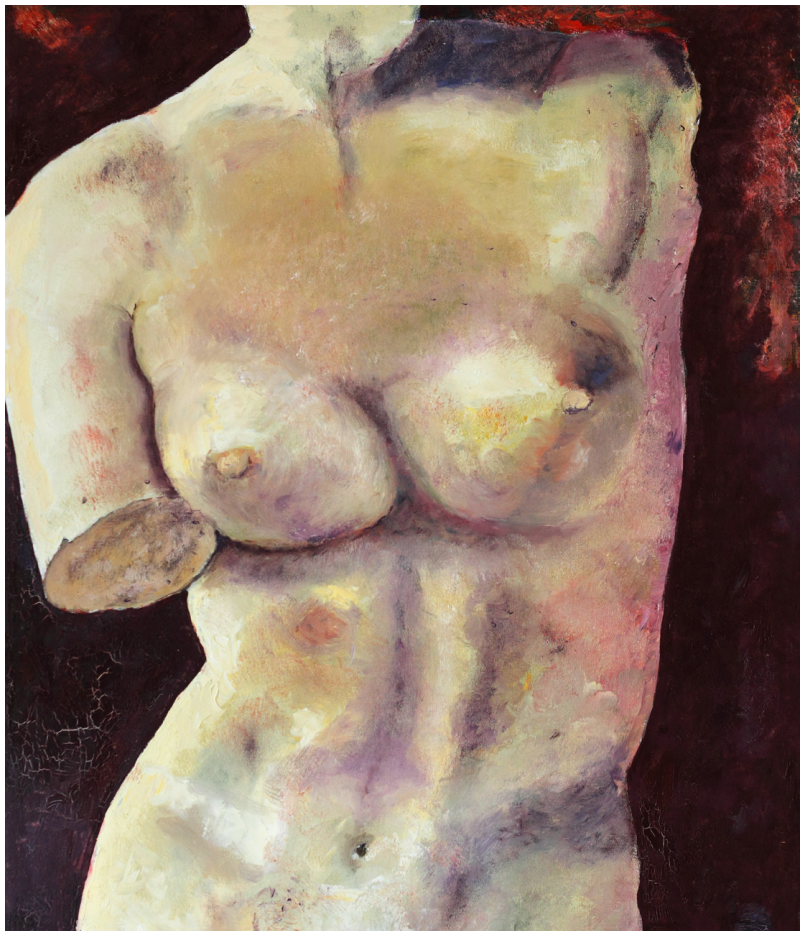
Thesis for doctoral degree (Ph.D.)
2017

Breast cancer in young women – aspects on mortality and local recurrence



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Karolinska Institutet

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Breast cancer in young women - aspects on mortality and local recurrence

av

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To women

ABSTRACT

The general aim of the thesis was to gain increased insight into the long-term prognosis for young women with breast cancer.

In a population-based cohort of 22,017 women with breast cancer, we studied prognosis by age. Women aged <35 (n=471), 35–39 (n=858) and 40–49 (n=4789) were compared with women aged 50–69. The cumulative 5-year relative survival ratio (RSR) and the relative excess risk (RER) of mortality were calculated. Women <35 years of age had a worse survival than middle-aged women, partly explained by a later stage at diagnosis. After correction for stage, tumor characteristics and treatment, young age remained an independent risk factor for death. The excess risk of death in young women was only present in stage I-II disease and was most pronounced in women with small tumors.

For in-depth studies on a large subpopulation from the original cohort (all 471 women aged <35 and a random sample of 700 women aged 35–69), we collected detailed data from the medical records, re-evaluated slides and produced TMAs from tumor tissue. Breast cancer-specific survival (BCSS), distant disease-free survival (DDFS) and locoregional recurrence-free survival (LRFS) by age were analysed. In a multivariate analysis, age <35 and age 35–39 years conferred a risk in LRFS but not in DDFS and BCSS. The age-related differences in prognosis were most pronounced in early stage luminal Her2-negative tumors, where low age was an independent prognostic factor also for DDFS (HR 1.87 (1.03–3.44)).

To study the importance of proliferation markers for the long-term prognosis in young women, protein expression of Ki-67, cyclin A2, B1, D1 and E1 was analysed in 504 women aged <40 and in 383 women aged ≥40. The higher expression of proliferation markers in young women did not have a strong impact on the prognosis. Proliferation markers are less important in young women, and Ki-67 was prognostic only in young women with Luminal PR+ tumors. Age <40 years was an independent risk factor of DDFS exclusively in this subgroup (adjusted HR 2.35 (1.22-4.50)). The only cyclin adding prognostic value beyond subtype in young women was cyclin E1.

In a cohort of 469 women aged <40 and 360 women aged ≥40 we examined whether Her2 status assessed by silver enhanced *in situ* hybridization (SISH) for all cases, would reveal a proportion of women undiagnosed by routine Her2 testing and whether this would affect their prognosis. With SISH testing for all women, the Her2-positive rate increased from 20.0% to 24.4% ($p<0.001$), and similarly for women aged <40 and ≥40 years. Young women had Her2+ breast cancer twice as often as middle-aged women. Her2 amplification was present in 4.6% of cases scored 0 with IHC, while the corresponding proportions for scores 1+, 2+ and 3+ were 36.0%, 83.7% and 96.8%, respectively. All Her2 amplified cases, both true positive and false negative, had a significantly worse BCSS than the true negative cases.

LIST OF SCIENTIFIC PAPERS

I. Fredholm H, Eaker S, Frisell J, Holmberg H, Fredriksson I*, Lindman H*. Breast Cancer in Young Women: Poor Survival Despite Intensive Treatment. PLoS ONE 2009; 4(11): e7695 (*contributed equally)

II. Fredholm H, Magnusson K, Lindström LS, Garmo H, Eaker Fält S, Lindman H, Bergh J, Holmberg L, Pontén F, Frisell J, Fredriksson I. Long-term Outcome in Young Women With Breast Cancer – a Population-based Study. Breast Cancer Res Treat 2016; 160(1): 131-143.

III. Fredholm H, Magnusson K, Lindström LS, Tobin NP, Lindman H, Bergh J, Holmberg L, Pontén F, Frisell J, Fredriksson I. Breast cancer in young women and prognosis – how important are proliferation markers? Submitted.

IV. Fredholm H, Pontén F, Hikmet Noraddin F, Bergh J, Frisell J, Holmberg L, Fredriksson I. Her2 positive breast cancer in young women – the prognostic impact of Her2 expression by immunohistochemistry versus silver in situ hybridization (SISH) in a population-based cohort. In manuscript.

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LIST OF ABBREVIATIONS

AI	Aromatase Inhibitor
BCS	Breast conserving surgery
BCSS	Breast cancer-specific survival
BI-RADS	Breast imaging reporting and data system
BMI	Body Mass Index
BRCA	Breast Cancer gene
CI	Confidence Interval
CISH	Chromogenic In Situ Hybridization
CBC	Contralateral Breast Cancer
CMF	Cyclophosphamide, Methotrexate and 5-Fluorouracil
DCIS	Ductal Cancer In Situ
DDFS	Distant disease free survival
EIC	Extensive Intraductal Component
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FFPE	Formalin Fixed Paraffin Embedded
FISH	Fluorescence In Situ Hybridization
GEP	Gene Expression Profiling
GnRH	Gonadotropin-Releasing Hormone
Her2	Human Epidermal growth factor Receptor 2
HR	Hazard Ratio
ICD	International Classification of Disease
IHC	Immunohistochemistry
LRFS	Locoregional-Recurrence Free
LR	Local Recurrence
LVI	Lymphovascular invasion
MRI	Magnetic Resonance Imaging
OFS	Ovarian Function Suppression
PABC	Pregnancy Associated Breast Cancer
PCR	Pathological Complete Response
PIN	Personal Identification Number
PR	Progesterone Receptor
QoL	Quality of Life
RER	Relative excess ratio
RSR	Relative survival ratio
SEER	Surveillance Epidemiology and End Results
SISH	Silver enhanced In Situ Hybridization
TAC	docetaxel, adriamycin and cyclophosphamide
TMA	Tissue Micro Array
TN	Triple-negative
vs	versus

BACKGROUND

Definition of young age

In the scientific literature, there are several definitions of young age in breast cancer: <35 years, <40 years, <50 years or premenopausal. In reality, it seems that age is a continuous variable and, the narrower definition applied, the larger will the age-related differences be. When diagnosed with breast cancer, women <35 and <40 years old share the same tumor characteristics and have a similar prognosis [1-3], whereas women 40-49 years old have the best survival rate from breast cancer [1,4]. Thus, a cut-off at age 50 will dilute the age-related differences. Age, rather than menopausal status, reflects the biological and aetiological entities associated with breast cancer in young women [5].

Epidemiology

The median age at a breast cancer diagnosis in Sweden is 63. The risk of being diagnosed with breast cancer (cumulative incidence) before age 75 is 10.5%, or 1 in 9.5 women. Breast cancer in young women is rare, with a cumulative incidence at age 45 of 1.1%, or 1 in 91 women [6]. In 2015, 417 women <40 years old (4.0% of all diagnosed cases) and 143 women <35 years old (1.5%) were diagnosed with breast cancer in Sweden [6]. That is in line with the rates in the Nordic countries as a whole (4.0%) [7], as well as in Europe (5.4%) and North America (5.6%) [8]. In South America, Asia and Africa, the longevity is shorter, which explains a larger proportion of young women with breast cancer in these parts of the world (**Figure 1**).

