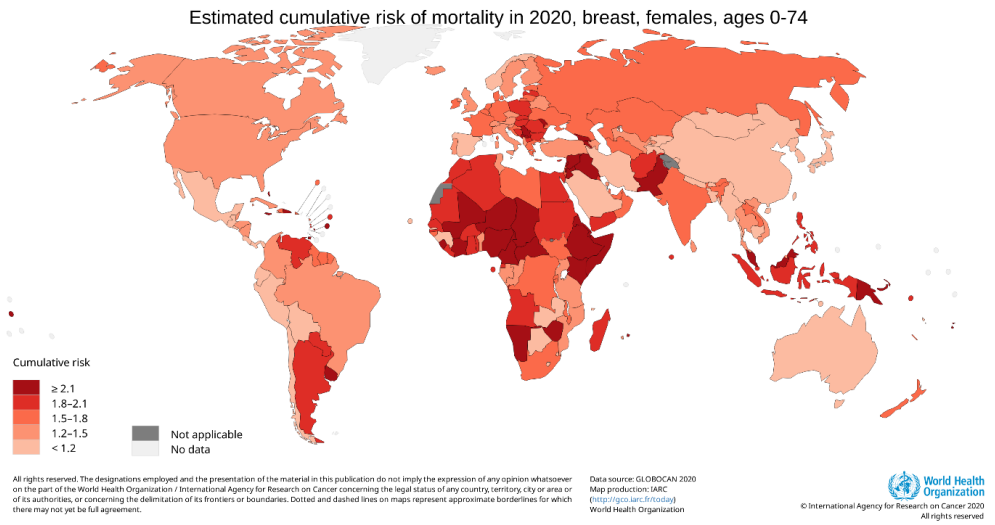

Figure 19: Mortality of breast cancer worldwide 2020 (1)

Aims

The main aim of this thesis was to study biomarkers with new and different approaches, and to investigate the properties of a new biomarker. More specifically, the aims were to investigate and to further optimize the use of current clinical biomarkers (Ki-67 and ER) and to investigate the relevance of *PAK1* CN in BC.

In Paper 1 we evaluated and counted cells expressing Ki-67 in BC patients using both conventional microscopy and digital image analysis.

In Paper 2 we studied *PAK1* CN in a series of BC patients to evaluate its potential as a biomarker with prognostic value.

In Paper 3 we studied ER expression in a series of BC patients to evaluate different levels of ER expression and their associations with tumour characteristics and time of diagnosis, and prognosis.

Materials and methods

Study populations

This study comprises women from three population-based surveys conducted in Trøndelag county, Norway. Information on BC incidence was obtained from the Cancer registry of Norway, date of death, and/or emigration was acquired from Statistics Norway, and causes of death from the Norwegian Cause of Death Registry. Pathology reports and FFPE tissue from the primary tumours and axillary lymph node metastases were retrieved from the Department of Pathology at St. Olav's Hospital, Trondheim University Hospital, Norway.

Tissue Microarray (TMA) blocks were made from the archival diagnostic tissue using the TissueArrayer Minicore with TMA Designer2 software (Alphelys). Three 1-mm diameter tissue cylinders from the periphery of the FFPE primary tumours and corresponding axillary lymph node metastases were transferred to TMA recipient blocks. TMA sections (4µm) were cut and stained. Reclassification of tumours into histological type and grade were determined on full-face sections for all cases in all three cohorts before reclassification into molecular subtypes was done based on biomarker assessment of TMAs (56, 182, 183)

Cohort 1: A population-based survey for the early detection of BC was conducted in the county of Nord-Trøndelag, Norway, between 1956 and 1959. The study included 25 727 women born 1886-1928 (184). These women were followed for BC occurrence, through linkage with data from the Cancer Registry of Norway. During the follow-up years, between 1961 and 2008, 1393 new BCs were registered. Of these, 909 cases were classified according to histological type, grade and molecular subtype(56). Patients were followed from time of BC diagnosis until time of death or until December 31st, 2015.

Cohort 2: The second survey was conducted between 1995 and 1997. In this study, all women in Nord-Trøndelag County aged 20 years or older were invited to participate in the second wave of the HUNT Study in Nord-Trøndelag (185). A total of 34 221 women born between 1897 and 1977 participated. From attendance until December 31st, 2009, 728 women were diagnosed with BC. Of these, 157 were already included in Cohort 1. Of the remaining tumours, 57 were unavailable for subtyping, resulting in a total of 514 tumours from Cohort 2 that were available for classification according to histopathological type, grade and molecular subtype(182). After diagnosis, these patients were followed until death from BC or death from other causes, or until December 31st, 2015.

Cohort 3: The cohort includes women who were born at E.C. Dahls Foundation Hospital, Trondheim, between 1920 and 1966. After excluding 524 twins, 111 triplets, 32 women with missing information on plurality, and 12 women whose identity could not be determined with certainty, 22 931 women born between 1920 and 1966 were eligible for BC follow-up until the end of 2015. Follow-up ended when a cancer (at any site) was diagnosed, at emigration or at death (from any cause), or on December 31st, 2015, whichever occurred first. During follow-up, a total of 870 women were diagnosed with BC. Among them, 598 were diagnosed at St. Olav's Hospital. Archival diagnostic tissue was available for all these 598 patients, and molecular subtyping was successful for 537 of these cases (183).

The study population for Paper I comprises 248 of the 654 BC patients with invasive carcinoma NST from Cohort 1, previously described by Engstrøm et al(56).

The study population for Paper II includes 512 BC cases from Cohort 1.

The study population for Paper III includes 1955 BC cases from all three cohorts.

Immunohistochemistry

For the study of Ki-67 (paper I), the Ki-67 antibody was applied (Clone MIB1, 35 mg/L, 1:100, Dako Denmark A/S, Glostrup, Denmark) on 4 µm thick full-face sections.

Digital image analysis

The Ki-67 IHC-stained slides were digitally scanned at 40X magnification with a resolution of 0.23 µm/pixel using Hamamatsu NanoZoomer S360 Digital Slide Scanner C13220-01 (Inter Instruments AS) at the Department of Pathology, St. Olav's Hospital, Trondheim University Hospital, Norway. The digital images were analysed for Ki-67 protein expression using the open-source, DIA software QuPath v. 0.1.2 (57).

Hamamatsu NanoZoomer S360 Digital Slide Scanner C13220-01

The Hamamatsu NanoZoomer S360 is an advanced digital slide scanner equipped with a manual slide loader system capable of scanning up to 360 standard slides, including corresponding meta-data. It delivers high-resolution images for detailed histopathological examintaion.

QuPath software

QuPath is an open-source digital pathology image analysis software developed at Queen's University, Belfast. It can be used for tumour identification and automated assessment of IHC-stained tissue (57, 186). Several studies have used QuPath as a tool for validation and systematic scoring of several biomarkers (118, 186, 187).

Fluorescence *in situ* hybridization

For the study of *PAK1* and CEP11 CN (paper II), FISH was done on TMA sections using DAKO Histology FISH Accessory Kit K 579911 according to the manufacturer's instructions. *PAK1* (3 µL, PAK1-20-RE, SpectrumRed fluorochrome Empire Genomics) and CEP11 (3 µL, CEP11 [D11Z19], SpectrumGreen fluorochrome, VYSIS) probes were used. Nuclei were stained with DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride).

Statistical analyses

In all three papers, survival analyses were carried out estimating Cumulative incidence of BC death and Cox proportional hazard ratios. In the first study on Ki-67 assessment using VA and DIA, we used Bland-Altman plot to compare the two methods.

Pearson's Chi^2-test

Pearson's Chi^2 analysis were used to assess associations between categories of VA and DIA (Ki-67 Low, Intermediate and High) (Paper I), *PAK1* CN (Paper II), ER levels (Paper III) and different tumour characteristics, and for assessment of associations between *PAK1* and *CCND1* CN (Paper II). The test estimates X^2 by comparing the observed and expected values in a two-by-two table and calculates a corresponding p-values. The Chi^2 test is not applicable for small populations and should not be used if n<5 (188).

Cumulative incidence of death from breast cancer and Gray's test

Cumulative incidence of death from BC was calculated for VA100, VA500, DIA100 and DIA500 (Paper I). In paper II cumulative incidence of BC death was calculated according to mean *PAK1* CN (<4, ≥4<6, ≥6; and <4, ≥4), and for Paper III according to ER levels both before and after 1995 (unlikely to have received, or likely to have received adjuvant hormone therapy). Death from other causes was treated as a competing event. Estimations of cumulative incidence curves of BC death was chosen since the method acknowledges that risk of death from cancer may be influenced by the risk of death from other causes (189). For example, a BC patient who dies from heart disease a year after her BC diagnosis, will not die of BC. If the risk of death by heart disease is high in a given population, it will affect the population's risk of death by cancer.

The cumulative incidence of death from BC can be defined as the risk of dying from BC over a given time (F(t)), given that the person has not died from other causes. When estimating cumulative incidence of death, the time given is divided into several time-intervals. F(t) sums up the probability of dying from BC in each time interval. In estimation of the risk of death in each given time interval, cumulative hazard for two competing risks (for example, death from BC and death from other

causes) are taken into the equation. Gray's test is used to test for equality between cumulative incidence curves (190).

Bland-Altman plot analysis

Bland Altman plots, or difference plots, is a graphical method to compare two measurement methods. Bland Altman analysis quantifies the agreement between two measurements by assessing the mean difference and creating limits of agreements (191). The plots are used to evaluate a bias between the mean differences between two sets of data, and to estimate an agreement interval for where 95% of the differences of the second method fall, compared to the first method. The Bland Altman plot provides a visual presentation of the difference between two measurements on the y-axis, and the average of the two measurements on the x-axis. This statistical method will not tell whether the limits are acceptable or not, it only defines the interval of agreement (192).

Bland-Altman plots were used to evaluate the agreement between methods in Paper I. We used VA500 as the reference measurement, and evaluated the agreement between the reference, and DIA100 and DIA500, by estimating the difference between the methods in relation to the mean value.

Cox proportional hazard ratios

The Cox proportional hazard model was used in all papers to compare the rates of death from BC in different categories. The hazard ratio (HR) is given as an unadjusted and adjusted estimate. Adjustments were made for for age, stage and histopathological grade. Hazard ratios were calculated as measures of relative risk of death from BC with 95% confidence interval (CI). In each calculation one of the categories, was defined as the reference group and compared to the other groups. For example, in Paper III, ER<1% was defined as the reference group, and ER≥1<10% and ER≥10% were compared to it. If the hazard ratio is less than 1 (the reference), then the risk of death is considered lower, or less likely to occur. If the HR is higher than 1, then the predictor is associated with increased risk of death, or more likely to occur. The confidence interval must be taken into account as well, because if the CI crosses 1, it is not statistically significant.

In the Cox proportional hazard model it is assumed that that HR of the exposure and covariates are constant over time (193). To check the proportional hazards assumptions over time, we made log minus log plots for all HR in Paper I-III and found no clear violations. All statistical analyses were performed using Stata version 17 (StataCorp LP, College Station, Texas, USA).

Summary of results

Paper I

A consecutive series of whole sections of 248 invasive carcinomas (NST) stained for Ki-67 protein expression using IHC, were assessed by traditional visual assessment in a light microscope, and then by DIA using QuPath. Five 100-cell increments were counted in hotspot areas in both methods and reported it as percentage Ki-67 positive nuclei pr 100 cells. We calculated cut-off levels based on the median of 500 cells for each method. The median for Ki-67 positivity were also calculated for each 100-cell increment. The statistical analyses include only the first 100-cell increment (VA100 and DIA100), and the total 500 cell (5 X 100 cells) (VA500 and DIA500). We used Pearson's Chi^2 test to study associations between counted cells and method, and tumour characteristics. In analysis of prognosis, we assessed cumulative risk of death and hazard ratios.

We found that the median Ki-67 level was higher using DIA compared to VA in the same tumours. The median values after counting 500 cells were 22.3% for VA, and 30% for DIA. While the proportion of Ki-67 positive tumour cells did not change substantially with increasing number of cells counted in VA, the number of cells counted affected the result when using DIA. The highest proportion of Ki-67 High cases were found when counting 100-200 cells using DIA. All of the counted 100-cell increments in both methods predicted poor prognosis in the highest Ki-67 levels, and with little difference between VA and DIA. The DIA100 group with Ki-67 High identified the largest proportion of histopathological grade 3 tumours, 70/101 cases (69.3%).

We showed that when assessing Ki-67 expression in BC using DIA, we identified higher levels of Ki-67 compared to VA of the same tumours. All counting methods predicted BC prognosis according to Ki-67 levels. However, there was no significant difference in prognosis between VA and DIA. We underline the importance of calibrating diagnostic cut-off levels upon introduction of new methodology.

Paper II

We studied *PAK1* copy number (CN) in a series of 512 BC tumours and their corresponding lymph node metastases. Fluorescence in situ hybridization for *PAK1* gene and CEP11 was performed on TMA slides. Copy numbers were estimated by counting the number of fluorescent signals for *PAK1* and CEP11 in 20 tumour cell nuclei. We studied associations between *PAK1* CN and proliferation status, molecular subtype and prognosis. In addition, we studied associations between *PAK1* and *CCND1* CNs (n=504), which are both located at the long arm of chromosome 11. Both genes encode proteins shown to activate ER. We used Pearson's Chi^2 test to study associations between *PAK1* CN

and tumour characteristics, and between *PAK1* and *CCND1* CNs. In the analysis of prognosis, we estimated cumulative risk of BC death and hazard ratios.

Copy number increase (mean *PAK1* CN ≥4) was found in 9.4% of tumours. Of these, 4.3% had mean *PAK1* CN ≥6. The HER2 type and Luminal B (HER2-) subtype had the highest proportion of cases with *PAK1* CN increase. We found association between mean *PAK1* CN ≥4 and high proliferation, and high histopathological grade, but not with prognosis. Of the cases analysed for both *PAK1* and *CCND1* CNs, 30/48 of cases with *PAK1* CN ≥4 (62.5%) also had *CCND1* CN≥4.

We conclude that *PAK1* CN increase is associated with aggressive tumour characteristics such as high histopathological grade and high Ki-67 protein expression, but not with prognosis.

Paper III

We studied ER expression in 1955 cases of BC, and associations between ER expression levels and tumour characteristics and prognosis. ER expression levels were divided into <1%; ≥1 <10%; ≥10%, and we paid special attention to the ER Low Positive (ER ≥1 <10%) cases. All cases were stratified into patients unlikely to have received adjuvant therapy according to treatment guidelines at time of diagnosis (before 1995), and those would have likely received adjuvant therapy (diagnosed in 1995 or later). ER levels were compared with time of diagnosis, histopathological grade, proliferation status, and molecular subtypes, using Pearson's Chi^2 test. In analysis of prognosis, we estimated cumulative risk of BC death and hazard ratios.

Of the 1955 included cases, only 65 (3.3%) were ER Low Positive. In patients diagnosed before 1995 2.1% were ER Low Positive. Among patients diagnosed in 1995 or later 4.2% were ER Low Positive. The highest proportion of ER Low Positive tumours was found among the Luminal B (HER2+) subtype, and grade 3 tumours. We found that the risk of death from BC was lower in ER Low Positive tumours and ER ≥10% tumours, compared to the ER negative (ER<1%) cases. Women with ER Low Positive diagnosed in 1995 or later had smaller tumours, and tumours of lower grade and lower proliferative status compared to ER Low Positive tumours diagnosed before 1995. We found no significant difference in prognosis when we compared ER Low Positive and ER ≥10% tumours among women diagnosed in 1995 or later.

We conclude that women with ER Low Positive tumours diagnosed in a time period when adjuvant therapy was available (1995 or later) had tumours of smaller size, lower grade, and lower proliferative status, and similar prognosis to those with ER≥10%, compared to women diagnosed earlier.

Discussion

This thesis is based on the work of three papers with the intention to study biomarkers with new and different approaches, and to investigate the properties of a new biomarker.

Despite improved molecular characterization and more precise prognostic and predictive biomarkers, there is still a need for greater precision in their assessment in order to further tailor treatment strategies for each individual patient. For the same reason, it is also necessary to continue searching for new biomarkers that will impact both prognostication and determine treatment options.

Discussion of main findings

We found clear differences in the median Ki-67 levels between VA and DIA, and this may reflect the respective methods ability to identify hotspot areas in tissue sections. This has been reported in similar studies of Ki-67 assessed using DIA (118, 187, 194-198). One study found that DIA is particularly effective in identifying hotspots, outperforming VA in assessing Ki-67 and mitotic counts (119).

To handle interlaboratory variation, the Expert Panel at the St. Gallen conference in 2015 suggested that the in-house median Ki-67 value at each laboratory should be the foundation for choice of cut-off values(199). More recently, the 17th St. Gallen International Breast Cancer Conference in 2021 proposed that Ki-67 expression should be used to determine treatment in ER-negative, HER2-negative BC in accordance with the guidelines from the International Ki67 Breast Cancer Working Group (108). The determination of cut-off levels is still challenging as reflected by these latest recommendations where only clearly low or clearly high levels of Ki-67 protein expression are considered to have clinical utility (108, 200). In 2014, Romero and co-workers suggested a stepwise counting strategy without fixed denominators, especially to target heterogenetic tumours with some highly proliferative hotspots (113). The International Ki67 Breast Cancer Working Group has proposed a standardized visual scoring method using a scoring app available online (108). Thus, the need for a standardized approach in the assessment of Ki-67 in BC has been recognized but not yet resolved.

Recent studies have suggested that downgrading of Ki-67 levels in some tumors may occur in VA when more than 2-300 cells are counted (113, 201). However, we found that there was little difference in the percentage of Ki-67 positive cells in each of the five 100-cell increments across cut-off levels using VA. This would imply that it may not be necessary to count more than 200-300 cells in VA. On the other hand, there was a clear fall in the number of Ki-67 High cases and a corresponding rise in the number of cases classified as Ki-67 Low with increasing cell counts using

DIA. Thus, using DIA, the highest proportion of Ki-67 positive cell nuclei is achieved by counting 100-200 cells in digitally identified hotspots. This appears to be in agreement with Romero et al.(113).

Digital image analysis could be used for assessment of other biomarkers such as ER which is currently assess by VA or "eyeballing" in diagnostics. Using DIA one could study different levels of ER expression and study associations between ER expression levels and tumour characteristics and prognosis. It would be interesting to further investigate ER cut-off levels with DIA calculations to see if it could stratify patients that would or would not benefit from endocrine treatment. One recent study of ER using DIA found that DIA accurately discriminates ER positive from ER negative cases and showed great concordance with pathologists' scores (202). Other studies have found that digital assessment of IHC staining is more reproducible than pathologists' visual scoring, suggesting that DIA is especially preferable in the assessment of large study populations and large tissue sections (203, 204). Sparse and/or low staining intensies may be overlooked by the pathologist using VA, and may be more easily detected by DIA (205, 206).

The most important finding in Paper I is the great difference between the median Ki-67 values assessed by VA and DIA. Determining cut-off values for treatment should be done for each method separately using robust end-point data such as treatment effect or breast cancer specific survival. Our findings emphasize the necessity to recalibrate cut-off levels whenever new assessment methodologies are introduceds.

Despite associations between *PAK1* CN increase and high histological grade and high proliferation, we did not find a statistically significant association between increased *PAK1* CN and prognosis. It would be interesting to study prognosis according to *PAK1* CN for each of the molecular subtypes separately. However, in the present study the number of cases in some of the molecular subtypes was too low to warrant further analyses of subgroups. The numbers of cases showing *PAK1* CN increase in primary tumours only, lymph node metastases only, or both, were too low to give reliable prognostic information.

Tamoxifen is an established hormonal therapy used in ER positive BC. Five years of tamoxifen therapy nearly halves the risk of BC recurrence among ER positive patients (207). Phosphorylation of ER by PAK1 may induce tamoxifen-resistance in ER positive tumours and tamoxifen itself may also increase nuclear PAK1 and PAK1 kinase activity (131, 136, 139). Patients with *PAK1* amplification are shown to have reduced benefit from tamoxifen and *PAK1* CN may therefore be a predictor of tamoxifen resistance (139). PAK1-inhibitors may be useful in ER positive

tumours, to improve the effect of tamoxifen among these cases (142, 208). In the era of personalized medicine, PAK1s influence on the effect of tamoxifen in BC makes it an interesting biomarker and potential therapeutic target for treatment (209).

PAK1 CN increase is found in all molecular subtypes of BC, except a subgroup of the triple negative subtype (the 5-negative phenotype), and occurs most frequently in the HER2 and Luminal B (HER2-) subtypes. It is associated with aggressive tumour characteristics such as high histopathological grade and high Ki-67 expression, but not with prognosis. It is co-amplified with *CCND1* in 62.5% of cases in our study. Co-amplification of genes located on the 11q13.3 amplicon has been found to be associated with poor prognosis in breast cancer, and co-amplification of the *CCND1-FGF* locus might decrease anti-tumour immune activity in breast cancer (141). The 11q13 region is amplified in approximately 15% of all breast tumors (139). Both *PAK1* and *CCND1* are genes that encodes proteins shown to activate ER, and amplification in at least one of the genes in ER-positive BC indicates a reduced recurrence-free survival (139). *PAK1* and *CCND1* CN should be studied further in a larger cohort with appropriate methods to investigate tamoxifen resistance mechanisms and possible treatment targets.

