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Ekholm, Maria

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Prognostic and Predictive Markers in Primary Breast Cancer

Quality Assurance and Long-term Effects of Adjuvant Tamoxifen Treatment

Maria Ekholm

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DOCTORAL DISSERTATION
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Faculty opponent
Lars Holmberg, Professor Emeritus
Faculty of Life Sciences and Medicine, King's College, London, England and
Department of Surgical Sciences, Uppsala University, Sweden

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Title and subtitle: Prognostic and Predictive Markers in Primary Breast Cancer - Quality Assurance and Long-term Effects of Adjuvant Tamoxifen Treatment

Abstract: Breast cancer is the most common cancer among women in Sweden and worldwide. When a patient is diagnosed with breast cancer, prognostic and predictive factors are used to estimate the prognosis and the likely benefit from different kinds of adjuvant therapy. A challenge in this setting is to identify patients with oestrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative breast cancers, for whom adjuvant endocrine therapy is sufficient and chemotherapy can be omitted. One of the most important markers used for this decision is Ki67, which reflects the degree of proliferation within the tumour. However, there are concerns about the reproducibility of Ki67 results between different laboratories as well as between different assessors. It is therefore important to continuously evaluate the laboratories' performance by participation in quality assurance schemes and to strive to improve all steps in the Ki67 analysis.

Study I: We evaluated a new rabbit monoclonal antibody (SP6) for immuohistochemical staining of Ki67, but in comparison with the gold standard antibody (MIB1), the agreement between assessors was not improved, nor was the prognostic value.

Study II: We investigated the quality of Swedish breast cancer biomarker analyses (ER, PR, HER2, and Ki67) in terms of agreement by using a well-known reference laboratory in Milan, Italy for comparison. We found very good agreement for ER, PR, and HER2. Agreement for Ki67 was good, which was better than expected; however, it decreased when applying laboratory-specific cut-offs.

Study III-IV: In the SBII2pre study, premenopausal patients with primary breast cancer (n=564) were randomised between 2 years of adjuvant tamoxifen and no systemic therapy (control). Study III includes long-term follow-up on mortality and the results showed that tamoxifen-treated patients with ER-positive tumours had a 27% reduced risk of breast cancer-related death after a median follow-up 26 years compared with patients in the control group. In Study IV, we show that tamoxifen-treated patients in the ER-positive subgroup had a reduced incidence of breast cancer-related events (38%) and distant recurrences (27%) after 30 years of follow-up compared with patients in the control group. The breast cancer-free interval was also significantly reduced for the period >15 to 30 years, indicating a 'carryover effect' of tamoxifen. In the ER-positive subgroup, tamoxifen was associated with shorter survival after distant recurrences (median, 29 months vs. 43 months). The incidence of contralateral breast cancer was reduced by 42% in patients allocated to tamoxifen, whereas there were no differences in the incidence of secondary non-breast malignancies.

Long-term follow-up of trials including adjuvant tamoxifen is essential to evaluate the overall effects of this medicine as late recurrences are common in ER-positive breast cancer, and this is particular important for for studies including premenopausal patients with long life expectancy.

Key words: breast cancer, prognostic, predictive, Ki67, quality assurance, tamoxifen, long-term follow-up

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Prognostic and Predictive Markers in Primary Breast Cancer

Quality Assurance and Long-term Effects of Adjuvant Tamoxifen Treatment

Maria Ekholm

LUND
UNIVERSITY

Department of Clinical Sciences, Lund
Division of Oncology and Pathology
Lund University, Lund, Sweden

Author: Maria Ekholm (MD)

Main supervisor: Professor Mårten Fernö
Department of Clinical Sciences, Lund
Division of Oncology and Pathology
Lund University, Sweden

Co-supervisors: Professor Lisa Rydén (MD)
Department of Clinical Sciences, Lund
Division of Surgery
Lund University, Sweden

Professor Olle Stål
Department of Clinical and Experimental Medicine and
Department of Oncology
Linköping University, Sweden

Cover by Tina Lindholm
Photo by Anna Hållams

Lund University
Faculty of Medicine
Department of Clinical Sciences Lund
Division of Oncology and Pathology

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If not now, then when…?

Contents

Abbreviations

AI	Aromatase inhibitor
BCFi	Breast cancer-free interval
BCS	Breast-conserving surgery
CBCM	Cumulative breast cancer-related mortality
CI	Confidence interval
CM	Cumulative mortality
CRF	Case report form
CTC	Circulating tumour cell
ctDNA	Circulation tumour DNA
DCIS	Ductal cancer *in situ*
DDFS/D-DFS	Distant disease-free survival
DFS	Disease-free survival
D-RFi	Distant disease-free interval
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ELISA	Enzyme-linked immunosorbent assay
ER	Oestrogen receptor
FFPE	Formalin-fixed paraffin-embedded
GnRH	Gonadotropin-releasing hormone
HER2	Human epidermal growth factor receptor 2
HG	Histological grade
HR	Hazard ratio
IEO	European Institute of Oncology
IHC	Immunohistochemistry
ISH	*In situ* hybridisation
LA	Local assessments
LBA	Ligand-binding assay
mAB	Monoclonal antibody
OFS	Ovarian function suppression
OS	Overall survival
PAI-1	Plasminogen activator inhibitor type-1
PR	Progesterone receptor
QA	Quality assurance
RA	Reviewed assessments
RabMAb	Rabbit monoclonal antibody
RFS	Recurrence-free survival
RR	Recurrence rate
SNP	Single-nucleotide polymorphism
TMA	Tissue microarray
uPA	Urokinase plasminogen activator

List of included papers

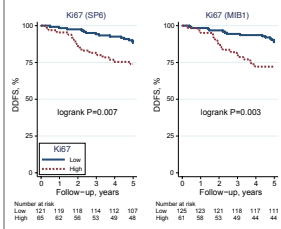
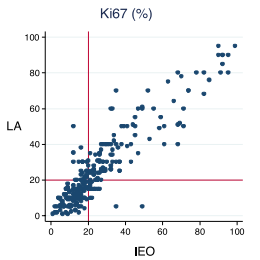
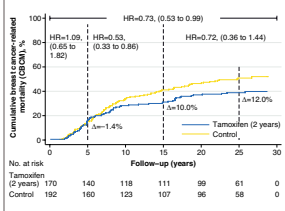
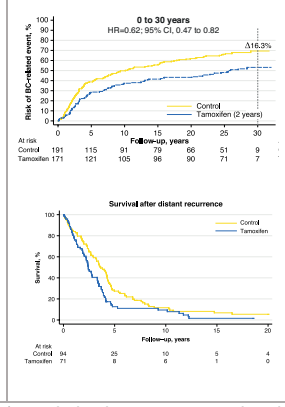
I. **Immunohistochemical Assessment of Ki67 with Antibodies SP6 and MIB1 in Primary Breast Cancer: a Comparison of Prognostic Value and Reproducibility.**
Ekholm M, Beglerbegovic S, Grabau D, Lövgren K, Malmström P, Hartman L, and Fernö M.
Histopathology. 2014 Aug;65(2):252-60.

II. **Highly Reproducible Results of Breast Cancer Biomarkers when Analysed in Accordance with National Guidelines – a Swedish Survey with Central Re-assessment.**
Ekholm M, Grabau D, Bendahl PO, Bergh J, Elmberger G, Olsson H, Russo L, Viale G, and Fernö M.
Acta Oncologica. 2015 Jul;54(7):1040-8.

III. **Two Years of Adjvuant Tamoxifen Provides a Survival Benefit Compared with no Systemic Treatment in Premenopausal Patients with Primary Breast Cancer: Long-term Follow-up (>25 years) of the Phase III SBII:2pre trial.**
Ekholm M, Bendahl PO, Ferno M, Nordenskjold B, Stal O, and Rydén L.
Journal of Clinical Oncology. 2016 Jul 1;34(19):2232-8.

IV. **Persistent Reduction of the Incidence of Breast Cancer-related Events, but Shorter Survival after Distant Recurrence, in Premenopausal Patients Randomized to Adjuvant Tamoxifen and Followed for Almost Three Decades.**
Ekholm M, Bendahl PO, Ferno M, Nordenskjold B, Stal O, and Rydén L.
Manuscript submitted

Thesis at a glance

Study	Aims	Methods	Results	Conclusion
I	To compare the antibodies SP6 and MIB1 for analysis of Ki67 in primary breast cancer in terms of prognostic value and reproducibility.	TMA sections with tumours from premenopausal node-negative patients were stained for Ki67, using the antibodies MIB1 and SP6, and then scored by different assessors.		SP6 was not superior to MIB1, but the two antibodies were comparable for prognostic use in primary breast cancer. The reproducibility was marginally better for MIB1.
II	To investigate the concordance for ER, PR, HER2, and Ki67 when routinely analysed in Swedish laboratories compared with at a central laboratory (IEO, Milan, Italy) (n=270).	IEO re-assessed the original Swedish glass slides and used the original tumour tissue blocks for new stainings and scoring.		The agreement was highly concordant for ER, PR, and HER2. Agreement was good for Ki67, but worsend when applying laboratory-specific cut-off values for the Swedish samples.
III	To investigate the long-term effects of 2 years of adjuvant tamoxifen vs. no sytemic treatment in premenopausal patients (n=564) in terms of overall mortality and breast cancer-related mortality.	Data were obtained from the Swedish Causes of Death Register. CM and CBCM were choosen as endpoints, and for the latter, competing risks were taken into consideration.		After a median follow-up of 26 years, the CBCM was 27% lower in patients with ER-positive tumours treated with 2 years of adjuvant tamoxifen, compared with patients in the control group. A non-significant benefit was also seen for CM.
IV	To investigate the long-term effects of 2 years of adjuvant tamoxifen vs. no sytemic treatment in premenopausal patients (n=560) in terms of breast cancer-related events and distant recurrences. Moreover, this study also aimed to study the effects on surival after distant recurrence and secondary malignancies.	Medical records were reviewed and complemetary data were retriewed from the Swedish Causes of Death Register and the Swedish Cancer Registry. BCFi and D-RFi were choosen as endpoints. The statistical analyses focused on the ER-positive subgroup.		Tamoxifen-treated patients had a reduced incidence of breast cancer-related events (38%) and distant recurrences (27%) after 30 years of follow-up compared with patients in the control group. Tamoxifen was associated with shorter survival after distant recurrence. I all patients, secondary malignancies were similar, except for a reduction of contralateral breast cancers.

Abbreviations: BCFi (breast cancer-free interval); CBCM (cumulative breast cancer-related mortality); CM (cumulative mortality); D-RFi (distant recurrence-free interval); IEO (European Institute of Oncology); ER (oestrogen receptor); HER2 (human epidermal growth factor receptor 2); PR (progesterone receptor); TMA (tissue microarray).

Populärvetenskaplig sammanfattning
(summary in Swedish)

Bröstcancer är den vanligaste cancersjukdomen hos kvinnor både i Sverige och i resten av världen och utgör ungefär en tredjedel av alla cancerfall. I Sverige diagnostiserades knappt 8300 kvinnor med bröstcancer under 2016. Medianåldern vid diagnos var 65 år och ungefär 4 % av de som insjuknade var yngre än 40 år. När en patient diagnostiseras med bröstcancer görs en bedömning av tumörens sjukdomsutbredning och dess biologiska egenskaper. Så kallade prognostiska faktorer används för att uppskatta prognos, dvs risken att drabbas av återfall, medan prediktiva faktorer används för att bedöma sannolikheten att en patient ska ha nytta av en specifik behandling. En majoritet av patienterna erbjuds återfallsförebyggande behandling med syfte att minska risken för återfall. Det kan vara svårt att avgöra om en patient med hormonkänslig bröstcancer utöver sedvanlig antihormonell behandling också ska erbjudas tilläggsbehandling med cytostatika. En av de viktigaste markörerna som används för dessa beslut är Ki67, en markör som speglar tumörens tillväxthastighet och där höga värden har visat sig vara associerat med högre risk för återfall. Ett problem är dock att reproducerbarheten av resultaten av Ki67-analyser kan variera mellan olika patologlaboratorier och mellan olika bedömare. För att säkerställa korrekta analysresultat är det därför viktigt att patologavdelningar kontinuerligt utvärderar sina analysmetoder och sina resultat, t.ex. genom att medverka i externa kvalitets-säkringsprogram.

Studie I

Flera ansträngningar har gjorts för att förbättra de olika stegen i Ki67-analysen. Utvärdering av nya antikroppar kan vara ett sätt att förbättra färgningen av tumörvävnaden, vilket därmed kan göra det enklare att bedöma Ki67.

I Studie I jämfördes den nyare antikroppen SP6 med den etablerade antikroppen MIB1 för immunohistokemisk färgning av Ki67 avseende samstämmighet mellan olika bedömare och möjlighet att förutsäga prognos.

Vi fann inte att SP6 ökade samstämmigheten mellan olika bedömare och de bägge antikropparna bedömdes vara likvärdiga för att bedöma prognos hos patienter med primär bröstcancer.

Studie II

I Studie II undersökte vi samstämmigheten avseende analysresultat för ett antal biomarkörer när de analyserats i svensk rutinsjukvård jämfört med i ett välkänt referenslaboratorium (European Institute of Oncology i Milano, Italien).

Vi fann hög överensstämmelse för följande markörer; östrogen receptorn (ER) (positivt vs. negativt), progesteron receptorn (PR) (positivt vs. negativt), human epidermal growth factor receptor 2 (HER2) (positivt vs. negativt) och Ki67 (högt vs. lågt). Överensstämmelsen avseende Ki67 försämrades när laboratoriespecifika gränsvärden användes för att definiera högt/lågt Ki67 för de svenska bedömningarna. Vidare noterades att en antikropp för infärgning av PR var associerat med generellt högre värden och några falskt positiva fall, vilket understryker vikten av deltagande i externa kvalitetssäkringsprogram för biomarkörer.

Antihormonell behandling har visat sig minska den relativa risken för återfall i bröstcancer med cirka 40%. I början på 1980-talet var nyttan av den antihormonella behandlingen tamoxifen till yngre kvinnor med bröstcancer inte fastställd och för att undersöka detta genomfördes en studie (SBII2pre) i de Sydöstra och Södra Sjukvårdsregionerna. Mellan 1984 och 1991 inkluderades 564 premenopausala patienter med primär bröstcancer och de randomiserades mellan 2 års behandling med tamoxifen och ingen systemisk behandling (kontroll). Eftersom sambandet mellan ER-positivitet och effekt av tamoxifen inte var helt klarlagd vid denna tidpunkt, inkluderades patienter oavsett tumörens hormonreceptorstatus. Eftersom det är vanligt med sena återfall vid ER-positiv bröstcancer, ibland flera decennier efter den primära diagnosen, är det viktigt med lång uppföljning vad gäller studier som omfattar denna patientgrupp.

Studie III

I Studie III undersökte vi med hjälp av datauttag från Dödsorsaksregistret om 2 års behandling med tamoxifen på lång sikt minskade risken att dö (oavsett orsak) respektive risken att dö pga. bröstcancer hos de premenopausala patienter med bröstcancer som ingick i SBII2pre studien.

Efter en medianuppföljning på 26 år fann vi i den ER-positiva subgruppen att patienter som behandlats med tamoxifen hade 27% lägre risk för bröstcancerrelaterad död jämfört med patienter i kontrollgruppen.

Studie IV

I Studie IV undersökte vi vilka långtidseffekter 2 års tamoxifenbehandling hade hos premenopausala kvinnor med ER-positiv bröstcancer avseende bröstcancerrelaterade händelser (lokalt, regionalt- och fjärrecidiv, bröstcancerrelaterad död och ny cancer i andra bröstet) respektive fjärrmetastaser. För att besvara frågan genomfördes

journalgranskning omfattande tiden från diagnos fram till november 2016. Dessutom inhämtades kompletterande uppgifter från Dödsorsaksregistret och Cancerregistret.

Efter 30 års uppföljning fann vi att tamoxifenbehandlade patienter hade 38% lägre incidens av bröstcancerrelaterade händelser och 27% lägre incidens av fjärrmetastaser jämfört med patienter i kontrollgruppen. Effekten avseende bröstcancerrelaterade händelser var också tydlig för intervallet mellan 15 och 30 år, vilket indikerar att nyttan av tamoxifen kvarstår många år efter avslutad behandling. Bland de patienter i den ER-positiva gruppen som drabbats av fjärrmetastaser fann vi att de som hade behandlats med tamoxifen hade signifikant kortare överlevnad jämfört med de som primärt inte fått någon behandling (median, 29 månader vs. 43 månader). Sett i hela patientgruppen var incidensen av cancer i det andra bröstet 42% lägre hos patienter som behandlats med tamoxifen, medan det inte sågs några skillnader avseende andra sekundära cancrar.

De viktigaste fynden i mitt avhandlingsarbete är att 2 års tamoxifenbehandling tydligt minskar risken att dö till följd av bröstcancer och att minskningen av bröstcancerrelaterade händelser är tydlig även 15 år efter den primära diagnosen. Förhoppningsvis kan dessa resultat uppmuntra patienter att fullfölja sin antihormonella behandling och de patienter som drabbas av mycket biverkningar kan kanske motiveras att ”härda ut” i åtminstone 2 år. Långtidsuppföljning är viktigt vid studier som rör ER-positiv bröstcancer med tanke på att det finns risk för sena återfall. Detta är särskilt viktigt vid studier som inkluderar yngre patienter med lång förväntad överlevnad, och när man studerar mediciner där nyttan av behandlingen kan ses lång tid efter att behandlingen avslutats. Vidare understryker våra fynd vikten av att alla laboratorier som analyserar biomarkörer vid bröstcancer deltar i externt kvalitetssäkringsarbete, detta för att möjliggöra korrekt bedömning av prognos och för att varje patient ska kunna erbjudas rätt behandling.

Introduction

Epidemiology

Incidence and mortality

Breast cancer is the most common cancer in females worldwide with approximately 1.7 million new cases and 520,000 deaths each year.[1] In Sweden, breast cancer accounts for approximately 30% of the cancers among women.[2] During 2016, 8,463 breast cancers were reported diagnosed in Sweden, 11% of which were *in situ* cancers. Median age at diagnosis was 65 years and 3.6% of the tumours were diagnosed in patients aged <40 years (see Figure 1).[3] In Sweden, the yearly age standardised incidence has increased from 1.6% based on the last 20 years to 2.6% based on the last 10 years, and the cumulative risk for being diagnosed with breast cancer before the age of 75 is now approximately 11%.[4] This increase is partly due to the fact that more tumours are reported per patient, as well as the increased participation in screening programs and improved imaging techniques.[4] The increase is also attributed to lifestyle changes, which are described in more detail in the 'Risk factors' section below. Despite the increasing incidence of breast cancer, the mortality rates in Sweden have remained stable, at approximately 1,400 deaths per year, indicating a slight decrease in mortality.[4] Similar scenarios have been observed in other countries and can be explained by, for example, improved adjuvant treatment and earlier detection.[5]

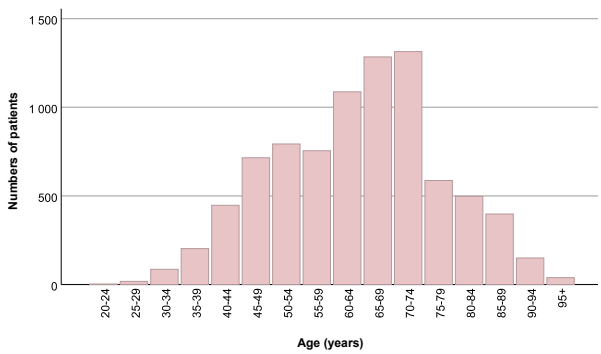


Figure 1. Number of patients diagnosed with breast cancer in Sweden during 2016, displayed by age categories.[3]

Risk factors

Although it is not clearly understood why some women develop breast cancer while others do not, there are well-established factors that have been shown to increase the risk of breast cancer. The incidence of breast cancer is generally higher in developed countries (i.e. North America, Northern and Western Europe, and Australia) compared with less developed countries (i.e. those in Africa and some parts of Asia).[1]

Women previously diagnosed with ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS) have an increased risk of developing invasive ipsilateral and contralateral breast cancer.[6] Moreover, patients with biopsy-confirmed benign breast disease, such as atypical hyperplasia, have been shown to have a four-fold increased risk of subsequent breast cancer.[7] High breast density according to mammography has also been associated with an increased breast cancer risk.[8]

Several risk factors are related to endogenous oestrogen exposure, including menstrual and reproductive events. Early menarche and late menopause prolongs the exposure to ovarian hormones and is associated with an increased risk of breast cancer.[9] A meta-analysis including eight Nordic studies confirmed that breast cancer risk is significantly and positively correlated to nulliparity, low parity, and older age at first birth.[10] Moreover, breastfeeding has been reported to reduce the breast cancer risk, particularly the breast cancer subtypes that are negative for hormone receptors.[11,12] There are conflicting results on whether oral contraceptives increase breast cancer risk. In some studies, a modest but increased risk was observed up to 10 years after cessation of oral contraceptive use,[13,14] whereas other studies showed similar incidences irrespective of former oral contraceptive use.[15,16] The use of hormonal replacement therapy (HRT) containing only oestrogen, is not associated with increased risk of breast cancer, whereas combined HRT almost triples the risk.[17] However, the increased risk does not seem to remain after cessation of treatment.[18]

Obesity is associated with an increased risk of breast cancer in postmenopausal women and this can partly be explained by the increased peripheral production of sex hormones in the adipose tissue.[19,20] Additional mechanisms underlying the link between obesity and breast cancer may include elevated levels of insulin and insulin-like growth factor-1 (IGF-1), which may increase the downstream signalling through the IGF-1 receptor, as well as reduce the production of sex hormone-binding globulin from the liver, and thereby increase the bioavailability of oestrogens in the blood. For premenopausal women, the opposite relationship between overweight and breast cancer risk has been observed.[21] The underlying reason is not known, but it has been suggested to be associated with more frequent anovulatory menstrual cycles.[22] Moreover, some evidence points towards an association between obesity and oestrogen receptor (ER)-negative breast cancer in premenopausal women.[23] Other factors associated with an increased risk of breast cancer are excess alcohol drinking and high-dose radiation to the chest at young age.[24,25] Physical activity, on the other hand, has been demonstrated to reduce the breast cancer risk.[26,27]

Hereditary breast cancer

BRCA is an acronym for the BReast CAncer gene, and people with germline mutations in *BRCA1* or *BRCA2*, have a lifetime risk of 50%–80% for developing breast cancer.[28] These mutations are also associated with an increased risk of ovarian cancer and pancreatic cancer, and males with *BRCA2* mutations have an increased risk of prostate cancer.[29] *PALB2* is another gene associated with an increased risk of developing breast cancer.[30] Other genes have been associated with a moderate increased risk, such as *CHEK2*, *ATM*, and *BRIP1*.[31] Moreover, some genes are involved in different cancer predisposition syndromes, which may increase the risk of several cancer forms including breast cancer; these genes include *TP53*, *PTEN*, *STK11*, *CDH1*, and *NF1*.[31] A single-nucleotide polymorphism (SNP) is a variation of one single nucleotide in the genome that is present in >1% of the population.[32] A SNP located within a gene, may result in an altered amino acid sequence, which in turn may affect the expression and/or function of the encoded protein. The presence of SNPs within key genes related to breast cancer may increase the risk of developing breast cancer.[33] In patients in which no disease-associated gene alterations can be identified, despite a considerable number of breast cancer cases in their family, there is a risk prediction model, BOADICEA, to estimate the risk for subsequent breast and ovarian cancer.[34] Patients with a calculated risk exceeding 20% during their remaining lifetime should be offered participation in screening programs from an earlier age or prophylactic mastectomy.[35]

Carcinogenesis

'The Hallmarks of Cancer'

Normal cells grow and reproduce in an orderly and controlled way. Cancer develops in a multistep process that involves the acquiring of genetic alterations that drive the progressive development from normal to malignant cells. Hanahan and Weinberg defined a series of underlying principles that characterised tumorigenesis, referred to as 'The Hallmarks of Cancer', which was first published in 2000 and later updated in 2011 (see Figure 2).[36,37] These principles are briefly summarised below.