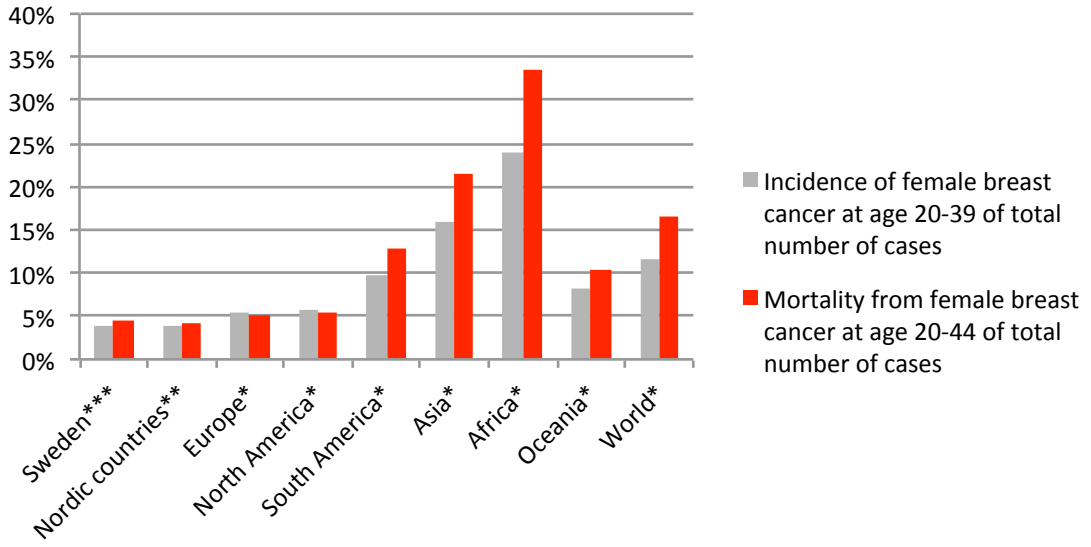


Figure 1. *Incidence of breast cancer at age 20-39 and mortality by geographic region. Grey bars indicate incidence, given as the proportion of all cases of female breast cancer. Red bars indicate mortality attributed to breast cancer, including a 5-year delay from diagnosis at age 20-44. *[8], **[7], ***[6,9]*

In Sweden, the incidence of breast cancer has increased by 1.6% per year during the last 20 years. For young women, however, there has been no increase, but rather a stabile incidence of 20-23 new cases per 100,000 women [6]. From a global perspective, there has been a dramatic increase in the incidence of breast cancer, with a rise of 43.0% from 2005 to 2015 [10]. This increase is due to population growth (12.6%), to change in age structure (15%) and to a true change in the incidence rate (14.9%). A study based on the Surveillance Epidemiology and End Results (SEER) data showed that between 2000 and 2009, the incidence of oestrogen (ER)+ breast cancer increased in women <40 years, while the incidence of ER- breast cancer decreased [11].

Despite breast cancer being rare in young women, it is the most frequent type of cancer diagnosed in this age group. In Swedish women aged 20-39, breast cancer accounted for 27.2% (386 of 1417) of all cancers diagnosed. The corresponding proportion for women of all ages was 31.4% (9730 of 30970) [6].

Although the incidence has increased over time, the mortality has decreased considerably over the last few decades due to early diagnosis and better treatment. In young women, however, breast cancer is still the leading cause of death from cancer and the third most common cause of death overall after suicide and road injuries [9].

Risk factors for developing breast cancer at a young age

Breast cancer in young women has a somewhat different panorama of risk factors to those in middle-aged women. Young women have a shorter exposure to risk factors associated with the disease, but they may instead be more sensitive to some risk factors. Many of these risk estimates concern young women in a wider perspective, defined as premenopausal women.

Previous chest radiotherapy

Women with previous radiotherapy at a young age (10-30 years) have about 6 times as high a risk to develop breast cancer as in the general population of young women [12,13]. They more often develop bilateral breast cancer and receptor negative tumors [12].

Family history

Family history is a strong risk factor for women <40 years old. The risk is higher with an increasing number of first-degree relatives with breast or ovarian cancer and if the relatives are young at diagnosis [14]. If a woman <40 years old has one relative with breast cancer before age 40, her risk is almost 6-fold, decreasing to 2-fold if the relative is ≥60 years old at diagnosis [15]. A positive family history is present in almost 50% of the young women with breast cancer [16], but it is not a risk factor for worse survival [17].

BRCA1/2 mutations

In the general population, *BRCA1/2* mutations are very rare. Only 0.2% of women are generally estimated to be *BRCA1/2* mutation carriers. Translated into the Swedish female population of 4.9 million women (2016), this means that 9800 women can be estimated to be *BRCA1/2* mutation carriers [18]. While the cumulative incidence of breast cancer for an unaffected 70-year-old woman is 10%, the risk is 6-fold (60%) for a *BRCA1* mutation carrier of the same age. The cumulative incidence of breast cancer for a 40-year-old woman is generally 1%, while for a *BRCA1* mutation carrier the corresponding risk is 20% (a 20-fold increase) [6,19].

Breast density

Breast density is a strong independent risk factor for the development of breast cancer, and is associated with a 4-fold increased risk of the disease [20,21]. Breast density is a risk factor for breast cancer for women both <50 and ≥50 years old, according to a meta-analysis [21]. Breast density seems to be a proxy for a combination of genetic, hormonal and lifestyle risk factors predicting breast cancer. Breast density decreases with increasing age, but a woman with high breast density at age 40 will persist with a high baseline density throughout life, indicating that a high risk for breast cancer later in life can already be identified at a young age [22]. Dense breast tissue is over-represented in young women. In a study of 7000 mammograms, 81% of the women <40 years old had dense breasts. The proportions for women aged 40-49, 50-59 and 60-69 years were 57%, 57% and 45%, respectively [23]. Dense breast tissue is related to both the risk of being young at the diagnosis of breast cancer, as well as the outcome of breast cancer, and thereby constitutes a classical confounder.

Breast density is highly inheritable. Twin-studies have identified about 60% of dense breast to be ascribable to inherited genetic factors [24]. Breast density also carries a risk for breast cancer in women with *BRCA* mutations, as they have a higher proportion of high breast density than non-carriers [25,26].

Reproductive factors

In a large meta-analysis of risk factors for breast cancer in women aged 40–49 years, reproductive factors contributed only modestly with a 1.0 to 1.5-fold increased risk. The following reproductive factors were defined as risk factors: early menarche, age ≥30 years at first full-term pregnancy, <3 births or nullipara [14]. Breastfeeding and the duration thereof reduce the breast cancer risk in young women [5,14,27].

Oral contraceptives increase the risk of breast cancer in young women. A lifetime use exceeding 15 years increases the risk 1.5-fold for women aged 20-44. Current use for 5 years or longer increases the risk for triple-negative (TN) breast cancer in women aged 20-39 almost 4-fold [28]. In vitro fertilization does not increase the risk of breast cancer [29].

Intrauterine exposure

High birthweight (≥4 kg versus <3 kg) increases the risk of breast cancer 6-fold [30], with a stronger association for premenopausal presentation [31]. The mechanism is thought to be an oestrogen-mediated stimulation of mammary stem cells [32]. Also tall height at 8 years of age is associated with an increased risk of both premenopausal and postmenopausal breast cancer [31].

Obesity

Obesity increases the risk of postmenopausal breast cancer, but it is a protective factor for premenopausal women [33,34].

Alcohol and smoking

Alcohol and smoking are not risk factors for breast cancer in women aged 40-49 [14].

Diagnostic procedures in young women

Clinical examination

A palpable breast mass or lump in the breast is most often the first symptom of breast cancer in young women. The first presentation can also be nipple discharge or a lump in the axilla [35]. In a study on women <35 years old with breast cancer, only 37% of the cases yielded a strong suspicion of cancer at palpation, while another 20% were undeterminable and 43% were considered to be benign (most frequently with a fibroadenoma-like palpation finding) [36]. In a population-based study, 98% of the women <40 years of age were detected clinically [37].

Breast imaging

Due to a higher proportion of patients with dense breast tissue among young women breast imaging is more difficult in young women than in older ones. In one study, the sensitivity of mammography was 30% in extremely dense breasts compared to 80% in predominantly fatty breasts [38]. Ultrasound has been shown to be superior as a first-line breast imaging technique up to age 45, correlating with a shift in the hormonal milieu by this age [39]. If ultrasound indicates a suspect malignancy (BI-RADS 3-5), the next step is a complementary mammography.