Breast cancer survival in Norway has increased since the mid-1990's as seen in the present and other studies (210). This may be ascribed to earlier detection (211, 212) and improved treatment (68, 70). The reduced risk of death observed between the two time-periods for all categories of ER expression, probably reflects earlier diagnosis with the introduction of mammography screening and the introduction of adjuvant treatment therapies in the mid-1990's. The change in prognosis observed across time for patients with ER Low Positive tumours may also be attributed to adjuvant therapy other than antihormonal treatment in addition to changing tumour characteristics such as smaller tumour size and lower histopathological grade.

ER status is an important prognostic factor and a predictor of the effect of endocrine treatment. ER signaling is a main driver of proliferation in ER Positive BCs, and inhibition of ER signaling has improved survival among ER Positive BC patients (69, 70). Studies suggest that selection of patients for endocrine therapy may need to be further personalized (74, 75, 213). While most ER positive BCs have high IHC scores, about 2-3% of cases are ER Low Positive (214-216). In the present study, 3.3% of the total number of cases were ER Low Positive. Tumours classified within the ER positive category, appear to have a risk profile more like that of ER-negative breast cancers (215). A recent study found no benefit of endocrine therapy in the ER <10% group compared to the ER >10% group (216). The lack of benefit of endocrine therapy in patients with low ER expression has also recently been shown in a meta-analysis, including more than 16,000 patients (217). The meta-

analysis indicated that primary BC patients with ER 1-9% gained no significant survival benefit from endocrine therapy, but had better prognosis than patients with cancers expressing ER<1% (217). In the present study, among patients diagnosed in 1995 or later, the ER Low Positive patient group had similar prognosis to those with ER ≥10%. The patients included in this study were diagnosed with BC between 1961 and 2012, and the ER >1% cut-off level for endocrine treatment was first introduced in Norway in 2011 after recommendations from ASCO/CAP (41). Therefore, the improved prognosis seen among ER Low Positive patients diagnosed in 1995 or later, can most likely not be attributed to endocrine treatment (218). Among women diagnosed in 1995 or later, we found a greater proportion of ER Low Positive tumours with smaller size, lower grade and lower proliferation compared to ER Low Positive tumours diagnosed before 1995. Thus, the improved prognosis may be attributed to factors other than endocrine treatment, such as earlier diagnosis due to the introduction of mammography screening and greater BC awareness among women. Determining endocrine treatment for patients with a diagnosis of ER Low Positive BC should be carefully considered in light of the potential risks and benefits of the treatment (215).

Cut-off controversies

Ki-67 is commonly used as a proliferative biomarker in clinical decision-making, to distinguish between the subtypes Luminal A (Low Ki-67) and Luminal B (High Ki-67). Ki-67 can provide both prognostic and predictive information (96, 106, 219). High Ki-67 score has been shown to be associated with poor prognosis (220). There has been considerable debate regarding Ki-67 counting methods and cut-off levels for prognostication and treatment decisions using this biomarker (51, 115, 221-225). As mentioned, the current guidelines for assessment of Ki-67 IHC underline that only positively stained nuclei should be counted in hotspot areas, and the number of counted nuclei should be between 500 and 1000 (34, 102). However, using DIA, it may be sufficient to count 1-200 cells in a digitally selected hotspot area to identify the greatest number of tumours with Ki-67 High. Stålhammar *et al* did a study in 2018 on proliferation markers, and found that DIA of Ki-67 performed better at mitotic count and phosphohistone H3 values with regard to prognostic value, especially in hot spots, compared to VA (119). Our findings underline the need for recalibration of established cut-off levels on the introduction of digital assessment. With the introduction of DIA, it could be possible to establish more reproducible and precise biomarker assessments for both established and novel biomarkers in BC, and thereby address the challenges of inter- and intraobserver variation (57, 58, 194).

There are no established guidelines for cut-off levels in the assessment of *PAK1* CN. We chose to follow *HER2* guidelines for categorizing CN, as in previous studies by our group (226-230). While we also registered CN of CEP 11, we did not calculate the ratio between CNs of *PAK1* and

CEP11 as this would have masked the true gene CN increase. Furthermore, we found that CEP11 CN increase was observed in only seven cases, of which only two were accompanied by CN increase of *PAK1*.

The 1% threshold for ER expression to justify endocrine therapy remains controversial. According to the St. Gallen 2019 Consensus Discussion on The Optimal Primary Breast Cancer Treatment it was stated that there is a need for better evaluation of ideal cut-offs for prescription of endocrine therapy for ER positive tumors, mainly with ER levels < 10%. With current assessment procedures in mind, which are mainly done by VA or "eyeballing", there is a need for a more reliable estimation of ER expression in BC. Of the experts that were present at the conference in 2019, 24% did not see that there was an ideal cut-off for ER status, whereas 38% would recommend prescription with levels of ≥10% (231). The Panel of the 17th St Gallen International Breast Cancer Consensus Conference in 2021 were also divided on the optimal ER threshold for initiation of endocrine therapy (43). However, the ASCO/CAP Expert Panel states that even though there are limited data on endocrine therapy benefit for cancers with 1% to 9% ER positive tumour cells, they should be reported as ER Low Positive and include a comment on the limited data on treatment benefits for these patients (71, 73).

It has been proposed that ER Low Positive tumours are more similar to the ER negative group, and therefore may not profit from endocrine therapy (213). Thus, cut-off levels should be further investigated in order to offer BC patients more personalized endocrine treatment, and to avoid over-treatment of patient less likely to respond (74, 232, 233).

Study population

The studies in this thesis include reliable information on BC incidence and follow-up data that were available from high-quality national registries like the Cancer Registry of Norway, the Cause of Death Registry and the Norwegian Patient register (234, 235). This enables comparability within the study population over time. As relapse of breast cancer can occur several years after initial diagnosis, data with long-term follow up has great value in breast cancer research. Unfortunately, data on relapse or disease recurrence was not available for the three cohorts in this thesis.

The patient cohorts included in the studies comprise only female residents of Trøndelag county, Norway born between 1886 and 1977. Distribution of molecular subtypes and varying prognosis for BC patients according to race have been studied and demonstrated in several studies (236-238). Therefore, the study population may not be representative for more ethnically diverse populations. However, we consider the size, stability and homogeneity of this study population a strength.

Materials and methods

Formalin-fixed paraffin-embedded tissue samples are easy to handle, store and are suitable for histological staining, IHC and *in situ* hybridization. In diseases whose treatment involves surgery, this sample type is most likely available. The BCs included in these studies cover a diagnostic timespan of nearly five decades, and we know that preanalytical conditions have varied during these years. Many of the tumours were diagnosed at a time before IHC was introduced as a method in pathology laboratories, and in our studies, IHC was done retrospectively for all cases. It is shown that valuable information can be drawn from archival tissue samples (239, 240). Studies by Dowsett et al. and Camp et al. have found that FFPE blocks are generally well preserved for several decades (241, 242). Ki-67 and ER IHC is robust in FFPE tissue (243, 244) and antigenicity is well preserved (242, 245, 246). Pre-analytical variables may affect the results of IHC staining (247, 248).

Tissue microarrays include only small tissue cylinders from the tumour and may not be representative of the whole tumour, particularly in breast cancer cases with known intra-tumour heterogeneity (164, 249). Thus, important information from the tumour may be lost. However, studies have shown that ER IHC carried out on TMA sections can provide similar information regarding clinical course as IHC on full face tissue sections (164, 250). TMA sections enables us to stain hundreds of tumour samples at the same time, under the same laboratory conditions at a relatively low cost.

The TMAs used in these studies comprise three 1mm in diameter tissue cylinders carefully selected from the periphery of each tumour. The tumour periphery is often the most proliferative area of the tumour. Breast cancer is known for its intra-tumour heterogeneity and the proliferative activity and biomarker expression may be different in other parts of the tumour. While this approach may not have captured the heterogeneity of the tumours sampled, it is probable that it has captured the most biologically active areas of the tumours (251).

In the study for Paper II we used FISH applied to TMAs. The method is available in most laboratories, as opposed to more expensive multigene assays. It enables us to assess the morphology of the section and ensure that only invasive tumour cell nuclei are analyzed. Despite this, FISH applied to tissue sections may lead to an underestimation of CN compared to analysis of whole nuclei, due to nuclear truncation (252). This would be of particular importance in cases with low CN increase. Preanalytical conditions will have varied considering that the cases included in the present study were diagnosed over decades. This could have affected the cases suitable for FISH analysis. While som tumour blocks from the 1960s and 1970s were discarded due to unsuccessful

FISH, probably due to fixation in unbuffered formalin, the vast majority of tissue samples were successfully hybridized.

Important information from tissue samples in FFPE blocks may be lost due to truncation of the tissue, both in full-face sections and TMA sections. Truncation refers to the three-dimensional information that may be lost in a two-dimensional cross section of a sample (Figure 20). In order to study biologically irregular tissue samples it is important that the tissue or cell component that is investigated is present in an adequate number; easily identifiable on the section; and of similar size and shape at different locations within the tissue (253). The two-dimensional tissue sections must in the best possible way be representative of the material.

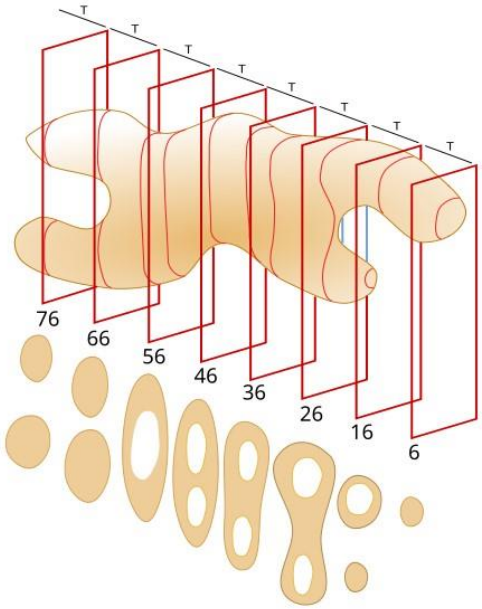

Figure 20: Illustration of the variation on cut slides from a irregular FFPE tissue sample.
Illustration obtained from Atlas of plant and animal histology. Histological techniques. Stereology. Retrieved (Oct 2023) from https://mmegias.webs.uvigo.es/02-english/6-tecnicas/ampliaciones/estereologia.php

Conclusions and future perspectives

This thesis provides information on new methodological approaches used on well-known biomarkers (Ki-67 and ER) and investigates *PAK1* CN in breast cancer.

In Paper I we found that when Ki-67 expression is assessed in BC using DIA, higher levels of Ki-67 were identified, compared to VA of the same tumours. We found no significant difference in prognosis between VA and DIA, when counting Ki-67. We suggest recalibration of diagnostic cut-off levels upon introduction of new methodology.

In Paper II we found that *PAK1* CN increase is associated with aggressive tumour characteristics such as high histological grade and high Ki-67 protein expression, but not with prognosis. We found co-amplification of *PAK1* and *CCND1* in 62.5% of *PAK1* amplified tumours.

In Paper III we concluded that women with ER Low Positive tumours diagnosed in a time period when adjuvant therapy was available had tumours of smaller size, lower grade, and lower proliferative status. Women with ER Low Positive tumours had similar prognosis to those with ER≥10% when diagnosed in 1995 or later, compared to those diagnosed earlier. In this thesis we conclude and underline the need for method-specific cut-off values to address the issues of inter- and intralaboratory differences. For both biomarkers Ki-67 and ER, there is a need for cut-off standardization, to make even more personalized treatment decisions. *PAK1* is interesting as a possible predictive biomarker for ER positive tumours that do not benefit from anti-hormonal treatment like tamoxifen. Blocking of *PAK1* may be useful in ER positive tumours, to improve the effect of tamoxifen in these cases. It could be interesting to study *PAK1* CNs in a large study population of ER Low Positive BCs. Identification and better understanding of the effects of new biomarkers like *PAK1* may help in the search for new therapy targets.

Some breast cancer patients maybe receiving endocrine therapy unnecessarily, with no effect on their prognosis. The ER Low Positive group of patients may not benefit from anti-hormonal treatment. According to current treatment strategies most ER Low Positive patients are treated with endocrine therapy for up to ten years based on “eyeballing” to establish a 1% cut-off for positivity. It could be useful to investigate ER expression levels using the DIA method and calibration of new, clinically relevant cut-off levels to further personalize anti-hormonal treatment strategies. Digital image analysis may have better reproducibility as it has defined limits for what is positive and what is not. The human eye in visual analysis is not as precise in finding low intensity staining in IHC compared to DIA, but on the other hand a pathologist can better interpret the total information from the tumour. In other words, DIA can be a means to define reproducible ER cut-off levels,

especially the ER Low Positive breast cancers, and maybe it would be possible to find the optimal cut-off where patients with ER positive breast cancers without doubt will benefit from anti-hormonal treatment.

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Paper I

Skjervold et al. Diagnostic Pathology (2022) 17:45
https://doi.org/10.1186/s13000-022-01225-4

Diagnostic Pathology

RESEARCH Open Access

Visual and digital assessment of Ki-67 in breast cancer tissue - a comparison of methods

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Abstract

Background: In breast cancer (BC) Ki-67 cut-off levels, counting methods and inter- and intraobserver variation are still unresolved. To reduce inter-laboratory differences, it has been proposed that cut-off levels for Ki-67 should be determined based on the in-house median of 500 counted tumour cell nuclei. Digital image analysis (DIA) has been proposed as a means to standardize assessment of Ki-67 staining in tumour tissue. In this study we compared digital and visual assessment (VA) of Ki-67 protein expression levels in full-face sections from a consecutive series of BCs. The aim was to identify the number of tumour cells necessary to count in order to reflect the growth potential of a given tumour in both methods, as measured by tumour grade, mitotic count and patient outcome.

Methods: A series of whole sections from 248 invasive carcinomas of no special type were immunohistochemically stained for Ki-67 and then assessed by VA and DIA. Five 100-cell increments were counted in hot spot areas using both VA and DIA. The median numbers of Ki-67 positive tumour cells were used to calculate cut-off levels for Low, Intermediate and High Ki-67 protein expression in both methods.

Results: We found that the percentage of Ki-67 positive tumour cells was higher in DIA compared to VA (medians after 500 tumour cells counted were 22.3% for VA and 30% for DIA). While the median Ki-67% values remained largely unchanged across the 100-cell increments for VA, median values were highest in the first 1-200 cells counted using DIA. We also found that the DIA100 High group identified the largest proportion of histopathological grade 3 tumours 70/101 (69.3%).

Conclusions: We show that assessment of Ki-67 in breast tumours using DIA identifies a greater proportion of cases with high Ki-67 levels compared to VA of the same tumours. Furthermore, we show that diagnostic cut-off levels should be calibrated appropriately on the introduction of new methodology.

Keywords: Ki-67, Cell proliferation, Immunohistochemistry, Digital pathology, Digital image assessment, Breast cancer

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Introduction

Sustained proliferative signalling is one of the hallmarks of cancer, as proposed by Hanahan and Weinberg in 2011 [1]. The nuclear antigen detected by the Ki-67 antibody is a marker of the growth fraction of a tumour. It is expressed in the G1, S, G2 and M phases of the cell cycle, but not in the resting phase, G0. While expression levels are low in G1 and S, they peak during G2 and M [2]. In breast cancer (BC), immunohistochemical (IHC) staining of the Ki-67 antigen is commonly used in the assessment of the proliferative activity of the tumour. It can provide information on prognosis and predict response to treatment in the adjuvant and neoadjuvant settings [3–6]. High Ki-67 score is associated with poor prognosis [7] but also a good response to chemotherapy [8, 9].

In molecular subtyping of BC, Ki-67 can be used to distinguish between Luminal A-like (Ki-67 low) and HER2 negative Luminal B-like (Ki-67 high) BC subtypes [10, 11]. While Luminal A patients generally have a good prognosis and may qualify for endocrine treatment only, Luminal B patients have a poorer prognosis and will often be given chemotherapy in addition. Thus, differentiation between these two subtypes has important therapeutic value [8, 10, 12].

Although the clinical validity of the Ki-67 Proliferation Index is accepted in BC, its clinical utility is still regarded as limited and there is a lack of consensus on the appropriate number of cells to count and cut-off levels for prognostication and treatment [13]. Furthermore, inter- and intra-observer agreement in the assessment of Ki-67 is poor [14–19].

Ki-67-staining is often heterogeneous within a tumour [20, 21]. In the assessment of Ki-67 IHC, only positively stained nuclei and mitotic figures should be scored, regardless of staining intensity, and between 500 and 1000 tumour cells should be counted in hotspot areas [22, 23]. According to the International Ki67 in Breast Cancer Working Group, Ki-67 levels between 5% and 30% are subject to considerable interobserver and interlaboratory variability. They suggest that only very low (< 5%) or very high (≥ 30) levels should be considered clinically actionable [13, 24]. To ameliorate issues of interlaboratory variation, the 14th St. Gallen International Breast Cancer Conference in 2015 proposed that the in-house median value at each laboratory should be used to determine cut-off values due to interlaboratory differences [17].

Several studies have suggested the use of automated digital image analysis (DIA) to improve reproducibility in the assessment of Ki-67. With the introduction of DIA, it should be possible to redefine interpretation algorithms for biomarker assessment for both established clinical and novel biomarkers in BC, and address the issue of inter- and intraobserver variation in the interpretation of these biomarkers [15, 18, 19, 25–29].

In this study we compared visual assessment (VA) and DIA of tissue sections stained for Ki-67 in a consecutive series of BCs. The aim was to identify the number of tumour cells necessary to count in each method to reflect the growth potential of a given tumour, as measured by tumour grade, mitotic count and patient outcome.

Materials and methods

Study population

The study comprises 250 BCs from a larger series of BC patients. The background population from which this series arises comprises 25,727 women born between 1886 and 1928 in Nord-Trøndelag County in Norway, who were followed for BC occurrence from 1961 to 2008. In total, 1379 cases of BC were diagnosed during follow-up, and 909 of these tumours were classified into six molecular subtypes using IHC and chromogenic *in situ* hybridization (CISH) as surrogates for gene expression analysis [30]. After diagnosis, all patients were followed until death from BC, or death from other causes or until December 31st, 2015 [30, 31].

In the present study, we included 250 consecutive cases of invasive carcinoma of no special type [32]. Two cases were excluded due to unsatisfactory staining (Fig. 1).

Immunohistochemistry

Full-face sections 4 μm thick, mounted on SuperFrost glass slides, were retrieved from storage (-20 °C). Paraffin was removed using TissueClear and sections were rehydrated with ethanol and water. Slides were heated at 60 °C for two hours and pretreated in a PT Link Pre-Treatment Module for Tissue Specimens (Dako Denmark A/S, 2600 Glostrup, DK) with a buffer (Low pH Target Retrieval Solution K8005) at 97 °C for 20 min. The Ki-67 antibody was applied (Clone MIB1, 35 mg/L, 1:100, Dako Denmark A/S, Glostrup, Denmark) in a DakoCytomation Autostainer Plus (Dako), with 40 min incubation time. Dako REAL™EnVision™ Detection System with Peroxidase/DAB+, Rabbit/Mouse (K5007), was used for visualization.

Digital image analysis

The IHC-stained slides were scanned at 40X magnification with a resolution of 0.23 μm/pixel using Hamamatsu NanoZoomer S360 Digital Slide scanner C13220-01 (Inter Instruments AS) at the Department of Pathology, St. Olav's Hospital, Trondheim University Hospital, Norway. The digital images were analysed for Ki-67 protein expression using the open-source, DIA software QuPath v. 0.1.2 [27].

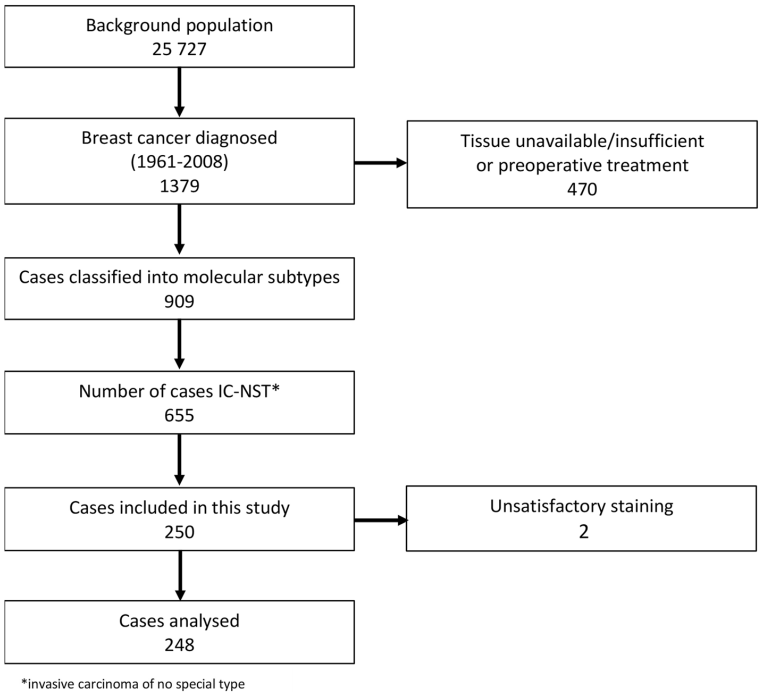

Fig. 1 Flowchart showing an overview of the cases included in this study

Training of the classifier

A separate series of 19 representative cases from the main cohort were used as a training set to train a two-class object classifier in QuPath after watershed nucleus detection [27]. The tumour area was delineated manually in the QuPath software. Cell nuclei (training objects) were selected and defined as either epithelial tumour cell nuclei or other (non-tumour cell nuclei or tumor stroma cell nuclei) in the whole slide images (WSI).