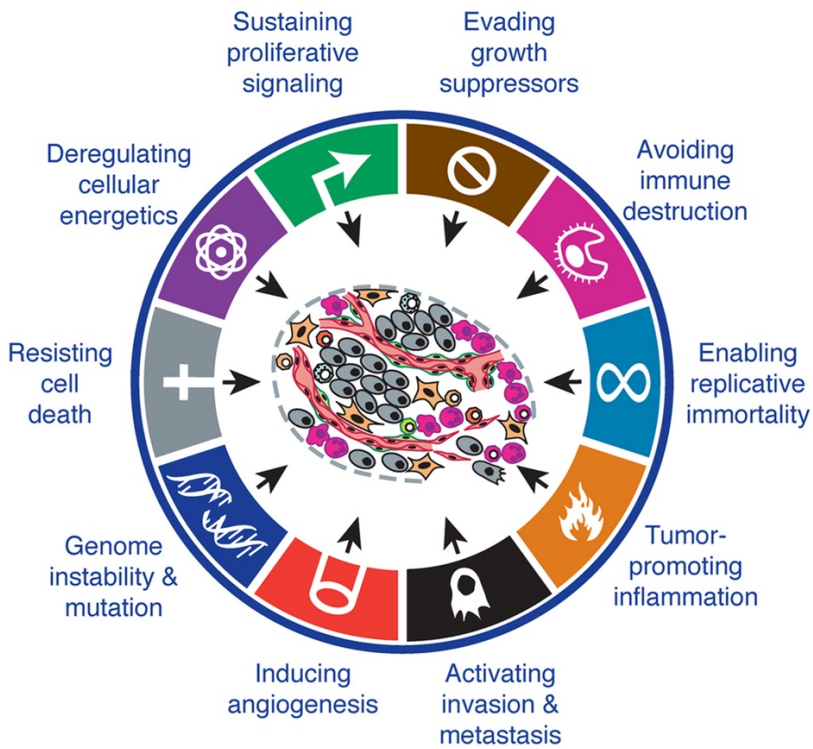


Figure 2. Hallmarks of cancer. Reprinted with permission from Elsevier: Cell, © 2011.[37]

Sustaining proliferative signalling

Normal cells require external growth signals, such as hormones and other molecules, to grow and divide, but growth is inhibited in the absence of growth factors. Cancer cells, however, can divide without external growth signals, as they may obtain capabilities to sustain proliferative signalling in different ways. Cancer cells may start to produce their

own growth factors (autocrine signalling) or stimulate normal cells to produce growth factors (paracrine signalling). Receptor proteins can be upregulated and overexpressed. Moreover, mutated receptors may activate downstream signalling pathways, despite the absence of ligand binding.[37]

Evading growth suppressors

Normal cells have highly controlled processes to prevent unregulated cell growth and division. In contrast, cancer cells often have acquired the capability to avoid anti-growth signals, for example by defects in tumour suppression genes or by ignoring contact inhibition from neighbouring cells.[37]

Resisting cell death

In normal cells, programmed cell death by apoptosis is used to eliminate defective cells. Cancer cells have various strategies to avoid apoptosis. One is the loss of the TP53 tumour suppressor function. *TP53* is a tumour suppressor gene, and the p53 protein has been described as 'the guardian of the genome', as it normally conserves the genome by preventing mutations. p53 acts through several mechanisms, one of which is the initiation of apoptosis.[38,39] Malignant cells may also increase the expression of anti-apoptotic regulators or downregulate pro-apoptotic factors to avoid apoptosis.[37]

Enabling replicative immortality

Normal cells are only able to divide a limited number of times, normally 60–70 doublings, before they enter a non-proliferative state (senescence) or die (crisis). The counting device for cell division is the telomere, which is a region of repetitive nucleotide sequences at the end of each chromosome. During each cell division, telomeres lose DNA at the tips and progressively shorten. The limited capacity for normal cells to divide before they become senescent is referred to as the 'Hayflick limit', which correlates with the length of telomeres.[40,41] Many cancer cells, however, are able to escape this limit by the upregulation of telomerase, an enzyme that maintains telomeres.[37] Hence, cancer cells can achieve unlimited replicative potential.

Inducing angiogenesis

Like normal tissue, tumours require blood vessels to secure the supply of oxygen and nutrients and to evacuate carbon dioxide and metabolic wastes. Angiogenesis, the process of new blood vessel development, is active during embryogenesis, but in adults it is only turned on during certain physiological circumstances, such as wound healing and pregnancy. In contrast, the 'angiogenic switch' is turned on early during cancer development and continues to remain activated, resulting in new vessels that help sustain tumour growth.[42] Several angiogenetic regulators, such as vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor (FGF), and thrombospondin-1

(TSP-1), are upregulated in cancer.[37] Upregulation of VEGF gene expression can be induced by growth factors or hypoxia.[43,44]

Activating invasion and metastasis

The 'invasion-metastasis cascade' involves the process of invasion and metastasis. This process begins with the local invasion of cancer cells, followed by the entry of cancer cells into vessels (intravasation), transition through the haematogenous and lymphatic systems to a distant site, exit from the vessel into the parenchyma of another tissue (extravasation), forming of tumour masses (micrometastases), and finally the growth into larger tumours (colonisation).[45] The process in which the cancer cells derive their ability to spread is referred to as the 'epithelial-mesenchymal transition' (EMT). However, to develop metastases at a distant site, the cancer cells have to regain their epithelial status and this is achieved through the reversed process, referred to as the 'mesenchymal-epithelial transition' (MET).[46] There are two distinct models describing tumour progression, carefully reviewed by Klein:[47]

- The linear progression model – according to this model, tumour cells grow and evolve in the primary tumour until they have derived certain properties that enable them to metastasise. This process is followed by the seeding of disseminated tumour cells (DTCs) to a distant site, where a secondary growth phase take place. Once the DTCs have adapted and formed a significant mass, this can provide further DTCs, which can give rise to new secondary metastases.
- The parallel progression model – according to this model, tumour cells that are still evolving may spread and cause metastases. This parallel spreading of tumours cells will result in a diversity of clones that may differ between different sites but also between metastases within the same organ.

In breast cancer, macroscopic metastases may arise a long time after the primary diagnosis.[48] This is likely a result of dormancy, in which tumour cells or micrometastases succeed to colonise tissues and form metastases after years or sometimes decades without disease progression.[49]

When 'Hallmarks of Cancer' was updated in 2011, two enabling characteristics were described that were essential for acquiring the hallmarks described above: 'Genomic instability and mutation' and 'Tumour-promoting inflammation'.

Genome instability and mutation

The genomic instability present in cancer cells contributes to more and more mutations and chromosomal rearrangements, which results in an evolution of the tumour cells in which they acquire the characteristics described under the different hallmarks. Genomic instability will therefore drive tumour progression.[37]

Tumour-promoting inflammation

Inflammation may result in release of bioactive molecules that can promote the different hallmarks described above. For example, these molecules can act as growth factors, pro-angiogenetic factors, activators of EMT, and degrading enzymes that drive invasion and angiogenesis. Additionally, the molecules released by inflammation may harm the DNA in nearby cells and thereby accelerate the genetic evolution of these cells.[37]

Another two new emerging hallmarks were also presented: 'Reprogramming energy metabolism' and 'Evading immune destruction'.[37]

Reprogramming energy metabolism

Under aerobic conditions, normal cells derive energy through the oxidation of glucose and adenosine triphosphate (ATP) is produced in the mitochondria of the cells. Under anaerobic conditions, metabolism is switched to glycolysis, which is independent of oxygen but produces far less ATP than during normal metabolism. The observation that cancer cells largely reprogram their metabolism to glycolysis was described by Otto Warburg in 1930.[50] To compensate for this loss of energy, cancer cells upregulate the transportation of glucose into the cytoplasm.[51] The rational for the metabolic switch is that it facilitates the synthesis of molecules required for new cells.[52] The increased uptake of glucose in cancer cells can be visualized in positron emission tomography (PET), in which a radiolabelled glucose analogue (18F-fluorodeoxyglucose, FDG), is used as tracer to distinguish malignant tumours from normal tissue.[53]

Evading immune destruction

The immune system is involved in recognizing and eliminating incipient cancer cells and thereby prevents the development of cancer. Cancer cells appear to have several strategies to avoid interaction with the immune system.[37]

Prognostic and predictive factors

A prognostic marker provides information on the probable outcome of a disease, while a predictive marker provides information regarding the probability to benefit from a certain treatment.[54] Breast cancer is a heterogeneous disease, and thus the risk of recurrence can differ significantly between patients. To estimate prognosis and to be able to recommend the best possible treatment for each patient, the use of prognostic and predictive factors are essential in breast cancer. These factors include:

- Patient-related factors, such as age and menopausal status
- Tumour stage, based on tumour size, involvement of regional lymph nodes and presence of distant metastases
- Biological characteristics of the tumour, such as histological grade (HG), proliferation, the expression of hormone receptors (ER and progesterone receptor (PR)), and the expression of human epidermal growth factor receptor 2 (HER2)

Some biomarkers, like ER and HER2, provide both prognostic and predictive information, and these are used to identify patients likely to benefit from endocrine therapy and anti-HER2 therapy, respectively.[54]

Age

Breast cancers in younger women are often associated with worse pathological features, including ER-negative status, HG3, and higher stage at presentation. After adjusting for pathology variables and adjuvant treatment in multivariable analysis, age remains to be a powerful prognostic factor.[55,56] A large retrospective study from the SEER database that included 243,000 women diagnosed between 1988–2003 revealed that women with stage I–II breast cancer aged <40 years were 39% more likely to die compared with those aged ≥40 years (hazard ratio (HR)=1.39; 95% confidence interval (CI), 1.34–1.45).[57] There are several studies reporting that the relationship between outcome and the patient age at diagnosis varies by tumour subtype. Sheridan et al. reported that after adjusting for adverse pathological factors and adjuvant treatment, age <40 years was associated with a significantly worse prognosis only for patients with luminal tumours (ER-positive/HER2-negative).[58] Similar results have been reported by others.[59,60]

Tumour stage

The TNM Classification of Malignant Tumours is a staging system that describes the stage of a cancer in solid tumours and it was first presented in the 1940s.[61] TNM was developed by The Union for International Cancer Control (UICC), but this classification is also used by the American Joint Committee on Cancer (AJCC). In the TNM staging system, T defines the size of the primary tumour, N describes the degree of regional lymph node involvement, and M describes the presence of distant metastasis. There are also prefix modifiers to address what the TNM classification is based upon: c=physical examination or imaging, p=pathological examination, y=stage assessed after neoadjuvant therapy, or a=autopsy.[62] A TNM classification based on pathological examination after neoadjuvant therapy would, for example, be referred to as ypT2N1M0. The combination of T, N, and M gives the anatomic stage of the disease. Not surprisingly, greater tumour size, more extensive involvement of the regional lymph nodes, and presence of distant metastases are all associated with a poorer prognosis.[63,64] The updated 8th edition of the AJCC Cancer Staging Manual includes some major changes. In this version, prognostic factors (ER, PR, HER2 status, HG, and multi-gene panel score) have been incorporated into the staging system to also include the biological features of the tumour.[65]

Stage migration

The TNM system criteria have varied over time. It is crucial to be aware that a given stage may have a different prognosis depending on which edition of the TNM classification it was based upon.[66] Most studies report patients according to the TNM classification used at the start of the study rather than the staging edition used at the time for publication.

Histologic classification

Tumour type

All tumours are classified according to the World Health Organization (WHO) classification system. Ductal carcinoma is the most common tumour type in breast cancer (70%–75%), followed by lobular carcinoma (5%–15%).[67] In the most recent WHO classification version from 2012, the term ductal has been omitted and ductal carcinoma is now referred to as invasive carcinoma of no special type (NST) or not otherwise specified (NOS); however, the term 'invasive ductal carcinoma' is still accepted as an alternative terminology option.[68,69] The more unusual tumour types, such as papillary, cribriform, apocrine, and adenoid cystic carcinoma, are often associated with a better prognosis, whereas metaplastic breast cancer confers a poorer prognosis.[70]

Histological grade (HG)

HG, or tumour grade, classifies tumours according to the degree of differentiation of the tumour tissue and it reflects how closely the tumour cells resemble normal cells when examined by microscope. In breast cancer, the HG system was first described by Bloom and Richardson in 1957 and later revised by Elston and Ellis in 1991.[71,72] This grading system contains evaluation of three parameters: tubule formation, nuclear pleomorphism, and mitotic count. Each factor is scored 1–3 and the scores are then added together to give the HG:

- Total score 3–5: Grade 1 (low grade or well differentiated)
- Total score 6–7: Grade 2 (intermediate grade or moderately differentiated)
- Total score 8–9: Grade 3 (high grade or poorly differentiated)

HG has repeatedly been shown to be a strong independent prognostic factor in primary breast cancer.[63,72,73]

Nottingham prognostic index (NPI) is a prognostic model based on tumour size, lymph node involvement, and tumour grade.[74] NPI can be used to estimate the risk of recurrence with short and long-term follow-up, and it has been validated in several large multicentre trials.[75-77] In recent years, the NPI+ was developed. This tool also includes biomarkers such as ER, PR, and different cytokeratins, as well as members of the epidermal growth factor receptor (EGFR) family, and thus also integrates the biological features of the tumour in the model.[78]

Steroid hormone receptors

In addition to ER and PR, the family of steroid hormone receptors (SHRs) also includes receptors for androgens, mineralocorticoids, and glucocorticoids.[79] All SHRs have a similar basic structure with several functional domains.[80] The structure of SHRs has been thoroughly reviewed[81] and includes the following domains (illustrated in Figure 3):

- The ligand-binding domain (LBD) located is in the C-terminal region
- The activation function 2 (AF-2), located within the LBD, is important for the ligand-mediated transcriptional activity
- The DNA-binding domain (DBD) binds to specific elements in the promoter regions of target genes
- The activation function 1 (AF-1) domain, located at the N-terminal region, can function as a ligand-independent transcriptional activator or synergise with AF-2

ER and PR modulate gene expression by both activation and repression of transcription.[82]

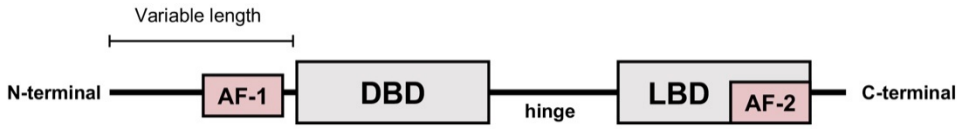


Figure 3. Molecular structure of a steroid hormone receptor.

Oestrogen receptor (ER)

The first evidence indicating that breast cancer may be oestrogen dependent was found in the 1890s, when oophorectomy was reported to lead to tumour regression in premenopausal patients with advanced breast cancer.[83] In 1960, Jensen and Jacobsen discovered what today is recognized as ERα, one of two main types of receptors for oestrogen.[84] By administering radiolabelled oestradiol to rats, Jensen and Jacobsen showed that oestrogen was retained in oestrogen-sensitive tissues, such as the uterus and the vagina, but not in tissues like muscles and liver. The authors thus concluded that oestrogen targets tissues expressing ER.[84] ERα was first cloned in 1986, and a decade later ERβ was discovered by Gustafsson and colleagues.[85,86] ERα and ERβ are encoded by two separate genes (*ESR1* and *ESR2*), located on different chromosomes.[87] ERα is clearly associated with proliferative effects in normal breast tissue as well as in breast cancer.[88] The prognostic and predictive values of ERβ expression still remain unclear, and contradictory results have been presented in clinical studies.[89,90] ERs are widely expressed in different tissue types outside the reproductive organs, and in addition to their key roles in the reproductive organs, these receptors also have biological effects in the musculoskeletal, cardiovascular, immune, and central nervous systems.[91-93]

ER signalling

The most potent form of oestrogen is 17β-oestradiol (E2), whereas the two metabolites of E2, oestrone (E1) and oestriol (E3), act as weaker agonists on ER.[94,95] When a ligand binds to the LBD in ER, this induces a conformal change of the receptor. The ligand-bound ERs then dimerise and bind either directly to oestrogen response elements (EREs) in the promoters of target genes in DNA (direct ligand signalling) or indirectly by binding to other DNA-bound transcription factors (tethered ligand signalling). Upon binding to DNA, co-activators or co-suppressors are recruited to activate or repress gene transcription.[89,96] Growth factor receptors may initiate ligand-independent intracellular signalling. Activated kinases phosphorylate ERs, which initiates dimerisation of the receptors, DNA binding, and gene regulation (growth factor signalling).[96] There is also growing evidence for rapid non-genomic complex ER signalling pathways (non-genomic signalling).[97] The different models for ER activation are illustrated in Figure 4. ER activation results in cell cycle progression and cell proliferation, and the increased cell division elevates the risk for replication errors, which increases the risk that the cell will acquire mutations that drive tumour growth.[98]

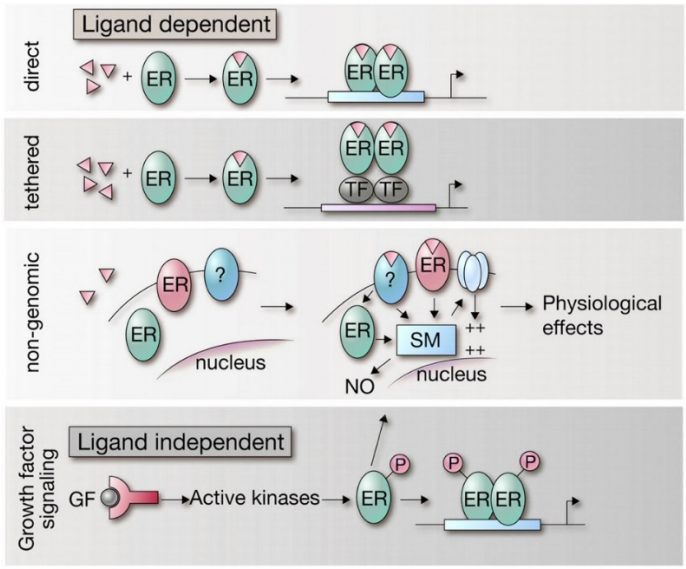


Figure 4. Different models of ER signalling. ER, oestrogen receptor; GF, growth factor; SM, second messengers; TF, transcription factor. Reprinted in adapted colours with permission from The American Physiological Society: Physiol Rev, © 2007.[96]

ERα as a prognostic and predictive marker

Approximately 75%–85% of all breast cancers express ER and are referred to as ER-positive breast cancers.[99] Recent population-based data indicate an even higher proportion of approximately 85%.[3] ERα is still the only reliable biomarker to predict the efficacy of endocrine therapy.[100] ERα is routinely analysed in breast cancer tumours and according to the Swedish guidelines, tumours are considered ER-positive if >10% of the nuclei are positively stained by immunohistochemistry (IHC).[101] International guidelines often use ≥1% as cut-off, as some studies have indicated a benefit from endocrine treatment also in patients with tumours with 1%–9% ER-positivity.[102-107] However, the benefit from endocrine therapy in this group has been questioned.[108,109] In the molecular setting, tumours with low ER expression show more similarities with ER-negative tumours.[110,111] High ERα expression is often associated with a more beneficial effect of endocrine treatment.[112,113] Moreover, a higher level of ERα is often associated with a favourable prognosis and a lower risk of recurrence and death from breast cancer, at least during the first 5 years after diagnosis. However, because ER-positive breast cancer is often associated with late recurrence (beyond 5 years), the prognostic value shifts and with longer follow-up, ERα-positivity is instead associated with higher risk of recurrence compared with ER-negative tumours.[114-118] In the following chapters, ERα will be referred to as ER.

Progesterone receptor (PR)

The two most common isoforms of progesterone receptors (PR-A and PR-B) are encoded by the same gene (*PGR*) and can act as homo- or heterodimers.[119,120] Normal breast tissue expresses roughly equal amounts of PR-A and PR-B, whereas the proportion of PR-A is largely increased in DCIS and invasive breast cancer.[121] Upon binding of the ovarian steroid hormone, progesterone, or by synthetic ligands (progestins), the receptors form dimers that can bind directly to DNA or via transcription factors.[122]

Approximately 60%–70% of all breast cancers are PR-positive, and ER and PR are most often co-expressed in early-stage breast cancer.[3,99] Tumours that are initially PR-positive may however lose PR expression during disease progression, resulting in a more aggressive disease with poorer outcome compared to tumours that remain ER- and PR-positive.[123,124] PR loss may be associated with upregulation of the PI3K pathway and subsequent downregulation of ER and PR expression results in a tumour that is less dependent on oestrogen signalling.[125,126] The subgroup of ER-negative/PR-positive tumours have been reported to represent <1%–3% of all breast cancers.[3,99,107] However, some claim that ER-negative/PR-positive tumours represent technical artefacts,[127,128] while others are convinced that this subgroup really exists.[129-131] In addition, studies that use gene expression analysis to study this subgroup have shown contradictory results.[110,132,133] Consequently, as the existence of this subgroup is controversial, tumours found to be ER-negative/PR-positive should be retested for ER and PR.

Tumours that express both ER and PR are often associated with better outcome, and PR is therefore considered a strong prognostic marker in breast cancer.[129,134,135] Historically, PR expression has been thought to indicate a functional ER pathway, and conversely, ER-positive/PR-negative tumours have been considered to have a non-functional ER pathway and therefore respond less to tamoxifen.[136] However, according to preclinical data, progesterone activation of PR modulates where ER binds to DNA, resulting in decreased activity of ER-mediated gene expression and reduced proliferation.[120] Several studies have demonstrated that PR expression is predictive for benefit of adjuvant tamoxifen.[137-139] However, in the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis from 2011, the efficacy of adjuvant tamoxifen in ER-positive patients was found to be independent of PR status.[112] Perhaps PR does not add value for tamoxifen efficacy, but these results may also be explained by the fact that approximately 50% of the patients included in the EBCTCG overview also received adjuvant chemotherapy and that the poorer outcome in patients with PR-negative tumours may have been counteracted by the positive effects of chemotherapy.[140] Moreover, the methodological problems associated with the early PR assays have also been suggested to partly explain these findings.[141,142] Consequently, there is a need for a better understanding of the role and function of PR in cancer cells as well as in normal cells.

Proliferation/Ki67

Sustained proliferative signalling is a hallmark of cancer, and proliferation is an important feature in invasive breast cancer.[37,143] There are several methods to assess proliferation in breast cancer, including thymidine labelling index, flow cytometric S-phase fraction, mitotic activity index, and assessment of proliferation-associated proteins, such as cyclin A and Ki67.[143-145] Increased proliferation strongly correlates with a poor prognosis in primary breast cancer, irrespective of the method used for evaluation.[143,146-151]

Ki67 is a protein expressed in the nuclei of proliferating cells and Ki67 can be detected during all phases of the cell cycle, except for G0.[152] Therefore, the proportion of Ki67-positive cells well reflects the proliferative status of the tumour. The Ki67 labelling index is defined as the percentage of Ki67-positively stained tumour cell nuclei in a specific area of the tumour.[153] Denckert et al. reviewed the predictive role of Ki67, and although most studies have shown an association between high Ki67 and response to neoadjuvant chemotherapy, no correlation has been established between high Ki67 and benefit from adjuvant chemotherapy.[154] Several studies have shown that a decline in Ki67 following neoadjuvant endocrine therapy may be translated into long-term outcome.[155,156] Ki67 has been increasingly used over the last decade and it is included in the St. Gallen surrogate classification for the intrinsic subtypes.[157] A Ki67 value of ≥20% has been proposed to define tumours with high Ki67.[134,157] However, a meta-analysis that included 64,196 patients from 41 studies concluded that Ki67 was prognostic for both distant disease-free survival (DFS) and overall survival (OS), even though the cut-off to define high Ki67 differed largely between studies (range: 10%–≥25%).[158] Ki67 is a continuous variable, and irrespective of which value represents the optimal cut-off, there are uncertainties about using Ki67 as a dichotomised variable to define high and low expression, especially as a great proportion of cases are located near the cut-off.[159,160] In the consensus document from the St. Gallen International Breast Cancer Conference in 2017, Ki67 is referred to as clearly high, clearly low, or intermediate.[105]

Despite the strong prognostic value of Ki67, there are concerns regarding the reproducibility of this marker.[153,161] If Ki67 is to be used for treatment decisions, it is important to use a standardised scoring method for Ki67 to ensure high interlaboratory reproducibility.[103,105] One working group (The International Ki67 in Breast Cancer Working Group) is aiming to homogenise Ki67 analysis and increase the scoring concordance for Ki67.[153,161,162] Notably, the recently published American Society of Clinical Oncology (ASCO) guidelines regarding breast cancer biomarkers advised against the use of Ki67 to guide decisions on whether a patient should be recommended adjuvant chemotherapy.[104]

Human epidermal growth factor receptor 2 (HER2)

HER2 is a receptor protein encoded by the gene *ERBB2*, which is located at chromosome 17 (17q12).[163] Amplification of this proto-oncogene and/or overexpression of the HER2 receptor is found in approximately 15% of primary breast cancers and is associated with aggressive tumour characteristics and a worse prognosis, at least in the absence of targeted treatment.[164-167] In addition, HER2-positivity is predictive for efficacy of anti-HER2 treatment. HER2 belongs to the HER family which also includes HER1/EGFR, HER3, and HER4 (Figure 5). Notably, no ligands have been identified for HER2.[168] HER2 can homodimerise with another HER2 or heterodimerise with any of the other three receptors, but HER2 is the preferred partner.[169] The HER2/HER3 heterodimer is considered the most potent combination to drive tumour progression.[170] HER3 lacks the intracellular active tyrosine kinase domain and thus is dependent on interaction with HER2 for signalling.[168] The dimerisation of receptors results in phosphorylation of the cytoplasmic domain, which initiates a variety of signals through the MAPK and PI3K/AKT pathways.[171] Activated HER2 signalling promotes cell proliferation and opposes apoptosis, thereby stimulating tumour growth (Figure 5).

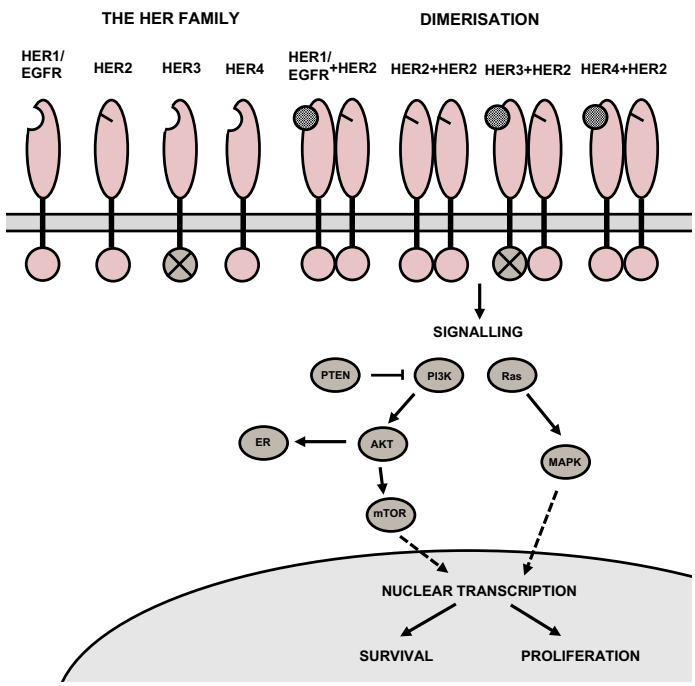


Figure 5. Illustration of the HER family, different forms of dimerisation, and an overview of HER2 signalling pathways. Ligands for HER1, HER3, and HER4 have been identified (notched receptors), while no ligands have been reported for HER2 (filled receptor). HER3 lacks the intracellular active tyrosine kinase domain (grey circle). HER, human epidermal growth factor receptor; HG, EGFR, epidermal growth factor receptor.