MRI does not improve primary breast cancer diagnostics in normal-risk women (median ages, 50–63 years), but it increases mastectomy rates with more harm than good [40]. Nor does an MRI assessment in the primary investigation prevent future local recurrences [41] when assessed in women aged 49–64. MRI has been shown to be beneficial as a screening method in young women with a high risk of breast cancer [42], but it has not been determined whether or not it is beneficial in the primary breast cancer diagnostics of normal-risk women <40 years old. In a small study on women <50 years old with breast cancer, MRI

did detect additional disease, leading to improved management in 44% of the cases, to no change in another 44%, and to excessively extensive surgery in 12% of cases [43]. A study on 440 women <56 years old with breast cancer randomized to MRI or not resulted in a 11% lower breast reoperation rate in the MRI group, but no differences in the overall mastectomy rate between the groups were noted [44].

New diagnostic tools, such as Automated Breast Ultrasound (ABUS), can probably improve early diagnosis in young women as it is more sensitive in dense breast tissue than conventional mammography. The technique has a standardized protocol and can be performed by medical personnel and then interpreted afterwards by the radiologist. Adding ABUS to screening mammography increased the cancer detection rate by 37% in an American study [45] and by 57% in a Swedish study [46].

Cytology and histopathology

The use of cytology and histopathology for confirmation as a part of the triple assessment is especially important in young women since both clinical examination and imaging have less sensitivity. Fine-needle aspiration has a high sensitivity (93–96%) in young women [36,47]. A histopathological confirmation to get information on tumor characteristics is important in young women as they have a large proportion of tumors that respond to neoadjuvant chemotherapy [48]. Knowledge of tumor characteristics at diagnosis is also important for raising the suspicion of the woman having a *BRCA* mutation, thereby gaining sufficient time for genetic testing before initiating radiotherapy.

Screening

Breast cancer screening is highly evidence-based and reduces breast cancer mortality by 25% when asymptomatic women aged 50–69 years are invited biannually [49]. As for younger women, several randomized trials from Sweden and the UK have shown the benefit of screening to be approximately 16% for women starting at age 40 with an interval between examinations of 12–18 months [50,51]. At long-term follow-up (24 years), the reduction of mortality increased to 40%, which is a larger reduction than in older women and can possibly be explained by a shorter screening interval in the younger age group [52].

General screening for women <40 years old is not an option because of the low prevalence of breast cancer in this age group. Young women can be included in high-risk screening programs if they meet any of following criteria: a lifetime risk of breast cancer exceeding 20–25%, being a carrier of a *BRCA* mutation, being a first-degree relative of someone carrying a *BRCA* mutation or having a history of chest radiation during ages 10–30. The screening modality for women <40 years old meeting these criteria is an annual MRI [42,53], whereas mammography does not seem to add any value in this context [54].

Tumor characteristics in young women

Histological type

Ductal carcinoma is the most common histological type in women <40 years old (nearly 90% of tumors) [37,55-58]. In women >60 years old, this proportion is about 70% [56]. Lobular carcinoma is uncommon in young women, but it increases in incidence with age (**Table 1**) [59]. A classic lobular carcinoma is ER+ (90%), PR+ (60-70%) and grade II with low Ki67 [60], characteristics that are less common in young women. Generally speaking, medullary carcinoma is uncommon, but it is associated with young age, high grade, TN subtype [61] and *BRCA1* mutation [62]. Histological type is associated with prognosis. On comparing histological type and the 21-gene recurrence score, tumors of the ductal type more often had a high-risk recurrence score, and those of the lobular type, a low-risk recurrence score [63].

Stage

Young women are being diagnosed with breast cancer at more advanced stages, with both larger tumors and, more often, axillary lymph node involvement [64-68]. Young women are not included in screening programs. They have tumors with a higher proliferation rate and thus faster growth. Women with breast cancer aged <40 have a 3-fold higher risk of being diagnosed with high-stage disease [69]. Stage is one of the strongest prognostic indicators in breast cancer [70] and age-related differences in breast cancer survival have been shown to be present only in the early stages of disease. In the SEER dataset, age-related survival differences were studied in women diagnosed with breast cancer during 1988–2003 (n = 240,012). The authors found a higher risk for women <40 years of age to die from breast cancer if they were in stages I and II [66]. In another registry-based study including women with breast cancer diagnosed during 2005–2009 (n = 59,191) and with a mean follow-up time of only 3 years, no age-related differences in prognosis by stage at diagnosis were identified [71].

Nodal stage, or the number of involved axillary lymph nodes, has been known for decades as the most significant prognostic indicator in breast cancer [72,73]. Nodal stage is distributed differently across subtypes in young women: among those with luminal tumors, 44% had positive lymph nodes at diagnosis, with Her2+ tumors 65%, and with TN breast cancer only 31% [55]. Basal-like tumors with a known inferior survival have a lower risk of being lymph node-positive at diagnosis, compared to luminal tumors (HR 0.5–0.6) [74]. Women with triple-negative, lymph node- negative breast cancer had a worse 10-year distant disease-free survival than those with Luminal A, node-positive disease (55% vs 88%) [75]. It seems that nodal stage remains prognostic in all subtypes. Nodal stage and subtype cannot replace one another as prognostic factors [76,77].

Grade

Grade is based on morphological features of the cancer cells, including scores on tubular formation, nuclear pleomorphism and mitotic count, summed up to grades I–III [78]. Young

women have a higher proportion of high-grade tumors than older women [55,57,64,66,68,71,79,80]. For women <40 years old, grade III versus grades I–II carry an independent risk for breast-cancer specific and overall survival [71], but not for local recurrence [80].

Table 1. *Tumor characteristics in women <35 and <40 years old at the diagnosis of breast cancer. The table is based on data from a literature overview including population-based studies and excluding duplicative publications based on the same cohort. Proportions are calculated on informative cases only.*

	Age <35 years			Age <40 years		
	%	n/total	Reference	%	n/total	Reference
Tumor <2 cm	51.9%	927/1786	55,57,79	48.5%	12020/12692	56-58,64,71
Node neg	51.2%	922/1802	55,57,79	52.7%	14614/27717	56-58,64,71
Grade III	57.5%	1109/1930	55,57,79	57.3%	6568/11462	3,57,80,56,64
ER+	77.1%	243/315	79	60.0%	12952/21592	3,5,56,57,58,80
PR+	67.9%	214/315	79	53.2%	2777/5220	3,5,56,57,80
Her2+	25.0%	282/1130	55,57,79	24.7%	1395/5651	3,5,56,57,80
Ki67>20%	77.1%	239/310	79	no data available		
HR+Her2-	45.7%	1102/2410	55,71,79	50.1%	2191/4377	71
HR+Her2+	19.5%	471/2410	55,71,79	18.5%	811/4377	71
HR-Her2+	11.7%	281/2410	55,71,79	10.7%	469/4377	71
TN	23.1%	556/2410	55,71,79	20.7%	906/4377	71
Ductal	89.4%	1628/1821	55,57,79	87.6%	19354/22085	56-58,80
Lobular	1.7%	31/1821	55,57,79	4.5%	984/22085	56-58,80
LVI pos	43.1%	394/915	57,79	43.0%	2069/4811	57,64,80
Multifocal	19.2%	158/825	55	29.7%	779/2624	80
EIC pos	no data available			20.3%	557/2746	80

IHC, immunohistochemistry, ER estrogen receptor, PR progesterone receptor, HR hormonal receptor (ER/PR), TN triple negative, LVI lymphovascular invasion, EIC extensive in situ component

Hormonal receptors

The oestrogen receptor is expressed in several human tissues and over-expressed in breast cancer cells [81]. The presence or absence of ER in a tumor reflects rather the growth rate than the metastatic potential, and thereby rather the length of distant disease-free survival than overall survival [82]. ER is a strong predictor of the response to endocrine therapy [83-85]. Compared to middle-aged and elderly women, young women generally have a lower protein and gene expression of ER [59,86,87]. In clinical practice, ER has been measured by means of immunohistochemistry (IHC) since the late 1990s, with a cut-off of 1% to define a positive expression [88]. The former cut-off of 10% was changed into 1% in 2010 [89], while publications suggest tumors with ER 1-9% compared to ER>10% have an inferior survival, less response to endocrine therapy and are more often seen in young women [90,91].

The progesterone receptor expression is induced by oestrogen signaling via ER [92]. PR is lower in postmenopausal women as oestrogen levels decrease after the menopause [93]. While ER is a strong predictor of endocrine therapy, the predictive role of PR has been more in dispute [94-96], and until recently, it has been considered a marker of ER function [97]. More recent data indicate that PR functions as a 'proliferative brake' in ER+ breast cancer [98]. ER+PR+ breast cancer is associated with a better outcome than ER+PR- tumors [99-101]. PR is measured by IHC and a cut-off of 20% has been shown to be optimal [102].

Her2

Human epidermal growth factor receptor 2 is a gene coding for a signalling network controlling cellular proliferation, apoptosis, tumor cell motility and capacity for metastatic dissemination [103]. Amplification of the gene produces correspondingly high Her2 protein levels, which are seen in about 15-20% of women with primary breast cancer and which correlates with a worse survival [104]. Her2-positivity is 1.5-2 times as common in young women [3,5,57,71,77,79,105,106]. Her2 is analysed by IHC and the amount of Her2 receptor protein on the cancer cell surface gives a score of 0 to 3+. If the score is 0 to 1+, it is considered negative, while a score of 2+ is equivocal and a score of 3+ is positive. For equivocal cases, a confirmatory analysis is done using an *in situ* hybridization method that detects amplification of the Her2 gene [107]. A Swedish national survey of the reproducibility of Her2 analyses using IHC and FISH showed high quality and reproducibility using TMA-based samples [108].