In the training set, stains were digitally separated using the colour deconvolution method and the automated "Estimate stain vectors" function in QuPath [27]. Watershed cell nucleus detection was performed and optimized visually using the following settings: Optical density (OD) sum; requested pixel size 0.4 μm; background radius 8.0 μm; median filter radius 1.5 μm; sigma 1.5 μm; min/max area 10/350 μm; threshold 0.02; maximum background intensity 3.0; and cell expansion 5 μm. Smoothing of object features (25, 50 and 100 μm) was applied. The threshold value for Ki-67-positivity (nucleus DAB OD mean) was assessed and adjusted manually, to best correspond to the visual perception of Ki-67 positivity in VA. Hence, the threshold was finally set to 0.15 nucleus DAB OD mean for all slides.

A cell nucleus detection object two-class Random Trees classifier (tumour cell nuclei vs. non-tumour cell nuclei) was trained using the default settings [27]. Training continued until visibly acceptable classification was achieved using 67% equally spaced train/test-split, resulting in approximately 85% accuracy. This was obtained using 7514 training objects and 135 object features from the 19 annotated images in the training set. The classifier was saved and applied to the watershed nucleus detections within the manually annotated tumor areas of all 248 cases in this study.

All nuclei in the tumour were detected by running positive cell nucleus detection provided by QuPath, and then sub-classified into epithelial tumour cell nuclei and other intra-tumoural nuclei by the trained classifier. Due to the heterogeneity of BC tissue, additional annotations were subsequently added to the classifier for most of the digital images until visually acceptable discrimination between epithelial tumour cell nuclei and all other nuclei was achieved for each WSI. Examples of annotation of training objects are shown in Fig. 2.

Digital Ki-67 hotspot identification

The tumour area in each of the 248 full-face sections was delineated manually by an experienced breast pathologist and the manual delineation was thereafter used to guide

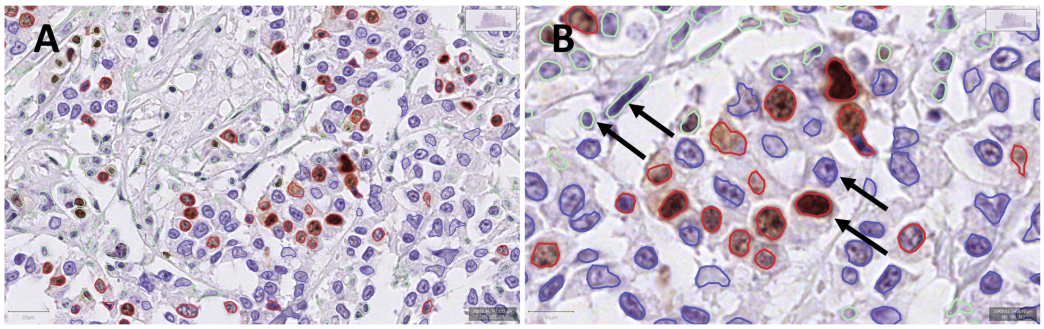

Fig. 2 **A** Overview image from QuPath showing cell nucleus detection and classification. **B** Arrows indicate elongated stromal nucleus and lymphocyte (green); Ki-67 positive tumour cell nucleus (red) and Ki-67 negative tumour cell nucleus (blue)

digital delineation of the tumour in the WSIs in the QuPath software. Ki-67 positive tumour hotspot areas were identified using a semi-automated approach by generating measurement heat maps in QuPath by visualizing nucleus DAB OD mean: Smoothed 50 μm. The heat maps were manually adjusted for each WSI to identify and annotate the area with the highest density of Ki-67 positive tumour cell nuclei (Fig. 3A-D). Areas with obvious artefacts resulting in false hotspots were manually excluded.

Scoring and reporting

Visual assessment

Visual assessment of Ki-67 proliferation rate was done using a brightfield microscope (Nikon Eclipse 80*i*) at 40x magnification. A total of 500 tumour cell nuclei (5 × 100) were counted in visually selected hotspot areas in each case, starting with the group of 100 cell nuclei which appeared to have the highest proportion of Ki-67 positive cells. The number of positive-staining tumour cell nuclei was recorded separately for each 100-cell increment counted.

Digital image analysis

All cases were assessed for Ki-67 expression using the QuPath software. Once the Ki-67 tumor hotspot was identified using the measurement heat map, five areas containing 100 tumour cell nuclei were manually delineated using the QuPath "brush tool". Counting started in the group of 100 nuclei that, within the identified

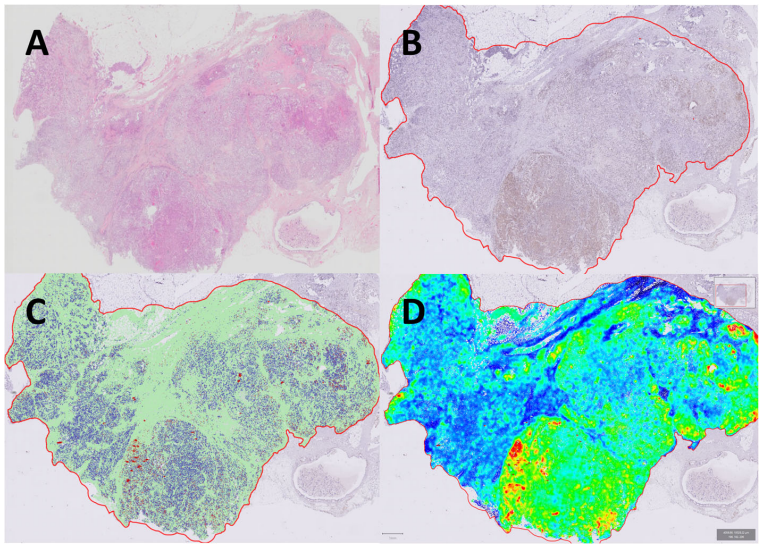

Fig. 3 **A** HES stained WSI; **B** IHC stained WSI (Ki-67) with manually delineated tumour area (red); **C** Cell detection within the tumor area and tumor (blue/red) and non-tumor (green) classified cells; **D** Measurement heat map showing Ki-67 hot-spots in red

hotspot, appeared to have the highest density of positive staining nuclei according to the heat map and continued in decreasing order of density until five sets of 100 nuclei were counted (Fig. 4).

Cut-off levels for Ki-67 Low/Intermediate/High positivity

We determined cut-off levels based on the median Ki-67 values for each method according to the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015 [17]. Ki-67 Low was defined as 10% points below the median, and Ki-67 High as 10% points above the median. Values falling between Low and High were classified as Intermediate. The median values of Ki-67 positivity using VA and DIA were calculated for 100 cells (VA100, DIA100); 200 cells (VA200, DIA200); 300 cells: (VA300, DIA300); 400 cells (VA400, DIA400); and 500 cells (VA500, DIA500) (Fig. 5). In the statistical analyses, only the results for VA/DIA100 and VA/DIA500 were used.

Statistical analyses

Tumour characteristics were compared using Pearson's Chi squared test across categories of VA and DIA (Low, Intermediate and High as described above) for 100 and 500 nuclei counted. Bland-Altman plots were used to evaluate the agreement between VA500 as the reference measurement, and DIA100 and DIA500, by estimating the difference between the methods in relation to the mean. Cumulative incidence of death from BC was calculated for VA100, VA500, DIA100 and DIA500, treating death from other causes as competing events. Gray´s test was used to compare equality between cumulative incidence curves. Cox proportional hazard analyses were used to estimate hazard ratios (HR) of BC death, with censoring at death from other causes. Harrell's C-test was used to compare the predictive ability of VA100, VA500, DIA100 and DIA500. All analyses were performed using Stata v. 16.0 (StataCorp LP, College Station, Texas, USA).

Results

Patient and tumour characteristics are presented in Table 1. Of the 248 patients evaluated in this study, 108 had died of BC and 124 had died of other causes by the end of follow-up. There were 16 (6.5%) histopathological grade 1, 131 (52.8%) grade 2, and 101 (40.7%) grade 3 tumours.

Cut-off levels for Low/Intermediate/High Ki-67 positivity

Cut-off levels for Ki-67 positivity were calculated for both VA and DIA according to the median Ki-67 values after 500 tumour cell nuclei were counted (VA500, DIA500). The median Ki-67 level was 22.3% for VA500 and 30.0% for DIA500, as shown in Fig. 5. Thus, for the present study, cut-off levels for VA were set at < 12.3% (Ki-67 Low), ≥ 12.3 ≤ 32.3% (Ki-67 Intermediate) and > 32.3% (Ki-67 High). For DIA, cut-off levels were set at < 20.0% (Ki-67 Low), ≥ 20.0 ≤ 40.0% (Ki-67 Intermediate) and > 40.0% (Ki-67 High).

In VA, there was no clear difference between the median values of the five cumulative 100-cell increments (VA100-VA500) (range 22.3-23.2%). Using DIA, the median value for both DIA100 and DIA200 was 34.0%, falling to 30.0% at DIA500. Cumulative median values for all 100-cell increments (both VA and DIA) are shown in Fig. 5.

Visual assessment

Using the VA median-derived cut-off levels, 48 cases (19.4%) were classified as Ki-67 Low at VA100, falling to 44 (17.7%) at VA500. Twelve cases were upgraded from Ki-67 Low at VA100 to Ki-67 Intermediate at VA500. None were upgraded from Low to High. Similarly, a total of 123 cases (49.6%) were classified as Intermediate

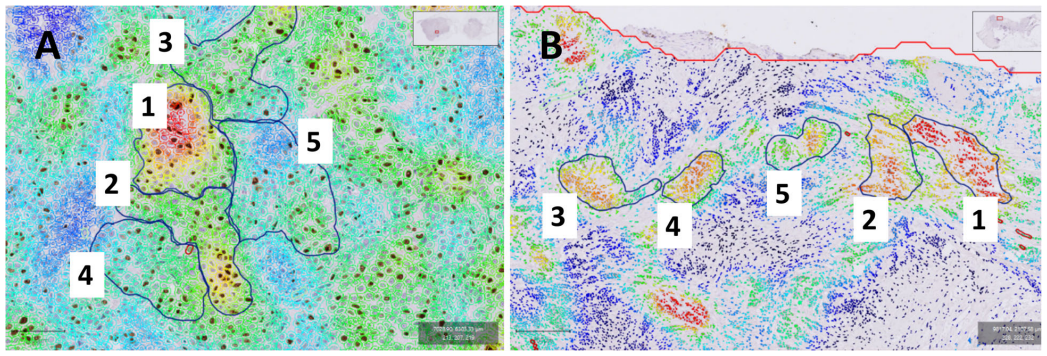

Fig. 4 **A** and **B** Hotspot identification and delineation images from QuPath. Areas of 100 tumour cell nuclei ordered from the area with the highest proportion of Ki-67 positive tumour cell nuclei [1] to the lowest [5]

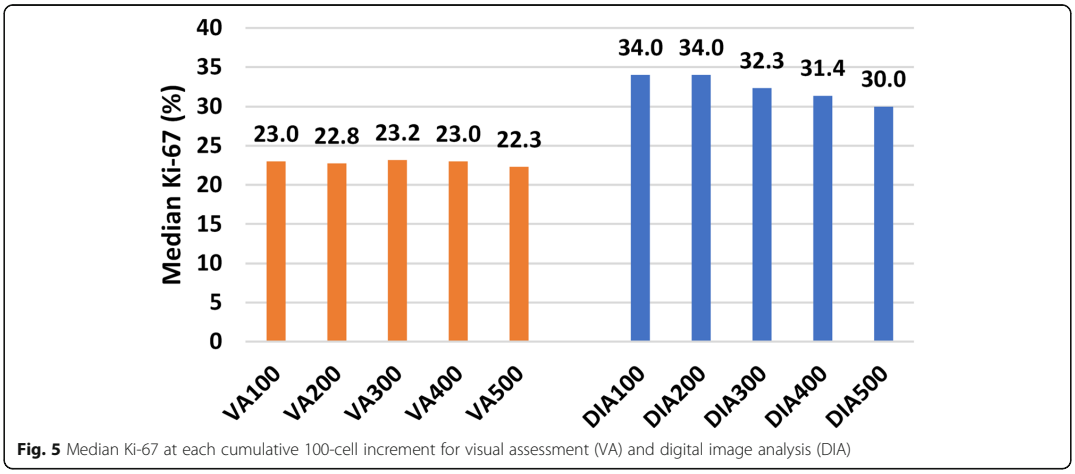

Fig. 5 Median Ki-67 at each cumulative 100-cell increment for visual assessment (VA) and digital image analysis (DIA)

at VA100, rising to 132 (53.2%) at VA500. Eight of these cases were downgraded from Intermediate at VA100 to Low at VA500, and eight were upgraded to High. A total of 77 cases (31.4%) were classified as High at VA100 falling to 72 (29.0%) at VA500. Thirteen cases were downgraded from High at VA100 to Intermediate at VA500, and none were downgraded to Low (Fig. 6).

Digital image analysis

Using the DIA median-derived cut-off levels, 44 cases (17.7%) were classified as Low at DIA100, rising to 75 (30.2%) at DIA500. Thus, with increasing number of cells counted a further 31 cases (12.5%) were classified as Low. None were upgraded from Low to Intermediate at DIA500. One hundred and four cases (41.9%) were classified as Intermediate at DIA100, falling to 94 cases (37.9%) at DIA500. Thirty cases were downgraded from Intermediate at DIA100 to Low at DIA500. None were upgraded to High. One hundred cases (40.3%) were classified as High at DIA100, falling to 79 (31.9%) at DIA500. Twenty-six cases were downgraded from High at DIA100 to Intermediate at DIA500. None were downgraded from High to Low (Fig. 6).

The numbers of cases classified as Low were similar in VA100 (48 cases), VA500 (44 cases) and DIA100 (44 cases) but increased at DIA500 (75 cases). The number of cases classified as High was greatest at DIA100 (100 cases), falling to levels comparable with VA100 (77 cases) and VA500 (72 cases) at DIA500 (79 cases) (Table 1; Fig. 6).

Ki-67 and histopathological grade

Grade 1

Among the 16 Grade 1 tumours, six (37.5%) tumours were classified as Ki67 Low at VA500. Five cases were classified as Low at DIA100 rising to nine (56.3%) at DIA500 (Table 1).

Grade 2

Of the 131 Grade 2 tumours, 13 (9.9%) were classified as High at VA500. Using DIA, 30 (22.9%) were High at DIA100 falling to 21 (16%) at DIA500. A higher number of Grade 2 tumours were classified as Intermediate in VA compared to DIA (Table 1).

Grade 3

Of the 101 Grade 3 tumours, 59 (58.4%) were classified as High at VA500. Using DIA, 70 (69.3%) were High at DIA100, falling to 58 (57.4%) at DIA500. The number of Grade 3 tumours classified as Low was greatest at DIA500 (12 (16%)) (Table 1).

Ki-67 and mitotic count

There was a clear association ($p < 0.001$) between high mitotic count (> 14.5 mitoses/10 HPF) and Ki-67 High across all counting modalities. The highest number of cases were observed at DIA100 where 51 of 62 (82.3%) cases with high mitotic count were classified as Ki-67 High (Table 1).

Ki-67 and prognosis

There was no clear association between Ki-67 cell counts and risk of death. By the end of follow-up, 108 (43.5%) patients had died of BC.

For VA100 High, the cumulative risk of death from BC during the first five years after diagnosis was 32.5% (95% CI 23.3–44.2), and 46.8% (95% CI 36.4–58.5) 10 years after diagnosis.

For VA500 High, the corresponding risks were 37.5% (95% CI 27.5–49.7) and 48.6% (95% CI 37.8–60.7),

Table 1 Patient and tumour characteristics according to Ki67 visual assessment (VA) and digital image analysis (DIA) of full face tissue sections

Total study population		VA categories 100 cells (median 22.3%)				VA Categories 500 cells (Median 22.3%)				DIA Categories 100 cells (Median 30%)				DIA Categories 500 cells (Median 30%)			
		< 12.3	> 12.3- < 32.3	> 32.3	χ^2	≤ 12.3	> 12.3- < 32.3	≥ 32.3	χ^2	≤ 20	> 20- < 40	≥ 40	χ^2	≤ 20	> 20- < 40	≥ 40	χ^2
N (%)	248	48	123	77		44	132	72		44	104	100		75	94	79	
Mean age at diagnosis range (42–95) (SD)	69.9 (10.9)	69.7 (11.0)	69.8 (11.5)	70.2 (9.9)		71.7 (11.3)	69.0 (10.9)	70.6 (10.5)		71.0 (10.5)	69.4 (11.2)	69.9 (10.8)		70.5 (11.0)	69.4 (11.2)	70.0 (10.5)	
Mean follow-up, years (SD)	10.9 (9.6)	12.3 (10.3)	11.3 (9.6)	9.4 (9.1)		12.6 (10.4)	11.4 (9.5)	8.9 (9.1)		11.7 (9.4)	11.5 (10.1)	9.9 (9.1)		11.0 (10.0)	12.1 (9.5)	9.4 (9.3)	
Deaths from breast cancer (%)	108 (43.6)	19 (39.6)	46 (37.4)	43 (55.8)	< 0.001	17 (38.6)	49 (37.1)	42 (58.3)	< 0.001	15 (34.1)	39 (37.5)	54 (54.0)	< 0.001	28 (37.3)	38 (40.4)	42 (53.2)	< 0.001
Deaths from other causes (%)	124 (50)	25 (52.1)	69 (56.1)	30 (39.0)		22 (50.0)	75 (56.8)	27 (37.5)		25 (56.8)	57 (54.8)	42 (42.0)		41 (54.7)	49 (52.1)	34 (43.0)	
Histologic grade (%)																	
I	16 (6.5)	6 (12.5)	10 (8.1)	0	< 0.001	6 (13.6)	10 (7.6)	0	< 0.001	5 (11.4)	11 (10.6))	0	< 0.001	9 (12.0)	7 (7.5)	0	< 0.001
II	131 (52.8)	38 (79.2)	72 (58.5)	21 (27.3)		34 (77.3)	84 (63.6)	13 (18.1)		34 (77.3)	67 (64.4)	30 (30.0)		54 (72.0)	56 (59.6)	21 (26.6)	
III	101 (40.7)	4 (8.3)	41 (33.3)	56 (72.7)		4 (9.1)	38 (28.8)	59 (82)		5 (11.4)	26 (25.0)	70 (70.0)		12 (16.0)	31 (33.0)	58 (73.4)	
Lymph node metastasis (%)																	
Yes	96 (38.7)	18 (37.5)	44 (35.8)	34 (44.2)	0.274	17 (38.6)	47 (35.6)	32 (44.4)	0.536	18 (41.0)	35 (33.7)	43 (43.0)	0.630	28 (37.3)	31 (33.0)	37 (46.8)	0.135
No	96 (38.7)	19 (39.6)	53 (43.1)	24 (31.2)		18 (40.9)	53 (40.2)	25 (34.7)		18 (41.0)	41 (33.7)	37 (37.0)		29 (38.7)	42 (44.7)	25 (31.7)	
Unknown histology	56 (22.6)	11 (22.9)	26 (21.1)	19 (24.7)		9 (20.5)	32 (24.2)	15 (20.8)		8 (18.2)	28 (27.0)	20 (20.0)		18 (24.0)	21 (22.3)	17 (21.5)	
Tumor size (%)																	
≤ 2 cm	113 (45.6)	19 (39.6)	65 (52.9)	29 (37.7)	0.317	17 (38.6)	70 (53.0)	26 (36.1)	0.162	21 (47.7)	52 (50.0)	40 (40.0)	0.863	33 (44.0)	48 (51.1)	32 (40.5)	0.562
> 2-≤ 5 cm	34 (13.7)	6 (12.5)	15 (12.2)	13 (16.9)		5 (11.4)	14 (10.6)	15 (20.8)		6 (13.6)	12 (11.5)	16 (16.0)		8 (10.7)	15 (16.0)	11 (13.9)	
Uncertain, but > 2 cm	30 (12.1)	5 (10.4)	15 (12.2)	10 (13.0)		7 (15.9)	13 (9.9)	10 (12.1)		5 (11.4)	11 (10.6)	14 (14.0)		9 (12.0)	11 (11.7)	10 (12.7)	
Uncertain	71 (28.6)	18 (37.5)	28 (22.8)	25 (32.5)		15 (34.1)	35 (26.5)	21 (29.2)		12 (27.3)	29 (27.9)	30 (30.0)		25 (33.3)	20 (21.3)	26 (32.9)	
Stage (%)																	
I	114 (46.0)	19 (39.6)	66 (53.7)	29 (37.7)	0.061	18 (40.9)	69 (52.3)	27 (37.5)	0.288	20 (45.5)	52 (50.0)	42 (42.0)	0.599	33 (44.0)	51 (54.3)	30 (38.0)	0.071
II	101 (40.7)	20 (41.7)	46 (37.4)	35 (45.5)		20 (45.5)	48 (36.4)	33 (45.8)		20 (45.5)	39 (37.5)	42 (42.0)		32 (42.7)	35 (37.2)	34 (43.0)	
III	17 (6.9)	4 (8.3)	8 (6.5)	5 (6.5)		4 (9.1)	8 (6.1)	5 (6.9)		2 (4.6)	7 (6.7)	8 (8.0)		7 (9.3)	2 (2.1)	8 (10.1)	
IV	13 (5.2)	4 (8.3)	1 (0.8)	8 (10.4)		1 (2.3)	5 (3.8)	7 (9.7)		1 (2.3)	4 (3.9)	8 (8.0)		1 (1.3)	5 (5.3)	7 (8.9)	
Unknown	3 (1.2)	1 (2.1)	2 (1.6)	0		1 (2.3)	2 (1.5)	0		1 (2.3)	2 (1.9)	0		2 (2.7)	1 (1.1)	0	