Additional prognostic markers

Urokinase plasminogen activator and plasminogen activator inhibitor type-1

Urokinase plasminogen activator (uPA) is an enzyme that is involved in cancer invasion and metastasis by its conversion of plasminogen to plasmin, resulting in the initiation of a proteolytic cascade.[172] High levels of uPA and its inhibitor plasminogen activator inhibitor type-1 (PAI-1) are clearly associated with poor prognosis in primary breast cancer and high levels of these markers are predictive for benefit of adjuvant chemotherapy.[173-176] Today these markers are not widely used nor included in routine breast cancer pathology. Analysis of uPA and PAI-1 include enzyme-linked immunosorbent assay (ELISA), which require rather large amounts fresh or fresh frozen tumour tissue.[173] However, Lang et al. have presented promising data showing good correlation between IHC and ELISA determination of uPA and PAI-1.[177]

Vascular invasion

Vascular invasion (VI) is a marker of metastatic potential, and the presence of VI has been shown to be an independent negative prognostic factor in primary breast cancer.[178,179] Although the prognostic role for VI has been known for more than four decades, VI does not have a distinct role in the clinic. This may partly be explained by the difficulties associated with pathological diagnosis of VI.[180,181]

Circulating tumour cells and circulation DNA

During recent years, there has been an increased interest in the use of liquid biopsies. The occurrence of circulating tumour cells (CTCs) has been shown to be associated with worse prognosis in primary breast cancer.[182] Circulating tumour DNA (ctDNA) is fragmented DNA released from the tumour or from CTCs following apoptosis, and detection of ctDNA has been shown to clearly correlate with an increased risk of distant recurrence.[183] Analysis of CTCs and/or ctDNA is currently included as a translational endpoint in many breast cancer trials, but is currently not recommended in clinical routine.[104]

Prognostic gene signatures

There are several prognostic assays based on gene expression, and the most commonly used assays are further described in the chapter 'Breast cancer subtypes'.

Time-dependence of different prognostic markers

Hilsenbeck and colleagues demonstrated that the HR for most of the prognostic factors in breast cancer vary over time.[114] For example, ER-positivity was shown to be associated with a good prognosis during the first years after diagnosis, with a HR <1.0.

After 3 years, however, the HR shifted and rose >1.0, indicating a poorer prognosis with longer follow-up. Time-dependent variation of the prognostic value for some of the markers described above was also studied in a meta-analysis including 12 studies and more than 10,000 patients with long-term follow-up.[118] Table 1 lists some of the data from the meta-analysis.

Table 1. Multivariate period-specific all-cause mortality hazard ratios (95% CI). Modified table reprinted with permission from PLOS Medicine, © 2010.[118]

	Year after diagnosis				
	0–2	**2–4**	**4–6**	**6–10**	**10–15**
Age, years					
<40	0.69 (0.49–0.98)	1.09 (0.87–1.37)	1.14 (0.84–1.55)	0.83 (0.62–1.12)	0.68 (0.44–1.05)
40–49	0.63 (0.48–0.84)	0.77 (0.64–0.93)	0.83 (0.64–1.06)	0.66 (0.53–0.82)	0.51 (0.38–0.68)
50–59	1.00	1.00	1.00	1.00	1.00
≥60	1.74 (1.36–2.22)	1.26 (1.04–1.52)	1.64 (1.31–2.06)	1.79 (1.49–2.14)	2.05 (1.63–2.58)
Node-positive	2.64 (2.12–3.27)	2.42 (2.09–2.82)	1.86 (1.55–2.23)	1.56 (1.35–1.82)	1.40 (1.15–1.70)
ER-positive	0.55 (0.42–0.71)	0.76 (0.63–0.91)	1.31 (1.02–1.68)	1.63 (1.29–2.07)	1.24 (0.91–1.69)
PR-positive	0.36 (0.27–0.47)	0.62 (0.52–0.74)	0.74 (0.60–0.91)	1.04 (0.87–1.23)	1.16 (0.92–1.37)
HER2-positive	1.21 (0.95–1.52)	1.50 (1.27–1.78)	1.55 (1.23–1.96)	1.35 (1.07–1.69)	0.96 (0.67–1.37)

Abbreviations: CI, confidence interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

Methods for analysis of ER, PR, Ki67, and HER2

Cytosol-based ligand-binding methods

The first methods for analysis of hormone receptors in breast cancer included biochemical ligand binding assays (LBAs), in which the amount of receptor was measured by its binding to a radiolabelled ligand.[184] The methods used for quantification were sucrose density gradient centrifugation (SDG), dextran-coated charcoal (DCC) assay with Scatchard analysis, and isoelectric focusing (IF).[185,186] In the 1980s, the enzyme immunoassay (EIA) method became increasingly used; this cytosol-based method used monoclonal antibodies instead of radiolabelled ligands.[187] The LBAs and EIAs were not always standardised, and at the time these methods were used, it was not mandatory to participate in quality assurance (QA) programs.[142] LBAs are technically challenging and have some major drawbacks. First, endogenous hormones or anti-oestrogens like tamoxifen may result in occupancy of receptors, which may render false negative or falsely low receptor values.[188,189] Second, LBAs require a large amount of fresh frozen tissue, which may not only be composed of tumour tissue but also of normal cells and stroma.[106] Moreover, it is not possible to address the heterogeneity of hormone receptor expression in the tumour.[189]

Immunohistochemistry

In the early 1990s, immunohistochemistry (IHC) began to replace LBAs, and a decade later IHC was approved for routine clinical use by College of American Pathologists (CAP) and ASCO.[190,191] IHC-based techniques can measure both unoccupied and occupied receptors and only require small amounts of formalin-fixed paraffin-embedded (FFPE) tissue. Microscopic visualization can ensure that assessment is restricted to invasive tumour tissue and reveal heterogeneity.[185,192] IHC is currently used for the assessment of ER, PR, Ki67, and HER2, and there are guidelines for assessment to ensure reproducible and reliable results.[102,153,193] IHC includes the use of monoclonal antibodies (mABs) for detection/staining of the biomarker of interest. mABs are a population of identical antibodies that all recognize the same specific epitope of an antigen. mABs are commonly produced in mice but may also be produced in rabbits or other species. Rabbits generally produce high affinity antibodies, and therefore rabbit mABs (RabMAbs) tend to have higher sensitivity, but without loss of sensitivity, compared with the corresponding mouse mABs.[194,195]

Principles of IHC

The final result of a biomarker analysed by IHC depends on a multistep process, including a pre-analytical phase, an analytical phase, and an interpretative phase. This process is described in The IHC Guidebook, published by DAKO,[196] and is briefly summarised below:

Pre-analytical phase

1. Samples from the tumour tissue may be taken with a core biopsy, a small biopsy, or by resection of the whole tumour with adjacent tissue.
2. To stop degradation and preserve the structure of the tissue, the tissue should be fixed as soon as possible. If the amount of tissue is large, it should be sliced to ensure penetration of the fixative. The preferred fixative is neutral buffered formalin and fixation for 24–72 hours results in crosslinks of proteins.
3. After fixation, the tumour is dehydrated through ethanol and then embedded in paraffin to enable sectioning and long-term storage.
4. The formalin-fixed, paraffin-embedded tissue is sectioned into 4–5-μm-thick slices and mounted onto glass slides.

Analytical phase

5. To perform analyses, the paraffin must be removed. Dewaxing is conducted by immersing the sections into a dewaxing solution such as xylene.
6. During fixation, proteins may undergo conformal changes that may mask their epitope. To unmask these unique epitopes, heat or enzymatic degradation is used in a process referred to as 'antigen retrieval'.
7. The sample is then incubated with the primary antibody, which binds to the specific antigen in the tumour sample. To ensure correct staining with high specificity, positive and negative controls should be included.
8. A detection system, which for example may include a secondary antibody and a colour system, is used to visualize the antigen/antibody complex.

Interpretative phase

9. The staining pattern is then assessed using the biomarker-specific guidelines for interpretation.

In situ hybridisation

In situ hybridisation (ISH) is used to evaluate gene amplifications, deletions, translocations, and chromosomal copy number changes in cells.[196] In fluorescent ISH (FISH), locus-specific DNA probes are used and the probes are visualized using a fluorescence microscope.[197] There are also ISH methods with probes using other substances for visualization, such as silver (silver-enhanced ISH, SISH) and chromogen (chromogenic ISH, CISH). Both of these ISH methods have the advantage that a light microscope can be used for visualization and that specimens can be archived for re-assessment.[198]

Analysis of breast cancer biomarkers

Analytical validity corresponds to how accurately an assay detects a biomarker, whereas clinical validity refers to how well the assay can predict the clinical outcome. In the end, the most important is clinical utility, i.e. that the assay provides information that makes it possible to improve the management of the disease.[104]

ER and PR

ER and PR status is determined by IHC, and there are guidelines that define the pre-analytical, analytical, and interpretative routines to secure the analytical validity.[102] The clinically relevant cut-off for ER-positivity was defined by LBAs, based on the response to endocrine therapy.[199] When IHC replaced the LBAs, there were few clinical trials that directly validated the results against benefit from adjuvant therapy.[200] Instead, most studies compared the two methods and it was assumed that a good correlation was enough to secure validity for the IHC cut-off.[102] Immunohistochemical assessment of ER and PR includes determination of the percentage of positively stained nuclei in the tumour. According to international guidelines, the cut-off for ER/PR-positivity is defined as ≥1% positive cells, whereas >10% is used in the Swedish guidelines.[101,102] The reason behind the lower cut-off is to avoid withholding endocrine treatment from patients that might benefit from this therapy; however, doctors should discuss the pros and cons of medication with patients with weakly positive breast tumours.[102] To better classify the weakly positive tumours, other methods are available that also evaluate the intensity of the staining, such as the Allred score and the H-score.[106,201,202]

Ki67

The Ki67 labelling index is the proportion of Ki67-positive tumour nuclei within a specific area of the tumour.[153] Several antibodies are available for the immunohistochemical staining of Ki67, and MIB1, a mouse mAB, is the most validated and commonly used.[153,203] There is no clear consensus regarding the assessment of Ki67, but according to The International Ki67 in Breast Cancer Working

Group, the invasive edge of the tumour should be scored and at least 500 malignant cells (preferably 1,000 cells) should be counted.[153] In the St. Gallen International Breast Cancer Conference consensus statement from 2013, ≥20% was proposed as cut-off to define high Ki67, whereas the use of laboratory-specific cut-off was discussed in 2015.[157,204] In the St.Gallen consensus statement from 2017, however, Ki67 is only referred to as clearly high, intermediate, or low.[105] Despite the strong prognostic value of Ki67, the reproducibility between different laboratories has been shown to be unsatisfactory as a result of differences in the pre-analytical or analytical practices.[153,161] Deficient reproducibility between assessors has also been reported, indicating issues regarding differences in the interpretative part of the analysis.[205,206]

HER2

The diagnostic assays available for assigning HER2 status are IHC and ISH (see Figure 6). IHC uses mABs to quantify the amount of HER2 protein on the surface of the tumour cells. Assessment of IHC results in a score (0, 1+, 2+, or 3+) based on the proportion of stained cells in combination with the intensity of the staining. If the score is 0 to 1+, the tumour is considered HER2-negative, and if the score is 3+ it is defined as HER2-positive. Tumours with a score of 2+ are considered equivocal and thereby require additional testing using ISH. In FISH, two locus-specific DNA probes are used: one for *ERBB2* and the other for the centromere of chromosome 17 (CEP17).[197] Some tumour cells may have an increased number of chromosome 17, with or without concurrent amplification of the *ERBB2* gene, and this is referred to as chromosome 17 polysomy.[207] ISH quantifies copy number changes of the *ERBB2* gene, and tumours are considered HER2-positive if the HER2/CEP17 ratio ≥2.0 or if the tumours have an average *HER2* gene copy number ≥6 signals per cell (i.e. amplification).[193,208] However, we can expect updated guidelines for the assessment of HER2 during 2018.

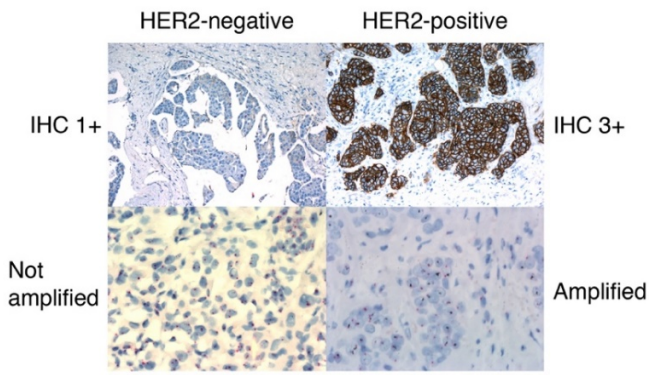


Figure 6. HER2 assessment by IHC (top row) and SISH (bottom row) in breast cancer tumour tissue. Pictures obtained from the Department of Pathology, Ryhov County Hospital, Sweden. HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; SISH, silver-enhanced in situ hybridisation.

Digital image analysis

To improve the interobserver and interlaboratory reproducibility of breast cancer biomarkers, especially for Ki67, several research groups have investigated the possibility of using digital image analysis for assessment of these biomarkers.[209-211] However, despite the benefit of image analysis, there are still controversies regarding the implementation of these analyses in clinical routine.[102,212-214]

Quality assurance of breast cancer biomarkers

As biomarkers form the basis for estimation of prognosis and treatment recommendations in breast cancer, it is of utmost importance that the results from these analyses are reliable and maintain good quality. The results from tumour assessments should be identical irrespective of in which laboratory the analyses are performed. Apart from following established guidelines for each biomarker, laboratories have to work with QA. All methods and technical equipment have to be continuously evaluated and it is mandatory for laboratories to participate in external QA programs.[196] There are several organisations that perform external proficiency testing, such as Nordic Immunohistochemical Quality Control (NordiQC), United Kingdom National External Quality Assessment Scheme for Immunocytochemistry (UK NEQAS), and CAP,[215-217] as well as smaller national initiatives. External QA often entails that tissue samples are sent out to several laboratories for staining according to the local routine procedures and that the laboratories then return their results and/or glass slides for comparison. Participation in external QA programs provides independent, objective, and impartial feedback on the laboratory's performance, enabling them to identify weaknesses and take appropriate action.

Tissue microarray

A tissue microarray (TMA) is a paraffin block in which multiple tissue cores are assembled in an array fashion. The technique to insert several tissue samples in one block was first introduced in 1986 and is now frequently used.[218] An advantage of TMAs is that it allows several tumour samples to be assessed by IHC or ISH on the same section, which is time-sparing and cost-effective, as less amounts of antibodies and reagents are needed. Moreover, more tissue from the original block can be conserved and the staining conditions will be similar for all cores.[219] The major disadvantage is that only a small portion of the tumour is included in the TMA, and in case of heterogeneity, this may render false results.[196,220] TMA has been validated to have an accurate agreement with whole sections for ER, PR, HER2, and Ki67.[221-223] The construction of a TMA is illustrated in Figure 7.

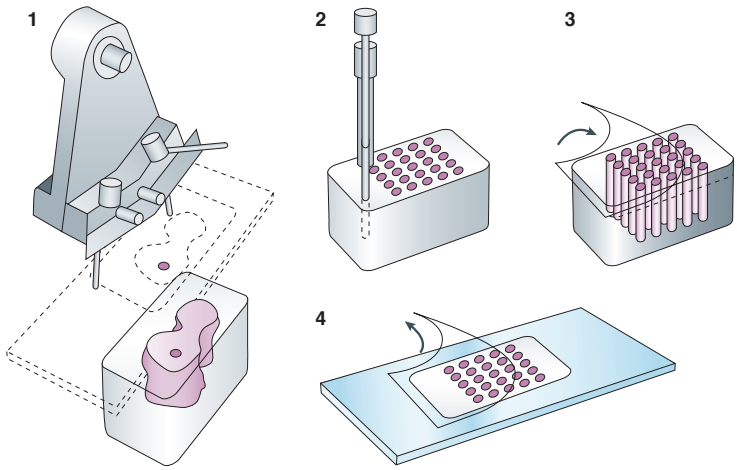


Figure 7. Schematic illustration of tissue microarray (TMA) construction. Part of the original figure is reprinted with permission from Macmillan Publishers Ltd: Nat Rev Drug Discov, © 2003.[224]

Breast cancer subtypes

Molecular intrinsic subtypes

DNA is the genetic material for organisms and it consists of a double-stranded molecule composed of nucleotides. DNA contains genes that are transcribed into mRNAs, which are subsequently translated into proteins.[225] The first reports that classified breast cancer into molecular intrinsic subtypes based upon patterns of gene expression were published almost two decades ago.[226,227] There are four major intrinsic molecular subtypes in breast cancer: luminal A, luminal B, HER2-enriched, and basal-like.[226] However, through next generation sequencing, a much higher number of subtypes with distinct features have been revealed.[228] The intrinsic subtypes have been repeatedly shown to be independent predictors of prognosis in breast cancer.[229-231] The characteristics of the main subtypes are briefly described below.

Luminal A

The luminal A subtype has an expression profile similar to that found in the luminal breast epithelium of normal cells, corresponding to high expression of ER-related genes and low expression of genes related to proliferation as well as HER2.[226,227] These tumours are sensitive to endocrine manipulation, but are less sensitive to chemotherapy.[232,233] Luminal A tumours represent approximately 30%–50% of all tumours, and this subtype is associated with a favourable prognosis.[150,234-238]

Luminal B

Like in luminal A, the luminal B subtype has high expression of *ESR1*.[134] Luminal B tumours, however, have low expression of other luminal genes and a relatively high expression of genes related to proliferation, whereas the expression of HER2-related genes may vary.[239] These tumours also have a higher number of mutations and chromosomal copy number changes across the genome compared with luminal A.[240] Luminal B represents approximately 20% of breast cancer cases and is associated with a relatively high risk of relapse.[234,235,237,238]

HER2-enriched

HER2-enriched breast tumours exhibit high expression of genes associated with HER2 and proliferation, whereas they tend to have an intermediate expression of luminal genes (e.g. *ESR1* and *PGR*).[239] HER2-enriched tumours represent approximately 15% of all breast cancers and notably, these tumours are not always HER2-positive by IHC/ISH and.[234-236] In the absence of anti-HER2 therapy, these tumours are associated with a poor prognosis.[237,238]

Basal-like

Basal-like tumours are characterised by low expression of genes related to ER and HER2 and high expression of proliferation-related genes, and these tumours have high genomic instability.[241,242] Basal-like tumours are associated with high HG and are frequently triple negative (ER-/PR-/HER2-), CK5/6-positive, and/or EGFR-positive by IHC.[243] However, basal-like and triple negative tumours are not synonymous, and only approximately 70% of triple negative tumours are basal-like according to gene expression.[242] The basal-like subtype represents approximately 10%–20% of all breast tumours and is associated with a poor prognosis.[234-238,241]

Two additional subtypes have also been described.

Normal-like

Normal-like tumours often share the same biomarker expressions as the luminal A subtype, i.e. ER-positive and/or PR-positive, HER2-negative, and low Ki67. Genetically, their expression pattern resembles that of normal breast cells and the prognosis has been described as slightly worse than that of luminal A.[244] However, some studies have suggested that this subtype is an artefact from the presence of normal breast cells within the tumour sample.[245]

Claudin-low

Like basal-like tumours, the claudin-low subtype shows high genomic instability. These tumours are low differentiated and approximately 50% are triple negative.[236] Because there is no clinical indication to define claudin-low as a separate subgroup, most of these tumours are classified as basal-like.[245]

The distribution of the subtypes differs across different age categories, and whereas luminal tumours are common in all age groups, the basal-like/triple negative and HER2-enriched/HER2-positive subtypes are proportionally more common in younger women compared with older women.[58,234]

Clinicopathological surrogate definitions for the intrinsic subtypes

Several studies have attempted to establish a clinicopathological surrogate definition for the intrinsic subtypes using the routine pathological markers: ER, PR, HER2, and Ki67.[134,150,246] A surrogate definition were also included in the St. Gallen International Breast Cancer Conference consensus statement from 2013 using Ki67 (high/low) and PR (high/low) to separate luminal A-like tumours from luminal B-like tumours (Table 2).[157]

Table 2. Definition of the clinicopathological surrogate definition of the intrinsic breast cancer subtypes according to St. Gallen 2013.[157]

Intrinsic subtype	Clinicopathological surrogate definition	Characteristics
Luminal A	Luminal A-like	ER-positive and high PR HER2-negative Low Ki67
Luminal B	Luminal B-like/HER2-negative	ER-positive HER2-negative At least one of the following: high Ki67 negative or low PR
	Luminal B-like/HER2-positive	ER-positive HER2-positive Any Ki67 Any PR
ErbB2 overexpression	HER2-positive/non-luminal	ER-negative and PR-negative HER2-positive
Basal-like	Triple negative	ER-negative and PR-negative HER2-negative

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
*No role for genomic assays in pathological low risk cases (pT1a-b), pN0, ER high, and grade 1).

In the St. Gallen consensus statement from 2017, the surrogate definition to distinguish between the two luminal subtypes is more imprecise: Luminal A-like tumours have high ER/PR and clearly low Ki67 or low grade, whereas luminal B-like tumours have lower ER/PR, clearly high Ki67, and high grade. There is no definition for the intermediate group and the use of molecular assays is highlighted (Table 3).[105]

Table 3. Definition of the breast cancer subtypes according to St. Gallen consensus statement 2017.[105]

Clinical grouping	Immunohistochemistry	Genomic assay
Triple negative	Negative ER, PR, and HER2	-
Hormone receptor-negative and HER2-positive	ER- and PR-negative HER-positive	-
Hormone receptor-positive and HER2-positive	ER- and/or PR-positive ≥1% HER-positive	-
Hormone receptor-positive and HER2-negative	ER- and/or PR-positive ≥1%	*
Luminal A-like	High ER/PR, clearly low Ki67, or grade 1	'Good' according to genomic assay if available
Intermediate	Uncertancies persist about risk and degree of responsivness to endocrine and cytotoxic therapies	'Intermediate' according to genomic assay if available
Luminal B-like	Lower ER/PR, clearly high Ki67, or grade 3	'Bad' according to genomic assay if available

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
*No role for genomic assays in pathological low risk cases (pT1a-b), pN0, ER high, and grade 1).

Swedish guidelines for subtype classification

In 2014, Maisonneuve and colleagues proposed a new clinicopathological surrogate definition for luminal breast cancers.[247] The study included 9,415 patients with ER-positive/HER-negative tumours and the authors divided Ki67 into three categories; <14% (low), 14%–19% (intermediate), and ≥20% (high). With a median follow-up of 8.1 years, the authors showed that low Ki67 was associated with a good prognosis and high Ki67 was associated with a poor prognosis, irrespective of PR expression. However, in the group with intermediate Ki67, patients with tumours expressing ≥20% PR had a significantly better outcome than patients with PR-negative/PR-low tumours. Moreover, the authors showed that HG3 tumours generally were associated with a poor prognosis, whereas HG1 tumours were associated with a good prognosis. This has also been shown by Ehinger and colleagues.[248] The updated Swedish guidelines for surrogate classification of the intrinsic subtypes, developed by our group, are based on the principles described by Maisonneuve, with the addition of grade.[247,249] A tumour classified as luminal A-like should not be high grade, and vice versa, a tumour classified as luminal B-like should not be low grade. If so, the tumour should be re-assessed by the pathologist. If the grade remains, the tumour should be re-classified according to HG (Figure 8).

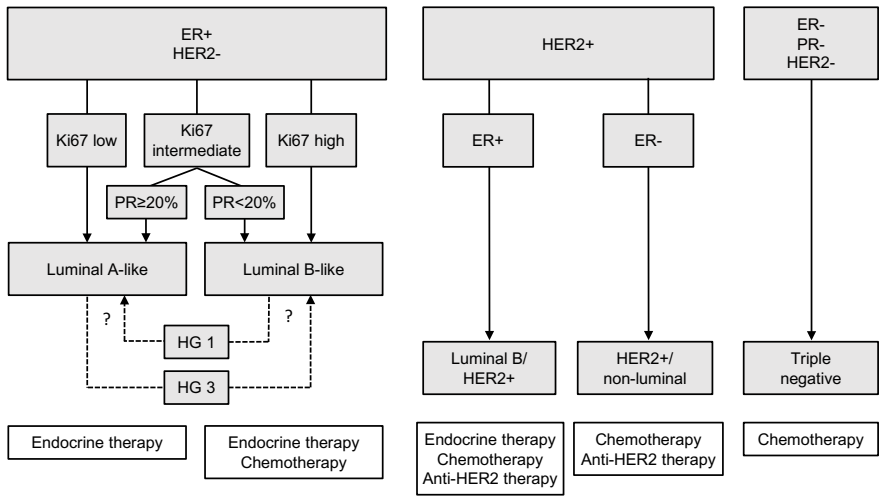


Figure 8. Surrogate subtype classification for the intrinsic subtypes according the Swedish guidelines 2018.[249] ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HG, histological grade; PR, progesterone receptor.

Commercial prognostic multi-gene assays

Following the gene expression era, a wide range of prognostic multi-gene assays have been developed that can be used to identify patients for whom chemotherapy can be omitted, based on the estimated the risk of recurrence. In these tests, which are performed on tumour tissue, the expressions of a selected number of genes are compared with the expressions in normal cells using different kinds of gene expression techniques. Common for all assays are that they were originally developed using a retrospective cohort of patients with clinical outcome data and that they were later retrospectively and prospectively validated in other cohorts. A short description of three of the most common tests is included below.