Proliferation

Proliferation is a hallmark of cancer [109] and is prognostic in most cancer types. Proliferation markers are highly expressed in young women with breast cancer regardless of whether they are estimated by gene expression [59,110], grade [3,57,67,105], Ki-67 [79,111,112] or cyclins [113-117]. In clinical treatment decision-making for women with luminal (Her2-negative) breast cancer, proliferation is heavily relied on, and is most often measured by Ki-67 when gene-expression analyses are not available [48].

Ki-67 is a protein expressed during the whole cell cycle, except in G_0 [118]. In clinical practice, it is used to separate Luminal A from Luminal B subtype [48]. In order to find an IHC substitute for the molecular intrinsic subtypes, Cheang et al. compared gene expression-based subtypes with IHC and found a Ki-67 level of 14% to best define the cut-off between the Luminal A and Luminal B subtypes [119]. The best Ki-67 cut-off is still the subject of a major debate worldwide. According to the latest St Gallen Guidelines, a Ki-67 of 20–29% should be interpreted by local laboratory references, while a value of <10% is definitely considered low and >30% high [48].

Cyclins are a family of proteins that regulate cellular growth and division in both normal and malignant cells [120]. The regulation is mediated by cyclin-dependent kinases [121]. Cyclins

display subtype-specific expression in breast cancer [122-125] and are generally expressed in higher levels in young women [114-117,126].

Lymphovascular invasion

LVI is overrepresented in young women [57,64,127,128] (**Table 1**). LVI is a strong independent marker of a worse breast cancer-specific survival [129,130] and locoregional recurrence in young women [131,132]. LVI has also been shown to be predictive of the response to neoadjuvant chemotherapy [133], but, in treatment decisions, it is not in and of itself considered to be sufficient to move patients from a low-risk group to a high-risk group [134].

Subtype

When analysed with microarray-based gene expression, breast cancer comprises a heterogeneous group of tumors with different distinct molecular features. The intrinsic molecular subtypes were originally described by Perou and Sorlie et al. in year 2000 [135]. Based on hierarchical clustering on fresh-frozen tumor material, they identified four distinct subtypes with distinct gene expression patterns: Luminal, Her2-enriched, Basal-like and normal-breast-like. Subsequent studies led to the Luminal subtype being divided into two, Luminal A and Luminal B [136], and to additional molecular subtypes being found, including the claudin-low [137,138] and the molecular apocrine subtypes [139].

Molecular subtypes have later been translated into corresponding IHC subtypes (**Figure 2**), which is not a perfect match [138], but still provides a good estimation of prognosis [140,141]. When using IHC-based subtypes the biggest challenge is to separate Luminal A from Luminal B since they have divergent prognoses and treatments. Tumors expressing ER+PR-Her2- have a worse prognosis than ER+PR+Her2- tumors and are thereby considered to be a Luminal B subtype, no matter what level of Ki-67 is [142-144].

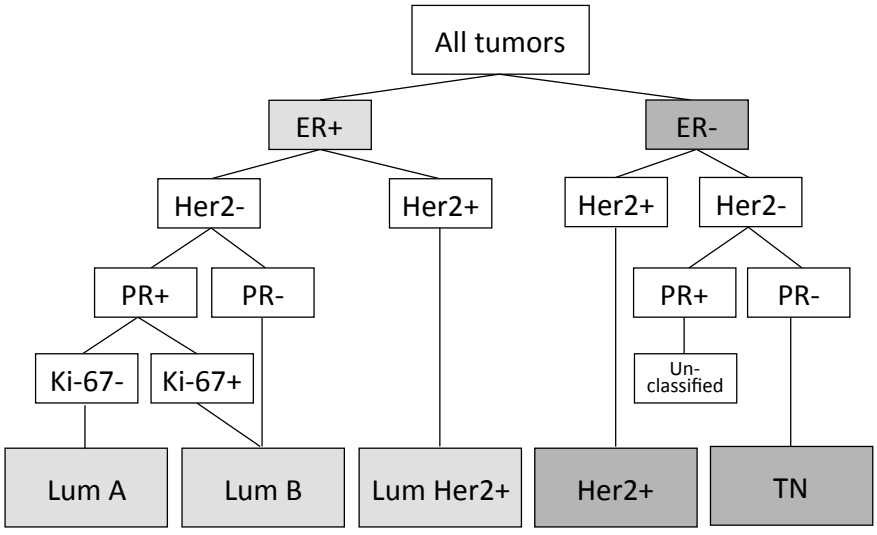


Figure 2. *Subtypes with IHC according to St Gallen Guidelines 2015.*

The Luminal A subtype is twice as common in older women as in younger ones [3,56,57,77,79,105,145]. Almost 50% of young women have a luminal breast cancer, and a

large proportion of them have high proliferation and high grade. Only a small proportion of tumors in the young will be of the Luminal A subtype when subtyping is based on IHC (**Table 1**). Luminal A is associated with the best survival of all subtypes in both young and older women [56,71]. In a study on prognosis in a large American population database, young age constituted an independent risk for breast cancer death in Luminal A disease (in this study, defined by ER+/PR+ and grade I-II) [3].

The proportion of luminal tumors of the Luminal B type is higher in young women than in older women [57,105]. Young women with tumors of the Luminal B subtype have a worse survival compared to older women [105,146,147]. In the gene-derived intrinsic molecular subtypes, the Luminal B subtype includes approximately 30% Her2+ tumors [112,119,148]. These Luminal Her2+ tumors are distinctively different from Luminal Her2- tumors since they can receive targeted therapy with trastuzumab and they should not be inappropriately classified as Luminal B [119].

The Her2+ subtypes are 1.5–2 times more frequent in young women than in older women [56,57,79,105,149]. The prognosis is similar for both young and older women [79,105,150].

TN breast cancer is twice as frequent in young women as in older ones [3,5,56,57,77,79,105]. There are no age-related differences in the prognosis within the TN subtype [151,152].

Tumors expressing ER-PR+Her2- have not been considered to belong to a separate reproducible subtype [95,153,154] although evidence for the opposite has also been published [155]. If they do indeed exist, ER-PR+Her2- tumors are uncommon and represent between 1% and 4% of all tumors [77,155] and are somewhat more common in young women [156].

***BRCA* associated breast cancer**

In a Swedish study on unselected women with breast cancer, the prevalence of BRCA mutations was only 0.4% [157]. Since *BRCA*-associated breast cancer penetrates at a low age, the prevalence in young women is much higher. In a Swedish population-based study, 9% of the women <41 years old at diagnosis had a *BRCA1/2* mutation [16]. In a prospectively collected American cohort where the indication for testing was breast cancer at age <40 years, the prevalence of *BRCA1/2* was 12.4% [158]. In an unselected breast cancer population, 12.2% of the women ≤45 years old tested positive for the *BRCA1/2* mutation [159]. In a large population of women subjected to genetic testing (n = 35,000), 14.4% of those <40 years old had a pathogenic variant of one of the 25 most common inherited breast cancer genes (in women aged <40, approximately 65% had *BRCA* mutations) [160]. In the subgroup of women with TN breast cancer, the corresponding prevalence was 24.8%. *BRCA1/2*-associated breast cancers are more often of the TN subtype and grade III [159]. All women with breast cancer <40 years of age should be recommended testing for *BRCA* mutations [161].

Treatment

Breast surgery

The proportion of young women having a mastectomy is higher (45-50%) than in older women (35%) [37,55]. Breast-conserving surgery (BCS) in women <40 years old is associated with a somewhat higher risk of local recurrence (LR) than mastectomy, but the type of surgery does not affect either distant disease-free survival or overall survival [80,162,163]. Multifocal lesions and extensive DCIS are more frequent in young women [55,80], which can affect re-excision rates, which are known to be higher in young women due to both tumor biology and smaller breast volumes [164]. It is of extra concern to have free margins after BCS in young women. Invasive cancer cells present on inked margins increased the LR rate 4-fold in women <40 years old, but not in women >40 years old [165]. Women operated on with BCS have a better quality of life than those who had a mastectomy and, with the long life expectancy of young women with breast cancer, BCS should be the first option whenever suitable [166]. In a cohort study of 965 women<40 years old, the 15-year outcomes concerning LR, distant metastases, breast cancer survival and overall survival were similar between those operated on with mastectomy and those operated on with BCS followed by whole breast radiotherapy [167].

Immediate breast reconstruction is safe in young women and should be offered to all women having the indication for mastectomy. Relative contraindications are locally advanced breast cancer involving skin or thoracic wall at diagnosis, grave psychiatric disease, smoking and a high BMI. Inflammatory breast cancer is an absolute contraindication [168]. Planned postmastectomy radiation therapy influences the cosmetic outcome after implant-based reconstruction, but most often, it still gives satisfactory results [169]. A systematic review has concluded that implant reconstruction should be advised prior to radiotherapy, while it is slightly better to perform autologous reconstruction after radiotherapy [170].