Table 1 Patient and tumour characteristics according to Ki67 visual assessment (VA) and digital image analysis (DIA) of full face tissue sections *(Continued)*

Total study population		VA categories 100 cells (median 22.3%)				VA Categories 500 cells (Median 22.3%)				DIA Categories 100 cells (Median 30%)				DIA Categories 500 cells (Median 30%)			
		< 12.3	> 12.3- < 32.3	> 32.3	χ^2	≤ 12.3	> 12.3- < 32.3	≥ 32.3	χ^2	≤ 20	> 20- < 40	≥ 40	χ^2	≤ 20	> 20- < 40	≥ 40	χ^2
Molecular subtype (%)																	
Luminal A	110 (44.4)	36 (75.0)	63 (51.2)	11 (14.3)	< 0.001	36 (81.8)	68 (51.5)	6 (8.3)	< 0.001	36 (81.8)	55 (52.9)	19 (19.0)	< 0.001	60 (80.0)	41 (43.6)	9 (11.4)	< 0.001
Luminal B (HER2-)	82 (33.1)	6 (12.5)	40 (32.5)	36 (46.8)		3 (6.8)	45 (34.1)	34 (47.2)		2 (4.6)	29 (27.9)	51 (51.0)		4 (5.3)	37 (39.4)	41 (51.9)	
Luminal B (HER2+)	28 (11.3)	5 (10.4)	11 (8.9)	12 (15.6)		2 (4.6)	14 (10.6)	12 (16.7)		4 (9.1)	11 (10.6)	13 (13.0)		6 (8.0)	9 (9.6)	13 (16.5)	
HER2 type	12 (4.8)	0	5 (4.1)	7 (9.1)		0	3 (2.3)	9 (12.5)		0	6 (5.8)	6 (6.0)		1 (1.3)	5 (5.3)	6 (7.6)	
TN	16 (6.4)	1 (2.1)	4 (3.2)	11 (14.3)		3 (6.9)	2 (1.5)	11 (15.3)		2 (4.6)	3 (3.0)	11 (11.0)		4 (5.4)	2 (2.2)	10 (12.6)	
Ki67 TMA high/low (%)																	
Ki67 < 15%	125 (50.4)	41 (85.4)	70 (56.9)	14 (18.2)	< 0.001	41 (93.2)	75 (56.8)	9 (12.5)	< 0.001	42 (95.5)	62 (59.6)	21 (21.0)	< 0.001	69 (92.0)	45 (47.9)	11 (13.9)	< 0.001
Ki67 ≥ 15%	123 (49.6)	7 (14.6)	53 (43.1)	63 (81.8)		3 (6.8)	57 (43.2)	63 (87.5)		2 (4.6)	42 (40.4)	79 (79.0)		6 (8.0)	49 (52.1)	68 (86.1)	
Mitoses/10 HPF median (IQR p25, p75) p25 = 3 p50 = 8 p75 = 14.5																	
Mitoses/10 HPF quartiles (%)																	
≤ 3	72 (29.0)	33 (68.8)	35 (28.5)	4 (5.2)	< 0.001	29 (65.9)	41 (31.1)	2 (2.8)	< 0.001	26 (59.1)	36 (34.6)	10 (10.0)	< 0.001	44 (57.9)	21 (22.6)	7 (8.9)	< 0.001
> 3-≤8	67 (27.0)	9 (18.8)	49 (39.8)	9 (11.7)		11 (25.0)	49 (37.1)	7 (9.7)		11 (25.0)	41 (39.4)	15 (15.0)		21 (27.6)	38 (40.9)	8 (10.1)	
> 8-≤14.5	47 (19.0)	6 (12.5)	19 (15.5)	22 (28.6)		4 (9.1)	23 (17.4)	20 (27.8)		6 (13.6)	16 (15.4)	25 (25.0)		10 (13.2)	17 (18.3)	20 (25.3)	
> 14.5	62 (25.0)	0	20 (16.3)	42 (54.6)		0	19 (14.4)	43 (59.7)		1 (2.3)	11 (10.6)	50 (50.0)		1 (1.3)	17 (18.3)	44 (55.7)	

Abbreviations: *SD* standard deviation, *HER2* human epidermal growth factor receptor 2, *TN* triple negative phenotype, *TMA* tissue microarray, *HPF* high power fields

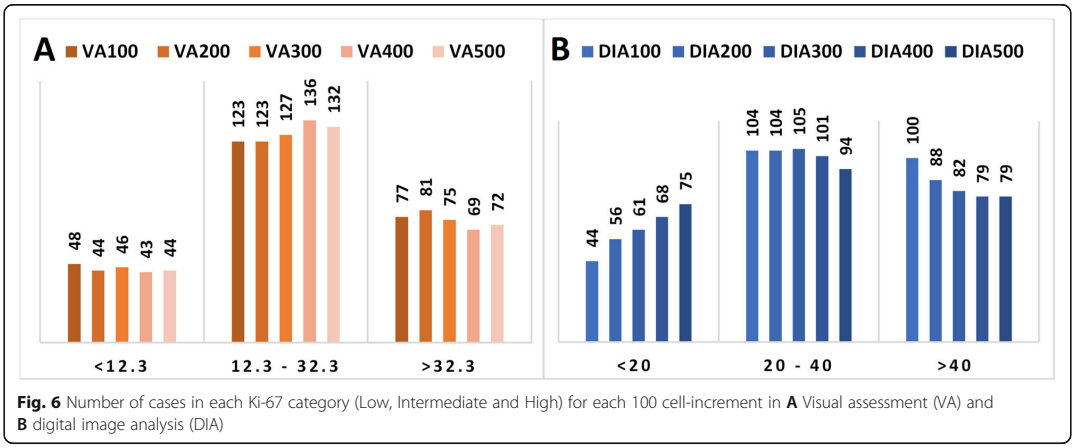

Fig. 6 Number of cases in each Ki-67 category (Low, Intermediate and High) for each 100 cell-increment in **A** Visual assessment (VA) and **B** digital image analysis (DIA)

respectively. Using VA500 Low as the reference, the rate of death from BC was unchanged for VA500 Intermediate but was higher for VA500 High (HR 1.94 ((95% CI 1.1–3.4))(Table 2; Fig. 7A).

For DIA100 High, the cumulative risk of death from BC during the first five years after diagnosis was 31.0% (95% CI 22.9–41.1) and after 10 years 44.0% (CI 34.9–54.3).

For DIA500 High, risk was 32.9% (CI 23.7–44.4) within the first five years, and 44.3% (CI 34.2–55.9) within the first 10 years.

Using DIA100 Low as the reference, the rate of death from BC was unchanged for DIA100 Intermediate but was higher for DIA100 High (HR 1.80 (95% CI 1.02–3.19), Table 2; Fig. 7B).

Comparison of methods

The Bland-Altman plots show that both DIA100 and DIA500 were clearly correlated to VA500. However, the mean values for Ki-67 using DIA (100 and 500) were on average higher than those for VA500, and the differences between DIA and VA500 increased with increasing mean values (Fig. 8). Harrell's C test showed no clear difference in predictive ability between the VA and DIA methods. A Cox model including grade and DIA100 correctly predicted survival times in 61% of cases, compared to 60% of cases for models combining grade and any one of the other three methods (VA100, VA500 and DIA500).

Discussion

In this study we compared Ki-67 protein expression in IHC-stained BC tissue sections assessed by DIA using the QuPath platform, and by VA according to current recommended guidelines [22, 23]. We found that the median Ki-67 level was higher using DIA compared to VA. We show that while the proportion of Ki-67 positive tumour cells did not change substantially with increasing number of cells counted using VA, the number of cells counted did impact the result when using DIA. Furthermore, the highest proportion of patients with Ki-67 High tumours was found when 1-200 cells were counted using DIA. All counting methods predicted a poor prognosis for patients with the highest Ki-67 levels, but with little difference between the methods.

Gerdes proposed in 1984 that, with the help of the monoclonal antibody Ki-67, we now had a simple means of estimating the growth fraction of a given subset of human cells. This would be of particular interest in tumour diagnostics since the proportion of proliferating cells in given neoplasms would be of prognostic value and could contribute to the determination of treatment strategies [2]. Ki-67 is now used as a prognostic marker and may also be used as a predictive marker of response to chemotherapy [7–9]. There has been considerable debate regarding counting methods and cut-of levels for both prognostication and determination of treatment [10, 16, 33–37].

At the St. Gallen conference in 2015, it was proposed that the in-house median value at each laboratory should

Table 2 Risk of death from breast cancer according to Ki-67 level and counting procedures, expressed as cumulative incidence and hazard ratios of death from breast cancer

		VA100			**VA500**	
	Low	*Intermediate*	*High*	*Low*	*Intermediate*	*High*
Cum. inc. 5 years, % (95% CI)	18.8 (10.2–32.9)	19.5 (13.5–27.7)	32.5 (23.3–44.2)	18.2 (9.5–33.1)	17.4 (11.9–25.1)	37.5 (27.5–49.7)
Cum. inc. 10 years, % (95% CI)	22.9 (13.4–37.6)	28.5 (21.3–37.3)	46.8 (36.4–58.5)	25.0 (14.7–40.6)	27.3 (20.5–35.7)	48.6 (37.8–60.7)
HR (95% CI) [1]	1.00	1.01 (0.59–1.72)	1.73 (1.01–2.98)	1.00	1.01 (0.58–1.76)	1.94 (1.1–3.42)
Harrell's C [1]		0.58			0.59	
h (95% CI) [2]	1.00	0.92 (0.53–1.60)	1.4 (0.76–2.58)	1.00	0.96 (0.55–1.68)	1.65 (0.85–3.19)
Harrell's C [2]	0.60			0.60		
		DIA100			**DIA500**	
	Low	*Intermediate*	*High*	*Low*	*Intermediate*	*High*
Cum. inc. 5 years, % (95% CI)	15.9 (7.9–30.5)	19.2 (12.9–28.2)	31.0 (22.9–41.1)	21.3 (13.7–32.4)	17.0 (10.8–26.3)	32.9 (23.7–44.4)
Cum. inc. 10 years, % (95% CI)	22.7 (12.9–38.1)	26.9 (19.5–36.6)	44.0 (34.9–54.3)	28.0 (19.3–39.6)	27.7 (19.8–37.9)	44.3 (34.2–55.9)
HR (95% CI) [1]	1.00	1.14 (0.63–2.06)	1.80 (1.02–3.19)	1.00	1.00 (0.62–1.64)	1.60 (0.99–2.58)
Harrell's C [1]		0.58			0.57	
h (95% CI) [2]	1.00	1.08 (0.59–1.97)	1.48 (0.78–2.82)	1.00	0.93 (0.56–1.53)	1.27 (0.72–2.22)
Harrell's C [2]	0.61			0.60		

[1] Unadjusted. [2] Adjusted for tumour grade (1, 2 or 3). *CI* Confidence interval, *Cum. inc.* Cumulative incidence, *HR* Hazard ratio

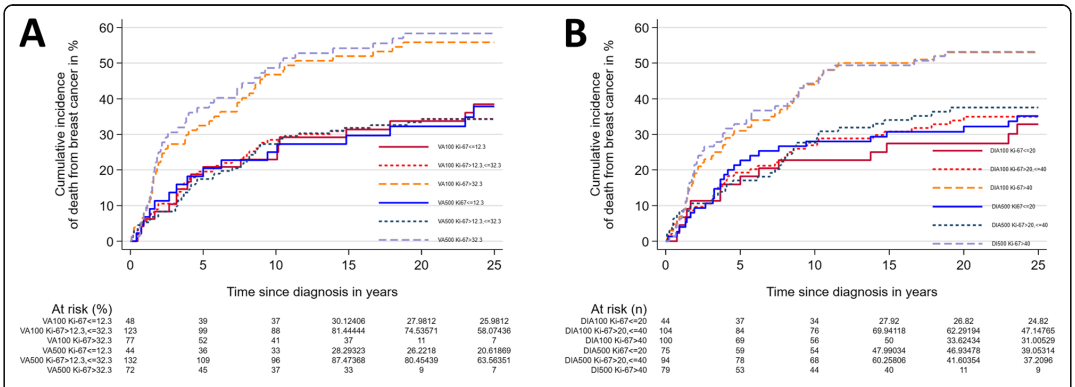

Fig. 7 Cumulative incidence of death from breast cancer. **A** Visual assessment (VA), Grays' test for VA100 (Low, Intermediate and High) $P = 0.1062$; Grays' test for VA500 (Low, Intermediate and High) $P = 0.0500$. **B** Digital image analysis (DIA), Gray's test for DIA100 (Low, Intermediate and High) $P = 0.3335$; Grays' test for DIA500 (Low, Intermediate and High) $P = 0.0796$

be used to determine cut-off values to offset interlaboratory differences [17]. More recently, the 17th St. Gallen International Breast Cancer Conference proposed that Ki67 should be used to determine treatment in estrogen receptor-negative, HER2-negative T1-2N0-1 BC in accordance with the International Ki67 Breast Cancer Working Group. The determination of cut-off levels is still challenging as reflected by these latest recommendations where only clearly low or clearly high levels of KI67 protein expression are considered to have clinical utility [13, 24]. Romero and co-workers suggested in 2014 a stepwise counting strategy without fixed denominators, especially to target heterogenetic tumours with some highly proliferative hotspots [29] and the International Ki67 Breast Cancer Working Group has proposed a standardized visual scoring method using a scoring app available online [13]. Thus, the need for a standardized approach in the IHC assessment of Ki-67 in BC has been recognized.

In this study, we found clear differences in the median levels of Ki-67 positivity between VA and DIA (VA500 (22.3%) and DIA500 (30%)) reflecting the respective method's ability to identify hotspot areas in the tissue section. This is in agreement with previous studies [38–41]. Still, others have reported no real differences between the two methods [38, 41–44]. In the present study, the threshold set for OD sum in DIA and thus the ability to digitally detect positive Ki-67 staining, was set close to the pathologist's threshold for positive staining before commencement of classifier training and digital assessment. The difference between the median values in VA and DIA, suggests that there is need for

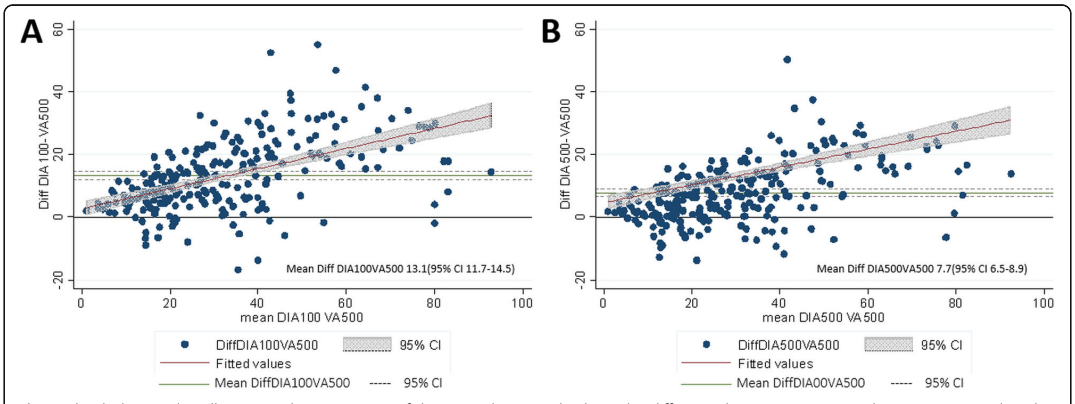

Fig. 8 Bland-Altman plots illustrating the agreement of the VA and DIA methods. **A** The difference between DIA100 and VA500 compared to the mean of DIA100 and VA500. **B** The difference between DIA500 and VA500 compared to the mean of VA500 and DIA500

calibration of cut-off levels according to the method employed. The Bland-Altman plot [45, 46] shows that the methods perform quite similarly but that DIA in general reported higher levels of Ki-67 positivity compared to VA. Introduction of DIA for the assessment of Ki-67 in our hands would thus require recalibration of cut-off levels in order to correspond to established clinically actionable Ki-67 levels. This underlines the importance of understanding the consequences the introduction of a new method may have on patient treatment. However, Harrell's C test [47] and risk-of-death analyses did not show any clear difference between methods in their ability to predict survival.

Recent studies have suggested that downgrading of Ki-67 levels in some tumors may occur in VA when more than 2-300 cells are counted [29, 48]. However, in the present study we found that there was little difference in the percentage of Ki-67 positive cells in each of the five 100-cell increments across cut-off levels using VA. This would imply that it may not be necessary to count more than 2-300 cells in VA. On the other hand, there was a clear fall in the number of Ki-67 High cases and a corresponding rise in the number of cases classified as Low with increasing cell counts using DIA. Thus, using DIA, the highest proportion of Ki-67 positive cell nuclei is achieved by counting 1-200 cells in digitally identified hotspots. This appears to be in agreement with Romero et al. [29]. In our hands, a significantly higher number of grade 3 tumours was found in DIA100 High compared to VA100 High, VA500 High and DIA500 High ($p < 0.0001$). Thus, we show that declining Ki-67 levels are more likely to occur using DIA compared to VA. A greater number of deaths from BC was seen at DIA100 Ki-67 High compared to DIA500 Ki-67 High (54 vs. 42 cases; 50.0% vs.38.9%). In comparison, for VA, the difference in the numbers of deaths from BC between the VA100 Ki-67 High group and VA500 Ki-67 High group were negligible (43 vs. 42 cases; 40.0% vs. 38.9%).

The cases included in our study were diagnosed with BC over a timespan extending from 1961 to 2008, and pre-analytical conditions may have varied. Ki-67 IHC is robust in formalin-fixed, paraffin-embedded tissue [49, 50] and antigenicity is well preserved, though staining intensity is prone to be reduced with increasing storage-time [51–53]. In the present study, staining intensity was not assessed. The international Ki-67 in Breast Cancer Working Groups has expressed concern about Ki-67 assessment of tissue stored in paraffin-blocks for more than five years, because of the degradation of the epitope in paraffin blocks. The exact mechanisms of the Ki-67 epitope degradation are not yet fully explored and there is still concern about the precision of the assessment. They recommend that the internationally standardized laboratory guidelines (ASCO and CAP) for HER2 and hormone receptors should also be applied to Ki-67 IHC [13]. Variation in tissue processing, staining reagents, laboratory protocols, and digitization procedures, may all contribute to variability in the interpretation of IHC in both conventional VA and DIA. Standardization of the preanalytical and analytical phases of tissue processing would greatly contribute to the creation of a more robust classifier for the digital analysis, although BC's inherent heterogeneity would still remain a challenge [21, 54, 55]. In the present study, we included only invasive cancer (not otherwise specified). The classifier would require further development to reliably identify tumour cell nuclei morphologies such as those typical of lobular carcinoma. We found that some tissue slides were not suitable for DIA due to artefacts such as tissue folds, damaged tissue, or inadequate staining.

Studies comparing the QuPath platform with other digital analysis platforms have shown good reproducibility and functionality [38, 56]. One study comparing DIA using QuPath with VA shows that QuPath gave stronger prognostic stratification than the manual method [57]. The QuPath software was developed to improve the efficiency, objectivity, and reproducibility of digital histopathology, as well as biomarker analysis using digital images [27]. In the present study a greater number of cases were classified as either Low or High using QuPath DIA compared to conventional VA. Using the Ventana Virtuoso platform, Kwon et al., reported high concordance between VA and DIA, and stronger accuracy using DIA in the High Ki-67-group (≥ 20%) compared to the low Ki-67-group (≤ 10%). They also found that DIA is more useful in the borderline cases between cut-off levels citing observer variation as a greater challenge in these cases [55].

The initial regions of interest on the WSIs were manually delineated using the brush tool in QuPath. This approach was time-consuming, and automatic tissue detection or WSI annotation would be preferable. The first 100-cell increment counted by DIA was visually selected within the area of the tumour with the highest expression of Ki-67 in the heat map. To identify these hotspots, we created measurement maps for nucleus DAB OD mean with 50 μm smoothing. In this process we were aware that tissue folds, ink debris and abundant lymphocytes could result in higher OD in non-relevant areas. Thus, the measurement map method for detecting hotspots may not be suitable in sections with too many such irregularities and artefacts. We noted that membranous staining presented a greater challenge to the QuPath software than to experienced pathologists. A pathologist will ignore non-relevant staining, while the software will detect anything with color, unless the classifier is trained to ignore it.