Oncotype DX

Oncotype DX involves measurement of the expression of 16 prognostic/predictive genes and five reference genes using FFPE tumour tissue and qRT-PCR. Recurrence score (RS), which is based on an algorithm that includes the mRNA expression of the 16 genes, estimates the 10-year risk of distant recurrence in patients with node-negative ER-positive/HER2-negative tumours treated with endocrine therapy on a scale of 0 to 100: <18 indicates low risk, 18–31 indicates intermediate risk, and >31 indicates high risk of recurrence.[250] Several retrospective studies have shown that Oncotype DX is prognostic also for patients with ≤3 positive lymph nodes.[251-253] Oncotype DX is currently prospectively evaluated in the TAILORx trial, which includes node-negative patients (ClinicalTrials.gov identifier: NCT00310180):

RS <11 (16%): received endocrine therapy

RS >25: received chemotherapy and endocrine therapy

RS 11–25: randomly assigned to receive endocrine therapy +/- chemotherapy

The first report from this study showed that the 3-year DFS was excellent (98%) in the group with low RS.[254] Notably, the values that define the low, intermediate, and high risk groups in TAILORx differ from the values described for RS when used in clinical routine. The main purpose of TAILORx is to investigate whether patients with an intermediate RS benefit from adding chemotherapy to the endocrine treatment. RxSPONDER is another prospective trial that included patients with 1–3 positive lymph nodes and a RS ≤25 who were randomised to endocrine therapy +/- chemotherapy (ClinicalTrials.gov identifier: NCT00310180).

MammaPrint

MammaPrint was developed based on tumours from patients with early breast cancer and ≤3 positive lymph nodes, regardless of hormone receptor expression and HER2 status. MammaPrint is a DNA-microarray assay that classifies patients to have a 'good prognosis' or 'poor prognosis' based on the expression of 70 genes.[255-257] MammaPrint has also been prospectively evaluated in the MINDACT trial, in which each patient's risk was assessed by both clinical risk (using an older version of Adjuvant online) and genomic risk (using MammaPrint). Patients with discrepant results between these two estimations were randomised to receive chemotherapy or not. At 5 years, the survival without distant recurrence rate for patients with high clinical risk and low genomic risk was 94.7% (95% CI, 92.5–96.2). Patients with high risk according to genomic risk but low risk based on the clinical risk were reported to have a similar outcome, but more patients could be spared chemotherapy when using MammaPrint.[258]

PAM50/risk of recurrence score/Prosigna

PAM50 was developed to classify tumours according to the intrinsic subtypes (i.e. luminal A, luminal B, HER2 enriched, and basal-like) and is based on the expression of 50 different genes.[229] The risk of recurrence score (ROR)/Prosigna includes an algorithm that includes the intrinsic subtype, a proliferation score, and tumour size. The patient receives a value between 0 and 100 and is assigned to either low, intermediate, or high risk of recurrence.[229] Several studies have validated PAM50/ROR to be useful in patients (N0 and N+) treated with endocrine therapy.[230,253,259] To date, PAM50/ROR has not been validated in any prospective randomised trial.

Additional assays

Additional assays are available, such as the Breast Cancer Index and Endopredict;[260,261] however these are not further described in this thesis.

Concordance

MammaPrint vs. *IHC*

There may be discrepancies between the subtype provided by molecular assays and the one derived based on traditional immunohistochemical markers. Viale et al. published a paper based on the MINDACT trial, demonstrating that more patients were classified as luminal A-like tumours by molecular subtyping compared with the pathological subtyping using the surrogate classification from St. Gallen 2013 (63% vs. 47%, respectively).[262] In this study, the molecular subtyping re-stratified 54% of the patients assessed as luminal B-like according to the pathological subtyping to the luminal A-like subtype, with a comparable outcome at 5 years.[262] However, DFS at 5 years is not an optimal endpoint for this subgroup, and longer follow-up is needed to clearly evaluate this comparison.

PAM50 vs. *IHC*

Bastien and colleagues analysed 814 tumours with PAM50 and IHC/ISH and found that only 77% of the ER-positive/HER2-positive tumours were classified as HER2-enriched. Within the triple negative subgroup, 57% were classified as basal-like and 30% as HER2-enriched.[245] Consequently, this may lead to uncertainties regarding as to which treatment the patient most likely will benefit from.

Comparison between different genomic assays

Prat et al. compared six different genomic signatures (Oncotype DX, MammaPrint, and PAM50-ROR, among others) in cohorts of women with ER-positive tumours treated with tamoxifen. When including two signatures at a time in the multivariate analysis, most assays were shown to provide independent prognostic information.[263] Another study by Iwamoto and colleagues compared six genomic signatures in the same cohort of patients and the authors reported poor to good agreement (kappa 0.24–0.70) for pairwise agreement.[264] Moreover, 30% of the patients assigned as high risk according to MammaPrint were classified as low risk by Oncotype DX.[264] In a recently published paper from Bösl and colleagues, tumours were assessed with MammaPrint and Endopredict and the overall concordance was only 66%, which resulted in different treatment recommendations for 38% of the patients.[265]

In conclusion, it is important to be aware of the fact that different genomic assays provide different information, that their correlation is modest, and that patient risk predictions may vary between different assays. Consequently, these assays are not to be considered interchangeable, and the choice of assay should be based on available evidence to assure its clinical validation and utility.[104]

Endocrine therapy in primary breast cancer

Neoadjuvant and adjuvant treatment

Although most patients present with localised breast cancer and may be cured with local therapy, distant recurrences are common and often result in death from the disease. To decrease the risk of recurrence, patients may be recommended neoadjuvant and/or adjuvant therapy, based on prognostic and predictive factors as well as co-morbidity.

Neoadjuvant therapy

Neoadjuvant therapy refers to the treatment given prior to surgery for the primary tumour (i.e. preoperative therapy). Neoadjuvant therapy is increasingly used in breast cancer and aims to downstage the tumour, allowing less extensive surgery, but also aims to eliminate micro-metastatic disease and thereby reduce the risk of recurrence and breast cancer-related death. This strategy enables early evaluation of the effect of the administered drugs *in vivo*. The neoadjuvant concept is increasingly used in breast cancer trials, as it provides good opportunities for translational research and has the advantage of rapid assessment of tumour response to different drugs. However, randomised trials have demonstrated similar DFS and OS irrespective of if the systemic therapy is delivered pre- or postoperative.[266,267] Pathologic complete response (pCR) after neoadjuvant therapy is most clearly correlated to long-term outcome in the triple negative and HER2-positive/non-luminal subtypes, whereas the correlation to prognosis is less evident in the other subgroups.[268]

Adjuvant therapy

Adjuvant therapy may be given in addition to surgery. The purpose is to eradicate cancer cells or micro-metastatic disease that may remain in the patient's body to reduce the risk of recurrence and breast cancer-related death. The benefit of adjuvant therapy was first reported in the 1970s.[269,270] Adjuvant therapies are often associated with side effects, potential risks, and costs. Therefore, recommendations regarding adjuvant therapy include an estimation of the individual patient's risk of recurrence and potential benefit from the different treatments available, based on prognostic and predictive markers, as described previously. Adjuvant therapy may include any of the following, alone or in combination: chemotherapy, anti-HER2 therapy, endocrine therapy, bisphosphonates, and radiotherapy.

Tamoxifen

Tamoxifen was first developed in the 1960s, but the intended use as a post-coital contraceptive failed.[271] In 1973, tamoxifen was instead approved for treatment of advanced breast cancer, and a decade later, adjuvant tamoxifen was shown to improve recurrence-free survival (RFS) in postmenopausal women with primary breast cancer.[271,272] Because the early EBCTCG overview failed to demonstrate benefit in younger women, this recommendation was not extended to include premenopausal women until the 1990s.[273,274] The first adjuvant trials included tamoxifen treatment for 1–2 years, but in the late 1990s, 5 years of treatment was shown to be superior to shorter treatment.[272,275-279] According to the EBCTCG overview from 2011, 5 years of tamoxifen reduced the recurrence rate (RR) by half during the treatment period and by one third in the subsequent 5 years, whereas no effect was seen for the period thereafter compared with no endocrine therapy. At 15 years, this corresponded to an absolute risk reduction of 13.2%. Breast cancer mortality was reduced by almost one third throughout the first 15 years with an absolute benefit of 9.2%.[112] The ATLAS trial, which investigated the benefit of extended tamoxifen treatment to 10 years, demonstrated an absolute reduction in breast cancer mortality of 2.8% at 15 years of follow-up.[280] The aTTom trial provided similar results.[281] The phenomenon in which the reduction of breast cancer events continues after discontinuation of treatment is often referred to as the 'carryover effect'. So far, ER is the only established predictive marker for the efficacy of tamoxifen treatment.[100,112] Several studies have demonstrated that PR adds predictive value for the efficacy of tamoxifen in patients with ER-positive tumours;[137-139,141] however, the EBCTCG meta-analysis from 2011 did not support this finding.[112]

Mechanisms of action

Tamoxifen is an oestrogen-like drug that inhibits oestrogen-stimulated growth in breast cancer by competitively binding to ER.[282] The binding of tamoxifen induces a conformal change of the receptor, which recruits different corepressors to the promoter regions of target genes and results in an inhibition of the oestrogen-dependent growth-signalling pathway.[98,283] Although tamoxifen acts as an antagonist in breast tissue, it has agonistic effects in tissues like bone and endometrium, resulting in increased bone mineralization and endometrial hyperplasia.[98] Tamoxifen belongs to a group of drugs called selective oestrogen-receptor modulators.[284]

Side effects

In addition to decreasing the risk of breast cancer recurrence, tamoxifen also reduces the long-term risk of contralateral breast cancer by 38%–50%.[285,286] Ongoing studies are currently investigating to what extent lower doses of tamoxifen may reduce the risk

of breast cancer in women with dense breasts.[287] Moreover, tamoxifen has been reported to reduce cardiovascular events as well as the risk of lung cancer.[288,289]

As tamoxifen can bind to ER in other tissues besides breast, it may also be associated with unwanted side effects. Commonly reported side effects include menopausal symptoms, such as hot flashes, vaginal dryness, mood swings, nausea, and low libido. Less common, but serious side effects include increased risk of endometrial cancer and venous thrombosis.[112] For some women, the side effects can negatively affect their quality of life, making it hard to adhere to the prescribed therapy. A study, based on the Swedish Prescribed Drug Register, reported that after 3 years, almost one third of the patients had discontinued their adjuvant treatment.[290]

Tamoxifen resistance

ER-positivity is a predictive marker for tamoxifen efficacy for most patients, but despite the positive effects observed in tamoxifen trials, a considerable number of patients suffer from relapse as a result of inadequate effects from this therapy.[100,112] The term 'de novo resistance' indicates that a tumour is irresponsive to endocrine treatment from the start, whereas acquired resistance is developed after an initial response to endocrine therapy.[291] Some tumours are completely insensitive to all kinds of ER-targeted therapy, whereas others may be resistant to specific endocrine drugs but respond to others, indicating that they are still ER-dependent.[292]

Loss or modification of ER expression

A proportion of patients whose primary tumours are ER-positive will have ER-negative recurrences.[293,294] ER expression may also be lost during tamoxifen treatment.[295] However, the majority of tumours with acquired tamoxifen resistance, for example as a result of acquisition of mutations in ER, remain to express ER but will be insensitive to anti-oestrogens.[296,297] In addition, epigenetic changes, such as CpG island hypermethylation, may inactivate *ESR1*.[298] Still, approximately 20% of the patients who relapse during tamoxifen will respond to subsequent treatment with an aromatase inhibitor (AI) or fulvestrant.[299,300] The duration of response tends to shorten along with a decline of ER expression, indicating a shift towards alternative pathways for disease progression.[291,301,302] Importantly, however, therapies acting on pathways that downregulate ER may be associated with a restoration of ER expression and recovery of ER sensitivity.[303,304] PR loss is even more frequent than loss of ER, resulting in a more aggressive disease with poorer outcome compared with tumours that remain positive for ER and PR after developing resistance to endocrine therapy.[123] Moreover, PR loss may be associated with upregulation of the PI3K pathway and subsequent downregulation of ER and PR expression, resulting in a tumour that is less dependent on oestrogen signalling.[125,126]

Alterations in co-regulatory proteins

When oestrogen binds to ER, co-activators are recruited and the transcription of target genes is enhanced. In contrast, tamoxifen recruits corepressors upon binding to ER, resulting in the repression of ER target genes.[305] Altered expression of different coregulators may impact the effects of tamoxifen.[306] In HER2-positive breast tumours, coactivator proteins may be recruited upon binding of tamoxifen to ER, resulting in opposing agonistic effects.[305]

Alterations in cell cycling signalling molecules

Several cellular factors are involved in regulation of the cell cycle. Upregulation of positive regulators, such as MYC and cyclin E1 and D1, or downregulation of negative regulators may promote proliferation and lead to endocrine resistance.[291,307]

Growth factor receptor pathways

Several cellular pathways can promote survival and proliferation despite the simultaneous inhibition of the ER pathway. ER-negative tumours frequently overexpress growth factor receptors such as EGFR or HER2.[308] Cross-talk may occur between any of the following receptor families: EGFR/HER, IGF receptor (IGFR), VEGF receptor (VEGFR), or FGF receptor (FGFR); this cross-talk can be increased by amplification or overexpression of the receptors, or by increased levels of their specific ligands.[291] Activation of the PI3K pathway can also be achieved by mutations in key genes such as *PIK3CA* or *PTEN*.[309]

Tumour microenvironment and host-associated mechanisms of resistance

During recent years, increasing evidence has shown that stromal cells, the extracellular matrix, growth factors, and cytokines, as well as hypoxia and acidity may be involved in the development of endocrine resistance.[291]

Pharmacological mechanisms

Cytochrome P450 2D6 (CYP2D6) is involved in the metabolism of tamoxifen to its most active metabolite endoxifen.[310] CYP2D6 activity may be inhibited, partly or completely, as a result of drug interactions or the presence of genetic variants (SNPs), resulting in low levels of endoxifen and consequently no or inadequate effects of tamoxifen.[311]

Aromatase inhibitors (AIs)

Mechanisms of action

AIs deprive oestrogen levels by inhibiting the aromatase enzyme, which converts androgens into oestrogens (aromatisation). AIs include non-steroidal inhibitors, such as letrozole and anastrozole, which bind to the aromatase enzyme via reversible competition, and irreversible steroidal inhibitors, such as exemestane, which form a permanent and de-activating bond with the aromatase enzyme.[312] The side effects associated with AI treatment are similar to those seen in patients treated with tamoxifen, but arthralgia is more frequent in AI users.[313] In premenopausal women, oestrogen is produced mainly in the ovaries and the production is regulated by hypothalamus and the pituitary gland through a feedback system including gonadotropin-releasing hormone (GnRH) and follicle-stimulating hormone (FSH).[314] A temporary inhibition of the oestrogen production increases the levels of GnRH and FSH and therefore AIs have to be combined with ovarian suppression by a GnRH-analogue, oophorectomy, or radiation, to safely lower oestrogen levels in premenopausal patients.[315,316] In postmenopausal women, oestrogens are produced by aromatization of androgens to oestrogens in peripheral tissue such as adipose tissue and skin.[314] The oestrogen synthesis pathway for pre- and postmenopausal women is illustrated in Figure 9.

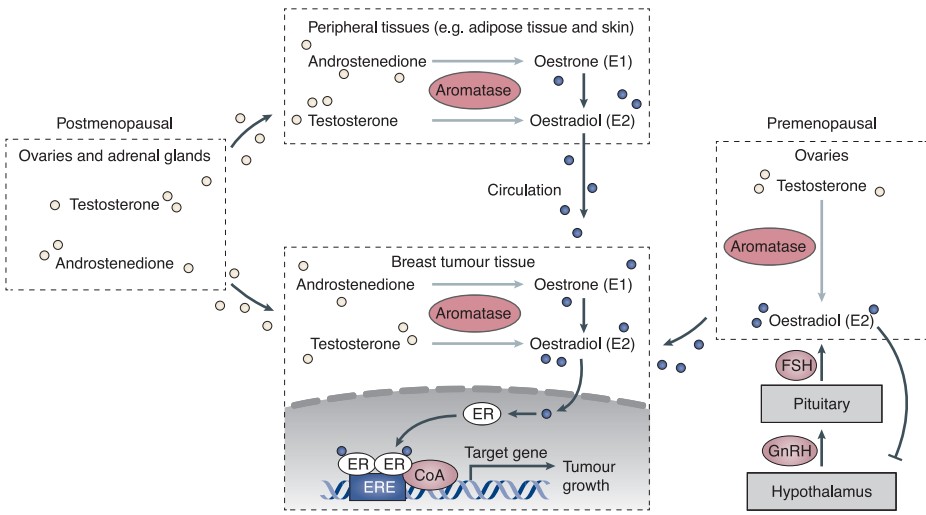


Figure 9. Oestrogen synthesis in pre- and postmenopausal women. Reprinted in adapted colours with permission from Macmillan Publishers Ltd: Nature Reviews Cancer, © 2015.[314]

Tamoxifen vs. *AI*

Over 8,000 patients were included in the BIG 1-98 trial and randomised to either tamoxifen for 5 years, an AI for years 5 years, tamoxifen for 2 years followed by an AI for 3 years, or an AI for 2 years followed by tamoxifen for 3 years. In comparing the monotherapy arms, after a median follow-up of 25.8 months, the 5-year DFS was significantly better for patients treated with an AI compared with tamoxifen (84.0% vs. 81.4%, respectively; HR=0.81, 95% CI, 0.70–0.93).[317] With a median follow-up of 8.7 years, a similar benefit was seen for DFS (HR=0.86, 95% CI, 0.78–0.96) and OS (HR=0.86, 95% CI, 0.76–0.98). When adjusting for cross-over, the benefit from AI treatment was increased. Sequential treatment did not improve outcome but was comparable to letrozol in monotherapy.[318] Long-term follow-up data, with a median follow-up time of 12 years, were presented as a poster at the San Antonio Breast Cancer Symposium in 2016 and showed no significant differences between any of the arms compared with 5 years of tamoxifen.[319] Similar results were reported for the monotherapy arms from the ATAC/LATTE study, in which no significant differences regarding breast cancer events and deaths were observed between the anastrozol and the tamoxifen arm after a median follow-up of 10 years.[320]

In 2015, EBCTCG published a meta-analysis comparing AI and tamoxifen that included nine studies and 31,920 postmenopausal patients with ER-positive breast cancer who received adjuvant endocrine therapy according to one of the following schemes: AI 5 years, tamoxifen 5 years, tamoxifen for 2–3 years followed by an AI up to 5 years, or AI for 2 years followed by tamoxifen for 3 years. AI was shown to reduce the risk of recurrence by 30% compared with tamoxifen during the treatment period, but no significant differences were seen after cessation of treatment. Breast cancer mortality was reduced both during and after the treatment period. Based on results from previous EBCTCG reports, the authors concluded that compared with no endocrine therapy, 5 years of an AI reduced the RR by two thirds during the treatment period and by about one third during years 5–9 (proportionally). The relative reduction of breast cancer mortality rate was estimated to be approximately 40% throughout the first 10 years.[321]

In conclusion, AI provides a benefit over tamoxifen regarding breast cancer events and breast cancer mortality during the treatment period and the subsequent years, but no differences are yet seen with longer follow-up.

AI resistance

The mechanisms of AI resistance are somewhat similar to those seen in tamoxifen resistance, such as loss of ER expression and cross-talk between growth factor receptor signalling and ER. However, there are some additional mechanisms worth highlighting:

ESR1 alterations

ERS1 mutations are very rare in primary breast cancer, whereas they occur in approximately 20% of metastatic tumours, especially in tumours that have progressed during AI treatment.[322,323] AI resistance may also result from amplification or translocation as well as epigenetic silencing of *ESR1*.[298,324]

Activated PI3K pathway

The PI3K-AKT-mTOR pathway is often altered in breast cancer.[325] Many studies are currently investigating PI3K pathway-targeting agents and potential predictive markers for such treatment.[314]

Induction of stem cell-like features

Breast cancer stem cells often show no or low ER expression and increased PI3K pathway signalling, and acquired AI resistance may be a result of tumour cells having evolved cancer stem cell-like properties.[314]

Extended adjuvant endocrine therapy

The ATLAS trial, which investigated extended tamoxifen treatment to 10 years in patients who were recurrence-free and had completed 5 years of tamoxifen, demonstrated a further reduction of breast cancer recurrence and breast cancer mortality in the arm with extended therapy. However, these effects were only evident after year 10: year 5–9 RR=0.90, 95% CI, 0.79–1.02 and ≥10 years RR=0.75, 95% CI, 0.62–0.90.[280] Similar results were reported from the aTTom trial.[281] Currently, prolonged tamoxifen treatment may be considered after 5 years of tamoxifen for pre/perimenopausal patients with higher risk of recurrence and tolerable side effects.[105]

In the MA17 trial, postmenopausal patients treated with tamoxifen for 5 years were shown to benefit from extended treatment with an AI for another 3–5 years.[326,327] The MA17.R trial investigated the effect of extending AI treatment to 10 years and showed significant improvement of DFS at 5 years (HR=0.66, 95% CI, 0.48–0.91). However, only recurrence and contralateral breast cancer were counted as events in the primary endpoint, and when death was included in the endpoint, no significant differences could be observed between the two treatment arms. Moreover, it is worth noting that 70% of the patients included in MA17.R had also received 5 years of tamoxifen prior to the start of AI treatment.[328]

No studies have investigated the benefit of tamoxifen after the completion of 5 years of an AI.

Ovarian function suppression

Ovarian function suppression (OFS) can be achieved by surgical oophorectomy, radiation ovarian ablation, or pharmacological treatments, such as GnRH agonists. Accortding to the updated results from the SOFT trial presented at the San Antonio Breast Cancer Symposium in 2017; with a median follow-up of 8 years, DFS was significantly improved by tamoxifen + OFS (HR=0.76; 95% CI, 0.62–0.93) and exemestane + OFS (HR=0.65; 95% CI, 0.53–0.81), compared with tamoxifen alone. Tamoxifen + OFS also improved OS (HR=0.67; 95% CI, 0.48–0.92).[329] An update on the combined analysis of the SOFT and TEXT trials was also reported at this meeting; with a median follow-up of 9 years, exemestane + OFS improved DFS and distant recurrence-free interval (D-RFi) compared with tamoxifen + OFS (DFS: HR=0.77; 95% CI, 0.67–0.90 and D-RFi: HR=0.80; 95% CI, 0.65–0.96). No differences were seen for OS, but longer follow-up is needed to evaluate the true effects on OS.[330] However, in ABCSG-12, there were no differences in clinical efficacy between OFS + AI and OFS + tamoxifen after a median follow-up of 8 years.[331] In case of an AI, clinicians should be aware of that the suppression of the ovaries may be incomplete and evaluate this by measuring oestradiol and gonadotropin levels before the monthly administration of GnRH agonist.[332]

Additional systemic adjuvant therapy

Chemotherapy

According to a meta-analysis from the EBCTCG, adjuvant chemotherapy provides an equal relative risk reduction of approximately one third across all prognostic subgroups.[333] Patients with higher risk of recurrence are usually recommended adjuvant chemotherapy, including an anthracycline-based regimen and most often a taxane.[105]

Anti-HER2 treatment

HER2-positive breast cancer usually requires the addition of anti-HER2 therapy to chemotherapy. The gold standard is the mAB trastuzumab every third week for one year, but in the neoadjuvant setting an additional antibody, pertuzumab, should preferably be added for a shorter period of time.[105,334-336]

Bisphosphonates

Several randomised clinical trials have investigated the benefit of bone-targeted therapy in the adjuvant setting; however, the results are conflicting.[337-339] To clarify the role of adjuvant bisphosphonates, EBCTCG conducted a meta-analysis in which adjuvant bisphosphonates were shown to reduce the rate of breast cancer recurrence in bone and reduce breast cancer mortality, but only in postmenopausal patients.[340]

Adherence

To ensure treatment efficacy, it is important to ensure that patients adhere to their prescribed medication. Unfortunately, non-adherence to adjuvant endocrine treatment is an issue, as a significant proportion of patients stop taking their treatment, most often as a result of side effects. According to a previous study, based on the Swedish Prescribed Drug Register, only 69% of the patients were adherent after 3 years.[290] However, we recently conducted a similar study in Region Jönköping, also using the Swedish Prescribed Drug Register, and found that the adherence exceeded 90% after both 3 and 5 years.[341] Our study was descriptive and did not investigate the reasons behind the good adherence. These findings warrant future population-based studies to identify predictors of good adherence and optimal follow-up routines. Moreover, such studies will help identify patients in need of additional support to manage their therapy.