Axillary surgery

Approximately 50% of women <40 years old with breast cancer have lymph node-positive disease at diagnosis [5,55,64,66,80], compared to 70% at ages >40 [5,56,58,66], thus corresponding to a larger proportion of young women being operated on with an axillary clearance. The prevalence of lymphoedema at 5 years after surgery is high in young women (32%), and two of the risk factors for persistent swelling of the arm are the number of axillary nodes removed and obesity [171].

Axillary management is in the process of changing towards less extensive surgery, and this will benefit young women with breast cancer. For women with a maximum of 2 macro-metastatic lymph nodes, it is now considered safe to avoid axillary dissection [172]. However, it is worth noting that, for women <40 years old, the probability of axillary recurrence is twice as high as in older women [57,173].

Chemotherapy

Due to stage and subtype distribution, young women often have indications for chemotherapy. Since the year 2000, chemotherapy has been given to more than 90% of women <35 years old with breast cancer [55,80].

In the 1995 St Gallen Guidelines, age <35 was defined as high risk, and thereby, in and of itself, an indication for chemotherapy, no matter the stage or tumor biology [174]. This was further strengthened by Kroman et al. in 2000 in a randomized study on CMF versus no systemic treatment showing that women <35 years old have a 2.2–fold relative risk of dying compared to women aged 45–49 in the non-systemic treatment arm, whereas no difference in survival was seen within the chemotherapy arm [2]. In the 2005 St Gallen Guidelines, age <35 was lowered to an intermediate risk, resulting in chemotherapy being optional in young women with endocrine responsive tumors [175]. In the guidelines from 2009, age itself was no longer a reason for chemotherapy [176]. In clinical practice, there has been a major change in the use of chemotherapy during the last few decades, regarding both the proportion of patients treated and indications for treatment, as well as the choice of regimens. In a population-based Dutch study [177], 90% of the chemotherapy-treated women in the year 2000 received cyclophosphamide, methotrexate and 5-fluorouracil (CMF), while almost none were given CMF in 2005. In 2005, 96% of the treated women were assigned to anthracyclin-containing regimens, which gradually decreased to 68% in 2008 when, instead, taxane-containing regimens became more common, increasing from 24% to 34% between 2005 and 2008.

Neoadjuvant chemotherapy has developed from being an option for inoperable, locally advanced disease, to being the first option in tumors measuring 3 cm or larger, in case of node involvement, or in case of Her2+ disease. Neoadjuvant chemotherapy offers a biology-based treatment with the possibility of evaluating treatment response, increasing the chances of successful BCS and obtaining the surrogate prognostic information concerning a pathological complete response (pCR). In a population-based cohort of women diagnosed in 2000-2008, chemotherapy was given neoadjuvantly to 15.5% of the women <40 years old [80]. Nationwide population data from Sweden in 2014 showed that neoadjuvant chemotherapy was used in 19% of women of all ages and, in some regions, in up to 26% of women with primary breast cancer [178].

Women <40 years old have a higher rate of pCR than older women, especially for luminal-Her2-negative breast cancer [179]. Neoadjuvant studies have facilitated the introduction of more modern chemotherapy regimens such as docetaxel, adriamycin and cyclophosphamide (TAC). The highest pCR rates after TAC treatment have been observed in women <40 years old with TN or grade III tumors [180]. Neoadjuvant studies on women with TN breast cancer carrying *BRCA1/2* mutations have been shown to be promising, with high pCR rates when platinum salts have been added to standard chemotherapy [181].

Endocrine therapy

Today it is beyond all doubt that all women with ER+ breast cancer should be treated with endocrine therapy [94,182]. In 1992, the recommendation for premenopausal women with low risk (node-negative tumors <1 cm found incidentally) was observation or tamoxifen, for intermediate risk (node-negative, tumors 1–2 cm), tamoxifen and for high-risk ER+ tumors, chemotherapy +/- tamoxifen and, for high risk ER- tumors, chemotherapy only [183].

The options for endocrine treatment available today are tamoxifen, aromatase inhibitors (AIs) and a combination of either one of them with gonadotropin-releasing hormone (GnRH) agonists for ovarian function suppression (OFS). The standard endocrine treatment recommendation for premenopausal women with luminal tumors is tamoxifen for 10 years. According to the EBCTCG meta-analysis, 5 years of tamoxifen reduces the risk of recurrence by 50% throughout the first 10 years, independently of age, PR status, nodal stage and use of chemotherapy [94] and also reduces the 15-year mortality by a third. Extended adjuvant endocrine therapy with prolonged tamoxifen has been proved to be efficient in two very large trials [184].

For high-risk women (<35 years old or still premenopausal after chemotherapy or multiple axillary metastases), the recommendation is tamoxifen or AI for 10 years in combination with OFS for 2 years [48]. In the randomized SOFT and TEXT trials, premenopausal women given endocrine treatment including 3 years of GnRH, had a superior disease-free survival but no survival benefit [185,186].

Resistance to endocrine therapy may be more common in young women. They have a higher proportion of the Luminal B subtype that confers less benefit from endocrine therapy compared to those with Luminal A subtype [187]. In women with ER+ tumors, given both chemotherapy and endocrine therapy, women aged 35–50 had a survival benefit not seen in women <35 years old [188]. In a Swedish trial, premenopausal women with ER+ tumors benefitted from adjuvant tamoxifen only when PR was >75% [189]. Young women are less compliant with endocrine therapy than older ones [190-192].

Anti-Her2 treatment

Trastuzumab, a monoclonal antibody against Her2, was introduced in Europe in the year 2000 and in the adjuvant setting in 2005 [193]. In a Dutch study on women <35 years old, trastuzumab was introduced as a part of treatment in 2005, with successively increasing use towards 2008 [55]. Anti-Her2 treatment should be offered to all patients with Her2+ tumors, without taking age or menopausal status into account [150]. International guidelines recommend that all women with Her2+ breast cancer should receive 1 year of trastuzumab, except in very small (≤5 mm) node-negative tumors where trastuzumab can be omitted [48].

Radiotherapy

Young women with breast cancer have a high risk of local recurrence (LR) which is ascribed to their higher proportion of characteristics associated with an increased risk of LR (Table 1). In addition to the ordinary dose of 50 Gy to the breast, young women are recommended an extra boost of 16 Gy to the tumor bed after BCS [194]. Boost decreases the 10-year LR rate in young women by 50%, but has no effect on survival. [195]. Tumors of the Her2+ subtypes, which are more common in the young women, may be associated with resistance to radiotherapy and thus a higher risk of LR [196].

Risk prediction models

Different tools have been developed to tailor the treatment decision and select women in need of chemotherapy and/or endocrine therapy. The clinical risk can be measured as follows:
The Nottingham Prognostic Index (NPI) [197,198](1982) uses tumor size, grade and lymph node status in an equation to indicate excellent, moderate and poor prognostic groups.
Adjuvant online! [199,200](2001) is an online tool using age, tumor size, number of positive axillary nodes, grade and ER status to provide an estimate of the 10-year outcome.
PREDICT [201-203](2010) is another online tool using age, mode of detection, tumor size, lymph node status, grade, ER, Her2, Ki-67 status and given treatment for prediction of prognosis.

Risk prediction models are generally well validated, but not for women <40 or >70 years old. Adjuvant online! has been shown to underestimate the risk of recurrence in up to 30% of women <40 years old [204]. PREDICT provides an accurate 10-year survival rate for women <40 years old, but the 5-year survival was overestimated for ER+ tumors and underestimated for ER- tumors [205].

The genetic risk can be measured by gene expression profiling (GEP) with the aim of improving decision-making regarding adjuvant chemotherapy in early breast cancer. Various commercial assays stemming from a common base of multiple genes expressing ER, Her2 and proliferation are available. Fresh-frozen tumor material or FFPE can be used with either deoxyribonucleic acid (DNA) microarrays or the reverse transcription-polymerase chain reaction (RT-PCR).
MammaPrint [206-208](2002) is a microarray-based assay of 70 genes allocating patients with early node-negative or node-positive breast cancer and endocrine treatment to a high-risk group (with additional benefit from chemotherapy) or a low-risk group (with no statistically significant additional benefit from chemotherapy).
Oncotype DX [110,209](2004) is an RT-PCR analysis based on 21 genes. It is used to predict the benefit of chemotherapy, as well as the likelihood of distant disease at 10 years

in women with ER+ early breast cancer. The Oncotype DX Recurrence Score is a score between 0 and 100 reflecting a successively higher risk of distant disease in both node-negative and node-positive disease. The Oncotype DX assay has mainly been validated in ER+, node-negative disease in patients with a median age of 50–60 years.

MapQuant DX [210](2006) uses 8 genes to produce a Genomic Grade Index that improves the histological grade classifications and divides histological grade II tumors into either grade I or grade III.

The *PAM50* [211](2009) assay is a minimal gene set based on 55 genes for classifying intrinsic-like subtypes of breast cancer. It produces a continuous risk score and also determines the benefit of chemotherapy in addition to endocrine therapy in postmenopausal women with ER+Her2-negative breast cancer. The evidence is mostly based on node-positive patients, and only one study included premenopausal patients.