In the present study, the QuPath-based DIA method entailed a considerable amount of manual adjustment, thus rendering it time-consuming and impractical for implementation in a clinical setting. Robertson et al. published a paper in 2020 that suggested that a digital global scoring of Ki-67 was a practical and clinically valid approach [58]. The International Ki67 in Breast Cancer Working Group discuss several methods including global score and hot spot score in addition to their own online scoring app giving a weighted global score based on the assessment of 100 cells in each of four areas in the tumour section (negligible, low, medium, or high). To the best of our knowledge, the latter has not achieved widespread acceptance. They point out that none of the current scoring systems achieved high analytical validity [13]. Global scoring was not evaluated in the present study. We chose to follow the guidelines for visual assessment of Ki-67 in BC currently in use in Norway, counting 500 cells in the area of the tumour with highest proliferation as assessed under the light microscope [23]. We used the same approach in the digital assessment. We acknowledge that this method may have drawbacks but in comparing the two methods our main finding remains that recalibration of cut-off levels is essential when introducing new methodology in the assessment of tissue biomarkers [23].

The number of cases in this study was limited and thus survival analyses should be interpreted with caution. Our results need to be validated in larger series of cases from other sources. However, the study clearly illustrates that new methodology in biomarker assessment requires recalibration of established cut-off levels.

Conclusions

In this study we show that assessment of Ki-67 in breast tumours using DIA identifies a greater proportion of cases with high Ki-67 levels compared to VA of the same tumours. Using VA, we found that the results do not change substantially with increasing number of cells counted. However, we propose that, using DIA, it may be sufficient to count 1-200 cells in a digitally selected hotspot area to identify the greatest number of cases with Ki-67 High tumours. Associations with survival should be interpreted with caution due to the limited number of cases and variation of pre-analytical conditions of the tissue samples in this study. Finally, our findings underline the need for recalibration of established cut-off levels on the introduction of new methodology.

Abbreviations

BC: Breast cancer; IHC: Immunohistochemistry/immunohistochemical; DIA: Digital image analysis; VA: Visual assessment; OD: Optical density; WSI: Whole slide images; CI: Confidence interval

Acknowledgements

The authors thank the Department of Pathology, St. Olav´s Hospital, Trondheim University Hospital for making the diagnostic archives available for the study and for digitizing histopathological slides, and the Cancer Registry of Norway for supplying the patient data.

Authors' contributions

Conceptualisation: AMB, AHS. Methodology: AHS, HSP, AMB. Formal analysis: AHS, SO, MV, AMB. Original draft preparation: AHS, AMB. Manuscript review and editing: AMB, AHS, MV, SO, HSP. The author(s) read and approved the final manuscript.

Funding

This present study has received financial support from the Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway. Previous data that is also included int study received financial support from the Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology and The Research Council of Norway.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to issues of sensitivity and limitations determined in the conditions for approval by the Regional Ethics Committee. However, they can be made available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Approval of this study was granted by the Regional Committee for Medical and Health Research Ethics, Central Norway (REK 836-09). The approval includes dispensation from the general requirement of patient consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 9 February 2022 Accepted: 12 April 2022
Published online: 06 May 2022

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Publisher's Note

Paper II

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RESEARCH ARTICLE

PAK1 copy number in breast cancer—Associations with proliferation and molecular subtypes

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Citation: Skjervold AH, Valla M, Ytterhus B, Bofin AM (2023) *PAK1* copy number in breast cancer—Associations with proliferation and molecular subtypes. PLoS ONE 18(6): e0287608. https://doi.org/10.1371/journal.pone.0287608

Editor: Elingarami Sauli, Nelson Mandela African Institute of Science and Technology, UNITED REPUBLIC OF TANZANIA

Received: January 30, 2023

Accepted: June 8, 2023

Published: June 27, 2023

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pone.0287608

Data Availability Statement: The datasets generated in the current study are not publicly available due to ethical and legal restrictions imposed by General Data Protection Regulations

Abstract

Introduction

P21-activated kinase 1 (*PAK1)* is known to be overexpressed in several human tumour types, including breast cancer (BC). It is located on chromosome 11 (11q13.5-q14.1) and plays a significant role in proliferation in BC. In this study we aimed to assess *PAK1* gene copy number (CN) in primary breast tumours and their corresponding lymph node metastases, and associations between *PAK1* CN and proliferation status, molecular subtype, and prognosis. In addition, we aimed to study associations between CNs of *PAK1* and *CCND1*. Both genes are located on the long arm of chromosome 11 (11q13).

Methods

Fluorescence *in situ* hybridization for *PAK1* and Chromosome enumeration probe (CEP)11 were used on tissue microarray sections from a series of 512 BC cases. Copy numbers were estimated by counting the number of fluorescent signals for *PAK1* and CEP11 in 20 tumour cell nuclei. Pearson's x^2 test was performed to assess associations between *PAK1* CN and tumour features, and between *PAK1* and *CCND1* CNs. Cumulative risk of death from BC and hazard ratios were estimated in analysis of prognosis.

Results

We found mean *PAK1* CN $\geq$4<6 in 26 (5.1%) tumours, and CN $\geq$ 6 in 22 (4.3%) tumours. The proportion of cases with copy number increase (mean CN $\geq$4) was highest among HER2 type and Luminal B ($HER2^-$) tumours. We found an association between *PAK1* CN increase, and high proliferation, and high histological grade, but not prognosis. Of cases with *PAK1* CN $\geq$ 6, 30% also had *CCND1* CN $\geq$ 6.

(GDPR), National health research legislation and the conditions for approval by the Regional Committee for Medical and Health Research Ethics, Midt-Norge (REK 836/2009), but may be available from the corresponding author on reasonable request and/or the Institutional Research Officer, Department of Clinical and Molecular Medicine, Faculty of Medicine, NTNU at postmottak@mh.ntnu.no.

Funding: The research leading to these results received funding from The Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology (NTNU), The Joint Research Committee between St. Olav's Hospital and the Faculty of Medicine and Health Sciences, NTNU (FFU), and the Department of Clinical and Molecular Medicine, NTNU. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Conclusions

PAK1 copy number increase is associated with high proliferation and high histological grade, but not with prognosis. *PAK1* CN increase was most frequent in the HER2 type and Luminal B (HER2$^-$) subtype. *PAK1* CN increase is associated with CN increase of *CCND1*.

Introduction

P21-activated kinases (PAK) are a family of serine/threonine protein kinases comprising six isoforms (*PAK1*–6). They are overexpressed in several human tumours, such as breast cancer (BC), colon cancer and lung cancer, and in neurofibromatosis [1]. The six PAK isoforms are subdivided in PAK1-3 (group I) and PAK4-6 (group II) [2, 3]. PAKs play a significant role in proliferation, cytoskeletal dynamics, and cell survival [1, 4]. Their roles in these cell processes make them potential therapeutic targets. More is known of the functions of PAK1 and PAK4, than of the other isoforms [5, 6].

PAK1 is located on chromosome 11 (q13.5-q14.1). Amplification of *PAK1* and high PAK1 protein levels are found in several human cancers, including BC [7–9], and are linked to aggressive tumour types, chemotherapy resistance and poor prognosis [4, 10–14]. In 2000, Mira *et al*. first discovered that *PAK1* had an important role in proliferation in BC cell lines [15]. Since then, *PAK1* has been found to be involved in many stages of the BC process and is known to regulate several signaling pathways. [4, 16–21]. *PAK1* amplification has recently been found to be significantly associated with reduced relapse-free survival of ER-positive BC patients [19]. *PAK1* is localized in the same chromosomal region as *CCND1*, 11q13 [22, 23]. Cyclin D1 (CCND1) has been found to be overexpressed in breast cancer, and *PAK1* is shown to regulate the expression of CCND1 in BC [8, 23].

In this study we aimed to assess *PAK1* gene copy number (CN) in a well-characterized series of primary BCs and their corresponding axillary lymph node metastases. We studied associations between *PAK1* CN and proliferation, molecular subtypes, and prognosis. In addition, we examined associations between CN of *CCND1*, assessed in an earlier study by our group [24], and *PAK1* CN.

Materials and methods

Study population

A population-based survey for the early detection of BC was conducted in the county of Nord-Trøndelag, Norway, between 1956 and 1959. The study included 25,727 women born 1886–1928 [25]. These women were followed for BC occurrence, through linkage with data from the Cancer Registry of Norway. During the follow-up years, between 1961 and 2008, 1379 new BCs were registered. Of these, 909 cases were included in the study population and were first reclassified into molecular subtypes in a previous published by our group in 2013 (Table 1) [26]. All patients were followed from time of diagnosis until death or December 31st, 2015.

For the present study, we performed fluorescence *in situ* hybridization (FISH) on tissue specimens from cases mainly diagnosed after 1985 (n = 558). Of these, 46 were excluded due to missing or insufficient tumour tissue (n = 25), or due to unsuccessful FISH (n = 21). Thus, 512 cases were suitable for assessment of *PAK1* and chromosome enumeration probe 11 (CEP11) CN in primary tumours (Fig 1). Of the 512 cases, 172 had lymph node metastases, and tissue from lymph node metastases was available for 143 cases. Cases with unsuccessful

Table 1. Reclassification of breast cancers into molecular subtypes [26].

Molecular subtype	Classified by
Luminal A	ER^+ and/or PR^+, $HER2^-$, Ki-67<15%
Luminal B ($HER2^-$)	ER^+ and/or PR^+, $HER2^-$, Ki-67≥15%
Luminal B ($HER2^+$)	ER^+ and/or PR^+, $HER2^+$
HER2 type	ER^-, PR^-, $HER2^+$
Basal-like	ER^-, PR^-, $HER2^-$, $CK5^+$ and/or $EGFR^+$
5-negative phenotype	ER^-, PR^-, $HER2^-$, $CK5^-$, $EGFR^-$

*ER = Oestrogen receptor, PR = Progesterone receptor, HER2 = Human epidermal growth factor receptor 2, CK5 = Cytokeratin 5, EGFR = Epidermal growth factor receptor 1

https://doi.org/10.1371/journal.pone.0287608.t001

FISH (n = 9) or insufficient amounts of tumour tissue (n = 11) were excluded. Hence, lymph node metastases from 123 cases were included in the analyses.

Specimen characteristics

The primary tumours were previously reclassified into histological type and grade according to present-day guidelines [26–28]. Tissue microarray (TMA) blocks were made using the TissueArrayer Minicore with TMA Designer2 software (Alphelys). Three 1-mm in diameter

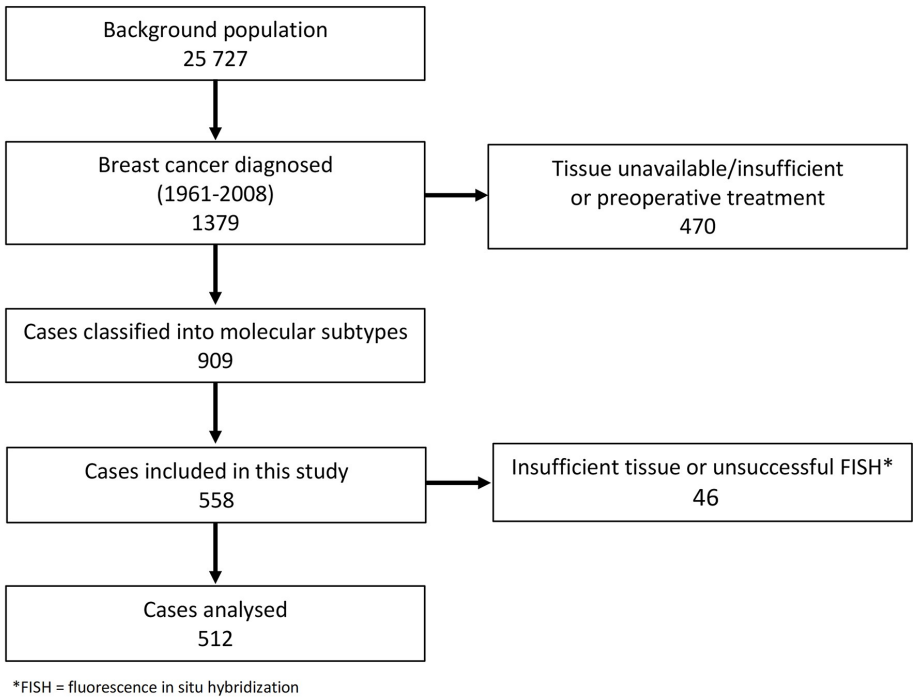

Fig 1. Overview of study population and cases included in this study.

https://doi.org/10.1371/journal.pone.0287608.g001

Table 2. Sources and dilutions of primary antibodies used for molecular subtyping [26].

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
Ki-67	MIB1	Dako	35 mg/l	1:100
CK5	XM26	Novocastra	50 mg/l	1:100
EGFR	2-18C9	Dako	Ready to use	No dilution

https://doi.org/10.1371/journal.pone.0287608.t002

tissue cylinders were extracted from the periphery of the primary tumour, and from lymph node metastases and transferred to TMA recipient blocks. Using sections from the TMAs, primary tumours were then reclassified into molecular subtypes using immunohistochemistry (IHC) and chromogenic *in situ* hybridization (CISH) as previously described (Table 1). Briefly, Oestrogen Receptor (ER), Progesterone Receptor (PR), the proliferation marker Ki-67, Cytokeratin 5 (CK5) and Epidermal Growth Factor Receptor 1 (EGFR) were assessed using IHC, and Human Epidermal Growth Factor Receptor 2 (HER2) was assessed using both CISH and IHC [26] (Table 2). In a previous study of *CCND1* CN, FISH was used to target *CCND1* and CEP11, using Dako Histology FISH Accessory Kit K 579911 probes for *CCND1* (3 μL, Empire Genomics) and CEP11 (1 μL, Abbott/VYSIS) [24].

Fluorescence in situ hybridization

For the present study of *PAK1* and CEP11 CN, FISH was done using DAKO Histology FISH Accessory Kit K 579911 according to the manufacturer's instructions. TMA sections were preheated at 60˚C for 1–2 h, then de-waxed and rehydrated. The slides were then boiled in a microwave oven for 10 min. in pretreatment solution and washed in DAKO wash buffer (2x3min.) after cooling (15 min.), followed by protein digestion in pepsin solution (37˚C, 25 min.). After protein digestion, the slides were washed in DAKO wash buffer (2x3 min.), dehydrated (2 min. in 70%, 85% and 95% ethanol), then air-dried for 15 min. at room temperature.

PAK1 (3 μL, PAK1-20-RE, SpectrumRed fluorochrome Empire Genomics) and CEP11 (3 μL, CEP11 [D11Z19], SpectrumGreen fluorochrome, VYSIS) probes were mixed with hybridizing buffer (9 μL, Empire Genomics) and applied to TMA slides according to the manufacturer's instructions. Coverslips were then applied to the slides, sealed with DAKO coverslip sealant, and the slides were dried for 20 min. After drying, denaturation was performed at 83˚C for 3 min., followed by hybridization at 37˚C overnight using DAKO hybridizer. Post-hybridization washes were done in 0.4 X SSC/ 0.3% NP-40 stringent wash buffer at 72˚C (2 min.) and 2 X SSC/ 0.1% NP-40 wash buffer at room temperature (1 min.). Slides were then dried at 37˚C for 15 min., DAPI II VYSIS (15 μl, no 06J50-001) was applied. The slides were then coverslipped and stored at −20˚C.

Scoring and reporting

A fluorescence microscope (Nikon Eclipse 90i) was used for counting *PAK1* and CEP11 CN. For each case, all available tissue spots were examined and the number of fluorescent signals for *PAK1* and CEP11 were counted in 20 well-preserved, non-overlapping tumour cell nuclei. Mean *PAK1* and CEP11 CNs was calculated for tumours and lymph node metastases and were first categorized as <4 and ≥ 4. In addition, to distinguish between low-level CN gain and high-level gain or gene amplification, we also subdivided CN into three categories: <4; $\geq 4 < 6$; and ≥ 6 according to guidelines for categorizing *HER2* CNs [29], a strategy which has been

used in previous studies of other genes by our group [24, 30–32]. The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) were followed [33].

Statistical analyses

Pearson's chi square test was used to compare tumour characteristics across categories of *PAK1* mean CN. Cumulative incidence of death from breast cancer was estimated, and Gray's test was used to compare equality between cumulative incidence curves. Cox proportional hazard analyses were used to estimate hazard ratios (HR) of breast cancer death with 95% confidence intervals (CI). The analyses were adjusted for age (≤ 49, 50–59, 60–64, 65–69, 70–74, ≥ 75), stage (I–IV), histological grade (1–3), and Ki67 status (</≥ 15%). Adjustments were made for each variable separately, and for age, grade, and stage combined. No clear violations of proportionality were observed in log minus-log plots. All statistical tests were two-sided and statistical significance was assessed at the 5% level. We used Stata 16 (Stata corp., College station, TX, USA) in the statistical analyses.

Ethics statement

This study was granted approval including dispensation from the general requirement of informed consent, by the Regional Committee for Medical and Health Research Ethics, Midt-Norge (REK 836/2009). All methods were carried out in accordance with relevant guidelines and regulations (The Declaration of Helsinki and national regulations (ACT 2008-06-20 no. 44: Act on medical and health research (the Health Research Act)).

Results

Patient and tumour characteristics for the 512 patients included in the present study are given in Table 3. The mean age at diagnosis was 75.4 years (range 41–96) and the mean follow-up after diagnosis was 9.1 years (SD = 7.2). At end of follow-up, 35.4% of patients had died from BC and 54.3% had died from other causes.

PAK1 and CEP11 copy number, and histological grade and proliferation

PAK1 CN ≥4 was found in 48 (9.4%) tumours (Table 3, Fig 2). Of these, 26 (5.1%) cases had mean CN ≥4<6, and 22 (4.3%) had mean CN ≥6. While 147/464 (31.7%) cases with CN <4 were grade 3, 22/48 (45.8%) cases with CN ≥4 were grade 3 (p = 0.037). We found no significant associations between *PAK1* CN increase and high histological grade using three categories of mean *PAK1* CN (Table 3).

PAK1 CN ≥4 was associated with high Ki-67 (≥15%). Of cases with *PAK1* CN <4, 178/464 (38.4%) had Ki-67 ≥15%, compared to 26/48 (54.2%) among those with *PAK1* CN ≥4 (p = 0.033). No association between *PAK1* CN increase and Ki-67 status was found when *PAK1* CN was subdivided into three categories. The median mitotic count was higher in cases with mean *PAK1* CN ≥4, compared to cases with mean CN <4 (8 mitoses/10 high power fields [HPF] and 5 mitoses/10 HPF, respectively). The proportion of cases with mitotic counts in the upper quartile was also higher for cases with mean *PAK1* CN ≥4, compared to those with mean CN <4 (106/464 [22.8%] and 14/48 (29.2%), respectively (p = 0.162)) (Table 3). Only seven cases showed CEP11 CN increase. Five of these were in cases with *PAK*1 CN <4. Of the 26 cases with *PAK1* CN ≥4<6, only two were accompanied by CEP11 CN increase (≥4<6). Of the 22 cases with *PAK1* CN ≥6, none had concurrent CN increase of CEP11.

Table 3. Patient and tumour characteristics according to *PAK1* copy number.