Endpoints in breast cancer trials

The primary goal of randomised adjuvant breast cancer trials is to improve OS, characterised as the time from randomisation to death (all causes). Because patients may survive years, and sometimes even decades, after being diagnosed with stage IV breast cancer, there is a need for surrogate endpoints for OS, such as DFS or RFS. By using surrogate endpoints, the benefit of a drug can be evaluated more quickly and it is possible to reduce the number of patients included in randomised trials, which substantially reduces the costs associated with clinical trials. A major concern, however, is that that the definition used for these time-to-event (TTE) endpoints may differ between trials, which limits the interpretation and comparisons between different studies.[342-344] With the aim of rectifying TTE endpoints, The STEEP System was proposed by Hudis et al. in 2007, which included a proposal for standardised definitions for efficacy endpoints in adjuvant breast cancer trials.[342] In 2015, the DATECAN (Definition for the Assessment of Time-to-event Endpoints in CANcer trials) initiative presented guidelines for TTE endpoints in breast cancer that were based on a literature review followed by consensus in an international multidisciplinary panel of experts (Table 3).[343,344]

Setting	Recommended Time-to-event endpoint	Causes of death included in definition					Clinical events included in definitions							
		From breast cancer	From non-breast cancer cause	Related to protocol treatment	From any cause	From unknown cause	Invasive ipsilateral breast tumor recurrence / progression	Local Invasive recurrence / progression	Regional Invasive recurrence / progression (M+ : Regional progression)	Invasive contra lateral breast cancer	Appearance/ occurrence of metastases/ Distant recurrence	Second primary invasive cancer (non-breast cancer)	Ipsilateral DCIS	Contra lateral DCIS
Non metastatic	*BCSS*	X		NC										
	iDFS	X	X	X	X	X	X	X	X	X	X	X		
	D-DFS	X	X	X	X	X					X			
	D-RFS	X	X	X	X	X					X			
	RFS	X	X	X	X	X	X	X	X		X		X	
	L-RFS	X	X	X	X	X	X	X	X				X	
	RFi	X					X	X	X		X		X	
	BCFi	X					X	X	X	X	X		X	X
	D-RFi	X									X			
Metastatic	*PFS*	X	X	X	X	X	NA	NA	X		X			
	TTP	X					NA	NA	X		X			

NOTE 1: It was recommended NOT to include the following events in any of the time to event endpoints: loss to follow-up.

BCSS, breast cancer-specific survival; iDFS, invasive disease-free survival; D-DFS, distant disease-free survival; D-RFS, distant relapse-free survival; RFS, relapse-free survival; L-RFS, locoregional relapse-free survival; RFi, recurrence-free interval; BCFi, breast cancer-free interval; D-RFi, distant recurrence-free interval; PFS, progression-free survival; TTP, time to progression; NC = No consensus.

Table 3. Clinical events to be included in the definitions of time-to-event end points in randomised clinical trials assessing treatments for breast cancer according to DATECAN guidelines. Reprinted with permission from Oxford University Press, Annals of Oncology, © 2015.[343,344]

Aims and hypotheses

Short background to Study I and II

Because the biomarkers ER, PR, HER2, and Ki67 are essential for prognosis and treatment prediction in primary breast cancer, it is of the utmost importance to ensure that all laboratories that analyse these markers provide reliable results. Provided that a laboratory follows guidelines and continuously works with QA, including participation in external proficiency testing programs, the reproducibility for ER, PR, and HER2 is often satisfactory. However, for Ki67, there are still concerns regarding the reproducibility between different laboratories as well as between different assessors.[153,161,205,206] Several efforts have therefore been made to improve the different steps of the Ki67 analysis. Evaluation of newer antibodies may be one way to improve the staining, thereby making it easier to assess Ki67. RabMAbs have been reported to have higher sensitivity without loss of specificity compared with their corresponding mouse mABs and may theoretically be a better option for Ki67 assessment.[194,195] Moreover, these biomarkers are used to subgroup tumours according to the surrogate classification of the intrinsic subtypes. Because the recommended adjuvant systemic therapy generally differ between the different subtypes, an agreement analysis of the different subtypes reflects the clinical consequences of incorrect biomarker analyses, which ultimately affects patients.

Study I

The **aim** of this study was to compare the newer RabMAb SP6 with the gold standard mouse mAB MIB1 for analysis of Ki67 in primary breast cancer in terms of reproducibility between assessors and prognostic value.

We expected that Ki67(SP6) would be superior to Ki67(MIB1) regarding reproducibility between assessors and that Ki67(SP6) and Ki67(MIB1) would have a similar prognostic value.

Study II

The **aim** of this study was to investigate the concordance between Swedish pathology departments and the well-known reference laboratory European Institute of Oncology (IEO), Milan, Italy, for routine analysis of ER, PR, Ki67, and HER2. Moreover, this

study aimed to examine the concordance for the breast cancer subtypes based on the St. Gallen 2013 surrogate classification for the intrinsic subtypes.

We expected that the quality of the Swedish results would be very good for ER, PR, and HER2, but low/moderate for Ki67. Moreover, we expected that the possibility to distinguish between high and low Ki67 would improve when using laboratory-specific cut-offs for Ki67. Finally, we expected that the concordance of the breast cancer subtypes according to the St. Gallen 2013 classification, using a combination of all markers, would be moderate/good.

Short background for Study III and IV

Tamoxifen is an anti-oestrogen that is well known to improve the outcome for patients with ER-positive breast cancer. As its mechanism of action is independent of ovarian function, tamoxifen is the preferable option for most premenopausal women. As ER-positive breast cancer is associated with late recurrences, long-term follow-up is essential to evaluate the long-term effects, especially in younger women with long life expectancy.

Study III

The **aim** of this study was to investigate if 2 years of adjuvant tamoxifen significantly decreased the cumulative breast cancer-related mortality (CBCM) and the cumulative mortality (CM) in premenopausal patients with ER-positive breast cancer compared with no systemic treatment, after the complete long-term follow-up and for specified time intervals.

We expected that tamoxifen would decrease mortality for both endpoints in patients with hormone receptor-positive tumours.

Study IV

The **aim** of this study was to investigate if 2 years of adjuvant tamoxifen significantly decreased the incidence of breast cancer-related events and distant recurrences in premenopausal patients with ER-positive breast cancer compared with no systemic treatment after long-term follow-up of almost three decades and for specified time intervals. Moreover, this study aimed to investigate the effects of tamoxifen on secondary malignancies and survival after distant recurrences.

We expected that tamoxifen would significantly decrease breast cancer-related events and distant recurrences after the complete long-term follow-up in the ER-positive subgroup. Moreover, we expected to see these positive, but not necessarily significant, effects for the separate intervals as result of the 'carryover effect' associated with tamoxifen.

Methods

Patients

Study I

Study I includes 237 premenopausal patients with node-negative breast cancer who were included in the SB91B trial, a prospective study which aimed to investigate the prognostic value of flow cytometric S-phase fraction.[147] Between 1991 and 1994, approximately 60% of all premenopausal patients with node-negative breast cancer diagnosed in the South Healthcare region were included in this study and the corresponding figure for patients with tumours large enough to allow biomarker analyses (and thereby inclusion) was approximately 75%.[147] Patients underwent either breast-conserving surgery (BCS) (n=172) or modified radical mastectomy (n=65), including axillary dissection. Those who underwent BCS were also included in the SweBCG 91RT trial, which investigated the benefit of adjuvant radiotherapy, and were thereby randomised between radiotherapy (50 Gy in 25 daily fractions) (n=110) or no radiotherapy (n=62).[345] Due to narrow margins, radiotherapy was also administered to seven patients who underwent modified radical mastectomy. Twenty-seven women received adjuvant systemic therapy, either chemotherapy (n=21) and/or tamoxifen (n=7). One patient underwent oophorectomy. When first reported, this study demonstrated that S-phase fraction and uPA were predictors for distant recurrence in this cohort.[147]

Study II

Study II includes the 2014 SweQA (Swedish Quality Assurance in Breast Cancer) QA project, in which Swedish pathology departments were invited to participate (n=28). Twenty-seven laboratories, covering around 98% of the primary breast cancer surgery in Sweden, accepted the invitation. The laboratories were instructed to identify the first breast carcinoma diagnosed after day 15 in each month during 2012, except for in July and December, and send the original pathology reports together with the originally stained slides as well as the FFPE tumour tissue blocks that were analysed in the clinical setting (n=270) to Equalis.[346] No information regarding tumour stage or patient-related characteristics was collected.

Study III and IV

Study III and IV include patients who were included in the SBII2pre trial, which aimed to study the effect of adjuvant tamoxifen in premenopausal patients with breast cancer. Between 1984 and 1991, the study included 564 patients in the South Healthcare Region (n=427) and South-East Healthcare Region (n=137). The inclusion criteria were premenopausal patients with stage II breast cancer (UICC TNM Classification, 3rd edition, 1982); patients were included irrespective of hormone receptor status. No more than one year since the last menstrual period was allowed. The exclusion criteria included bilateral breast cancer, metastatic disease, or a history of other malignancies. Patients were randomised to 2 years of tamoxifen (n=276) (South Healthcare Region: tamoxifen 20 mg × 1; South-East Healthcare Region: tamoxifen 40 mg x 1) or no systemic treatment (n=288). The median age for the included patients was 45 years (range 25–58). The clinical characteristics were similar between the two groups, with the exception of slightly larger tumours in the tamoxifen group. Eight patients (<2%) received adjuvant chemotherapy and/or ovarian suppression. Patients who underwent BCS and/or were node-positive received adjuvant radiotherapy according the clinical standard of that time. In Study IV, four patients were excluded from the cohort and one patient was transferred from the control group to the tamoxifen group. This is described further in the chapter 'Methods'. Consequently, 564 patients were included in Study III and 560 patients were included in Study IV.

Ethics

Study I

This study is covered by the approval received by the Ethics Committee of Skåne University Hospital, Lund, Sweden, in 2001 (LU 240-01).

Study II

QA projects do not require ethical approval. After presenting the study design to the scientific secretary of the Ethics Committee in Lund, Sweden, it was confirmed that an application for Study II could be omitted.

Study III and IV

The original study was approved by the Ethics Committees of Lund University and Linköping University; however, we have not been able to locate the documents from this time. The first report of the study, including new biomarker analyses and follow-up data, was approved by the Ethics committees of Lund (Dnr LU 240-01) and Linköping Universities (Dnr Linkoping 01-134) in 2001.

Because Study III included retrieval of data from the Swedish Causes of Death Register and deceased patients are not covered by the Swedish Personal Data Act, no updated application was needed for Study III.

The review of the medical records and retrieval of data from the Swedish Cancer Register and the Swedish Causes of Death Register, respectively, that was necessary for the long-term follow-up in Study IV was approved by the Ethics Committee in Lund 2015 (Dnr 2015/350). An amendment to retrieve data on all malignancies from the Swedish Cancer Registry was approved in 2017 (Dnr 2017/35).

Biomarker analyses

Study I

A TMA with two 0.6 mm core biopsies from each tumour was constructed as previously described.[347] Analyses of HG, ER, PR, HER2, and Ki67(MIB1) have also been described earlier.[147,347]

Ki67 staining with the RabMAb SP6

Four-µm-thick sections were cut from the TMA and mounted on glass slides. Deparaffinisation and antigen retrieval was performed in PT-LINK (DAKO, Glostrup, Denmark) using a buffer at pH 9 (K5007; DAKO). The slides were heated to 98°C for 20 min, cooled to 65°C, rinsed, and put in an Autostainer Plus (DAKO) for IHC. Blocking was performed with peroxidase solution for 5 min. The slides were incubated with a 1:200 dilution of SP6 for 30 min (RM-9106; Neomarkers, Fremont, CA, USA). EnVision Polymer (K5007; DAKO) was used as secondary reagent and was applied for 25 min. For visualization, Peroxidase/DAB (K5007; DAKO) was used twice for 5 min. The slides were counterstained with haematoxylin and then dehydrated through ethanol to xylene and mounted with Pertex.

Scoring of Ki67 (MIB1 and SP6) by three assessors

Ki67(MIB1) was scored in 2008 by three investigators (DG, KL, and MK), as described in the previous study by Klintman et al.[347] Ki67(SP6) was scored in 2011 by three investigators (DG, KL, and SB); the first two of these investigators also scored Ki67(MIB1), as stated above. DG was an experienced pathologist and MK is a medical oncologist who performed the scoring during her time as PhD student. KL and SB are laboratory technicians.

The core with the highest percentage of positively stained invasive tumour cells was chosen for scoring. Two hundred nuclei were evaluated in hotspots (except in five samples, which had less than 200 cells available) and the staining intensity was disregarded. KL, MK, and SB used the counting method and counted the positively stained nuclei one by one, whereas DG instead scored the Ki67 staining into the following intervals; ≤1%, 2%–5%, 6%–10%, 11%–20%, 21%–30%, 31%–40%, 41%–50%, 51%–60%, 61%–70%, 71%–80%, 81%–90%, and 91%–100%.

Cut-off defining high and low Ki67

As DG was the most experienced assessor, her assessments were used for the prognostic analysis. The upper limit in the interval was used for calculation of Ki67 in the statistical analyses. In the study by Klintman and co-workers as well as in Study I, the seventh decile was used to define high Ki67, which for Ki67 (MIB1) corresponded to a cut-off of >20%.[347] This cut-point was selected because it was demonstrated as the most

optimal in a previous Swedish study investigating proliferation markers in primary breast cancer.[149] When >20% was applied as cut-off for Ki67(MIB1) in Study I, 33% (n=186) of the tumours were classified as high proliferating. The cut-off for Ki67(SP6) was chosen to achieve as similar a proportion of high-proliferation tumours as possible; this corresponded to a cut-off of >20% and a proportion of tumours with high Ki67 of 35%.

Study II

Swedish Laboratories

All tumour biomarkers (ER, PR, HER2 (IHC/ISH), and Ki67) were assessed according to each laboratory's clinical routines, based on the Swedish guidelines, 2014, without the knowledge of a subsequent external control.[348] These results are referred to as the local assessments (LAs).

European Institute of Oncology (IEO)

The original glass slides were sent to Equalis and then further distributed to IEO in Milan, Italy.[346] At IEO, the samples were re-evaluated for ER, PR, HER2 (IHC), and Ki67 by an experienced pathologist (LR), according their local guidelines. These results are referred to as the reviewed assessments (RA). The FFPE tumour blocks were also delivered to IEO, where 4-µm-thick sections were cut, stained, and scored regarding ER, PR, HER2 (IHC), and Ki67 by the same pathologist (LR), using their local guidelines. These results are referred to as IEO.

Cut-off values

ER, PR, HER2, and Ki67 were all used as dichotomised variables (positive vs. negative and high vs. low). For defining ER- and PR-positivity, we chose the international established cut-off of ≥1% of positively stained tumour nuclei. As a result of how ER and PR were reported according to Swedish guidelines, we had to use >1% as cut-off for LA. Because of the costs of ISH analyses, ISH was only performed as part of clinical routine in Sweden (LA), and these results were used as gold standard for tumours scored as 2+. The definitive results for HER2 (IHC +/- ISH) was referred to as the final HER status. For Ki67, both >20% and laboratory-specific cut-offs were used for LA, whereas >20% was used as cut-off for RA and IEO. Based on the former Swedish guidelines, the laboratory-specific cut-off values were defined as the 67th percentile based on the first 100 breast carcinomas at each laboratory each year (see Table 5).[348]

St. Gallen clinicopathological subtype classification 2013

This comparison was undertaken to reflect on the clinical consequences followed by differences in the results of the biomarker analyses. The final HER2 status was used to designate HER2 status and >20% defined both high Ki67 and PR (Table 4).

Table 4. The clinicopathological surrogate definitions of the intrinsic subtypes of breast cancer used in Study II, based on St. Gallen 2013.[157]

Intrinsic subtype	Clinicopathological surrogate definition	Characteristics
Luminal A	Luminal A-like	ER-positive and high PR HER2-negative Low Ki67
Luminal B	Luminal B-like/HER2-negative	ER-positive HER2-negative At least one of of the following: high Ki67 negative or low PR
	Luminal B-like/HER2-positive	ER-positive HER2-positive Any Ki67 Any PR
ErbB2 overexpression	HER2-positive/non-luminal	ER-negative and PR-negative HER2-positive
Basal-like	Triple negative	ER-negative and PR-negative HER2-negative

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
Cut-off for ER, LA: >1%
Cut-off for RA and IEO: ≥ 1%
Cut-off for PR (high vs. low): >20% (the cut-off used in the original paper by Prat et al.[134])
Cut-off for Ki67 (high vs. low): >20% (the cut-off used at IEO)

Table 5. Instruments, pH, antibodies, and local cut-offs (Ki67) used in the immunohistochemical methods in the pathology departments participating in this study

	ER and PR					Ki67				HER2		
Lab	Instrument	pH (ER)	Antibody (ER)	pH (PR)	Antibody (PR)	Local cut-off (%)	Instrument	pH	Antibody	Instrument	pH	Antibody
IEO	DAKO Autostainer		PharmDx kit*		PharmDx kit*	>20	DAKO Autostainer		MIB1	DAKO Autostainer		HercepTest
1	Ventana U	H	SP1	H	1E2	>20	Ventana U	H	MIB1	Ventana U	H	4B5
4	Leica Bond	H	6F11	L	16/SAN27	-	-	-	-	-	-	-
5	DAKO Autostainer	H	EP1	H	636	>30	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
7	Ventana XT	H	SP1	H	1E2	>29	Biocare	L	MIB1	Ventana XT	H	Pathway
8	Ventana U	H	SP1	H	1E2	>20	Ventana U	H	MIB1	Ventana U	H	4B5
9	DAKO Autostainer	H	EP1	H	636	>31	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
10	Ventana U	H	SP1	H	1E2	>31	Ventana U	H	30-9	Ventana U	H	4B5
11	Ventana XH	H	6F11	H	1E2	>25	Ventana XH	H	MIB1	Ventana XH	H	4B5
12	Ventana U	H	SP1	H	1E2	>25	Ventana U	H	30-9	Ventana U	H	Pathway
13	DAKO Autostainer	H	EP1	H	636	>30	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
14	Ventana U	H	SP1	H	1E2	>39	Ventana U	H	30-9	Ventana U	H	4B5
15	DAKO Autostainer	H	EP1	H	636	>25	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
16	Ventana XT+U	H	SP1	H	1E2	>25	Ventana XH+U	H	MIB1	Ventana XT+U	H	4B5
17	Ventana U	H	SP1	H	1E2	>25	Ventana U	H	MIB1	Ventana U	H	Pathway
18	Ventana XT+U	H	SP1	H	1E2	>35	Ventana XH+U	H	30-9	Ventana XT+U	H	4B5
19	Ventana XT	L	SP1	L	1E2	>30	Biocare	L	MIB1	Ventana XH	L	Pathway
20	DAKO Autostainer	H	6F11	H	636	-	DAKO Autostainer	H	MIB1	DAKO Autostainer	L	HercepTest
21	DAKO Autostainer	H	EP1	H	636	-	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
22	Ventana U	H	SP1	H	1E2	>29	Ventana U	H	MIB1	Ventana U	H	Pathway
23	Ventana XT	H	SP1	H	1E2	>25	Ventana XT	H	MIB1	Ventana XT	H	Pathway
24	Biocare	H	SP1	H	16	>20	Biocare	L	MIB1	Biocare	H	4B5
25	Ventana U	H	SP1	H	1E2	>30	Ventana U	H	MIB1	Ventana U	H	Pathway
26	Ventana XT+U	H	SP1	H	1E2	>19	Ventana XT+U	H	MIB1	Ventana XT+U	H	Pathway
27	DAKO Autostainer	H	EP1	H	16	>34	DAKO Autostainer	H	MIB1	DAKO Autostainer	L	HercepTest
28	DAKO Autostainer	H	EP1	H	636	>30	DAKO Autostainer	L	MIB1	DAKO Autostainer	L	HercepTest
29	BondMax	H	6F11	H	16/SAN27	>20	BondMax	H	MIB1	BondMax	H	c-erB-2
31	Ventana U	H	SP1	H	1E2	>35	Ventana U	H	MIB1	Ventana U	H	4B5

* PharmDx kit: DAKO ER/PgR PharmDx Kit (link) cod. SK310 (ER: clone 1D5 + ER-2-123 and PR: clone PgR 1294)
Abbreviations: H, high; L, low.

Study III and IV

Biomarker analyses

For the majority of patients, hormone receptor analysis was performed using LBA following the primary surgery (ER, n=457; PR, n=449). FFPE tumour tissue was collected during 2002 for the majority of patients (n=500). TMAs were constructed and ER, PR (both IHC), and HER2 (IHC and ISH), as well as HG, were assessed as previously described.[349] ER and PR were assessed in categories and >10% was used as the cut-off for defining ER/PR-positivity. A satisfactory agreement of approximately 90% has been reported between IHC and cytosol-based methods.[200,350] As IHC is considered the routine method for assessment of ER and PR, we chose to use IHC data and results from the cytosol-based methods were only used in patients in which IHC data were missing. Using this approach, hormone receptor data were available for 533 (95%) of the included patients.

Follow-up

Study I

The SB91B study included yearly follow-up to 10 years, including physical exam and mammography.[147] Medical records were reviewed during 1998 and in 2004 to increase the follow-up time. Although the median follow-up for patients who did not suffer from distant recurrence or death was 10.3 years, the follow-up in Study I was restricted to 5 years as most of the prognostic factors included are associated with non-proportional hazards with longer follow-up.[114,118,147,347]

Study II

Not applicable.

Study III

The SBII2pre study included regular follow-up to 10 years, including physical exam, mammography, and chest X-ray, according to a predefined protocol. For the purpose of Study III, information on the date and cause of death was retrieved from the Swedish Causes of Death Register by April 2014. Deaths were defined as breast cancer-related in cases in which breast cancer was registered as an underlying or contributing cause of death. Because there is a delay in the data reported in the register, the cause of death was missing for five patients and these were therefore annotated as having an unknown cause of death.

Study IV

Study IV includes long-term follow-up data on recurrence (local/regional/distant), contralateral breast cancer, breast cancer-related death, and secondary malignancies. To obtain these data, all medical records were reviewed from diagnosis until the last recorded healthcare contact or death. The review process included visits at hospitals in Jönköping, Linköping, Kalmar, Växjö, Ljungby, Karlskrona, and Halmstad, as well as the Regional Archives in Linköping and Lund. Until the beginning of the 21st century, most medical records consisted of paper journals and/or microfilm and the review included records from Surgical and/or Oncology Departments. Thereafter, computerised records became increasingly common. The audit was performed by me using a predefined case report form (CRF). In addition to information on the breast cancer events described above, data regarding the primary diagnosis, the randomisation process, and the adjuvant therapy were collected. In case of recurrence, the site of the metastases was noted, as well as the first line treatment and time to first progression.

Ten patients were found to have moved to another area or lacked an updated healthcare contact by the hospital records. For these patients, the CRF was sent to the Department of Surgery or Department of Oncology at their current hospital or to their general

practitioner, together with a description of the project and a copy of the ethical approval. Three patients had emigrated and their follow-up time was therefore censored at the time of emigration.

Complementary data on secondary malignancies (breast and non-breast) and death were retrieved from the Swedish Cancer Registry and the Swedish Cause of Death Register by January 2017. The data cut-off date for events was set at 30 November 2016. In cases with discrepancies between the register data and the data collected during the review, the latter were considered most reliable. In cases with uncertainties, patients were discussed within the study group without revealing the treatment arms.

All information was collected on paper CRFs and the data were then input to an electronic CRF, constructed in Epidata, version 2.0.8.56 r1286 (www.epidata.dk). All data were thereafter imported into the statistical package STATA for further processing.

Endpoints and statistics

Study I

Prognostic analyses

The prognostic comparison of the two antibodies was based on the scoring results from the pathologist (DG) and therefore 186 patients were included in these analyses (see Figure 10). Distant disease-free survival (DDFS) at 5 years was chosen as primary endpoint and first events included distant recurrence and/or death from breast cancer. According to the nomenclature in the DATECAN guidelines, this equals distant disease-free interval (D-RFi).[343,344] DDFS was estimated according to the Kaplan–Meier method and the log-rank test was used to compare the incidence of this event in different strata. Cox regression analysis was used to calculate HRs in univariable and multivariable analyses. When using the Cox model, proportional hazards during the follow-up time is assumed. If this assumption is violated and ignored, there is a risk to overestimate the strength of prognostic markers with shorter follow-up and underestimate important transient effects with longer follow-up. The follow-up was restricted to 5 years, as most of the included biomarkers are associated with non-proportional hazards beyond 5 years.[114,118]

Reproducibility analyses

Agreement (%) and Cohen's kappa statistics were used to evaluate the agreement between the assessors for each antibody. The kappa measure takes into account the probability that two tests may be assessed equally by chance. The kappa value (κ) ranges from -1 to +1, where κ of 1 represents perfect agreement and κ of 0 represents the amount of agreement expected by chance conditional of the marginal totals. A value below 0 indicates that the agreement is worse than what is expected by chance, a scenario that is unlikely in practice.[351] The following intervals are frequently used to judge this chance-corrected measure of agreement:[352]

κ = <0.20, poor
κ = 0.21–0.40, fair
κ = 0.41–0.60, moderate
κ = 0.61–0.80, good
κ = 0.81–1.00, very good

To be able to analyse the agreement for the scoring results of the two antibodies, results from all six assessors are necessary, and therefore only 168 patients could be included in the agreement analyses (Table 6 and Figure 10).

Table 6. Ki67 assessments by different assessors for the antibodies MIB1 and SP6 included in Study I.

	MIB1	SP6
DG	+	+
KL	+	+
MK	+	-
SB	-	+

Study II

Reproducibility analyses

Cohen's kappa statistics were used for the pairwise comparison of the results from LA, RA, and IEO. It is important to keep in mind that κ values are dependent on the prevalence of the different categories.[353] For example, despite a high percentage agreement, it is difficult to reach a high κ value for a marker like ER, as the vast majority of patients are ER-positive, compared with a marker with more evenly distributed values, such as Ki67. Moreover, it is more difficult to reach a high κ value for comparisons including several categories, such as the 2013 St. Gallen subtype classification.

Wilcoxon matched pairs signed-rank test

The Wilcoxon matched pairs signed-rank test was used in a post-hoc analysis following the finding that one of the antibodies used for PR seemed to provide falsely low/negative results. This test ranks the absolute differences within each matched pair (PR for RA vs. PR for IEO) and compares the sum of the ranks for e.g. the negative differences to the corresponding expected rank sum under the null hypothesis of no systematic difference to investigate whether a specific antibody was associated with generally lower PR values.