Local recurrence

Young age is an independent risk factor for LR, after both breast-conserving surgery and mastectomy [196,212-217]. The risk of having an LR due to young age is twice as high as that in middle-aged women [212,218]. The younger the patient, the higher the risk of LR. The risk for women aged <35 is higher than for women aged 35–39 [215]. The risk of LR in women <40 years old was 3.4% after 5 years and, in women >40 years old, 1.6% [196]. Established risk factors for LR are late stage diagnosis, high grade, subtype distribution, dense breast tissue [219], LVI, multifocality and the presence of extensive DCIS [218]. All of these characteristics are common in young women (**Table 1**). The subtypes associated with the highest risk of LR in young women are the Her2+ and TN subtypes [55,196,220].

Owing to a more biology-influenced approach in treatment decisions, the introduction of trastuzumab and taxanes, improvements in diagnostics and radiotherapy, the incidence of LR has decreased substantially over time and is now well under 1% per year also for the youngest women [55,221].

Distant recurrence and mortality

Young age is an independent risk factor for distant recurrence [57,216], but it seems to be restricted to early stages of the disease and to the luminal subtypes [3,79,147,152,188,222]. The majority of recurrences in young women are distant, not local. Among women <40 years old, 4.8% had an LR, versus 26.1% with a distant recurrence, at a median follow-up of 7 years [80]. In line with the observed decrease in LR with more modern systemic therapy, the incidence of distant recurrence has also decreased in young women. In 2003, women <35 years old had a 5-year distant recurrence rate of 17.8%. From 2004 to 2008, the corresponding rates were 19.2%, 14.6%, 8.2%, 8.1% and 10.0%, respectively [55].

There are no age-specific patterns of metastatic behaviour. Subtype-specific trends show a low frequency of metastases in women after Luminal A tumors. The metastases that occur are mostly bone metastases associated with a favourable prognosis. Her2+ and TN breast cancer have the highest risk of distant metastases, often visceral metastases occurring during the first three years [223,224].

Contralateral breast cancer

Young age at breast cancer diagnosis entails a risk for a later (metachronous) contralateral breast cancer (CBC) together with a *BRCA* mutation, positive family history, high breast density, lobular breast cancer, TN breast cancer, large tumor size and positive axillary lymph nodes [225-228]. The cumulative risk of CBC at 10 years is 4% for a 60-year-old woman and 6% for a 40-year-old woman, with a decreasing trend with modern systemic therapies [225,226]. Endocrine therapy decreases the risk of CBC by 50% [83]. Not only invasive breast cancer but also DCIS increases the risk of CBC. In a Swedish population-based study especially women aged <40 were at risk of a new contralateral event [229]. The majority of young women affected with primary unilateral breast cancer overestimate their risk of CBC. The risk of CBC is, in fact, similar (0.7% per year) [226] to the risk of a LR at the primary site (0.6% per year) [196]. Still, the risk of having distant metastases is much higher (2.8% per year) [55]. A study on the chromosomal relationship between the primary tumor and the CBC showed 1 in 10 CBC to be rather a distant spread instead of a new primary one [230]. Bilateral breast cancer at presentation (synchronous) is uncommon (fewer than 2%) and associated with older age [226,228]. Prophylactic mastectomy decreases the risk of CBC, but with no evidence of improved survival [231].

Fertility and pregnancy

Chemotherapy and endocrine therapy-induced amenorrhoea is common in young women [232]. The treatment's effects on fertility must be discussed with the young patient as soon as possible after the diagnosis. Early referral to a fertility clinic is strongly advised to discuss methods of fertility preservation. The available options for fertility preservation are ovarian stimulation with letrozole (AI), followed by egg retrieval for cryopreservation or *in vitro* fertilization for embryo cryopreservation. A third option is ovarian tissue harvesting and cryopreservation for later re-implantation [233].

Whether or not the use of GnRH agonists during chemotherapy preserve ovarian function has been investigated in several studies. According to two recent meta-analyses, GnRH appears to improve ovarian function and the ability to achieve pregnancy following chemotherapy [234,235].

Pregnancy-associated breast cancer (PABC) is defined as breast cancer detected during pregnancy or within one or two years since delivery. Every 4th breast cancer patient <35 years old and every 10th before age 40 is diagnosed during pregnancy. The incidence is increasing as women are postponing childbirth to later in life [236]. Women with PABC are more often diagnosed in advanced stages. Compared to other young women characterized by comparable age, stage and tumor biology, the prognosis does not differ [237,238].

Breast cancer treatment during pregnancy should not be inferior to that in the non-pregnant setting to spare the child [239]. The indications for breast surgery are the same as for non-pregnant women, but a sentinel node biopsy without blue dye is recommended. Endocrine therapy [240], trastuzumab [241] and radiotherapy [239] are contraindicated, whereas chemotherapy during the second and third trimester is well tolerated with no detrimental effect on the foetus. During the first trimester, chemotherapy is contraindicated due to a relatively high risk of congenital malformations. Women with breast cancer during pregnancy should be managed within a multidisciplinary team also including an obstetrician and a neonatologist. Delivery is recommended at full term after gestational week 37 [242].

Pregnancy after breast cancer does not impair the long-term prognosis, but rather has a protective effect on survival [243,244]. The best timing of pregnancy after ER+ breast cancer has not yet been established. There is an ongoing prospective study on women <42 years with ER+ breast cancer and a pregnancy desire (called POSITIVE) [245]. After 18–30 months of endocrine therapy, the treatment is interrupted for up to two years to enable a pregnancy.

Quality of Life

For everyone diagnosed with cancer, the diagnosis will have a huge impact on their daily life with physical, emotional, social and existential concerns. Young women with breast cancer have a worse Quality of Life (QoL) than middle-aged and older women, and the duration of reduced QoL is longer [246,247]. The QoL is even worse for young women affected by breast cancer than by other cancers [248] and a possible explanation could be that breast cancer treatment has a large impact on body image and sexual functioning and entails a premature menopause. Women with children and women with earlier psychiatric illness have a lower QoL [248]. To improve their QoL, young women with breast cancer should be offered interventions specifically designed to target their specific needs [166].

AIMS OF THE THESIS

The general aim of the thesis was to gain increased insight into the long-term prognosis for young women with breast cancer.

More specifically, the aims were to:

- study the long-term outcome by age for women diagnosed with breast cancer and to investigate the extent to which a worse prognosis for women aged <35 can be explained by stage, tumor characteristics, heredity, parity and treatment

- gain further insight into the biology behind the age-related differences in the prognosis for breast cancer

- find new prognostic markers that can help us to determine which of the young women that have an excess risk of recurrence

- assess the expression of proliferation markers in relation to age and subtype in order to clarify whether cyclins add prognostic information to the standard biomarkers in young women

- to compare Her2-status generated by testing *Her2* gene copy numbers (with silver *in situ* hybridization) and by Her2 protein expression (using immunohistochemistry) in a population-based cohort with a long-term follow-up and to find out whether either test method is superior for predicting the prognosis in young women who have a known overrepresentation of Her2-positive breast cancer.

PATIENTS AND METHODS

Studies I–IV are based on the same cohort identified from the regional Breast Cancer Quality Registries in Stockholm-Gotland and Uppsala-Örebro. We used the Swedish Personal Identification Numbers (PIN), assigned to all Swedish residents, for linkage between all registers used [249].

Data sources

The National Breast Cancer Quality Registry

The National Registry is based on joint information from the 6 different regional breast cancer quality registries in Sweden. It is updated continuously by matching with the Total Population Register, The Swedish Cancer Registry and The Swedish Causes of Death Registry. The National Registry contains prospectively collected data on patient, tumor and treatment characteristics and has a high validity. Since 2008, this registry has been organized on a web-based platform, INCA. In 2015, the coverage was 97% [178]. In the two regions from which data for our study cohort were taken, the Stockholm-Gotland and the Uppsala-Örebro regions, the Breast Cancer Quality Registries were started in 1976 and 1992, respectively. These two regions cover about 43% of the Swedish population [18].

The Swedish Cancer Registry

The Swedish Cancer Registry, located at the National Board of Health and Welfare, was established in 1958 and includes data on all diagnosed malignant (and some benign) primary tumors in Sweden. Reporting to this registry is mandatory by law, and since both the handling clinician and pathologist are each required to make the report, the coverage is very high, being >98% for breast cancer [250,251]. Individuals are identified by their PIN and tumors are coded with the International Classification of Disease (ICD) code. This registry includes data on the tumor site and histology, as well as dates, diagnostic methods and the name of the hospital providing the diagnosis.

The Swedish Causes of Death Registry

This nationwide registry, also located at the National Board of Health and Welfare, collects information on causes of death of Swedish residents who die in Sweden or abroad. This registry was initiated in 1751, digitalized in 1952, and its completeness is >99% [9]. This registry includes data on date of death, place of residence, underlying cause of death and contributing causes of death. Individuals are identified by their PIN and the cause of death coded with the ICD code.