	Total study population	Mean *PAK1* copy number, three categories				Mean *PAK1* copy number, two categories		
		<4	≥4 to <6	≥6	p value (χ^2)	<4	≥4	p value (χ^2)
N (%)	512	464 (90.6)	26 (5.1)	22 (4.3)		464 (90.6)	48 (9.4)	
Mean age at diagnosis, years (SD)	75.4(41–96) (8.2)	75.5 (8.1)	75.2 (7.3)	74.3 (10.0)		75.5 (8.1)	74.8 (8.6)	
Mean follow-up, years (SD)	9.1 (7.2)	9.0 (7.0)	9.6 (6.5)	9.0 (7.5)		9.0 (7.0)	9.3 (6.9)	
Deaths from breast cancer (%)	181 (35.4)	161 (34.7)	9 (34.6)	11 (50.0)		161 (34.7)	20 (41.7)	
Deaths from other causes (%)	278 (54.3)	255 (55.0)	15 (57.7)	8 (36.4)		255 (55.0)	23 (47.9)	
Histological grade (%)								
I	56 (10.9)	55 (11.9)	0 (0)	1 (4.6)	0.082	55 (11.9)	1(2.1)	0.037
II	287 (56.1)	262 (56.5)	12 (46.2)	13 (59.1)		262 (56.5)	25 (52.1)	
III	169 (33.0)	147 (31.7)	14 (53.9)	8 (36.4)		147 (31.7)	22 (45.8)	
Lymph node metastasis (%)								
Yes	172 (33.6)	153 (33.0)	13 (50.0)	6 (27.3)	0.272	153 (33.0)	19 (39.6)	0.360
No	228 (44.5)	209 (45.0)	9 (34.6)	10 (45.5)		209 (45.0)	19 (39.6)	
Unknown histology	112 (21.9)	102 (22.0)	4 (15.4)	6 (27.3)		102 (22.0)	10 (20.8)	
Tumor size (%)								
≤2 cm	245 (47.9)	217 (46.8)	16 (61.5)	12 (54.6)	0.327	217 (46.8)	28 (58.3)	0.516
>2 cm, ≤ 5 cm	95 (18.6)	88 (19.0)	4 (15.4)	3 (13.6)		88 (19.0)	7 (14.6)	
>5 cm	10 (2.0)	9 (1.9)	1 (3.9)	0 (0)		9 (1.9)	1(2.1)	
Uncertain, but >2 cm	63 (12.3)	60 (12.9)	3 (11.5)	0 (0)		60 (12.9)	3 (6.3)	
Uncertain	99 (19.3)	90 (19.4)	2 (7.7)	7 (31.8)		90 (19.4)	9 (18.8)	
Stage (%)								
I	242 (47.3)	221 (47.6)	9 (34.6)	12 (54.6)	0.027	221 (47.6)	21 (43.8)	0.117
II	218 (42.6)	198 (42.7)	14 (53.9)	6 (27.3)		198 (42.7)	20 (41.7)	
III	27 (5.3)	22 (4.7)	3 (11.5)	2 (9.1)		22 (4.7)	5 (10.4)	
IV	23 (4.5)	22 (4.7)	0 (0)	1 (4.6)		22 (4.7)	1 (2.1)	
Unknown	2 (0.4)	1 (0.2)	0 (0)	1 (4.6)		1 (0.2)	1 (2.1)	
Molecular subtype (%)								
Luminal A	272 (53.1)	251 (54.1)	11 (42.3)	10 (45.5)	0.649	251 (54.1)	21 (43.8)	0.375
Luminal B ($HER2^-$)	121 (23.6)	105 (22.6)	8 (30.8)	8 (36.4)		105 (22.6)	16 (33.3)	
Luminal B ($HER2^+$)	42 (8.2)	39 (8.4)	1 (3.9)	2 (9.1)		39 (8.4)	3 (6.3)	
HER2 type	27 (5.3)	23 (5.0)	3 (11.5)	1 (4.6)		23 (5.0)	4 (8.3)	
5NP	11 (2.2)	11 (2.4)	0 (0)	0 (0)		11 (2.4)	0 (0)	
BP	39 (7.6)	35 (7.5)	3 (11.5)	1 (4.6)		35 (7.5)	4 (8.3)	
Histological type (%)								
Invasive carcinoma NOS	353 (69.0)	318 (68.5)	19 (73.1)	16 (72.7)	0.593	318 (68.5)	35 (69.0)	0.273
Lobular carcinoma	66 (12.9)	61 (13.2)	2 (7.7)	3 (13.6)		61 (13.2)	5 (10.4)	
Tubular carcinoma	1 (0.2)	1 (0.2)	0 (0)	0 (0)		1 (0.2)	0 (0)	
Mucinous carcinoma	24 (4.7)	23 (5.0)	1 (3.9)	0 (0)		23 (5.0)	1 (2.1)	
Medullary carcinoma	14 (2.7)	10 (2.2)	3 (11.5)	1 (4.6)		10 (2.2)	4 (8.3)	
Papillary carcinoma	25 (4.9)	23 (5.0)	1 (3.9)	1 (4.6)		23 (5.0)	2 (4.2)	
Metaplastic	8 (1.6)	8 (1.7)	0 (0)	0 (0)		8 (1.7)	0 (0)	
Other	21 (4.1)	20 (4.3)	0 (0)	1 (4.6)		20 (4.3)	1 (2.1)	
Ki67 high/low (%)								
Ki67 <15%	308 (60.2)	286 (61.6)	12 (46.2)	10 (45.5)	0.104	286 (61.6)	22 (45.8)	0.033
Ki67 ≥15%	204 (39.8)	178 (38.4)	14 (53.9)	12 (54.6)		178 (38.4)	26 (54.2)	
Mitoses/10 HPF, median (IQR p25, p75)	5 (1, 12)	5 (1, 11)	9 (3,20)	6 (2, 12)		5 (1, 12)	8 (2.5, 16.5)	

(*Continued*)

Table 3. (Continued)

	Total study population	Mean *PAK1* copy number, three categories				Mean *PAK1* copy number, two categories		
		<4	≥4 to <6	≥6	p value (χ^2)	<4	≥4	p value (χ^2)
Mitoses/10 HPF, quartiles (%)								
≤1	136 (26.6)	128 (27.6)	6 (23.1)	2 (9.1)	0.025	128 (27.6)	8 (16.7)	0.162
>1 ≤5	133 (26.0)	123 (26.5)	1 (3.9)	9 (40.9)		123 (26.5)	10 (20.8)	
>5 ≤12	123 (24.0)	107 (23.1)	9 (34.6)	7 (31.8)		107 (23.1)	16 (33.3)	
>12	120 (23.4)	106 (22.8)	10 (38.5)	4 (18.2)		106 (22.8)	14 (29.2)	

Abbreviations: SD = standard deviation, HER2 = human epidermal growth factor receptor 2, 5NP = 5 negative phenotype, BP = basal phenotype, HPF = high power fields

https://doi.org/10.1371/journal.pone.0287608.t003

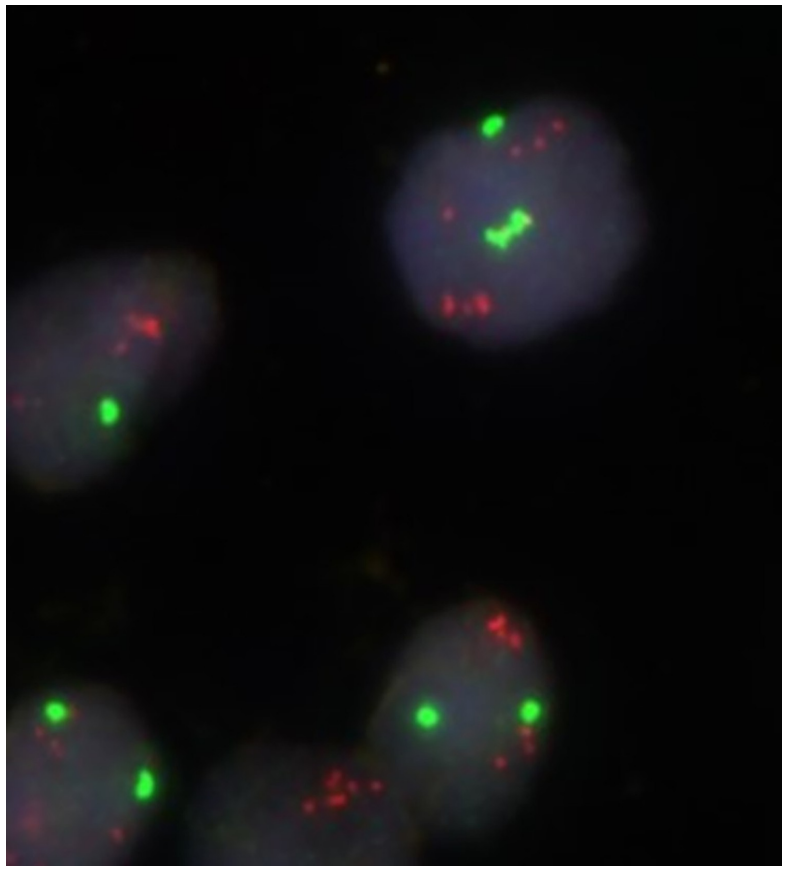

Fig 2. Fluorescence *in situ* hybridization using probes for CEP11 (fluorochrome SpectrumGreen) and *PAK1* (fluorochrome SpectrumRed). Fig 2 showing 2–3 copies of CEP11 and 6–8 copies of *PAK1* in each tumour cell nucleus.

https://doi.org/10.1371/journal.pone.0287608.g002

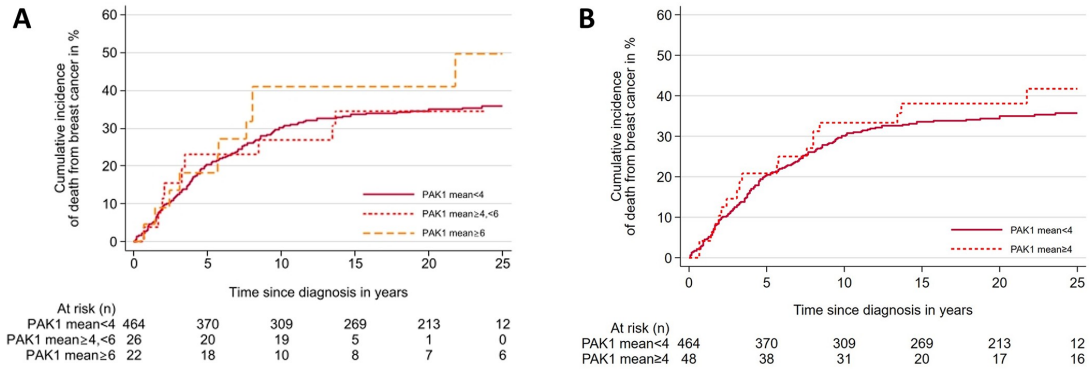

Fig 3. Cumulative incidence of death from breast cancer according to mean *PAK1* copy number in primary breast cancer tumours. Cumulative incidence curves show no significant association between *PAK1* copy number and risk of death. A) Mean *PAK1* copy number <4, ≥4<6 and ≥6. p = 0.39. B) Mean *PAK1* copy number <4 and ≥4. p = 0.42.

https://doi.org/10.1371/journal.pone.0287608.g003

PAK1 copy number and molecular subtypes

Copy number increase of *PAK1* was found in all molecular subtypes, except the 5-negative phenotype (5NP). The highest proportion of cases with *PAK1* CN ≥4 was found in the HER2 type, followed by Luminal B (HER2$^-$). Of a total of 27 cases of the HER2 type, four (14.7%) had *PAK1* CN ≥4, one of which (3.7%) had *PAK1* CN ≥6. In Luminal B (HER2$^-$), 16/121 (13.2%) had *PAK1* CN ≥4, and of these, 8/121 (6.6%) had *PAK1* CN ≥6. Among Luminal B (HER2$^+$) cases, 3/42 (7.1%) showed PAK1 CN ≥4 (Table 3).

PAK1 and prognosis

The cumulative risk of death from BC during the first 5 years after diagnosis was 20.3% (95% CI 16.9–24.2) for cases with mean *PAK1* CN <4, 23.1% (95% CI 11.1–44.3) for cases with CN ≥4<6, and 18.2% (95% CI 7.2–41.5) for cases with CN ≥6 (Fig 3, Table 4). During the first 10 years after diagnosis, the cumulative risk of death from BC was 30.1% (95% CI 26.1–34.5) for cases with mean *PAK1* CN <4, 26.9% (95% CI 13.9–48.3) for cases with CN ≥4<6, and 40.9% (95% CI 23.8–63.9) for cases with CN ≥6. In the Cox regression analyses using mean *PAK1*

Table 4. Absolute and relative risk of death from breast cancer according to mean PAK1 copy number/tumour cell nucleus in primary tumours.

	Mean *PAK1* copy number		
	<4	≥4<6	≥6
Cumulative risk after 5 years (%) (95% CI)	20.3(16.9–24.2)	23.1 (11.1–44.3)	18.2 (7.2–41.5)
Cumulative risk after 10 years (%) (95% CI)	30.1 (26.1–34.5)	26.9 (13.9–48.3)	40.9 (23.8–63.9)
HR unadjusted (95% CI)	1.0	0.9 (0.5–1.8)	1.4 (0.8–2.7)
HR adjusted for age (95% CI)	1.0	0.9 (0.5–1.8)	1.5 (0.8–2.7)
HR adjusted for stage (95% CI)	1.0	0.8 (0.4–1.6)	1.7 (0.9–3.2)
HR adjusted for grade (95% CI)	1.0	0.8 (0.4–1.6)	1.4 (0.8–2.6)
HR adjusted for Ki-67 (95% CI)	1.0	0.8 (0.4–1.7)	1.3 (0.7–2.3)
HR adjusted for age, stage, and grade (95% CI)	1.0	0.8 (0.4–1.5)	1.7 (0.9–3.2)

Abbreviations: HR = Hazard ratio, CI = confidence interval

https://doi.org/10.1371/journal.pone.0287608.t004

Table 5. ***PAK1*** **copy number in primary tumours and corresponding axillary lymph node metastases.**

	Mean *PAK1* copy number in primary tumours (%)			
Mean *PAK1* copy number in lymph node metastases (%)	**<4**	**≥4<6**	**≥6**	**Total**
<4	103 (94.5)	6 (66.7)	0	109
≥4<6	5 (4.6)	3 (33.3)	2 (40)	10
≥6	1 (0.9)	0	3 (60)	4
Total	109	9	5	123
	Mean *PAK1* copy number in primary tumours (%)			
Mean *PAK1* copy number in lymph node metastases (%)	**<4**	**≥4**		**Total**
<4	103 (94.5)	6 (42.9)		109
≥4	6 (5.5)	8 (57.1)		14
Total	109	14		123

https://doi.org/10.1371/journal.pone.0287608.t005

CN <4 as the reference, no significantdifference was observed in the rate of death from breast cancer for cases with *PAK1* CN increase (HR 1.4 [95% CI 0.8–2.7]) for cases with mean *PAK1* copy number ≥6). Fourteen of the 123 cases for which lymph node metastases were available had *PAK1* CN ≥4 in the primary tumour. Of these, 8 also had *PAK1* CN ≥4 in the corresponding lymph node metastasis. Of the five cases with *PAK1* CN ≥6 in the primary tumour, 3 also had *PAK1* CN ≥6 in the corresponding lymph node metastasis (Table 5).

PAK1 and *CCND1*

Among the 512 cases included in this study, *CCND1* CN status was available for 504 cases [24]. A total of 84/504 cases showed *CCND1* CN ≥4 and 40 of these had ≥6 copies of *CCND1*/nucleus (Table 6). Of the 22 patients with *PAK1* CN ≥6, 12 (54.6%) cases also had *CCND1* CN ≥6. Of the 48 cases with *PAK1* CN ≥4, 30 (62.5%) cases also had *CCND1* CN ≥4. However, 54 cases had *CCND1* CN ≥4 without a corresponding increase in *PAK1* CN and 18 cases showed CN increase ≥4 for *PAK1* without CN increase of *CCND1* (Table 6).

We found no significant difference in the cumulative risk of death from BC between cases with CN ≥4 of *PAK1* alone, CN ≥4 *CCND1* alone, and cases with CN ≥4 for both *PAK1* and *CCND1* combined (Fig 4). Similarly, The Cox regression analysis using combined PAK1 CN <4 and CCND CN <4 as the reference value, showed no significant difference in the rate of death from BC between the three groups of patients with copy number increase (Table 7).

Table 6. ***PAK1*** **and** ***CCND1*** **copy numbers in primary tumours.**

	Mean *PAK1* CN in primary tumours (%)				
Mean *CCND1* CN	**<4**	**≥4<6**	**≥6**	**Total**	
<4	402 (88.2)	11 (42.3)	7 (31.8)	420	p<0.001
≥4<6	31 (6.8)	10 (38.5)	3 (13.6)	44	
≥6	23 (5.0)	5 (19.2)	12 (54.6)	40	
Total	456	26	22	504	
	Mean *PAK1* CN in primary tumours (%)				
Mean *CCND1* CN	**<4**	**≥4**		**Total**	
<4	402 (88.2)	18 (37.5)		420	p<0.001
≥4	54 (11.8)	30 (62.5)		84	
Total	456	48		504	

https://doi.org/10.1371/journal.pone.0287608.t006

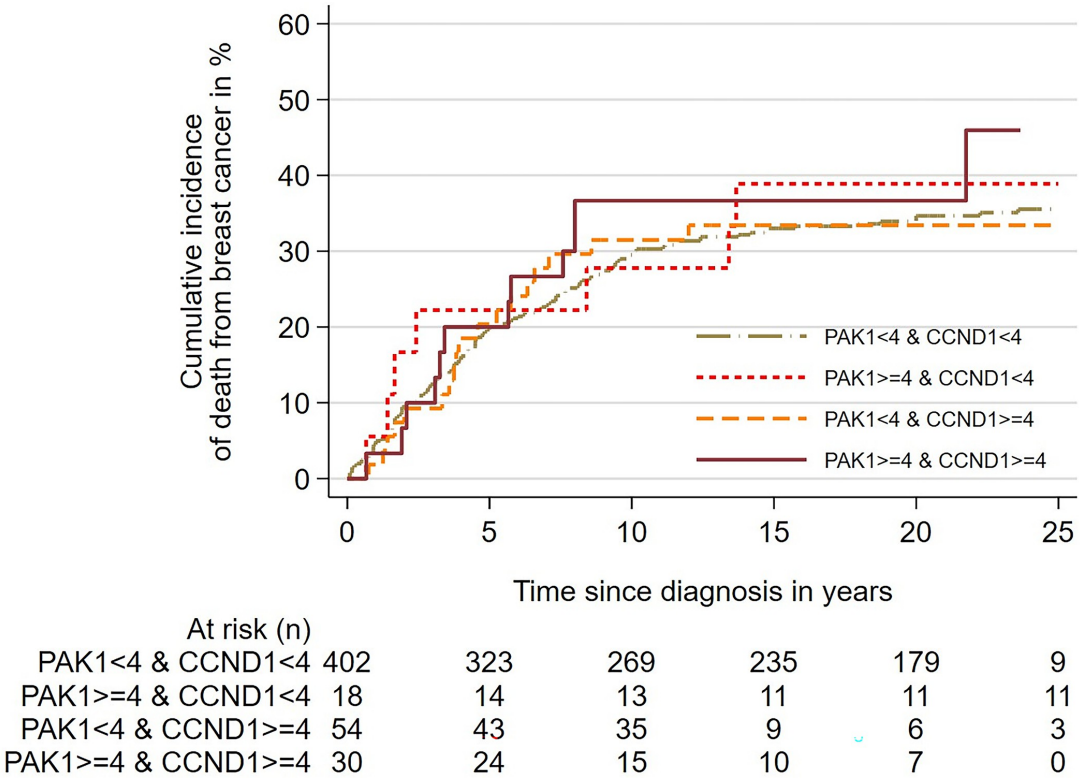

Fig 4. Cumulative incidence of death from breast cancer according to copy numbers of *PAK1* and *CCND1*, and co-amplification of *PAK1* and *CCND1*. Cumulative incidence curves show no significant association between *PAK1* copy number, CCND1 copy number, and co-amplification of *PAK1* and *CCND1*, and risk of death. p = 0,81.

https://doi.org/10.1371/journal.pone.0287608.g004

Discussion

In this study of 512 primary BC tumours, we found *PAK1* CN ≥4 in 48 (9.4%) cases, of which 22 cases showed high grade CN increase of *PAK1* CN ≥6. We found an association between *PAK1* CN ≥4, and high Ki-67 (≥15%) and high histological grade. The highest proportion of

Table 7. Relative risk of death from breast cancer according to copy numbers of *PAK1* and *CCND1*, and co-amplification of *PAK1* and *CCND1*.

Copy number of PAK1 and CCND1	Hazard ratio		
	HR	CI	p-value
PAK1 CN<4 & CCND1 CN<4 (reference value)	1.0		0.872
PAK1 CN≥4 & CCND1 CN<4	1.3	0.6–2.6	
PAK1 CN<4 & CCND1 CN≥4	0.9	0.6–1.5	
PAK1 CN≥4& CCND1 CN≥4	1.1	0.6–2.0	

Hazard ratio = HR, Confidence interval = CI

https://doi.org/10.1371/journal.pone.0287608.t007

cases with increased CN of *PAK1* (≥4) was found in the HER2 type and Luminal B (HER2$^-$) breast cancer subtype. Concurrent CN increase (≥4) of *PAK1* and *CCND1* was observed in 30 cases. Of the 123 cases with available lymph node metastases, only three cases had *PAK1* CN ≥6 in both the primary tumour and the corresponding lymph node metastases.

The cohort of Norwegian BC patients from which the cases of this study are derived is well-described, with mean follow-up of nine years. Since recurrence and death from BC may occur many years after the primary diagnosis, long-term follow-up is important in studies of prognostic markers. While recurrence data was unavailable to us, long-term survival data is complete, enabling us to assess the influence of biomarkers on prognosis. Histological typing and grading of all cases in this cohort were revised by experienced pathologists according to current guidelines. All biomarkers were stained at the same laboratory, and the same antibodies, cut-off levels and algorithm for molecular subtyping were used for all cases in the cohort [26].

In this study we used FISH on TMAs. TMAs provide the opportunity to efficiently study biomarkers in a large number of samples simultaneously under similar laboratory conditions at a relatively low cost. FISH is a method available in most laboratories, contrary to multigene assays. It enables us to assess the morphology of the section and ensure that only invasive tumour cell nuclei are assessed. Despite this, FISH applied to tissue sections may lead to an underestimation of CN compared to analysis of whole nuclei, due to nuclear truncation [34]. This would be of particular importance in cases with low CN increase. Preanalytical conditions will have varied considering that the cases included in the present study were diagnosed over decades. This could have affected the cases suitable for FISH analysis. However, few cases were discarded due to unsuccessful FISH. There are no established guidelines for cut-off levels in the assessment of *PAK1* CN. We chose to follow *HER2* guidelines for categorizing CN, as in previous studies by our group [24, 29–32]. While we also registered CN of CEP 11, we did not calculate the ratio between CNs of *PAK1* and CEP11 as this would have masked the true gene CN increase. Furthermore, we found that CEP11 CN increase was observed in only seven cases, of which only two were accompanied by CN increase of *PAK1*.