Study III

Endpoints

CM was used as primary endpoint in Study III. Because of the long-term follow-up, the risk of death from other causes was very high in this cohort, and therefore CBCM was chosen as secondary endpoint.

Survival analyses

The Kaplan–Meier method and log-rank test was used to estimate CM in the two treatment arms, whereas CBCM was estimated by a method taking death from other causes into consideration. In this method, described by Marubini et al., the cumulative incidence of breast cancer-related death is estimated in a way that takes the so-called competing risk, here all other causes of death, into account.[354] Cox regression analysis, stratified by healthcare region, was used to compare CM and CBCM in the two treatment arms. In the CBCM analysis, the follow-up was censored at the time of death from other causes, so-called cause-specific Cox regression.

We chose to mainly focus on the ER-positive subgroup, as ER-negative tumours do not respond to tamoxifen.[112] In the SBII2pre cohort, there is a relatively high number of ER-negative/PR-positive tumours (n=25; 4%). Because the existence of this subgroup has been questioned,[127] we chose to exclude tumours belonging to this subgroup. As PR has been shown to be predictive for tamoxifen efficacy in this cohort in a previous study,[138] we also included the ER-positive/PR-positive subgroup for the main analyses. For ER-positive tumours, the homogeneity of treatment effect across subgroups of prognostic factors was evaluated in Cox models with interaction terms.

Time-dependent analyses

In studies with long-term follow-up, non-proportional hazards are the rule rather than the exception. The follow-up time was therefore divided into three different intervals: 0–5 years, > 5–15 years, and >15 years. These intervals were chosen as RFS and OS at 5 years were the endpoints used in the original study and because the EBCTCG overview from 2011 reported follow-up at 15 years.[112,349] Smoothed hazard plots were drawn to illustrate the change in incidences during the follow-up period.

Study IV

Endpoints

The endpoints were chosen according to the recommendations in the DATECAN guidelines (see Table 3).[343,344] Because of the long-term follow-up, we found it reasonable to not include non-breast cancer-related deaths or secondary malignancies as events. Breast cancer-free interval (BCFi) and D-RFi were therefore used as primary and secondary endpoints and were decided upon before the start of data analysis. BCFi included any of the following events: local, regional, or distant recurrence; breast cancer-related death; or contralateral breast cancer (invasive or DCIS). D-RFi included distant recurrence or breast cancer-related death as first event. Because patients may suffer from near-simultaneous events, we followed the recommendation provided by Hudis et al. and considered events within 2 months as synchronous.[342] Near-simultaneous events were ranked according to a hierarchy of prognosis from worst to

best: distant recurrence > regional recurrence > local recurrence > contralateral breast cancer.

Survival analyses

Except for BCFi, which was also analysed for all patients (n=560) and for the ER-negative subgroup (n=150), we choose to focus solely on the ER-positive subgroup. The same method as in Study III was used to estimate the cumulative incidences of the events for each of these two endpoints.[354] For D-RFi, contralateral breast cancer was regarded as a competing risk, as we could not rule out that a subsequent distant recurrence may be a result of the secondary breast cancer. Cox regression analyses, stratified by healthcare region, were used to compare BCFi and D-RFi between the two treatment arms.

Subgroup analyses

To investigate the effect of tamoxifen across subgroups of prognostic factors, Cox models with interaction terms were used.

Time-dependent analyses

For the time-dependent analyses, we used the same time intervals as used in Study III.

Secondary malignancies

For each of the two treatment arms, secondary malignancies were reported as frequency (n) and incidence (per 1,000 patient-years), with the latter included because of the better survival and thereby longer time at risk in the tamoxifen treatment arm.

Flowcharts

Study I

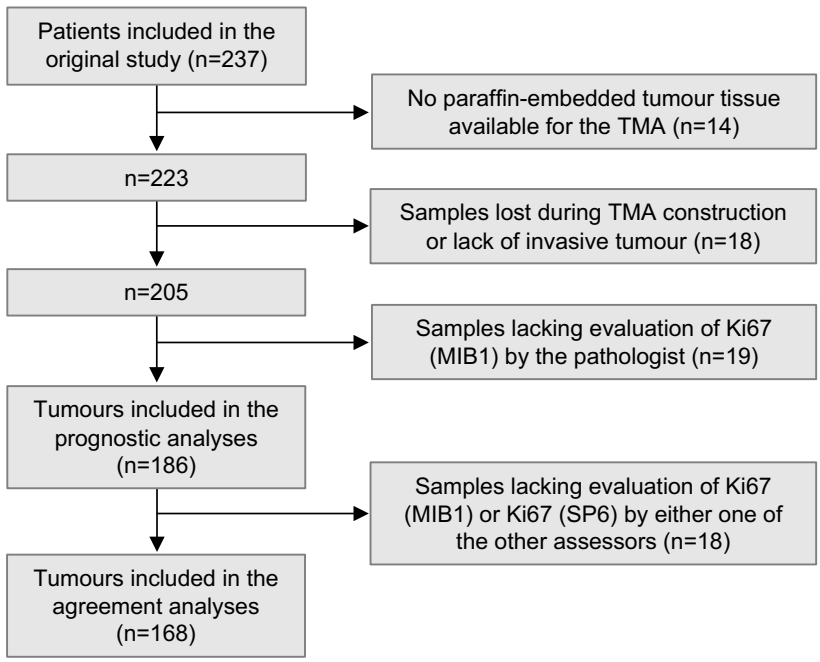


Figure 10. Flowchart for Study I. TMA, tissue microarray.

Study II

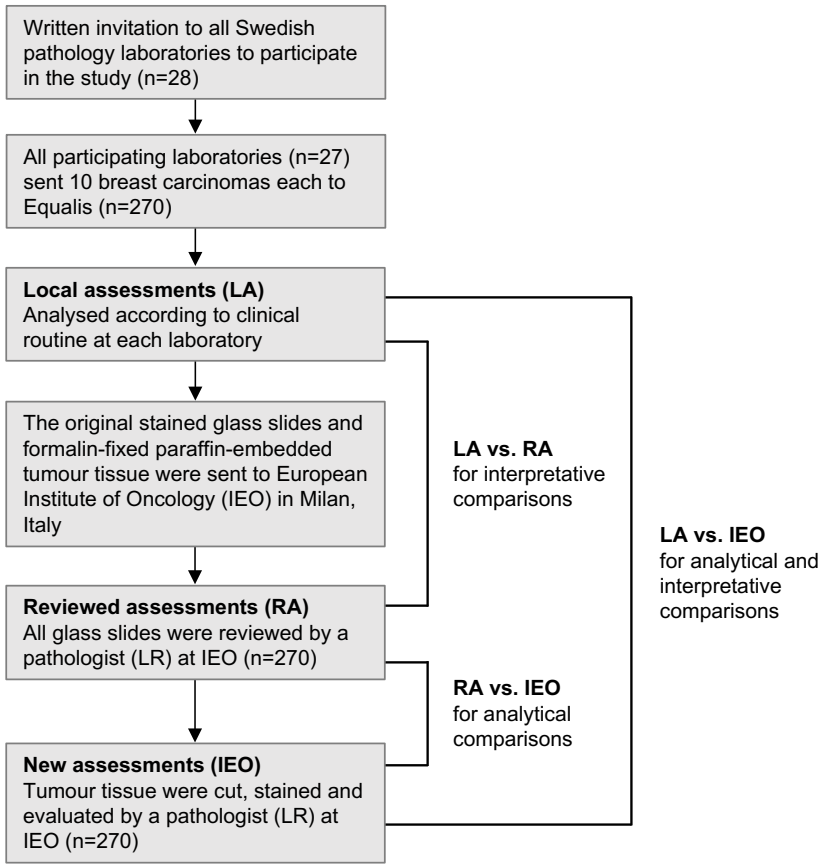


Figure 11. Flowchart for Study II.

Study III

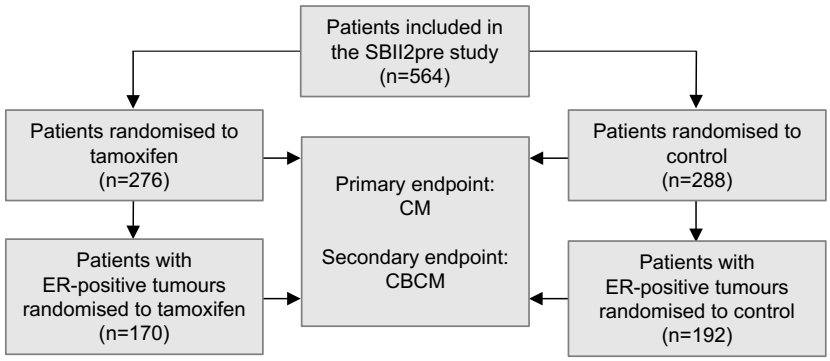


Figure 12. Flowchart for Study III. CBCM, cumulative breast cancer-related mortality; CM, cumulative mortality; ER, oestrogen receptor.

Study IV

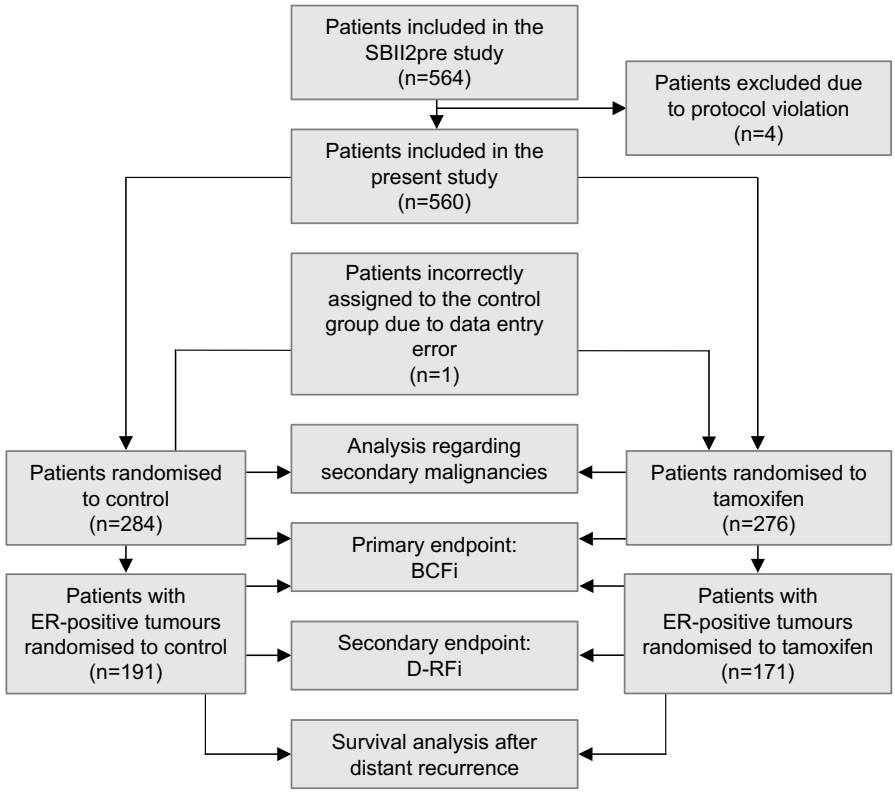


Figure 13. Flowchart for Study IV. BCFi, breast cancer-free interval; D-RFi, distant recurrence-free interval; ER, oestrogen receptor.

Results

To make this section as clear and illustrative as possible, the results from the different studies are presented using figures and tables. Unlike what is customary in the results section in published studies, I have also included explanatory background information to help the reader to put the results into context.

Study I

Reproducibility analyses

For the reproducibility analyses, only tumours with scoring results from all six assessors were included (n=168). The cut-off for defining high Ki67 differed between the two antibodies and among the three assessors. For Ki67(MIB1), the cut-off was higher for DG than for KL and MK. For Ki67(SP6), however, the cut-off was higher for KL and SB than for DG. For KL, the cut-off between Ki67(MIB1) and Ki67(SP6) differed as much as 15 percentage units. The cut-off values and the proportions of high proliferating tumours determined by each assessor are displayed in Table 7.

Table 7. Cut-off values defining high Ki67 and the proportion of high proliferating tumours for the different assessors for the antibodies MIB1 and SP6 (n=168).

	MIB1	SP6
DG		
Cut-off for high Ki67	>20%	>20%
Proportion of tumours with high proliferation	34%	38%
KL		
Cut-off for high Ki67	>14%	>29%
Proportion of tumours with high proliferation	33%	38%
MK		
Cut-off for high Ki67	>18%	NA
Proportion of tumours with high proliferation	34%	
SB		
Cut-off for high Ki67	NA	>26%
Proportion of tumours with high proliferation		38%

The pairwise agreement for each antibody, in which all assessors' scoring results were compared one by one, was marginally better for Ki67(MIB1) compared with Ki67(SP6) (Table 8).

Table 8. Pairwise agreement between assessors for Ki67 (high vs. low) for the antibodies MIB1 and SP6 (n=168).

Ki67(MIB1)		Ki67(SP6)	
DG vs. KL	92%, κ=0.83	DG vs. KL	88%, κ=0.75
DG vs. MK	93%, κ=0.84	DG vs. SB	89%, κ=0.77
KL vs. MK	95%, κ=0.88	KL vs. SB	87%, κ=0.72
Overall agreement: 92%–95% (κ=0.83–0.88)		Overall agreement: 88%–89% (κ=0.72–0.77)	

Prognostic analyses

Tumours with Ki67 assessments for both antibodies by the pathologist (DG) (n=186) were compared, and after 5 years of follow-up, 31 patients had been diagnosed with distant recurrence. DDFS for Ki67 high vs. low at 5 years was similar for both antibodies: Ki67(MIB1), 72% vs. 89% and Ki67(SP6), 74% vs. 88%, respectively (Figure 14 illustrates the Kaplan–Meier curves for DDFS at 5 years, based on Ki67 (high vs. low) for each antibody and the DDFS figures are displayed in Table 9). Ki67 was found to be prognostic for DDFS when assessed by both antibodies, however not strictly significant in the multivariable analysis (the different HRs are displayed in Table 9). HER2 was most prognostic in terms of HR, followed by age, PR, Ki67, HG, and tumour size (data not shown).

Table 9. Distant disease-free survival (DDFS) at 5 years based on Ki67 assessed with MIB1 and SP6 by the pathologist (DG) (n=186).

	Ki67(MIB1) low	Ki67(MIB1) high	Ki67(SP6) low	Ki67(SP6) high
	Descriptive survival analysis (95% CI)			
DDFS at 5 years	89% (82–93)	72% (59–82)	88% (81–93)	74% (61–83)
	Univariable Cox regression analysis			
HR (95% CI) P-value	1.0	2.8 (1.4–5.7) 0.004	1.0	2.5 (1.3–5.2) 0.001
	Multivariable Cox regression analysis (adjusted for age, tumour size, and HER2 status)			
HR (95% CI) P-value	1.0	2.0 (0.93–4.5) 0.074	1.0	2.2 (0.97–4.8) 0.058

Abbreviations: CI, confidence interval; HR, hazard ratio.

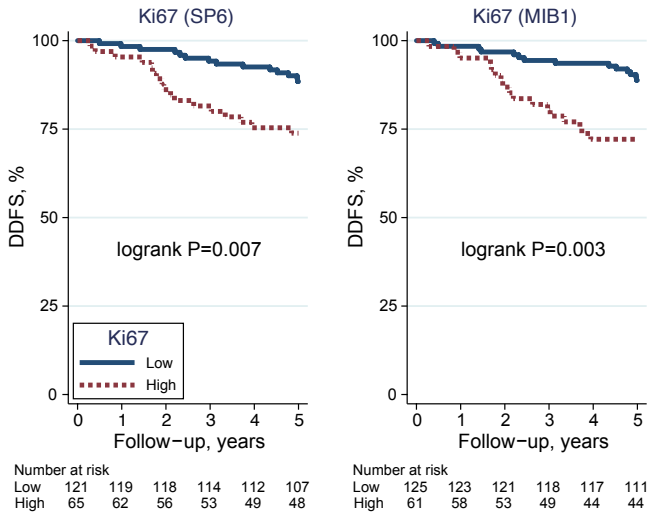


Figure 14. Kaplan–Meier curves illustrating DDFS at 5 years of follow-up based on Ki67(SP6) and Ki67(MIB1) (high vs. low) assessed by the pathologist (DG) (n=186). Reprinted with permission from John Wiley and Sons: Histopathology, © 2014.[355 (Study I)]

Study II

Agreement analyses for breast cancer biomarkers

The IEO results were considered the gold standard and the agreement analyses provide information on the quality of the Swedish routine biomarker analyses. Almost perfect agreement was observed for ER, PR, and HER2 final status, whereas HER2/IHC and Ki67(>20%) showed substantial agreement. Notably, agreement was only moderate when using laboratory-specific cut-offs for Ki67 for LA. The pairwise comparisons (LA vs. RA, LA vs. IEO, and RA vs. LA) for all biomarkers are illustrated in Table 10.

Table 10. Results from the agreement analyses for ER, PR, HER2, and Ki67 for tumours with complete data for all three assessments.

	LA vs. RA	LA vs. IEO	RA vs. IEO
	ER (positive vs. negative) (n=270)		
Agreement κ value	99% 0.96	99% 0.95	98% 0.91
	PR (positive vs. negative) (n=268)		
Agreement κ value	98% 0.94	95% 0.85	97% 0.91
	HER2/IHC (0/1+ vs. 2+/3+) (n=256)		
Agreement κ value	86% 0.66	85% 0.64	96% 0.91
	HER2 final status (positive vs. negative) (n=248)		
Agreement κ value	99.6% 0.98	99.6% 0.98	100% 1.0
	Ki67 (high vs. low; cut-off >20%) (n=265)		
Agreement κ value	89% 0.77	85% 0.70	94% 0.89
	Ki67 (high vs. low; laboratory-specific cut-off) (n=240)		
Agreement κ value	83% 0.64	80% 0.57	-

Abbreviations: LA, local assessments; RA, reviewed assessments; IEO, European Institute of Oncology; ER, oestrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridisation.

Exploratory analysis on the significance of antibody for PR staining results

The reason for this analysis was that seven of the 13 tumours that showed discrepant results for PR were assessed as positive according to both LA and RA, but negative according to IEO. These seven tumours were all from departments that used the Ventana 1E2 ready-to-use (RTU) antibody kit. To further investigate this in a post-hoc analysis, tumours from the laboratories using the Ventana 1E2 RTU antibody were identified (n=158) (Table 5). PR levels from the Ventana 1E2 RTU stainings were compared with PR levels from the PharmDx kit (clone PgR1294) (used at IEO), when scored by the Italian pathologist (LR) (e.g. RA vs. IEO). The results showed a statistically significant shift towards higher PR values for tumours stained with the Ventana 1E2 RTU antibody (P=0.03; Wilcoxon matched pairs signed-rank test).

The St. Gallen 2013 clinicopathological surrogate definition of the intrinsic subtypes

To investigate the clinical consequences of incorrect biomarker analyses, we undertook a comparison of the different breast cancer subtypes classified according to the St. Gallen 2013 surrogate definition (Table 4).[157] Tumours with complete data for all four biomarkers, including laboratory-specific cut-off values for Ki67, by LA, RA, and IEO were included in this analysis (n=233). The agreement was 91% (κ=0.86) for LA vs. RA, 88% (κ=0.81) for LA vs. IEO, and 94% (κ=0.91) for RA vs. IEO. The vast majority of discrepant tumours were found in the luminal A-like and luminal B-like subgroups (Table 11).

Table 11. Agreement between local assessments (LA) and European Institute of Oncology (IEO) for the surrogate definitions of the intrinsic subtypes, based on the 2013 St. Gallen classification, displayed by number (n=233).

		IEO				
		Luminal A-like	**Luminal B-like**	**Luminal B-like/ HER2-positive**	**HER2-positive/ Non-luminal**	**Triple negative**
LA	**Luminal A-like**	83	10	0	0	0
	Luminal B-like	17	78	0	0	1
	Luminal B-like/ HER2-positive	1	0	20	0	0
	HER2-positive/ Non-luminal	0	0	0	6	0
	Triple negative	0	0	0	0	17

Study III

Median follow-up time

By the data cut-off (April 2014), there were 314 deaths, 262 of which were considered breast cancer-related (breast cancer as the underlying or contributing cause of death). The median follow-up for patients that were still alive was 26 years.

Cumulative mortality (CM) and cumulative breast cancer-related mortality (CBCM)

For all patients (n=564), tamoxifen was associated with a substantial but non-significant reduction of CM and CBCM, whereas no benefit was shown for patients with tumours negative for ER and PR (n=153) (Table 12). CM and CBCM were also reduced by tamoxifen in the ER-positive subgroup (CM: HR=0.77; 95% CI, 0.58–1.03; *P*=0.075; CBCM: HR=0.73; 95% CI, 0.53–0.99; *P*=0.046) and in patients with tumours positive for both ER and PR (CM: HR=0.73; 95% CI, 0.54–0.98; *P*=0.034; CBCM: HR=0.70; 95% CI, 0.51–0.97; *P*=0.030). The effects of tamoxifen on CM and CBCM for the different subgroups in terms of HR are displayed in Table 12. Cumulative incidence curves for CM and CBCM for the ER-positive and the ER-negative/PR-negative subgroups are illustrated in Figure 15.

Table 12. Cox regression analyses of the effect of tamoxifen in all patients in different subgroups according to hormone receptor status with cumulative mortality (CM) and cumulative breast cancer-related mortality (CBCM) as endpoints.

	CM	**CBCM**
	Hazard ratio, (95%CI), *P-value*	
All patients (n=564)		
Control	1.0	1.0
Tamoxifen	0.82 (0.66–1.02), *P*=0.080	0.81 (0.63–1.03), *P*=0.090
ER+ (any PR) (n=362)		
Control	1.0	1.0
Tamoxifen	0.77 (0.58–1.03), *P*=0.075	0.73 (0.53–0.99), *P*=0.046
ER+ and PR+ (n=322)		
Control	1.0	1.0
Tamoxifen	0.73 (0.54–0.98), *P*=0.034	0.70 (0.51–0.97), *P*=0.030
ER- and PR- (n=153)		
Control	1.0	1.0
Tamoxifen	0.89 (0.59–1.34), *P*=0.57	0.96 (0.51–1.51), *P*=0.87

Abbreviations: CI, confidence interval; ER, oestrogen receptor; PR, progesterone receptor.

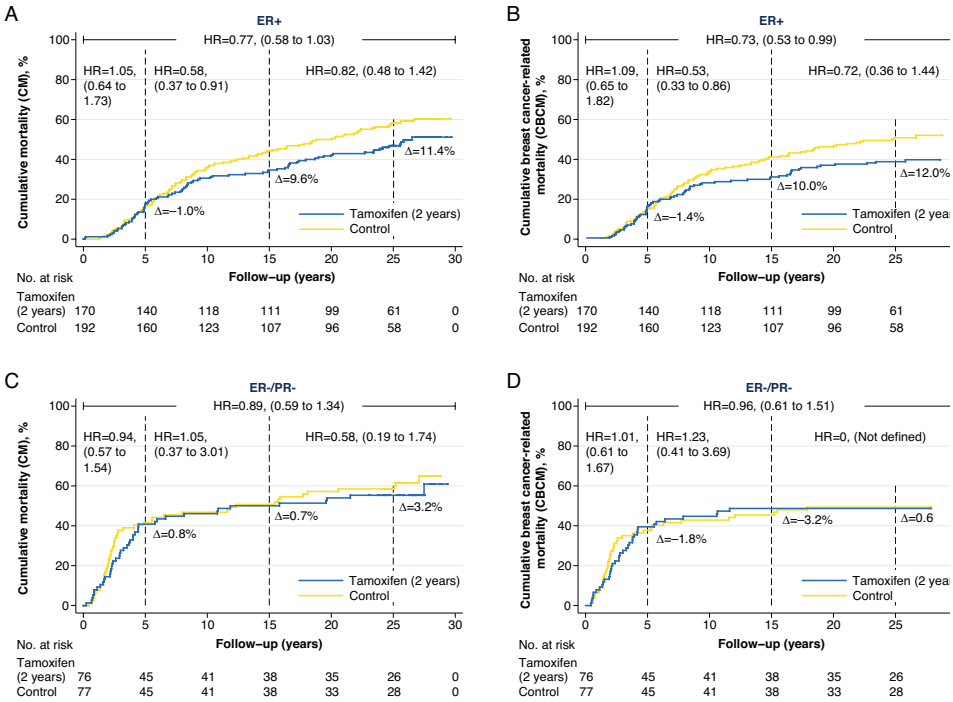


Figure 15. Cumulative mortality (CM) and cumulative breast cancer-related mortality (CBCM) according to treatment arm for patients with (A, B) oestrogen receptor (ER)-positive tumours and (C, D) ER-negative/progesterone receptor (PR)-negative tumours. Dashed vertical lines indicate the time intervals for which separate analyses of tamoxifen effect were carried out, and the follow-up times at which absolute differences in mortality were evaluated. HR, hazard ratio; 95% confidence intervals in parentheses. Reprinted with permission from American Society of Clinical Oncology, All rights reserved: Journal of Clinical Oncology, © 2016. [140 (Study III)]

Time-dependent analyses for patients with ER-positive tumours

The absolute difference between the two treatment arms, measured by percentage units, increased with longer follow-up for both endpoints; CM: 0–5 years: Δ=-1%, >5–15 years: Δ=9.6%, >15 years=11.4%; and CBCM: 0–5 years: Δ=-1.4%, >5–15 years: Δ=10.0%, and >15=12.0%; however, the difference was not significant for the interval >15 years (Figure 15). The HRs for the different time intervals are displayed in Table 13.

Table 13. Cox regression analysis of the effect of tamoxifen in ER-positive patients with cumulative mortality (CM) and cumulative breast cancer-related mortality (CBCM) as endpoints, displayed for the separate time intervals.