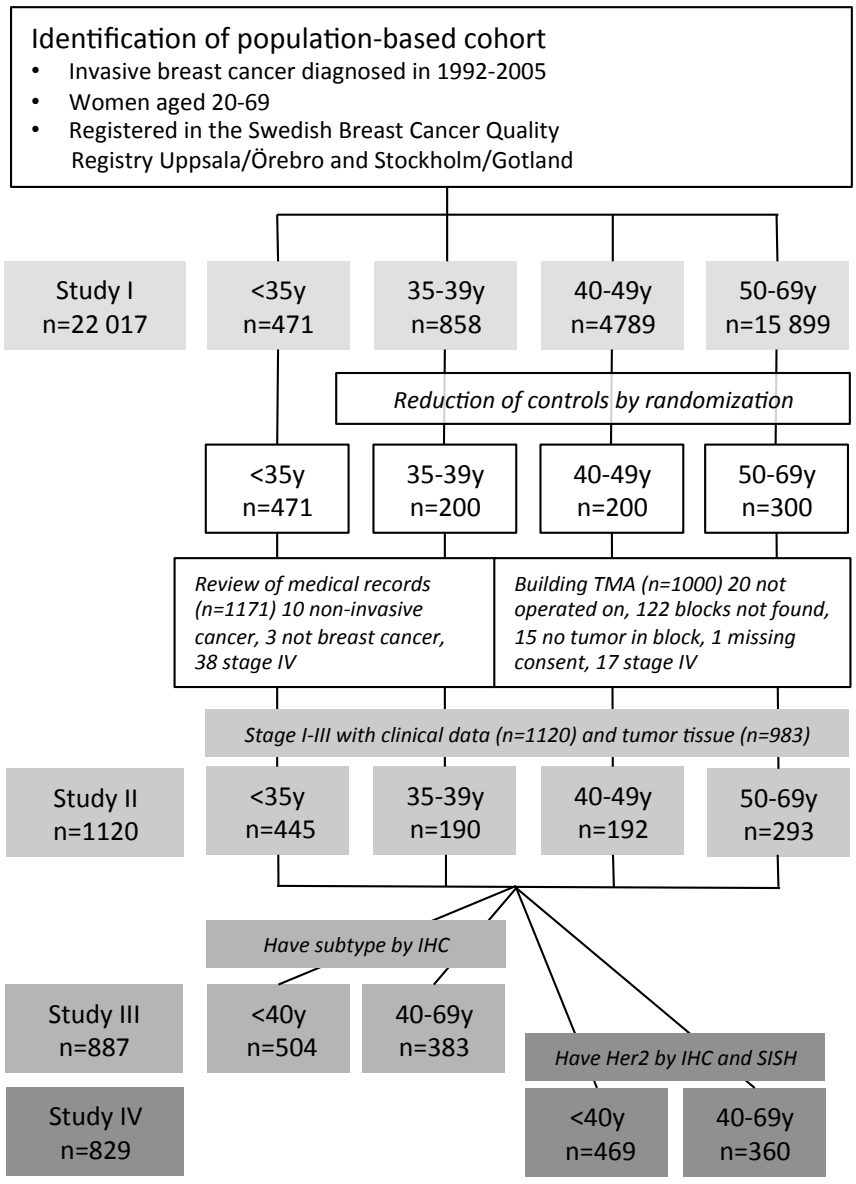
The Total Population Registry

This registry held by Statistics Sweden contains civil registration information on all private individuals in Sweden including PIN, sex, births, marriage and partnership, address, country of birth and assessed personal income tax [252]. Information on migration in and out of Sweden is available in the registry.

Study population

For the purpose of this thesis, all women aged 20–69 diagnosed during 1992–2005 with a primary invasive breast cancer (unilateral or synchronous bilateral) in the Stockholm-Gotland and Uppsala-Örebro regions were identified from the regional Breast Cancer Quality Registers. These women were included in the study cohort from which all studies (I–IV) in this thesis were derived (**Figure 3**).

Figure 3. *Flow-chart of Studies I-IV.*



Exclusion criteria were earlier breast cancer (n=617) and less than a one-month follow-up (n=52). In case of synchronous bilateral disease (n=260, 1.2%) the one in the most advanced stage was chosen as the index tumor. After exclusions, the cohort (**Study I**) consisted of 22,017 women, with 11,527 women from the Stockholm-Gotland region and 10,490 from the Uppsala-Örebro region. Women in the cohort were divided into four age groups: 20–34 (n = 471), 35–39 (n = 858), 40–49 (n = 4789) and 50–69 years (n = 15,899).

In **Study II,** registry data were supplemented with information from medical records and analyses of tumor tissue. All women <35 years old at diagnosis (n=471) from Study I were compared to randomly sampled groups of women aged 35–39 (n=200), 40–49 (n=200) and 50–69 (n=300) from the same cohort (**Figure 3**). The size of the sample was decided after a power calculation based on effect sizes from Study I, with the aim to over-sample young women aged 35 or older, but still with a reasonable possibility of collecting detailed clinical data and tumor tissue for cases and the comparison group. To reach a power of 80% at a 95% significance level, we needed 326 individuals to detect a difference in breast cancer-specific survival and 262 individuals to detect a difference in locoregional recurrence-free survival. After exclusions (3 wrongly registered as having breast cancer, 10 with DCIS, but wrongly registered as having invasive breast cancer), the new smaller cohort, still with its population-based origin, consisted of 1171 women (**Table 2**).

Table 2. *Comparison of cohort in Study I and II.*

	Study I	**Study II**
Age distribution	n (%)	n (%)
20-34 years	471 (2,1)	471 (40)
35-39 years	858 (3,9)	200 (17)
40-49 years	4789 (21,8)	200 (17)
50-69 years	15,899 (72,2)	300 (26)
total	22,017 (100)	1171 (100)
Median age	years	years
20-34 years	31	31
35-69 years	55	49
35-39 years	37	37
40-49 years	45	45
50-69 years	59	59

	Study I	**Study II**
Region/County	n (%)	n (%)
Sthlm+Gotland	11,527 (52)	662 (57)
Uppsala/Örebro	10,490 (48)	509 (43)
Uppsala	1545 (7)	80 (7)
Södermanland	1525 (7)	67 (6)
Värmland	1648 (7,5)	75 (6)
Örebro	1425 (6,5)	78 (7)
Västmanland	1420 (6)	61 (5)
Dalarna	1457 (7)	86 (7)
Gävleborg	1470 (7)	62 (5)

Women with DCIS are also included in the Breast Cancer Quality Register but were not selected for this cohort. In our sample of the cohort, we found the accuracy of registration to be 99.7% (1168/1171). As survival was the main outcome, we restricted further analyses to stage I–III disease, leaving out 38 women with stage IV disease at diagnosis. The final study base for Study II consisted of 1120 women. Stage IV was defined as the presence of a distant metastasis at diagnosis or within three months of the diagnosis. TNM staging was done according to UICC criteria [253].

In **Study III,** we related the expression of proliferation markers to subtype and age. All women from Study II with information on subtype were included in Study III (n=887).

In **Study IV**, we investigated whether or not added information on Her2 by SISH could explain survival differences between young and older women. All women from Study II included in TMA (n=983) except those without information on Her2 obtained either by using Silver *in Situ* Hybridization (SISH) or IHC (n=154) were included, leaving 829 women in the cohort.

Methods

Clinical data (Studies I-IV)

For **Study I,** the two regional Breast Cancer Quality Registers in Stockholm-Gotland and Uppsala-Örebro regions were combined into one large dataset. The registers contained slightly different variables, but most variables were joined and merged into one dataset (**Table 3**). Missing data were presented and there was no use of imputation. Data in the registers on multifocality, grade, ER and PR were incomplete to such an extent that they were deemed unreliable.

In **Study II** detailed clinical information was collected from medical records after ethical approval and local permission from supervisors at all involved clinics. New data were collected blinded from the individual data retrieved from Study I, as presented in **Table 3**.

Table 3. *Variables collected for Studies I-IV.*

Study I	**Registry** Age, county of residency, date of diagnosis, menopause status, mode of detection, contralateral breast cancer, multifocality, tumor side, tumor size, number of lymph nodes examined, number of positive lymph nodes, TNM-stage, grade, ER, PR, treatment (intention to treat data); type of breast and axillary surgery, radiotherapy, chemotherapy, endocrine therapy
Study II	**Medical records** Age, length, weight, parity, pregnancy association, heredity, mode of detection, tumor size, lymph node status, grade, ER, PR, proliferation, Her2, EIC, multifocality, LVI, histological type. Given treatment; type of breast and axillary surgery, surgical margins, re-excisions, profylactic surgery. Type and length of chemotherapy, endocrine therapy and radiotherapy. Treatment of LR. Date and location of contralateral breast cancer, LR, distant recurrence. Date and cause of death, last day of follow-up. **Pathology review** Grade, LVI, lymphocyte infiltration, cancer in situ component, tumor invasion front **TMA** ER, PR, Ki-67, Her2 (IHC, for 2+ confirmation of Her2 gene by SISH), EGFR, CK 5/6, 14, 17
Study III	**TMA** ER, PR, Ki-67, Her2, Cyclin A2, cyclin B1, cyclin D1, cyclin E1
Study IV	**TMA** ER, PR, Ki-67, Her2, Her2 SISH, CK 5/6

EIC and multifocality were extracted from the pathology reports. EIC was defined as >25% of the tumor consisting of DCIS with an intraductal component also beyond the edge of the invasive tumor. Multifocality was defined as two or more invasive tumor foci separated by at least 1 cm. For the 8 women lost to follow-up, the cause and date of death was retrieved from the Swedish Causes of Death Register. After data collection was completed, all of the included study subjects were anonymized, leaving only their given specific study code. The code key was kept separately. The cohort was recorded as a research registry according to the Swedish Data Protection Authority [254]. Clinical data retrieved for Study II was also used for **Studies III and IV**.