Tamoxifen is an established hormonal therapy used in ER positive BC. Five years of tamoxifen therapy nearly halves the risk of BC recurrence among ER positive patients [35]. Phosphorylation of ER by PAK1 may induce tamoxifen-resistance in ER positive tumours and tamoxifen itself may also increase nuclear PAK1 and PAK1 kinase activity [14, 23]. Patients with *PAK1* amplification have reduced benefit from tamoxifen and *PAK1* CN may therefore be a predictor of tamoxifen resistance [23]. Thus, PAK1-inhibitors may be useful in ER positive tumours, to improve the effect of tamoxifen in these cases [36].

Both *PAK1* and *CCND1* encode proteins shown to activate ER [23, 36]. Both are located on 11q13 and are thought to be frequently co-amplified. In this study, of the 504 patients analyzed for both *CCND1* and *PAK1*, 84 cases had CN ≥4 for *CCND1* and 48 with *PAK1* CN ≥4. A total of 30 (62.5%) cases had CN increase of both genes. These results are in accordance with the findings of others [23]. In the present study, co-amplification of *PAK1* and *CCND1* was not associated with prognosis.

The proportion of cases with increased *PAK1* CN in this study was lower compared to the results of previous studies [7, 8]. However, the mean age at diagnosis in our study was 75.4 years, which is high compared to other studies and higher than the mean age for diagnosis of breast cancer in Norway which is 62 years of age [37]. Fumagalli et al found CN increase in 11% of cases in a selected series of ER$^+$, metastatic breast cancer cases. In our series of cases, PAK1 CN increase was found among Luminal B HER2$^-$ and the HER2 type [38]. High proliferation rate and poor prognosis are found to be associated in BC [39, 40], and the prognostic effect of proliferation has been shown to vary with age, exerting a greater effect on prognossis among younger BC patients [41]. This may, in part, explain the discrepant results compared to

other studies of *PAK1* and further studies including a wider age range are warranted. Furthermore, the choice of method may also have contributed to these results. Tissue microarrays include only small tissue cylinders from the tumour and may not be representative of the whole tumour, particularly in cases with tumour heterogeneity [42, 43]. In the TMAs used in our study, tissue cylinders were extracted from the tumour periphery and are therefore not necessarily representative of other areas of the tumour. However, we considered the tumour periphery to be the region of greatest interest in the tumour given its greater proliferative activity [44] and its proximity to surrounding breast tissue. Furthermore, selecting tissue for TMA form the same region of all tumours contributes to a certain standardization of the material examined in the study.

Despite associations between *PAK1* CN increase and high histological grade and high proliferation, we failed to demonstrate a statistically significant association between increased *PAK1* CN and prognosis. It would be interesting to study prognosis according to *PAK1* CN for each of the molecular subtypes separately. However, in the present study the number of cases in some of the molecular subtypes was too low to warrant further analyses of subgroups. The numbers of cases showing *PAK1* CN increase in primary tumours only, lymph node metastases only, or both were too low to give reliable prognostic information. The frequency of *PAK1* CN change in this study was lower than the expression of established biomarkers, such as ER, PR and HER2 in BC. However, in an era of personalized medicine, its known influence on the effect of tamoxifen in BC makes it an interesting biomarker and potential target for treatment.

Conclusion

PAK1 CN increase is found in all molecular subtypes, except the 5-negative phenotype (5NP), and most frequently in the HER2 and Luminal B ($HER2^-$) subtypes. It is associated with aggressive tumour characteristics such as high histological grade and high Ki-67 protein expression, but not with prognosis. It is co-amplified with *CCND1* in a proportion of cases. Few cases showed *PAK1* CN increase in both the primary tumour and the corresponding lymph node metastases.

Acknowledgments

The authors thank the Department of Pathology, St. Olav´s Hospital, Trondheim University Hospital for making the diagnostic archives available for the study, and the Cancer Registry of Norway for supplying the patient data.

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Paper III

Breast Cancer Research and Treatment (2023) 201:535–545
https://doi.org/10.1007/s10549-023-07040-9

ORIGINAL LABORATORY INVESTIGATION

Oestrogen receptor low positive breast cancer: associations with prognosis

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Received: 27 April 2023 / Accepted: 5 July 2023 / Published online: 18 July 2023

Abstract

Purpose In this study of oestrogen receptor (ER) Low Positive breast cancers (BC) in three large cohorts of BC patients, we assess associations between levels of ER expression and tumour characteristics and prognosis.

Methods Cases were stratified into patients unlikely to have received adjuvant therapy according to treatment guidelines at time of diagnosis (before 1995), and those who could have received adjuvant therapy (diagnosed in 1995 or later). ER status was divided into $< 1\%; \geq 1 < 10\%; \geq 10\%$. Results were correlated with time of diagnosis, histopathological grade, proliferation status, and molecular subtypes, using Pearson's Chi-square test. For prognosis, hazard ratios and cumulative incidence of death from BC were used.

Results Of the 1955 tumours, 65 (3.3%) were ER Low Positive ($ER \geq 1 < 10\%$). Overall, the highest proportion of ER Low Positive tumours was observed among Luminal B (HER2+) subtype (9.4%) and grade 3 tumours (4.3%). The risk of death from BC was lower in ER Low Positive and $ER \geq 10\%$ compared to ER-negative cases. Compared to patients diagnosed before 1995, women diagnosed in 1995 or later showed a higher proportion of ER Low Positive BCs, and their tumours were of smaller size, lower grade, and lower proliferative status. There was no significant difference in prognosis compared to those with $ER \geq 10\%$ tumours.

Conclusion Women with ER Low Positive tumours diagnosed in a time period when adjuvant therapy was available had tumours of smaller size, lower grade, and lower proliferative status, and similar prognosis to those with $ER \geq 10\%$ compared to women diagnosed earlier.

Keywords Breast cancer · Oestrogen receptor · ER · ER low positive · Prognosis · Endocrine treatment

Introduction

Oestrogen receptor (ER) status plays an essential role in clinical decision-making and predicting outcome and treatment response for breast cancer (BC) patients [1]. According to current guidelines [2], patients with ER-positive tumours are considered eligible for endocrine therapy. Patients with ER-negative tumours are more likely to benefit from chemotherapy and generally have a poorer outcome than patients with ER-positive (ER+) tumours [3, 4].

Breast cancer differs from most tumours because of its dependence on female sex hormones for development and growth [5]. Expression of ER by immunohistochemistry (IHC) is seen in more than 70% of BC tumours [6]. The ASCO/CAP and current national BC guidelines state that BC tumours with $\geq 1\%$ positive staining tumour cell nuclei should be interpreted as ER+, and negative if $< 1\%$ of tumour cell nuclei express ER [2, 7]. However, the ASCO/CAP Expert Panel states that data on the effect of endocrine therapy for cancers with $ER \geq 1 < 10\%$ are limited. They suggest that samples with $ER \geq 1 < 10\%$ should be reported as ER Low Positive, with a comment mentioning the limited data available on the therapeutic benefit of anti-hormonal treatment for this group of patients [2]. According to the St. Gallen 2019 Consensus Discussion on The Optimal Primary Breast Cancer Treatment, there is a need for better evaluation of ideal cut-off levels for the prescription of endocrine

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therapy for ER + tumours, particularly for ER Low Positive cases [8–10].

In this study we examined expression levels of ER in BC tumours and associations between ER status and time of diagnosis, and tumour characteristics such as histopathological grade, molecular subtypes, proliferation and prognosis, with emphasis on ER Low Positive tumours.

Materials and methods

Study population

This study comprises women from three population-based surveys conducted in Trøndelag County, Norway. Information on breast cancer incidence was obtained from the Cancer Registry of Norway. Date of death, and/or emigration was obtained from the National Population Register and causes of death from the Norwegian Cause of Death Registry. Formalin-fixed, paraffin embedded (FFPE) tumour tissue from the primary tumours and corresponding pathology reports were retrieved from the Department of Pathology at St. Olav's Hospital, Trondheim University Hospital, Norway (Fig. 1).

Cohort 1: The cohort includes 25,727 women born 1886–1928 [11] invited to attend a population-based survey for the early detection of breast cancer conducted in Nord-Trøndelag County, Norway, between 1956 and 1959. During 47 years of follow-up (1961 to end of 2008), 1379 new BCs were registered among these women. In a previous study 909 of these tumours were classified according to histopathological type and grade and divided into molecular subtypes [12]. For one case ER status was missing, and this case was excluded from the present study, leaving 908 cases. After diagnosis, patients were followed until time of death from BC or death from other causes, or until December 31st, 2015.

Cohort 2: The second cohort comprises 34,221 women born between 1897 and 1977 and derives from the HUNT2 Study conducted between 1995 and 1997 in Nord-Trøndelag County, Norway [13]. From attendance until December 31st, 2009, 728 women were diagnosed with BC. Of these, 157 cases were already included in Cohort 1 and 57 were unavailable for subtyping. The remaining tumours ($n = 514$) from Cohort 2 were assigned histopathological type and grade and reclassified into molecular subtypes [14]. ER status was available for all 514 cases. After diagnosis, these patients were followed until time of death from BC or death from other causes, or until December 31st, 2015.

Cohort 3: The third cohort includes 22,931 women born at E.C. Dahl's Foundation, Trondheim, Norway between 1920 and 1966. During 52 years of follow-up (1961 to the end of 2012), a total of 870 women were diagnosed with BC. Among them, 598 were diagnosed at St Olav's Hospital, and histopathological typing, grading and molecular subtyping were successful for 537 of these cases [15]. ER status was available for 533 of these cases. After diagnosis, patients were followed until time of death from BC or death from other causes, or until December 31st, 2015.

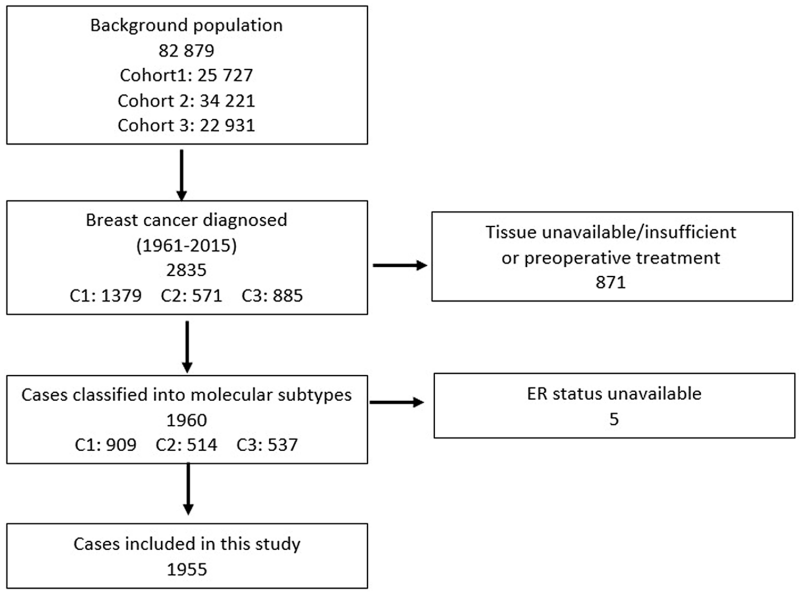

Fig. 1 Overview of the three cohorts of breast cancer patients included in the study

Specimen characteristics

Tissue Microarray (TMA) paraffin blocks were made from the archival tumour tissue using the TissueArrayer Minicore with TMA Designer2 software (Alphelys). Three 1 mm in diameter tissue cylinders from the periphery of the FFPE primary tumours were transferred to TMA recipient blocks. TMA Sections (4 µm) were cut and IHC-staining for ER was carried out within four weeks after sectioning. Between cutting and staining, sections were stored at − 20 °C. Staining intensity was not quantified in this study. Molecular subtypes for all cases in all three cohorts were determined using IHC and in situ hybridization in lieu of gene expression analyses, and have been published previously [12, 14, 15]. The IHC markers including ER are shown in Table 1.

Statistical analyses

For the present study, we divided ER expression into three categories (< 1%; ≥ 1 < 10%; ≥ 10%) and studied associations between ER expression and histopathological grade, molecular subtype, proliferation, and prognosis.

Pearson's chi square test was used to compare patient and tumour characteristics across categories of ER. In analyses of prognosis, we distinguished between women diagnosed before 1995 and women diagnosed in 1995 or later. This cut-off was used to approximate the gradual implementation of adjuvant treatment in Norway [14, 16]. Cumulative incidence of death from BC was estimated, with death from other causes as competing events. Gray's test was used to compare equality between cumulative incidence curves. Cox proportional hazard analyses were used to estimate hazard ratios (HR) of BC death with 95% confidence intervals (CI) within each diagnostic period, censoring at time of death from other causes. We adjusted for age, stage, histopathological grade, and for these variables combined. No clear violations of proportionality were found in log-minus-log plots. Statistical analyses were performed using Stata/MP version 17 (StataCorp LP, College Station, Texas, USA).

Table 1 Algorithm for reclassification of breast cancers into molecular subtypes [12]

Molecular subtype	Classified by
Luminal A	ER + and/or PR +, HER2-, Ki-67 < 15%
Luminal B (HER2-)	ER + and/or PR +, HER2-, Ki-67 ≥ 15%
Luminal B (HER2 +)	ER + and/or PR +, HER2 +
HER2 type	ER-, PR-, HER2 +
Five-negative phenotype	ER-, PR-, HER2-, CK5-, EGFR-
Basal phenotype	ER-, PR-, HER2-, CK5 + and/or EGFR +

ER Oestrogen receptor, *PR* Progesterone receptor, *HER2* Human epidermal growth factor receptor 2, *CK5* Cytokeratin 5, *EGFR* Epidermal growth factor receptor 1

Results

Patient and tumour characteristics for the 1955 patients included in the present study are shown in Table 2. Mean age at diagnosis was 67.3 years (SD: 12.8) and mean follow-up after diagnosis was 9.9 years (SD: 7.3). By end of follow-up, 545 (27.9%) patients had died from BC and 588 (30.1%) died from other causes. Of the 1955 tumours, 315 (16.1%) were ER < 1%, 65 (3.3%) were ER Low Positive (ER ≥ 1 < 10%) and 1575 (80.6%) were ER ≥ 10%. Of the 545 deaths from BC, 129 (23.7%) cases were ER < 1%, 16 (2.9%) were ER Low Positive and 400 (73.4%) were ER ≥ 10%.

ER categories and molecular subtypes

Of the 1955 tumours included in this study, 1669 (85.4%) were classified as one of the luminal subtypes (Luminal A, Luminal B (HER2-), or Luminal B (HER2 +)). Of these, 1640 were ER positive (ER ≥ 1%). Of the 180 cases of Luminal B (HER2 +), seven (3.9%) cases were ER < 1%, 17 (9.4%) were ER Low Positive and 156 (86.7%) were ER ≥ 10% ($p < 0.0001$). Among the 937 cases with Luminal A subtype, 10 (1.1%) cases were ER < 1%, 29 (3.1%) were ER Low Positive and 898 (95.8%) were ER ≥ 10%. Of the 552 Luminal B (HER2-) cases 12 (2.2%) cases were ER < 1%, 19 (3.4%) were ER Low Positive and 521 (94.4%) were ER ≥ 10%. Twenty-six cases with ER < 1% were classified as Luminal based on progesterone receptor (PR) positivity (Table 2).

ER categories, histopathological grade, proliferation, and histopathological type

In this study, 287 (14.7%) tumours were grade 1, 1015 (51.9%) were grade 2 and 653 (33.4%) were grade 3. The highest proportion of ER Low Positive (28/653 (4.3%)) was observed among grade 3 tumours ($p < 0.0001$). Of the 1057 cases with Ki-67 < 15%, 74 (7.0%) were ER < 1%, 31 (2.9%) were ER Low Positive, and 952 (90.1%) were ER ≥ 10%. Of the 898 cases with Ki-67 ≥ 15%, 241 (26.8%) were ER < 1%, 34 (3.8%) were ER Low Positive, and 623 (69.4%) were ER ≥ 10% ($p < 0.0001$). Similarly, of the 459 cases with ≤ 2 mitoses/10 High power fields (HPF) (p25), 23 (5.0%) were ER < 1%, 9 (2.0%) were ER Low Positive and 427 (93.0%) were ER ≥ 10% ($p < 0.0001$). Whereas, of the 875 cases with > 13 mitoses/10 HPF (p75), 215 (24.6%) were ER < 1%, 40 (4.5%) were ER Low Positive, and 620 (70.9%) were ER ≥ 10% ($p < 0.0001$). Of the 65 ER Low Positive cases, 50/1507 (3.3%) were invasive

Table 2 Patient and tumour characteristics according to ER categories

	Total study population	ER categories			
		< 1%	≥ 1 < 10%	≥ 10%	*p* value (χ^2)
N (%)	1955	315 (16.1)	65 (3.3)	1575 (80.6)	
Mean age at diagnosis, years (SD)	67.3 (12.8)	65.4 (14.0)	63.3 (13.9)	67.9 (12.4)	
Mean follow-up, years (SD)	9.9 (7.3)	8.4 (7.6)	10.3 (6.9)	10.2 (9.0)	
Alive Dec. 31st 2015 (%)	822 (42.1)	102 (32.5)	34 (51.5)	686 (43.6)	< 0.001
Deaths from breast cancer (%)	545 (27.9)	129 (41.0)	16 (24.6)	400 (25.4)	
Deaths from other causes or by the end of 2015 (%)	588 (30.1)	84 (26.7)	15 (23.1)	489 (31.1)	
Histopathological grade (%)					
I	287 (14.7)	13 (4.1)	6 (9.2)	268 (17.0)	< 0.001
II	1015 (51.9)	73 (23.2)	31 (47.7)	911 (57.8)	
III	653 (33.4)	229 (72.7)	28 (43.1)	396 (25.1)	
Tumour size (%)					
≤ 2 cm	1035 (52.9)	124 (39.4)	33 (50.8)	878 (55.8)	< 0.001
> 2 cm, ≤ 5 cm	391 (20.0)	75 (23.8)	15 (23.1)	301 (19.1)	
> 5 cm	24 (1.2)	9 (2.9)	3 (4.6)	12 (0.8)	
Uncertain, but > 2 cm	161 (8.2)	44 (14.0)	7 (10.8)	110 (7.0)	
Uncertain	344 (17.6)	63 (20.0)	7 (10.8)	274 (17.4)	
Stage (%)					
I	881 (45.1)	113 (35.9)	25 (38.5)	743 (47.2)	0.010
II	708 (36.2)	137 (43.5)	26 (40.0)	545 (34.6)	
III	98 (5.0)	23 (7.3)	3 (4.6)	72 (4.6)	
IV	72 (3.7)	14 (4.4)	2 (3.1)	56 (3.6)	
Unknown	196 (10.0)	28 (8.9)	9 (13.9)	159 (10.1)	
Molecular subtype (%)					
Luminal A	937 (47.9)	10 (3.2)	29 (44.6)	898 (57.0)	< 0.001
Luminal B (HER2-)	552 (28.2)	12 (3.8)	19 (29.2)	521 (33.1)	
Luminal B (HER2 +)	180 (9.2)	7 (2.2)	17 (26.2)	156 (9.9)	
HER2 type	108 (5.5)	108 (34.3)	0 (0.0)	0 (0.0)	
5NP	53 (2.7)	53 (16.8)	0 (0.0)	0 (0.0)	
BP	125 (6.4)	125 (39.7)	0 (0.0)	0 (0.0)	
Histopathological subtype (%)					
Invasive carcinoma (NOS[a])	1507 (77.1)	218 (69.2)	50 (76.9)	1239 (78.7)	< 0.001
Lobular carcinoma	210 (10.7)	17 (5.4)	8 (12.3)	185 (11.8)	
Tubular carcinoma	6 (0.3)	0 (0.0)	0 (0.0)	6 (0.4)	
Mucinous carcinoma	65 (3.3)	2 (0.6)	0 (0.0)	63 (4.0)	
Medullary carcinoma	60 (3.1)	38 (12.1)	4 (6.2)	18 (1.1)	
Papillary carcinoma	39 (2.0)	5 (1.6)	0 (0.0)	34 (2.2)	
Metaplastic	18 (0.9)	15 (4.8)	1 (1.5)	2 (0.1)	
Other	50 (2.6)	20 (6.4)	2 (3.1)	28 (1.8)	
Ki-67 low/high (%)					
Ki-67 < 15%	1057 (54.1)	74 (23.5)	31 (47.7)	952 (60.4)	< 0.001
Ki-67 ≥ 15%	898 (45.9)	241 (76.5)	34 (52.3)	623 (39.6)	
Mitoses/10 HPF, median (IQR p25, p75)	5 (2,13)	15 (7,29)	8 (4,17)	4 (1,10)	
Mitoses/10 HPF, quartiles (%)					
≤ 2	459 (23.5)	23 (7.3)	9 (13.9)	427 (27.2)	< 0.001
> 2, ≤ 5	275 (14.1)	23 (7.3)	6 (9.2)	246 (15.7)	
> 5, ≤ 13	342 (17.5)	54 (17.1)	10 (15.4)	278 (17.7)	
> 13	875 (44.9)	215 (68.3)	40 (61.5)	620 (39.5)	

[a]*NOS* Not otherwise specified

carcinoma NOS, 8/210 (3.8%) were lobular carcinoma, 4/60 (6.6%) were medullary carcinoma, and 1/18 (5.5%) was metaplastic carcinoma (Table2).