	Time period, years		
	0–5 (n=362)	>5–15 (n=300)	>15 (n=218)
	CM Hazard ratio, (95% CI), *P-value*		
ER+ (any PR) Control Tamoxifen	1.0 1.05 (0.64–1.73) *P*=0.84	1.0 0.58 (0.37–0.91) *P*=0.018	1.0 0.82 (0.48–1.42) *P*=0.49
	CBCM Hazard ratio, (95% CI), *P-value*		
ER+ (any PR) Control Tamoxifen	1.0 1.09 (0.65–1.82) *P*=0.76	1.0 0.53 (0.33–0.86) *P*=0.010	1.0 0.72 (0.36–1.44) *P*=0.35

Abbreviations: CI, confidence interval; ER, oestrogen receptor; PR, progesterone receptor.

Smoothed hazard plots showed increasing hazards for both endpoints up to 6 years, followed by a gradual decline (Figure 16).

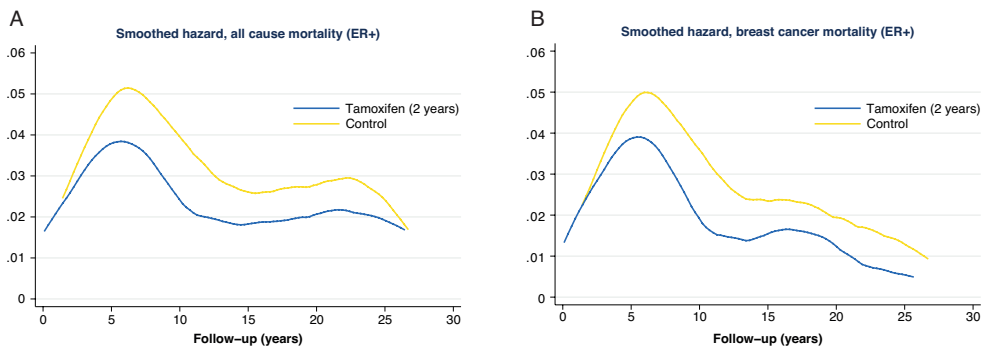


Figure 16. Smoothend hazard estimates for cumulative mortality (CM) and cumulative breast cancer-related mortality (CBCM) in patients with oestrogen receptor (ER)-positive tumours. Reprinted with permission from American Society of Clinical Oncology, All rights reserved: Journal of Clinical Oncology, © 2016.[140 (Study III)]

Study IV

During the thorough review of medical records, seven cases of protocol violation were identified. Two patients lacked invasive breast cancer and were found to have cystosarcoma and DCIS, respectively, and were therefore excluded. Two additional patients were excluded due to stage IV disease at the time of randomisation. Three patients were found to have stage III disease according to TNM, 3rd edition, based on a tumour size >5cm; however, this was considered a violation of minor importance and these patients were kept in the study. Moreover, these three patients were found to have tumours that were ER-negative or had missing values for ER, thereby not affecting the main results. During the audit, it was also noted that one patient, previously belonging to the control group, had been randomised to tamoxifen. This was considered a data entry error and this patient was transferred to the control group. Therefore, 560 patients were included in Study IV, with 276 were randomised to tamoxifen and 284 to control (Figure 13).

Median follow-up time and tamoxifen effect in all patients

By the data cut-off (November 30, 2016), the median follow-up was 27 years for the 168 patients who were still alive without any breast cancer-related events. Only four patients had a follow-up time shorter than 20 years, and for three of these patients this was the result of emigration.

In all patients (n=560), tamoxifen was associated with a reduced incidence of breast cancer-related events (HR=0.76; 95% CI, 0.61–0.94; P=0.011). No effect was seen in the ER-negative subgroup (n=150) (HR=1.05; 95% CI, 0.70–1.60; P=0.24).

Breast cancer-free interval (BCFi) in the ER-positive subgroup

For patients with ER-positive tumours, tamoxifen significantly prolonged BCFi at 30 years of follow-up (HR=0.62; 95% CI, 0.47–0.82; *P*=0.001). This positive effect was also observed for the different time intervals; 0–5 years: HR=0.67; 95% CI, 0.47–0.96; *P*=0.029; >5–15 years: HR=0.60; 95% CI, 0.35–1.04; *P*=0.068; and >15–30 years: HR=0.53; 95% CI, 0.28–0.98; *P*=0.042. Table 14 displays events and HRs by treatment arm, presented by category, for the full follow-up period and for each separate time interval. Figure 17 illustrates the cumulative incidence curves for BCFi by treatment arm, presented by category, for the full follow-up period and for each separate time interval.

Table 14. First breast cancer-related event, breast cancer-free interval (BCFi), and distant recurrence-free interval (D-RFi) in patients with ER-positive tumours, according to treatment arm for the different time intervals and for the full follow-up period.

	Time period, years			
	0–5	>5–15	>15–30	0–30
Patients at risk at the start of each interval, n				
Control	191	115	79	191
Tamoxifen	171	121	96	171
	Hazard ratio, (95% CI), *P-value*			
BCFi				
Control	1.0	1.0	1.0	1.0
Tamoxifen	0.67 (0.47–0.96) *P*=0.029	0.60 (0.35–1.04) *P*=0.068	0.53 (0.28–0.98) *P*=0.042	0.62 (0.47–0.82) *P*=0.001
D-RFi				
Control	1.0	1.0	1.0	1.0
Tamoxifen	0.80 (0.54–1.18) *P*=0.25	0.64 (0.35–1.17) *P*=0.15	0.62 (0.26–1.46) *P*=0.27	0.73 (0.54–0.99) *P*=0.043
	First breast cancer event, n (%)			
All breast cancer events				
Control	74 (39)	32 (28)	24 (30)	130 (68)
Tamoxifen	49 (29)	22 (18)	17 (18)	88 (51)
Breast cancer-related death*				
Control	0 (0)	0 (0)	0 (0)	0 (0)
Tamoxifen	1*(1)	0 (0)	0 (0)	1 (1)
Distant recurrence				
Control	55 (29)	22 (19)	11 (14)	88 (46)
Tamoxifen	44 (26)	17 (14)	9 (9)	70 (41)
Regional recurrence				
Control	6 (3)	0 (0)	1 (1)	7 (4)
Tamoxifen	0 (0)	0 (0)	1 (1)	1 (1)
Local recurrence				
Control	6 (3)	4 (3)	3 (4)	13 (7)
Tamoxifen	1 (1)	0 (0)	2 (2)	3(2)
Contralateral breast cancer (invasive and DCIS)				
Control	7 (4)	6 (8)	9 (11)	22 (12)
Tamoxifen	3 (2)	5 (4)	5 (5)	13 (8)

Abbreviations: CI, confidence interval; DCIS, ductal cancer *in situ*; ER, oestrogen receptor.
*Dead from pulmonary embolism with breast cancer as contributing cause of death.

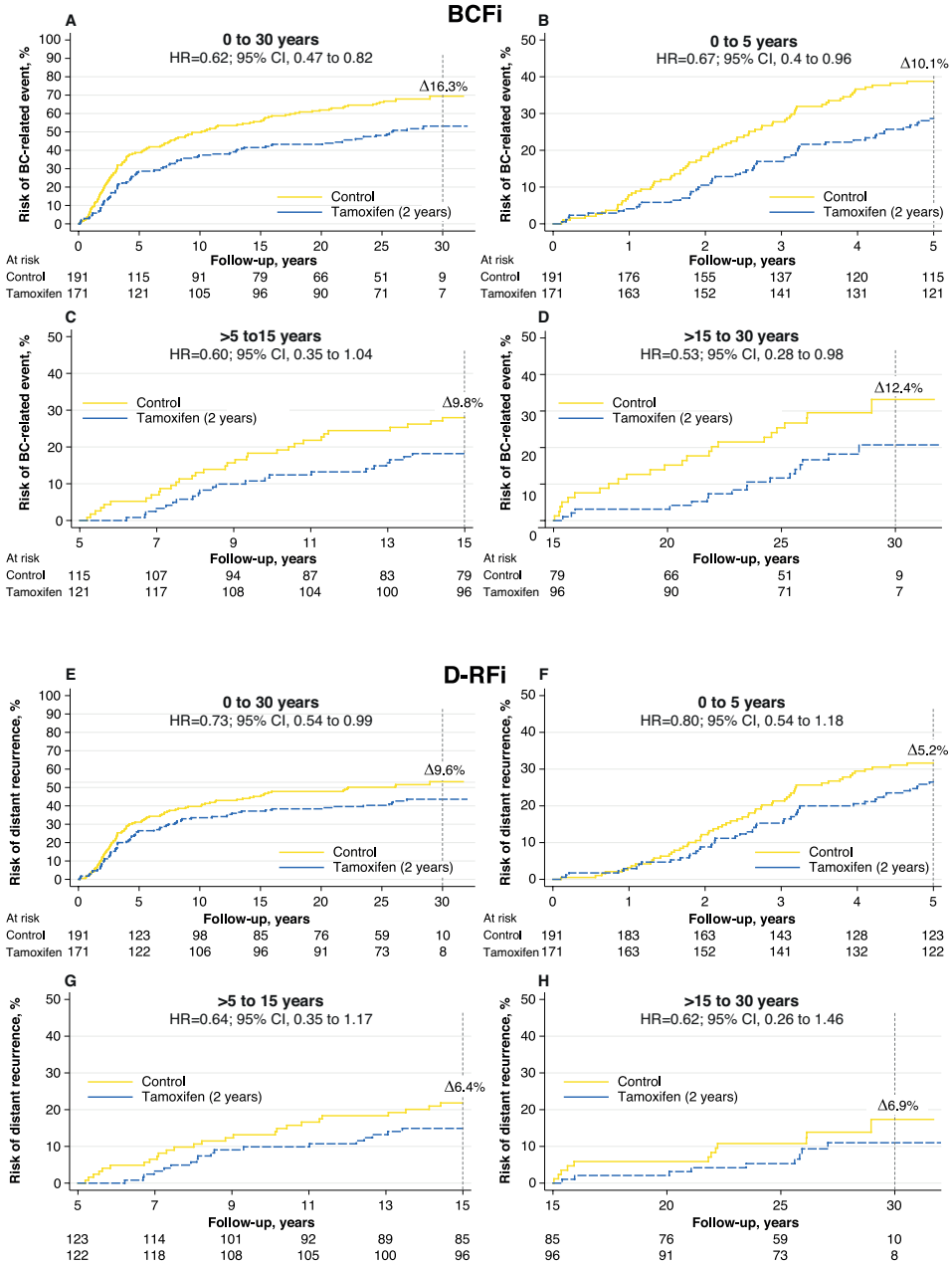


Figure 17. Cumulative incidence curves for breast cancer-free interval (BCFi) and distant recurrence-free interval (D-RFi) according to treatment arm for patients with oestrogen receptor (ER)-positive tumours at 30 years of follow-up (A, D) and for specified time intervals: 0–5 years (B, F), > 5–15 years (C, G), and >15–30 years (D, H). Δ is defined as the absolute difference in percentage units between the two arms at 5, 15, and 30 years. BC, breast cancer; HR, hazard ratio; CI, confidence interval.

Subgroup analyses for BCFi in patients with ER-positive tumours

The homogeneity of treatment effect across groups defined by prognostic factors (age, nodal status, tumour size, and histological grade) was analysed using Cox regression models with interaction terms. No significant interaction was found for any of the factors. The subgroup analyses are illustrated in Figure 18, together with the HRs.

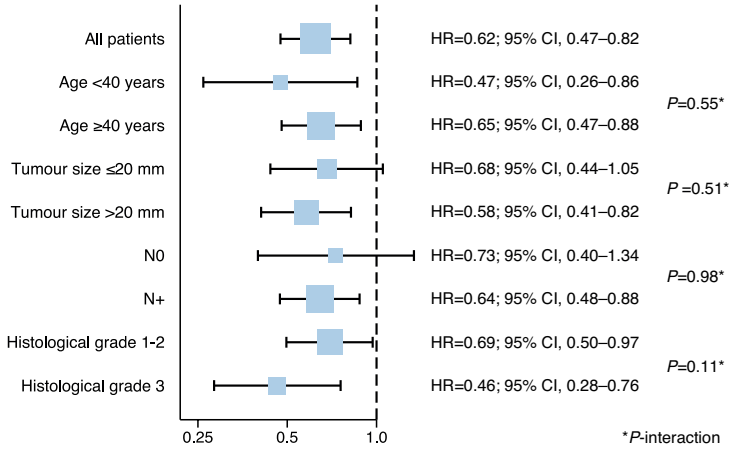


Figure 18. Forest plot showing subgroup analyses of tamoxifen vs. control regarding breast cancer-free interval (BCFi) in patients with oestrogen receptor (ER)-positive tumours at 30 years of follow-up. HR, hazard ratio; CI, confidence interval.

D-RFi in patients with ER-positive tumours, for the complete follow-up time and for specified time intervals

Tamoxifen prolonged D-RFi at 30 years of follow-up (HR=0.73; 95% CI, 0.54–0.99; *P*=0.043) and was associated with a non-significant increase of D-RFi for the separate time intervals: 0–5 years: HR=0.80; 95% CI, 0.54–1.18; *P*=0.25; >5–15 years: HR=0.64; 95% CI, 0.35–1.17; *P*=0.15, and >15–30 years: HR=0.62; 95% CI, 0.26–1.46; *P*=0.27 (Table 14 and Figure 17). We also performed a post-hoc analysis for D-RFi, in which the censoring of the follow-up for patients with contralateral breast cancer was omitted (n=35), and found marginally greater benefit for tamoxifen (HR=0.70; 95 % CI, 0.52–0.94; *P*=0.017).

Impact of adjuvant tamoxifen on survival after distant recurrence

Among the patients in the ER-positive subgroup who were diagnosed with distant recurrence (n=165), the median survival after distant recurrence was 29 months for patients in the tamoxifen group compared with 43 months in the control group. This corresponded to a 52% higher mortality for patients who received adjuvant tamoxifen (HR=1.52; 95% CI, 1.10–2.10; *P*=0.012). Kaplan–Meier estimates of OS after distant recurrence are illustrated in Figure 19.

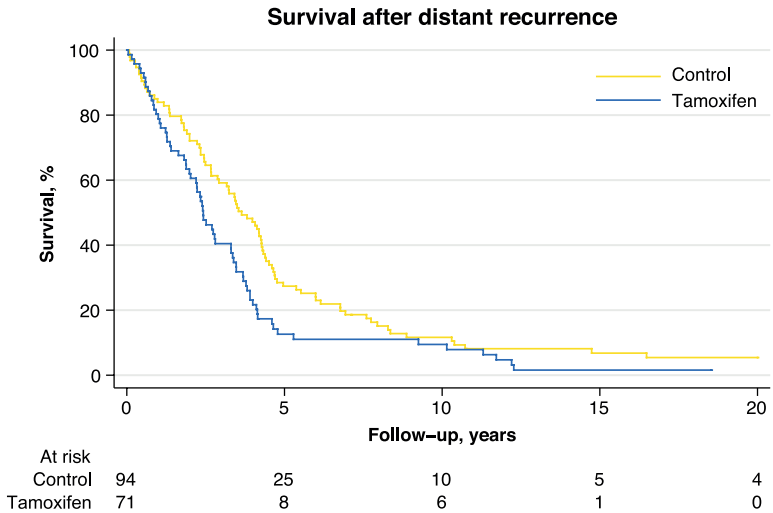


Figure 19. Kaplan–Meier estimates of overall survival after distant recurrence in patients with oestrogen receptor (ER)-positive primary tumours, according to treatment arm.

Surviving patients with no evidence of disease after distant recurrence

During the review of the medical records, two patients who were found to be alive with no evidence of disease despite that they were previously diagnosed with distant recurrence. Both patients had tumours that were negative for ER and PR. The first patient was diagnosed with lymph node metastases in the contralateral fossa/neck in 1990, confirmed by cytology. She was treated with high-dose cyclofosfamide and adriamycin for 6 months to complete remission. She had no evidence of disease at the last registered follow-up at the Department of Oncology in Gothenburg in May 2016 (visit due to an anal cancer). The second patient was diagnosed with bone metastasis and lymph node metastases in the contralateral fossa in 2002, the latter confirmed with cytology. She was treated with farmorubicin, capecitabine and a taxane for 6 months to complete remission. At her last follow-up at the Department of Oncology in Lund in June 2016, she had no evidence of disease.

Contralateral breast cancer and secondary non-breast malignancies

The frequency of secondary malignancies was investigated in the whole cohort (n=560), according to treatment arm. The incidence of contralateral breast cancer was reduced by 42% in tamoxifen-related patients compared with the control group, whereas there were no significant differences regarding the incidence of secondary non-breast malignancies (Table 15).

Table 15. Frequency and incidence of contralateral breast cancer and secondary malignancies (non-breast) in the control group and the tamoxifen group (all patients).

	Control group (n=284)		Tamoxifen group (n=276)	
Secondary malignancy	n	Incidence per 1000 patient-years	n	Incidence per 1000 patient-years
Contralateral breast cancer	39	8.65	24	5.02*
Secondary non-breast malignancy**	39		41	
Endometrial cancer	4	0.83	2	0.40
Ovarian cancer	3	0.63	5	1.01
Lung cancer	7	1.44	5	0.99
Gastrointestinal cancer	9	1.85	9	1.79
Urological cancer	2	0.41	5	0.99
Hematologic malignancy	3	0.62	3	0.59
Skin cancer	6	1.24	7	1.40
Other malignancy	5	1.04	5	0.99

*Hazard ratio=0.58; 95% confidence interval, 0.35–0.96; *P*=0.035
**Patients may have more than one secondary malignancy, but the same type of malignancy was only included once.

Discussion

Multi-gene assays are increasingly used for prognostication in primary breast cancer. However, due to the high costs, these tests are not available for most patients. Instead, decisions regarding adjuvant systemic therapy to a high extent rely on standard biomarkers, and it is therefore of utmost importance to ensure the accuracy of these analyses. Comprehensive international and national guidelines for analysis of hormone receptors and HER2 are aviliable.[101,102,193,208] In 2010, 'The International Ki67 in Breast Cancer Working Group' published a paper on proposed guidelines for Ki67 assessment.[153] However, these guidelines never became fully established. This group has since conducted a series of studies aiming to reduce the interlaboratory variability and improve the evaluation of Ki67.[161,162,356] Adherence to established biomarker guidelines is very important, however; it is equally important for laboratories to also participate in external QA programs to identify potential deficiencies in their analyses.

Study I

Despite the lack of consistent guidelines for Ki67 analysis and the fact that different cut-off values are used to differentiate between tumours with high and low proliferation, Ki67 is a strong prognostic marker in primary breast cancer.[154] In Study I, we therefore aimed to improve the Ki67 methodology by using the newer RabMAb SP6 for immunohistochemical staining. This antibody was chosen because RabMAbs generally have higher sensitivity without loss of specificity compared with their corresponding mouse mABs.[194,195] Moreover, Zabalgo and co-workers had previously reported that SP6 (RabMAb) was comparable to MIB1 (mouse mAB) for visual analysis of Ki67 but substantially better suited for image analysis because of less background staining.[357] We therefore hypothesised that SP6 would provide a more distinct staining, thereby making it easier to distinguish positive and negative nuclei, and that the reproducibility of the scoring between different assessors would be better for Ki67(SP6) than for Ki67(MIB1). Moreover, we expected that the prognostic value for Ki67 would be similar for the two antibodies.

We did not, however, find that the reproducibility between assessors was superior for Ki67(SP6) compared with Ki67(MIB1). High Ki67 was associated with a poorer

prognosis irrespective of the antibody used, but this was not significant in the multivariable analysis. We therefore concluded that SP6 was not superior to MIB1, but that the two antibodies were comparable for Ki67 analysis in primary breast cancer. The intensity of the staining was not assessed, but one of the assessors (KL) found the SP6 staining more distinct.

A major strength of this study is that SP6 was evaluated in a cohort of patients who were mainly untreated. Evaluation against clinical outcome is essential to be able to study the clinical utility of prognostic biomarkers.[102] We also confirmed that the cut-off defining high Ki67 may vary between different antibodies, which has also been shown by others.[358,359] Moreover, we showed that the cut-off for defining high Ki67 may vary between assessors, which emphasises the advantage of digital image analysis for Ki67 assessment to overcome the interobserver variability.[209,210,212,214]

TMA has been proven reliable for Ki67 assessment in primary breast cancer.[222,223] However, a TMA core only represents a small portion of the tumour and the biomarker expression may differ at different levels due to tumour heterogeneity. Because the antibody stainings were not performed at the same time, several sections had been cut between the sections that were stained using MIB1 and the sections that were stained using SP6, which may have affected the Ki67 results for the two antibodies. Another limitation is that the pathologist (DG) did not count Ki67-positive cells, but instead estimated Ki67 in percentage intervals; ≤1%, 2%–5%, 6%–10%, 11%–20%, 21%–30%, 31%–40%, 41%–50%, 51%–60%, 61%–70%, 71%–80%, 81%–90%, and 91%–100%. Pathologists often claim the inaccuracy of reporting Ki67 as an exact percentage figure, as there is a great insecurity in the scoring. However, the categorisation used by DG in this study resulted in slightly different proportions of high proliferating tumours for the two antibodies in the prognostic analyses. We were also not able to detect any difference in the cut-off values between the two antibodies for DG. The fact that it had been 3 years between the assessments for the two antibodies, may also have affected the results for DG and KL. In addition, the commonly used cut-off of >20% for defining high Ki67 is used to separate luminal A-like tumours from luminal B-like tumours in the ER-positive/HER-negative subgroup.[134,247] In this study, however, HER2-positive and the triple negative tumours were also included, which are both known to have higher degree of proliferation than luminal tumours.[360] For the prognostic analyses, it would have been more correct to exclude the HER2-positive and triple negative tumours. This would, however, have resulted in a cohort too small to be able to detect any statistical differences.

In a study recently published on behalf of the German Breast Screening Pathology Initiative, a TMA set of breast cancer tumours was sent out to 30 pathology departments for Ki67 staining according to their in-house protocol.[361] Seventy matched samples were then centrally assessed by one observer. The authors found considerable differences in median Ki67 values and the proportion of luminal A-like tumours

between the different laboratories. These differences remained significant when the laboratories using the same antibody (MIB1, 30-9, or SP6) were analysed separately. Furthermore, the study demonstrated that the variance for differences between the laboratories was higher than the differences between the different antibody clones.[361]

To conclude, the final result in a Ki67 analysis is dependent on each step of the pre-analytic phase, the analytic phase, and the interpretative phase. It is important to strive for optimising all of these steps, including the choice of antibody; however, for Ki67 analysis, the interpretative phase seems to have the largest impact on the final results. The best way forward may be to standardise common guidelines and to use laboratory-specific thresholds for Ki67 in combination with image analysis for scoring to obtain robust and reliable results for clinical use. Laboratory-specific cut-offs should preferably be clinically validated against patient outcome.[153,361]

Study II

Study II was undertaken to investigate the quality of Swedish biomarker results analysed in clinical routine, and we could show an excellent agreement for all pairwise comparisons of ER, PR, and final HER2 status (Table 10). Ki67 is known to be associated with poor reproducibility and therefore it was surprising that the overall agreement for this marker was substantial for all three comparisons (Table 10). For Ki67, the majority (>90%) of the discrepant tumours were located near cut-off (15%–25%), which is a problem often seen when a continuous variable is dichotomised.[159] As several studies, including Study I, have shown that different antibodies may render different cut-offs for Ki67, we were quite surprised by the fact that agreement was impaired when applying laboratory-specific cut-offs for Ki67.[358,359] Compared with the internationally established cut-off of >20% to define high Ki67, the cut-offs reported by the Swedish laboratories were generally higher (Table 5). This raised the question of the accuracy of the Swedish definition of high Ki67, which by then was defined as the 67th decile based on the first 100 breast carcinomas each year, irrespective of subtype.[348] As Ki67 is primarily used to separate luminal A-like from luminal B-like tumours, Ki67 guidelines should be based only on ER-positive/HER2-negative tumours. In addition, the former Swedish strategy was associated with statistical uncertainty, as the cut-off was based on only 100 cases.

The different breast cancer subtypes are generally recommended different adjuvant systemic therapy and to illustrate the clinical consequences of poor biomarker analyses, we also examined the agreement between the different breast cancer subtypes according to the St. Gallen 2013 classification.[157] As the agreement for the subtype classification was 88%, 12% of the patients would have been recommended different adjuvant systemic therapy if the tumour had been analysed at IEO compared with in Sweden. The majority of the discrepant cases were classified differently between the luminal A-like and the luminal B-like subtypes as a result of Ki67 values located around the cut-off. If we had been able to take other prognostic factors (e.g. T, N, and HG) as well as age and co-morbidity into account, the discrepancy regarding treatment recommendations would most probably have been substantially less than 12%.

A major strength with this study is that the Swedish samples (LA) were assessed as part of clinical routine and the pathologists had no knowledge of the subsequent central testing. The results can therefore be considered more reliable than in other QA trials, in which the laboratories are informed that their results will be subject to comparison. Most external quality assurance work performed by large organisations, like NordiQC and UK NEQAS, focus on the analytical phase by circulating tumour tissue sections for local staining at the different laboratories. By this approach, it is possible to detect differences in staining quality and to relate these differences to the different protocols, platforms, and antibodies used.[215,216,362] As highlighted by Focke et al., laboratories most

often optimise their immunohistochemical staining protocols to the pre-analytic protocols, and differences observed in external QA runs may therefore at least partly be explained by an interaction between the pre-analytic and analytic conditions.[361] There are also QA schemes comparing scoring results from centrally stained slides.[363] An additional strength of Study II is the study design, which enabled us to compare the analytical differences (RA vs. IEO), the scoring methods (LA vs. RA) as well as the overall comparison (LA vs. IEO) (Figure 11). We found that the discrepant results for ER and PR were mostly related to the analytic phase, while differences regarding HER2/IHC and Ki67 could be attributed to differences in the scoring. Another important finding was that the antibody used at 13 of the Swedish laboratories (Ventana 1E2 RTU kit) was found to be associated with false positive PR values and generally higher PR levels, compared with the PharmDX kit (clone PgR1294) used at IEO. Similar findings have been reported in other QA runs.[364,365] This emphasises the importance of participating in external QA programs, also for well-established biomarkers.

The high agreement for Ki67 found in this study has not been confirmed in other Ki67 QA trials.[361,366] As described in 'Clinicopathological surrogate definitions for the intrinsic subtypes' and further discussed in 'Future Perspectives', it is more reasonable to classify Ki67 into three categories (low, intermediate, or high) and not rely only on a Ki67 value in the intermediate range when making treatment decisions, but also use other markers.

In summary, as biomarker results form the basis for treatment decisions in breast cancer, participation in QA schemes is an important part of the breast cancer care, also for markers such as ER and PR. This is also highlighted in several guidelines.[102,105,367] To be able to evaluate both the analytical phase and the interpretative phase, the study design used in Study II should be more frequently used by others in future QA trials.

Study III

In Study III, we showed that 2 years of adjuvant tamoxifen significantly reduced the CBCM in the ER-positive subgroup after 26 years of follow-up. Considering the long-term follow-up, there is an increasing risk for non-breast cancer deaths among patients, and it was therefore not surprising that the primary endpoint, OS, did not reach statistical significance. The beneficial effect of tamoxifen was marginally better for patients with tumours positive for ER and PR. That PR is predictive for tamoxifen efficacy has been shown in several previous studies, including in this cohort.[137-139,141] This contrasts with findings in the EBCTCG overview from 2011, in which PR was not shown to add any predictive information over ER at 10 years of follow-up.[112] However, PR-negativity is known to be associated with a poorer prognosis and it is possible that the predictive value of PR is confounded by the widespread use (around 50%) of chemotherapy in this subgroup.

There are only a few other tamoxifen studies with a follow-up time exceeding 20 years. In the Swedish STO-3 study, postmenopausal women with node-negative breast cancer were randomly assigned to 2 years of tamoxifen or no treatment and those without recurrence after 2 years were further randomised to receive additional 3 years of therapy or to stop. By using an ultralow threshold for MammaPrint, Esserman and colleagues were able to identify a subgroup of patients with a disease-specific survival rate of 97% after 20 years of follow-up.[368] The Danish Breast Cancer Group (DBCG) reported long-term results from a study that had randomised high-risk postmenopausal patients with primary breast cancer to receive either tamoxifen (10 mg × 3 for 1 year) or no treatment.[369] After a median follow-up of 30 years, tamoxifen was associated with a reduction of the CBCM for patients with ER-positive tumours (re-assessed by IHC) (HR=0.62; 95% CI, 0.47–0.83; *P*=0.001). Competing risks were also taken into consideration and these results are in line with what was found in Study III. DBCG also published long-term results from a study comparing 2 years of tamoxifen (10 mg × 3) vs. no systemic therapy, with a median follow-up of 40 years.[370] Premenopausal and postmenopausal patients were included irrespective of hormone receptor status (n=317). All patients underwent mastectomy without any axillary dissection and therefore the nodal status for the included patients was not known. The hormone receptor status was determined by cytosol-based methods and ER status was only available for approximately 60% of the patients. After adjustment for baseline characteristics, tamoxifen was found to improve survival (HR=0.79, 95% CI, 0.63–0.99; *P*=0.04).

Study III has several strengths. First, it is a randomised trial in which the vast majority of the patients in the control arm were systemically untreated, allowing comparison with the natural cause of the disease. Second, Study III is one of the few tamoxifen studies that included only premenopausal patients; this is important, as tamoxifen is

still the most commonly used endocrine therapy in this group. Third, most of the tumours have been re-assessed for ER, PR, and HER2 using IHC and ISH, which are the methods currently used in clinical routine. Finally, the follow-up data were based on the Swedish Causes of Death Register, which has been shown to provide reliable data on causes of death in patients with breast cancer.[371,372]

There are also limitations in this study. First, the study was not powered to detect treatment effects in smaller subgroups of patients, and therefore we chose to focus on the ER-positive subgroup. In addition, only 67% of the patients with known hormone receptor status were defined as ER-positive, which is considerably lower than what is usually seen today.[3] This may partly be explained by the fact that cytosol-based data were used for the determination of ER-status in patients lacking IHC data (n=91, 16%), and this method classified even fewer patients as ER-positive. Furthermore, the proportion of ER-positive tumours is smaller in premenopausal women. The remaining discrepancy may be explained by better pre-analytical conditions and that the immunohistochemical techniques has improved. Moreover, the two healthcare regions used different tamoxifen doses (20 mg and 40 mg); however, these doses have been shown to be associated with similar benefits.[112,278] We chose to stratify for region in the statistical analyses to adjust for this as, well as for the different cytosol-based methods used for hormone receptor analyses.

In Study III, we stated that no patients were lost to follow-up. However, during the review of the medical records for Study IV, it was noted that three patients had emigrated and their follow-up should therefore have been censored at the time of emigration. The correct approach would have been to check the cohort against the Swedish Population Register before including all patients in the mortality analyses. When this was noted, the statistical analyses used in Study III were repeated and the results were not altered.

Study IV

Mortality is often considered the final endpoint in adjuvant studies, whereas endpoints like RFS and D-DFS are considered surrogate endpoints.[342-344] Although we presented long-term mortality data in Study III, it is also clinically important to report long-term results on breast cancer-related events such as local recurrence and contralateral breast cancer. Prevention of these events increases the chance of breast preservation and avoidance of additional adjuvant therapy, which is important for the individual patient. Because late recurrences are common in ER-positive breast cancer, long-term follow-up is important for this subgroup, particularly in premenopausal patients with long life expectancy.[48,116] Moreover, long-term follow-up is important in studies including drugs with possible 'carryover effects', such as tamoxifen. We therefore decided to review medical records and retrieve complementary data from national registers to update the SBIIpre study regarding all breast cancer-related events.

In Study IV, we focused on the ER-positive subgroup and found that at 30 years of follow-up, the patients in the tamoxifen arm had a 38% reduced incidence of breast cancer-related events, compared with those in the control arm. Tamoxifen was also shown to significantly reduce the incidence of distant recurrences by 27%. The benefit on BCFi was found significant also for the interval >15–30 years, indicating a long-lasting 'carryover effect' beyond 15 years, and to our knowledge, this has never been shown before. In the EBCTCG overview from 2011, no benefit from tamoxifen could be seen beyond 15 years.[112] However, a possible explanation may be that long-term follow-up for breast cancer-related events is inadequate in most of the included studies. Personal identity numbers and the high quality national registers are important to be able to carry out long-term follow-up, and therefore this is hard to achieve outside the Nordic countries.

Tamoxifen is the most commonly recommended adjuvant endocrine treatment in premenopausal women, whereas AIs have shown superiority over tamoxifen in postmenopausal patients.[317,321,373] According to the EBCTCG overview from 2015 comparing tamoxifen with AI treatment, the superiority in treatment efficacy for AIs was not significant after cessation of treatment.[321] Long-term follow-up results from the ATAC/LATTE and BIG1-98 studies, presented as abstracts at the San Antonio Breast Cancer Symposium 2016, also failed to show significant advantages for AI over tamoxifen regarding breast cancer events, with longer follow-up.[319,320] Considering these preliminary results, long-term follow-up of studies comparing tamoxifen and AIs is important.

Another interesting finding was that adjuvant tamoxifen was associated with shorter survival after distant recurrence in patients with ER-positive breast cancer. The median survival was 29 months in the tamoxifen group compared with 43 months in the control group. Based on register data, Kleeberg and colleagues found that adjuvant

chemotherapy was associated with shorter survival after distant recurrence, a phenomenon they referred to as ATRESS (adjuvant therapy-related shortening of survival).[374,375] ATRESS associated with chemotherapy has been repeatedly reported, mostly based on retrospective analyses including patients with metastatic breast cancer.[376-379] There are two previous studies on postmenopausal patients with recurrent disease that reported a negative impact on survival after adjuvant tamoxifen therapy.[380,381] A theoretical explanation may be that tamoxifen treatment given as adjuvant therapy eliminated endocrine-sensitive tumour cells and that subsequent metastases in tamoxifen-treated patients had a more adverse biology. Lindstrom et al. demonstrated that patients with ER-positive tumours treated with adjuvant endocrine treatment to a greater extent tended to have ER-negative metastases, contributing to an adverse outcome.[293] In the present study, distant recurrences were not routinely biopsied for most of the patients and we are therefore not able to provide information on the biology of the metastases. Despite these findings, the benefit of adjuvant endocrine therapy is indisputable and it improves OS, also after long-term follow-up.[112, Study III] However, based on our finding that adjuvant tamoxifen affected survival after distant recurrence, one should take into account the fact that previous adjuvant endocrine therapy may have a large effect on survival in advanced breast cancer trials. Moreover, it may be important to investigate what effects sequential endocrine therapy, extended endocrine therapy, and chemoprevention with tamoxifen may have on survival after distant recurrence.

As adjuvant therapy is given to prevent recurrence and death from a disease, it is important that the benefit from the drug is not negatively counterbalanced by an increased risk of other malignancies. Tamoxifen is associated with an increased risk of endometrial cancer,[112,289] but on the other hand, tamoxifen has been shown to reduce the incidence of lung cancer and contralateral breast cancer.[286,289] In Study IV, we could confirm that the incidence of contralateral breast cancer was considerably reduced for patients in the tamoxifen arm, whereas there were no obvious differences regarding the incidences of secondary non-breast malignancies (Table 15).

In prospective studies, there are strict protocols that cover all events and other details of interest; however, no studies include a pre-planned follow-up over several decades. For long-term follow-up of this kind, it is therefore necessary to review medical records and to collect information from available registers to find the information of interest. The audit preceding Study IV covered a follow-up time of more than 32 years, from the first patient included in 1984 to the data cut-off on November 30, 2016. The data collection was rather demanding, and for some patients, it was necessary to carefully review medical records from different departments, radiology and pathology reports, as well as correspondence between different departments/hospitals.

The greatest strength of this study is the high quality of the in-depth long-term follow-up data on breast cancer-related events. As the review was performed by only

one person (me, a medical oncologist), a consistent interpretation of data was guaranteed. In cases of uncertainty, patients were discussed within the study group without revealing information on the treatment arm. The previously mentioned DBCG studies only reported long-term follow-up on mortality, whereas the endpoint including breast cancer-related events was restricted to less than 10 years of follow-up.

In addition to the limitations discussed for Study III, four cases of protocol violation were identified during the review, and these patients were therefore excluded in Study IV. Moreover, one patient was transferred from the tamoxifen group to the control group due to previous data entry error. The number of patients and the size of the two treatment groups in Study IV therefore differ from Study III and the previous published papers from this cohort. During the audit, I reflected on the differences regarding good clinical practice used in the 1980s compared with current standards. While it is impossible to know what information patients orally received, it was apparent that the procedure was less developed and not as extensive as today.

Although current guidelines for premenopausal patients recommend 5–10 years of tamoxifen, sometimes with addition of OFS, and sometimes even OFS in combination with an AI, the results from Study III and IV are important. First, the results indicate that the positive effect on breast cancer-related events associated with tamoxifen is maintained over a long time. This finding is particularly important for younger patients with ER-positive tumours, as these patients have long life expectancy with an increased risk of late relapses.[112,116] Second, although >5 years of endocrine therapy is preferable, 2 years of adjuvant tamoxifen provides benefit regarding breast cancer-related events and survival.[Study III] Our results may therefore encourage patients experiencing difficulty in tolerating endocrine therapy to adhere to tamoxifen treatment for at least 2 years. Third, the evidence of the negative impact of adjuvant endocrine therapy on survival in the metastatic setting should be getting more attention, for example in advanced breast cancer trials. Finally, 2 years of tamoxifen also reduced the incidence of contralateral breast cancer. These findings are clinically relevant for young patients with an expected lifetime of decades, for which even a reduction in local recurrence and contralateral breast cancer contributes to a reduced risk of subsequent breast surgery and an improved overall outcome.

Conclusions

The specific conclusions from this thesis are as follows:

- The use of the SP6 antibody for immunohistochemical staining of Ki67 did not improve reproducibility between different assessors (Ki67 high vs. low) compared with the MIB1 antibody, and these antibodies are comparable for prognostication of DDFS in node-negative patients with primary breast cancer
- High quality in terms of agreement with the IEO results was seen for all biomarkers (ER, PR, final HER2 status, and Ki67) when analysed at Swedish pathology departments as part of clinical routine. The agreement worsened when laboratory-specific cut-off values were used for Ki67
- The use of the Ventana 1E2 RTU kit for PR staining may be associated with false PR-positivity and generally higher values of PR, emphasising the importance of participating in external QA programs, for PR as well as for other well-established biomarkers
- After a median follow-up of 26 years, 2 years of adjuvant tamoxifen reduced the risk of breast cancer-related death by 27% compared with no systemic treatment in premenopausal patients with ER-positive tumours
- In premenopausal patients with ER-positive tumours, 2 years of tamoxifen reduced the incidence of breast cancer-related events by 38% compared with no systemic therapy at 30 years of follow-up. Tamoxifen also reduced the incidence of distant recurrence in this subgroup by 27% compared with no systemic therapy. The beneficial effect on breast cancer-related-related events was significant also for the interval >15–30 years, indicating a persistent long-term 'carryover effect' of tamoxifen
- In ER-positive patients, tamoxifen was associated with shorter survival after distant recurrence compared with patients who received no systemic adjuvant therapy (median, 29 vs. 43 months)
- In all patients, the incidence of contralateral breast cancer was 42% lower in patients treated with 2 years of adjuvant tamoxifen compared with those in the control arm, whereas no significant differences were seen regarding the incidences of secondary non-breast malignancies

Future Perspectives

Validation of the new Swedish guidelines for surrogate subtype classification

The finding that the agreement for Ki67 was not improved when using laboratory-specific cut-offs in Study II made us question the Swedish Ki67 guidelines at that time. Moreover, a significant proportion of the Ki67 values are located near cut-off. Therefore, we proposed new guidelines for the surrogate classification of the breast cancer subtypes. Briefly, these guidelines are based on the publication from Maisonneuve and colleagues,[247] with the addition of HG, as described in the chapter 'Breast Cancer Subtypes'. Ki67 is classified into three categories and the laboratory-specific cut-offs are calculated from Ki67 data for ER-positive/HER2-negative tumours in the Swedish Breast Cancer Registry. For patients with intermediate Ki67, information on PR expression is included in the final estimation of the subtype. Moreover, HG is used as a control function to determine the final subtype (Figure 20).

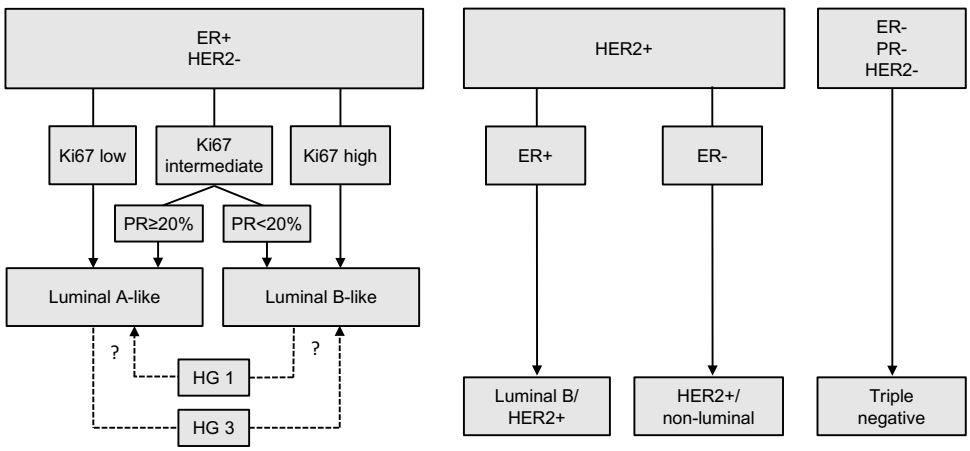


Figure 20. Surrogate subtype classification according to the Swedish guidelines, 2018.[249] ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HG, histological grade; PR, progesterone receptor.

There are several previously published papers that support the use of Ki67 in three categories. Denckert and colleagues emphasises that there is no optimal cut-off for Ki67, as it is a continuous variable. Moreover, they underline that treatment decisions should not rely on Ki67 in the intermediate range.[154] Shui et al. published a paper in which 160 breast cancers were scored in 10% intervals by five pathologists. When the intervals were categorised into three groups (≤10%, 11%–30%, and >30%), the authors could demonstrate a perfect correlation for the Ki67 low group and a substantial correlation for the Ki67 high group, whereas there was only a moderate correlation for the Ki67 intermediate group.[382] In another Ki67 QA trial that included up to 160 laboratories from 2010 to 2015, TMA slides from different tumours, including breast cancers, were distributed for staining and scoring according to local guidelines.[363] The Ki67 values were categorised into four different intervals (≤10%, >10%–15%, >15%–24%, and ≥25%) and the concordance was very good for the two groups with low and high Ki67, whereas there were considerably more discordant cases in the intermediate groups.[363] Moreover, Hida and colleagues evaluated the Ki67 'Eye-5 method', based on the estimated ratio of positive/negative cells. Patients with tumours scored as 1–2 had a significantly better RFS and OS compared with patients with tumours scored as 3–5. Moreover, by adding HG for tumours scored as 2–3, the intermediate group could be reduced from 62% to 26%.[383]

The new Swedish guidelines have been validated in two smaller patient cohorts (data not shown). However, as these guidelines will to a great extent influence the proportion of patients that will be recommended adjuvant chemotherapy after primary breast cancer, we have received permission to validate the new subtype classification guidelines in the Sweden Cancerome Analysis Network – Breast study (SCAN-B). We plan to compare the updated subtype classification with the PAM50 results for patients included in the SCAN-B project. The results will tell us whether the updated guidelines will be able to safely classify tumours as luminal A-like and luminal B-like, and if not, to what extent gene expression analyses will be needed to separate these two subgroups.

Further improvement of the surrogate subtype classification

The multi-gene assays, used for prognostication in primary breast cancer, include several genes related to proliferation.[229,250,255] In contrast, uPA and PAI-1 have been shown to provide prognostic information independent of proliferation in primary breast cancer.[147,384] As uPA is involved in cancer dissemination, the addition of this marker to the subtype classification may contribute additional important biologic information regarding the metastatic potential of the tumour.[172] Furthermore, uPA has been proven to be predictive for the efficacy of adjuvant chemotherapy.[173-176] A reasonable hypothesis would therefore be that the ability to predict outcome based on surrogate classification for the intrinsic subtypes, currently including ER, PR, HER2,

Ki67, and to some extent HG could be improved by the addition of uPA. To our knowledge, this has not previously been done, probably as the analysis requires fresh frozen tumour tissue. However, during recent years, data demonstrating good agreement between IHC in combination with image analysis and quantitative ELISA for uPA determination have been presented.[177] We therefore aim to compare uPA and PAI-1 assessed with IHC with the commonly used ELISA method in the same cohort as used in Study I. If we succeed, a future project would be to find a cohort in which the addition of uPA and PAI-1 to the subtype classification could be evaluated.

Personalised and response-guided treatment

The St. Gallen consensus statement from 2017 emphasises the importance of escalating and de-escalating treatments for early-stage breast cancer.[105] For several decades, an increasing proportion of patients have been recommended adjuvant therapy. Moreover, adjuvant chemotherapy often now includes both anthracyclines and taxanes, whereas endocrine treatment may be recommended for up to 10 years.[103,105] Within the field of breast surgery, increasingly smaller margins of tumour-free tissue have been accepted and omitting axillary dissection for patients with no palpable axillary adenopathy and ≤ 2 positive sentinel nodes is now considered safe in several countries.[385] The increased usage of neoadjuvant therapy has, among other things, led to more patients being eligible for BCS. Furthermore, the primary reason for the use of multi-gene assays is to identify patients with ER-positive/HER-negative tumours for whom adjuvant chemotherapy can be omitted.

To be able to de-escalate and escalate treatment in luminal breast cancers, I believe it is important to try to identify gene expression signatures that are prognostic for early and late recurrences. Even more important, there is a need for signatures that are predictive for systemic adjuvant therapy, including chemotherapy and different kinds of endocrine treatment. As has been demonstrated in this thesis, long-term follow-up is essential, at least in ER-positive breast cancer. Hence, I believe it is necessary to use previous randomised trials with long-term follow-up to identify these signatures. Esserman and colleagues have been able to identify a subset of patients in the STO 3 study with an excellent breast cancer-specific survival after > 20 years of-follow-up using an ultralow threshold of the 70-gene MammaPrint assay.[368] It may be possible to identify a group of patients among the premenopausal patients in the SBII2pre cohort who have very low risk of recurrence . We therefore plan to collect tumour tissue from the primary tumours for validated gene expression-based analyses with the aim to identify patients with a good long-term prognosis despite a rather advanced disease stage at diagnosis.

According to my personal opinion, neoadjuvant endocrine therapy is underused in luminal primary breast cancers. Besides, neoadjuvant chemotherapy is less effective in

low proliferating luminal breast cancers, compared with the other breast cancer subtypes.[386] In locally advanced tumours, it is therefore reasonable to start with the kind of therapy that is likely to give the greatest tumour shrinkage, i.e. endocrine therapy. This approach will also provide information on the *in vivo* response as well as the tolerability of the treatment. In case of insufficient response, it is possible to change therapy and re-evaluate the effect of the new therapy. If a therapy is going to be recommended for 5 or even 10 years, it is desirable to at least confirm that the primary tumour responds to the planned treatment. Moreover, the use of neoadjuvant endocrine therapy does not have to rule out the addition of adjuvant chemotherapy post-surgery in case of extensive disease or if it turns out that the patient does not tolerate endocrine treatment due to side effects. This is particularly important for the group that is recommended only endocrine treatment, because patients who disrupt their medication due to intolerable side effects otherwise risk being completely systemically untreated. Furthermore, I believe that neoadjuvant endocrine therapy is also suitable for small tumours, but perhaps 3 to 6 months may be sufficient for the smallest ones, whereas larger tumours should be treated until maximum response. Theoretically, the overall adherence may increase if the patient can see a clear tumour response related to the endocrine therapy.

If neoadjuvant endocrine treatment is to be used as a general strategy in luminal breast cancer, it will be necessary to continuously evaluate the treatment response, and probably not only by radiology. Dowsett and colleagues showed that the change in Ki67 after 2 weeks of endocrine treatment is prognostic for RFS.[156] The preoperative endocrine prognostic index (PEPI) score, based on tumour size, nodal status, ER, and Ki67, has also proven to be prognostic for RFS and breast cancer-specific survival.[155,387] Moreover, Larburu et al. presented a poster at the San Antonio Breast Cancer Symposium in 2017, where PAM50 was analysed at baseline and after neoadjuvant endocrine therapy, given for a median duration of 8 months.[388] The researchers found that the neoadjuvant therapy resulted in a change of the intrinsic subtype in 50% of the tumours. The correlation between the post PAM50/ROR and the PEPI score indicates that post PAM50/ROR may provide valuable prognostic information.[388]

In conclusion, the use of neoadjuvant endocrine therapy may reveal the tumours true capabilities for metastasis, recurrence, and death. This approach also enables change of therapy in cases of inadequate response, presence of a poor prognostic signature, or intolerability to endocrine therapy. However, in a recently published paper by Ellis and colleagues, the efficacy of subsequent chemotherapy was worse than expected in tumours with inadequate response to neoadjuvant endocrine therapy.[387] In the future, endocrine therapy may be combined with CDK4/6 inhibitors or other drugs targeting the PI3K/AKT/mTOR pathway, but the optimal therapy for these patients must be investigated in future clinical trials.

Liquid biopsies

Next generation sequencing has revealed heterogeneity between different tumour's in the same patient.[389] Moreover, single nucleus genome sequencing has also demonstrated a substantial heterogeneity between individual cells in the same tumour.[390] In addition, this heterogeneity evolves over time as the tumour grows and is under pressure from different treatments.[391] Quantification of the number of CTCs is a validated prognostic marker and change in CTC number is indicative of response to systemic therapy in metastatic breast cancer, whereas the role of characterisation of CTCs is to be settled.[392,393] In contrast, ctDNA, is mostly used to identify specific mutations in the tumour.[393] Liquid biopsies have several advantages compared with serial biopsies from one or a few metastatic lesions. First, a liquid biopsy is a non-invasive procedure that can be easily repeated. Second, the ctDNA and CTCs found in the circulating blood may better represent the tumour cells of interest. Third, liquid biopsies allow for continuous monitoring of the treatment efficacy.[393] It is now possible to isolate and sequence ctDNA in almost 90% of the patients with stage IV breast cancer and in approximately half of the patients with primary breast cancer.[394] I believe that in the future, we will use liquid biopsies for evaluation of treatment response, both in the adjuvant/neoadjuvant setting and during treatment for advanced disease. I also believe liquid biopsies will be used in the follow-up of primary breast cancer. An ongoing trial is investigating if the remaining ctDNA load following completion of neoadjuvant/adjuvant treatment is correlated to an increased risk of relapse, and moreover, if these patients benefit from additional therapy (ClinicalTrials.gov identifier: NCT03145961). A potential design for a future study could be to measure CTCs/ctDNA in patients with ER-positive high-risk tumours after completion of 5 years of endocrine therapy and to randomise patients without detectable CTCs/ctDNA between extended adjuvant endocrine therapy or to stop.

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