Comparisons between women diagnosed before 1995 and women diagnosed in 1995 or later

A total of 774 cases were diagnosed before 1995, and 1181 were diagnosed in 1995 or later. The distribution of cases according to time of diagnosis are shown in Table 3. Of women diagnosed before 1995, 352/774 (45.5%) died from BC during follow-up, as opposed to 193/1181 (16.3%) of those diagnosed in 1995 or later. Among women diagnosed before 1995, 152/774 (19.6%) tumours were ER < 1%, falling to 163/1181 (13.8%) among women diagnosed in 1995 or later. Similarly, 16/774 (2.1%) tumours were ER Low Positive before 1995, rising to 49/1181 (4.2%) in 1995 or later, and 606/774 (78.3%) cases diagnosed before 1995 were ER ≥ 10%, rising to 969/1181 (82.1%) among women diagnosed in 1995 or later. Furthermore, we found that 310/774 (40.1%) of tumours diagnosed before 1995 were ≤ 2 cm in diameter, rising to 725/1181 (61.4%) for tumours diagnosed in 1995 or later ($p < 0.0001$) (Table 3).

Characteristics of ER low positive tumours

The distribution of tumour characteristics in patients with ER Low tumours are shown in Table 4. There was a total of 65 (3.3%) ER Low Positive tumours in this study. Of these, 16 were diagnosed before 1995, and 49 was diagnosed in 1995 or later. Among the ER Low Positive tumours diagnosed before 1995, 8/16 (50%) died from BC during follow-up, as opposed to 8/49 (16.3%) of those diagnosed in 1995 or later. Among ER Low tumours, the proportion of tumours < 2 cm, rose from 31% in patients diagnosed before 1995 to 57% in those diagnosed in 1995 or later ($p < 0.0001$).

For all cases, there was a higher proportion of grade 1 tumours (17.2%), and a lower proportion of tumours with grade 3 (29.6%) among women diagnosed in 1995 or later, compared to women diagnosed before 1995 (Grade 1: 10.9%, Grade 3: 39.1% ($p < 0.0001$)). Among ER Low Positive cases, there was a higher proportion of grade 1 (12.2%) and 2 (53.1%) tumours among women diagnosed in 1995 or later, compared to the women diagnosed before 1995 (grade 1: 0%, grade 2: 31.2%). For grade 3 tumours the proportion of ER low tumours was lower when diagnosed in 1995 or later ($p = 0.04$) (Table 4).

For all cases, the proportion of Luminal A subtype was higher for women diagnosed in 1995 or later (52.5%) compared to those diagnosed before 1995 (41.0%). The proportion of Luminal B (HER2-) and HER2 subtypes was lower for women diagnosed in 1995 or later ($p < 0.0001$) (Table 3), compared to those diagnosed before 1995. Among ER Low Positive tumours, the proportion of Luminal A subtype rose from 25% in ER Low tumours diagnosed before 1995, to 51% when diagnosed in 1995 or later. The proportion of Luminal B (HER2 +) tumours was lower among the women diagnosed in 1995 or later (18.4%), than the women diagnosed before 1995 (50%) ($p = 0.037$) (Table 4).

ER categories and prognosis

Cumulative incidence of death by BC according to ER status is shown in Fig. 2. The risk of death from BC for all categories of ER expression was lower for women diagnosed in 1995 or later compared to women diagnosed before 1995 (Table 5). The cumulative risk of death from BC after 5 years, for women diagnosed before 1995, was 47.4% among cases with ER < 1%, 37.5% for cases with ER Low Positive and 20.8% for cases with ER ≥ 10%. Among women diagnosed with breast cancer in 1995 or later the cumulative risk of death from BC was 22.3% after 5 years for ER < 1%, and 8.3% for both the ER Low Positive and ER ≥ 10% group (Table 5). Thus, among patients diagnosed in 1995 or later, there was no clear difference in risk of death from BC between cases with ER Low Positive and ER > 10%.

Cox regression analyses showed that the risk of death was lower among patients with ER ≥ 10%, compared to those with ER < 1%, both among patients diagnosed before 1995, and among patients diagnosed in 1995 or later. The Cox analysis shows a lower relative risk of death from BC among patients with ER ≥ 10% tumours, compared to ER < 1% both before and after 1995. We observed a tendency towards a lower relative risk of death from BC among ER Low Positive, compared to ER < 1%. However, these findings were not statistically significant (Table 5).

Discussion

In this study of 1955 primary BC tumours, we found that 65 (3.3%) tumours fell under the ER Low Positive category. We found the highest proportion of ER Low Positive among Luminal B (HER2 +) tumours (9.4%). Among cases diagnosed before 1995, 2.1% were ER Low Positive rising to 4.2% among cases diagnosed in 1995 or later. We found an association between ER Low Positive and high histopathological grade, high Ki-67 levels and high mitotic count. However, the results did not show a significant association with prognosis.

Breast cancer survival in Norway has increased since the mid-1990s as seen in the present and other studies [17]. This may be ascribed to earlier detection [18, 19] and improved treatment [6, 20]. The reduced risk of death observed between the two time-periods for all categories of ER expression, probably reflects earlier diagnosis with the

Table 3 Patient and tumour characteristics among women diagnosed before 1995, or in 1995 and later

	Women diagnosed with BC before 1995 (%)	*p*-value	Women diagnosed with BC in 1995 or later (%)	*p*-value
Total cases (*n*)	774		1181	
Cohort 1 (*n* = 908)	661 (72.7)		248 (27.3)	
Cohort 2 (*n* = 514)	0 (0.0)		514 (100.0)	
Cohort 3 (*n* = 533)	113 (21.2)		420 (78.8)	
Mean age at diagnosis (SD)	69.5 (10.4)		65.4 (14.3)	
Mean follow-up-time (SD)	10.9 (9.7)		9.2 (5.0)	
Deaths by BC (%)	352 (45.5)	0.104	193 (16.3)	0.001
Deaths from other causes or by the end of 2015 (%)	364 (47.0)		224 (19.0)	
Alive at end of follow-up (31st Dec 2015)	58 (7.5)		764 (64.7)	
Oestrogen receptor (%)				
< 1% (%)	152 (19.6)	< 0.001	163 (13.8)	< 0.001
≥ 1 < 10% (%)	16 (2.1)		49 (4.2)	
≥ 10% (%)	606 (78.3)		969 (82.1)	
Tumour size				
≤ 2 cm (%)	310 (40.1)	0.023	725 (61.4)	< 0.001
> 2 ≤ 5 cm (%)	64 (8.3)		327 (27.7)	
Tumour size > 5 cm (%)	3 (0.4)		21 (1.8)	
Uncertain, but > 2 cm (%)	148 (19.1)		13 (1.1)	
Uncertain (%)	249 (32.2)		95 (8.0)	
Stage				
1	346 (44.7)	0.002	535 (45.3)	0.001
2	257 (33.2)		451 (38.2)	
3	47 (6.1)		51 (4.3)	
4	39 (5.0)		33 (2.8)	
Unknown	85 (11.0)		111 (9.4)	
Histopathological grade				
1	84 (10.9)	< 0.001	203 (17.2)	< 0.001
2	387 (50.0)		628 (53.2)	
3	303 (39.1)		350 (29.6)	
Histopathological type				
Invasive carcinoma (NOS)	566 (73.1)	< 0.001	941 (79.7)	< 0.001
Lobular carcinoma	96 (12.4)		114 (9.7)	
Mucinous carcinoma	27 (3.5)		38 (3.2)	
Medullary carcinoma	27 (3.5)		33 (2.8)	
Papillary carcinoma	21 (2.7)		18 (1.5)	
Metaplastic carcinoma	8 (1.0)		10 (0.9)	
Tubular carcinoma	2 (0.3)		4 (0.3)	
Other	27 (3.5)		23 (2.0)	
Molecular subtypes				
Luminal A	317 (41.0)	< 0.001	620 (52.5)	< 0.001
Luminal B (HER2-)	243 (31.4)		309 (26.2)	
Luminal B (HER2 +)	69 (8.9)		111 (9.4)	
HER2 type	63 (8.1)		45 (3.8)	
Five-negative phenotype	25 (3.2)		28 (2.4)	
Basal phenotype	57 (7.4)		68 (5.8)	
Mitoses/10 HPF, median (IQR p25, p75)	2 (7, 15)		4 (1, 10)	
Mitoses /10 HPF (%)				
≤ 2	203 (26.2)	< 0.001	256 (21.8)	< 0.001
> 2, ≤ 5	140 (18.1)		135 (11.5)	

Table 3 (continued)

	Women diagnosed with BC before 1995 (%)	*p*-value	Women diagnosed with BC in 1995 or later (%)	*p*-value
> 5, ≤ 13	202 (26.1)		140 (11.9)	
> 13	229 (29.6)		646 (54.9)	
Ki-67				
< 15%	377 (48.7)	< 0.001	680 (57.6)	< 0.001
≤ 15%	397 (51.3)		501 (42.4)	

NOS = Not otherwise specified, HPF = High Power Field

introduction of mammography screening and the introduction of adjuvant treatment therapies in the mid-1990s. The change in prognosis observed across time for patients with ER Low Positive tumours may also be attributed to adjuvant therapy other than antihormonal treatment in addition to changing tumour characteristics such as smaller tumour size and lower histopathological grade. However, a drawback of the present study was lack of availability of disease-free survival data.

ER status is an important indicator of prognosis and a predictor of the effect of endocrine treatment. ER signalling is a main driver of proliferation in ER Positive BCs, and inhibition of ER signalling has improved survival among ER Positive BC patients [6, 21]. Studies suggest that selection of patients for endocrine therapy may need to be further personalized [9, 22, 23]. While most ER + BCs have high IHC scores, about 2–3% of cases are ER Low Positive [10, 24, 25]. In the present study, 3.3% of the total number of cases were ER Low Positive. While these tumours are classified within the ER + category, their risk profile appears to be more like that of ER-negative breast cancers [24]. A recent study found no benefit of endocrine therapy in the ER < 10% group compared to the ER > 10% group [25]. The lack of benefit of endocrine therapy in patients with low ER expression has recently been shown in a meta-analysis, including more than 16,000 patients [26]. The meta-analysis indicated that primary BC patients with ER 1–9% gained no significant survival benefit from endocrine therapy, but manifested better overall prognosis than patients with cancers expressing ER < 1% [26]. In the present study, among patients diagnosed in 1995 or later, the ER Low Positive patient group had similar survival to those with ER ≥ 10%. The patients included in this study were diagnosed with BC between 1961 and 2012, and the ER > 1% cut-off level for endocrine treatment was first introduced in Norway in 2011 after recommendations from ASCO/CAP [27]. Therefore, the improved prognosis seen among ER Low Positive patients diagnosed in 1995 or later, can most likely not be attributed to endocrine treatment [28]. Among women diagnosed in 1995 or later, we found a greater proportion of ER Low Positive tumours with smaller size, lower grade, and lower proliferation compared to ER Low Positive tumours diagnosed before 1995. Thus, the improved prognosis may be attributed to factors other than endocrine treatment, such as earlier diagnosis due to the introduction of mammography screening and greater BC awareness among women. Determining endocrine treatment for patients with a diagnosis of ER Low Positive BC should be carefully considered in light of the potential risks and benefits of the treatment [24].

In the present study, the proportion of Luminal A tumours was higher among women diagnosed in the time period during which adjuvant treatment and earlier diagnosis became available, a finding previously observed by our group in an analysis of cohorts 1 and 2 [14]. It has been suggested that BC patients with ER Low Positive are more similar to the ER-negative group, and therefore may not profit from endocrine therapy [9]. Thus, it has been suggested that cut-off levels should be further investigated in order to offer BC patients personalized endocrine treatment [22, 29, 30]. In the present study we found that among cases diagnosed in 1995 or later, ER Low Positive cases showed a prognosis similar to that of ER ≥ 10% cases. However, the impact of hormonal therapy could not be assessed in this study, due to lack of individual information on treatment.

Similar to our findings, a recent study showed that ER Low Positive tumours were more frequently grade 3 and had a higher expression of Ki-67, compared to BCs with intermediate or high expression of ER [31]. Furthermore, they found that the expression of immune-related biomarkers in ER Low Positive was similar to that of ER-negative tumours. We observed four cases of medullary carcinoma and one metaplastic carcinoma among the ER Low Positive cases. When determining treatment for patients with ER Low Positive BC, it may be useful to consider including a panel of immune-related biomarkers.

The FFPE tumour tissue included in this study covered a diagnostic timespan of several decades, and preanalytical conditions may have varied over time. Many of the tumours were diagnosed at a time when ER IHC was not done in the diagnostic setting. However, valuable information can be drawn from archival tissue blocks [32, 33]. It has been shown that antigenicity is, for the most part, preserved in

Table 4 Patient and tumour characteristics among patients with ER Low Positive (≥ 1 < 10%) diagnosed before 1995, and in 1995 or later

	Women diagnosed with BC before 1995 (%)	Women diagnosed with BC in 1995 or later (%)	*p*-value
Total cases (*n*)	16	49	
Mean age at diagnosis (SD)	66.9 (12.8)	62.2 (14.2)	
Mean follow-up-time (SD)	10.8 (11.5)	10.2 (4.7)	
Deaths from breast cancer (%)	8 (50.0)	8 (16.3)	< 0.001
Deaths from other causes or by the end of 2015 (%)	7 (43.7)	8 (16.3)	
Alive at end of follow-up	1 (6.3)	33 (67.4)	
Tumour size			
≤ 2 cm (%)	5 (31.2)	28 (57.1)	< 0.001
> 2 ≤ 5 cm (%)	1 (6, 3)	14 (28.6)	
Tumour size > 5 cm (%)	0 (0.0)	3 (6.1)	
Uncertain, but > 2 cm (%)	6 (37.5)	1 (2.0)	
Uncertain (%)	4 (25.0)	3 (6.1)	
Stage			
1	5 (31.3)	20 (40.8)	0.001
2	2 (12.5)	24 (49.0)	
3	2 (12.5)	1 (2.0)	
4	2 (12.5)	0 (0.0)	
Unknown	5 (31.3)	4 (8.2)	
Histopathological grade			
1	0 (0.0)	6 (12.2)	0.041
2	5 (31.2)	26 (53.1)	
3	11 (68.8)	17 (34.7)	
Molecular subtypes			
Luminal A	4 (25.0)	25 (51.0)	0.037
Luminal B (HER2-)	4 (25.0)	15 (30.6)	
Luminal B (HER2 +)	8 (50.0)	9 (18.4)	
HER2 type	0 (0.0)	0 (0.0)	
5NP	0 (0.0)	0 (0.0)	
BP	0 (0.0)	0 (0.0)	
Mitoses/10 HPF, median (IQR p25, p75)	9.5 (5, 16.5)	8 (2, 17)	
Mitoses /10 High power field (HPF) p25 = 4, p50 = 8, p75 = 17 (ER Low)			
≤ 4/10 HPF	4 (25.0)	8 (16.3)	0.047
> 4 ≤ 8/10 HPF	3 (18.7)	5 (10.2)	
> 8 ≤ 17/10 HPF	5 (31.3)	5 (10.2)	
> 17/10 HPF	4 (25.0)	31 (63.3)	
Ki-67			
< 15%	5 (31.2)	26 (53.1)	0.129
≤ 15%	11 (68.8)	23 (46.9)	

paraffin blocks over decades but may decrease in sections stored over time, resulting in weaker staining [33–35]. We observed no apparent trend towards a negative result among the older specimens but felt it would be unwise to attempt to quantify staining intensity due to the varying preanalytical conditions over which we had no control.

Other strengths of this study include reliable information on BC incidence and follow-up data that were available from high-quality national registries like the Cancer Registry of Norway, the Cause of Death Registry and the Norwegian Patient register [36, 37] thus enabling comparability within the study population across time.

Using TMA sections enables us to stain hundreds of tumour samples at the same time, under the same conditions. The samples comprise a small amount of the original tumour tissue samples, compared to full-face sections. Thus, some important information from the tumour may be lost. However, it has been shown that IHC for ER carried out

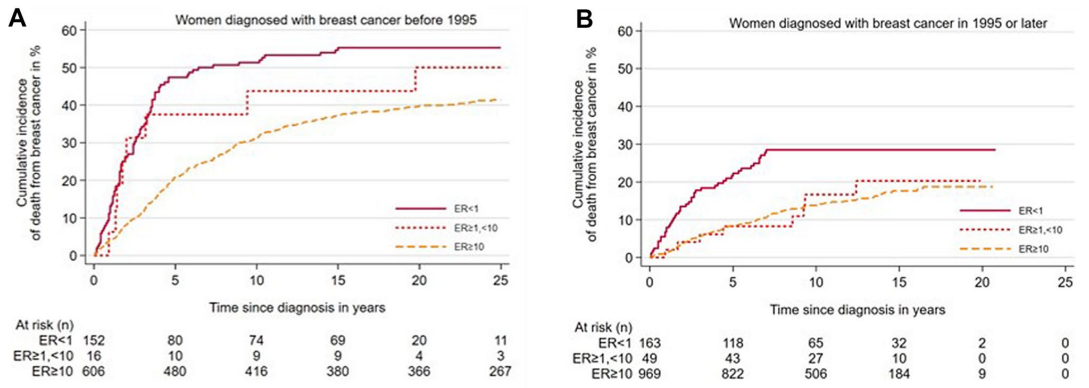

Fig. 2 Cumulative incidence of death from breast cancer according to oestrogen receptor (ER) levels. **A** Women diagnosed with BC before 1995. **B** Women diagnosed with BC in 1995 or later. Gray's test: $p < 0.0001$

Table 5 Absolute and relative risk of death from breast cancer according to ER levels, and breast cancer diagnosis before 1995 and in 1995 or later

	ER levels, diagnosis before 1995			ER levels, diagnosis in 1995 or later		
	< 1%	≥ 1 < 10%	≥ 10%	< 1%	≥ 1 < 10%	≥ 10%
Cumulative risk after 5 years (%) (95% CI)	47.4 (39.8–55.6)	37.5 (18.9–65.1)	20.8 (17.8–24.3)	22.3 (16.6–29.5)	8.3 (3.2–20-5)	8.3 (6.8–10.3)
Cumulative risk after 10 years (%) (95% CI)	51.3 (43.7–59.5)	43.8 (23.8–70.5)	31.4 (27.8–35.2)	28.5 (22.1–36.3)	16.7 (8.2–32.2)	13.8 (11.7–16.3)
HR unadjusted (95% CI)	1.0	0.8 (0.4–1.6)	0.6 (0.5–0.7)	1.0	0.5 (0.2–1.0)	0.5 (0.3–0.6)
HR adjusted for age (95% CI)	1.0	0.7 (0.3–1.8)	0.6 (0.4–0.8)	1.0	0.6 (0.3–1.3)	0.4 (0.3–0.6)
HR adjusted for stage (95% CI)	1.0	0.8 (0.3–1.9)	0.6 (0.4–0.7)	1.0	0.6 (0.3–1.2)	0.4 (0.3–0.6)
HR adjusted for grade (95% CI)	1.0	0.7(0.4–1.6)	0.7 (0.5–0.9)	1.0	0.6 (0.3–1.2)	0.6 (0.4–0.8)
HR adjusted for age, stage, and grade (95% CI)	1.0	0.7 (0.3–1.8)	0.7 (0.5–1.0)	1.0	0.9 (0.4–1.9)	0.5 (0.3–0.8)

ER Oestrogen receptor, *HR* Hazard ratio, *CI* confidence interval

on sections from TMAs can provide equivalent information regarding clinical endpoint when compared to IHC on full-face tissue Sections [38, 39]. Immunohistochemistry for ER on full-face tissue sections was not carried out in the present study.

Conclusion

Overall, ER Low Positive BCs exhibited many characteristics similar to ER-negative tumours and were frequently Luminal B (HER2+). Among women diagnosed in 1995 or later, the proportion of ER Low Positive BCs was higher than among women diagnosed before 1995 and ER Low Positive tumours diagnosed in 1995 or later were of smaller size, lower grade, lower proliferative status, and were more frequently Luminal A Women with ER Low Positive tumours had similar prognosis to patients with ER ≥ 10% when diagnosed in 1995 or later.

Acknowledgements The authors would like to thank the Department of Pathology at St. Olav´s Hospital, Trondheim University Hospital for making the diagnostic archives available for this project and the Cancer Registry of Norway for supplying the corresponding patient data.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. The first draft of the manuscript was written by Anette H Skjervold. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

Funding Open access funding provided by NTNU Norwegian University of Science and Technology (incl St. Olavs Hospital - Trondheim University Hospital). This present study has received funding from the Department of Clinical and Molecular Medicine, Norwegian University

of Science and Technology, Trondheim, Norway. Data included in this study received financial support from the Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology and The Research Council of Norway.

Data availability The datasets generated and/or analysed during this study are not publicly available due to issues of sensitivity and limitations determined in the conditions for approval by the Regional Committee for Medical and Health Research Ethics. However, the data may be made available from the corresponding author on reasonable request.

Declarations

Competing interest The authors declare that they have no competing interests.

Ethical approval This study and publication of its results was granted approval by the Regional Committee for Medical and Health Research Ethics, Central Norway (REK 836-09). The approval includes dispensation from the usual requirement of patient consent.

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ISBN 978-82-326-7754-2 (printed ver.)
ISBN 978-82-326-7753-5 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

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