Tumor tissue (Studies II-IV)

To retrieve tumor tissue, permissions from the 11 pathology departments involved were obtained according to the Law on Biobank for Research Purposes [255]. The collected tumor tissue was gathered in a sample collection at the Karolinska University Hospital Biobank.
When available, original haematoxylin and eosin stained sections were retrieved; otherwise, a re-sectioning and re-staining was preformed. The pathologist reviewed sections for grade according to Elston and Ellis [78], LVI, lymphocyte infiltration, cancer in situ component and tumor invasion front.
The pathologist marked tumor-representative areas on the section and tissue microarrays (TMAs) were generated from 1.0-mm cores in duplicate from each patient's archival FFPE tumor block. From the TMA blocks, 4 μm sections were cut and automated IHC was done using a Lab Vision Autostainer 480 (Thermo Fisher Scientific).
The IHC stained and mounted TMA sections, as well as whole tumor sections, were scanned at 20x magnification using a ScanScope XT System (Aperio Technologies, Vista, USA). The high-resolution, digital images of each tissue core were annotated with respect to the outcome of IHC staining. TMA production, IHC staining, section scanning and annotation were performed at the Human Protein Atlas facilities at Rudbeck Laboratory at Uppsala University and in accordance with their standards [256,257].

ER, PR, cyclins and cytokeratins (CK) were annotated at the following levels: 0-1%, 2-10%, 11-25%, 26-50%, 51-75% and >75%. Ki-67 were annotated at 0-1%, 2-10%, 11-14%, 15-20%, 21-30%, 31-40%, 41-50%, 51-75% and >75%. Her2 was annotated at 0, 1+, 2+, and 3+.

Her2 SISH was performed on an automated Ventana BenchMark ULTRA IHC/ISH Staining Module (Ventana Medical Systems, Inc., Tuscon, AZ, USA) and was encounted in <4 dots, 4–6 dots and >6 dots (including clusters). Cut-offs for defining positive expression and the antibodies and probes used are presented in **Table 4**.

Table 4. *Cut-offs for antibodies and probes used for protein and gene expression*

	Definition of pos staining			Ref	Antibody/probe/dilution
	Study II	Study III	Study IV		
ER	>10%	>1%	>1%	[88, 89]	M7047, 1:150, Dako, Glostrup, Denmark
PR	>10%	>25%	>25%	[102, 258]	M3569, 1:1000, Dako, Glostrup, Denmark
Ki-67	>20%	>14%	>14%	[48, 119]	M7240, 1:200, Dako, Glostrup, Denmark
Her2 IHC	3+, 2+ if SISH+	3+, 2+ if SISH+	3+, 2+ if SISH+	[107, 259]	A0485, 1:1000, Dako, Glostrup, Denmark
Her2 SISH	>6 dots	>6 dots	>6 dots	[107, 259]	INFORM HER2 Dual ISH DNA Probe Cocktail
Cyclin A2		>10%			CAB000114, 1:200, Novocastra, Germany
Cyclin B1		>10%			CAB000115, 1:1000, Transduction Lab, USA
Cyclin D1		>10%			CAB000024, 1:20, Novocastra, Germany
Cyclin E1		>10%			CAB000308, 1:200 Pharmingen, USA
CK5/6			>10%	[260]	M7237, 1:1000, Dako, Glostrup, Denmark

Out of the 1158 women included in the cohort for Study II, 20 were not operated on, 122 tumor blocks were not found, 15 blocks had no tumor left in them, and there was one missed consent to use tumor material. Thus, 1000 women were included in the TMA. For the analyses, only women with stages I–III were included (excluding 38 women with stage IV disease), leaving 983 women with tumor material available for analysis out of the 1120 potentially includable women (88%).

Definition of subtypes

For **Studies II–IV,** we used surrogate definitions based on central IHC re-evaluation of ER, PR, Ki-67 and Her2 according to the St Gallen Consensus Statement [48,261].

Luminal A	ER+, PR+, Her2- and Ki-67 low
Luminal B	ER+, PR+, Her2- and Ki-67 high or
	ER+, PR-, Her2- and any Ki-67
Luminal-Her2+	ER+ and Her2+, any PR or Ki-67
Her2+	ER-, PR- and Her2+, any Ki-67
Triple negative	ER-, PR- and Her2-, any Ki-67

Statistical analysis

Study I: The end-point was the 5-year relative survival ratio (RSR) [262]. The observation time was defined as the time between the breast cancer diagnosis (date taken from the Regional Breast Cancer Quality Registries) and death (date taken from the Total Population Registry). In the absence of events, patients were censored at the end of follow-up (31 December, 2006). The RSR was calculated by comparing observed survival with expected

survival of the general population, represented by all women matched for age, calendar period and county of residency.

To use relative survival in a large cohort like this has an advantage, cause of death is not required, and still can it provide a measure of mortality irrespective of cause of death. Data for calculating county-specific life tables were taken from Statistics Sweden 2008 [18].

To investigate the possible age-related differences in prognostic factors (tumor size, lymph node status and hormonal status) we used Fisher's exact test to test the independence between age and dichotomized variables. Excess mortality was modelled using the Poisson regression [263] to calculate differences in survival by age and important confounders such as stage, year of diagnosis, tumor size, lymph node involvement, grade, hormonal receptor status, multifocality, breast surgery and intended adjuvant systemic and locoregional treatment. Women aged 50-69 were used as a reference group. Cumulative RSR by stage was performed to study age differences across this variable. A multivariate analysis was performed, adjusted by year of diagnosis, stage at diagnosis and oncological treatment and stratified on tumor characteristics to evaluate the independent effect of age on survival.

Furthermore, we studied differences in survival between the age groups in stages I–IIb while adjusting for the potential determinants by modelling the excess mortality (RER) using a Poisson regression analysis. In order to assess the effect of the different variables separately, as well as in addition to each other, five separate models were constructed. SAS 9.1 software was used for all statistical analyses.

Study II: Endpoints were breast cancer-specific survival (BCSS), distant disease-free survival (DDFS) and locoregional recurrence-free survival (LRFS). All survival estimates were calculated from the date of diagnosis to the date of event or, in absence of an event, to the end of follow-up, recorded individually, but, at latest, the end of 2012. An event for BCSS was defined as death from breast cancer, for DDFS as a distant recurrence or death from breast cancer and, for LRFS, as a locoregional recurrence as the first event prior to distant recurrence. No censoring was done for invasive or non-invasive contralateral breast cancer.

Associations between variables were evaluated using the Pearson Chi-2 test. Survival curves were derived from Kaplan-Meier estimates [264] since death from other cause than breast cancer was uncommon in this population. Survival curves were compared using the log-rank test [265]. Cox proportional-hazard models were used to estimate univariate and multivariate hazard ratios (HRs) with 95% confidence intervals (CIs). IBM SPSS Statistics v22.0 (SPSS Inc., Chicago Illinois, USA) was used for all statistical analyses.

Study III: The endpoint was DDFS, as defined in Study II. All statistical methods used were the same as in Study II. IBM SPSS Statistics v24.0 (SPSS Inc. Chicago, Illinois, USA) was used for all statistical analyses.

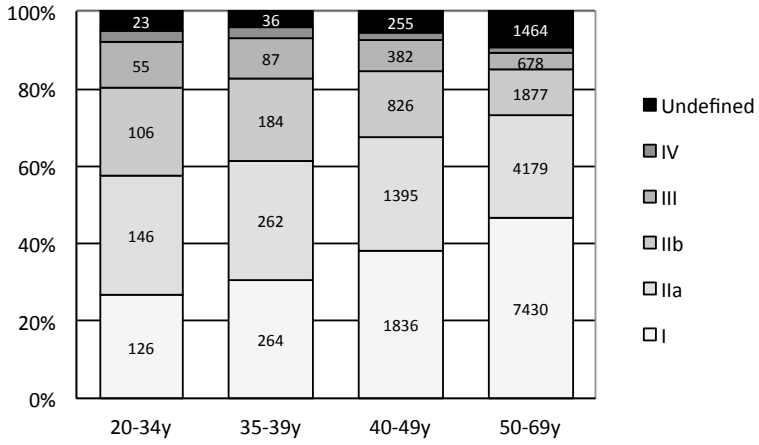
Study IV: The primary endpoint was the probability of Her2-positivity by IHC and SISH. The secondary endpoint was BCSS (as defined in Study II), predicted by either method. For probabilities of test accuracy, the likelihood ratio test was used and, for the calculation of post-test probabilities, Fagan´s nomogram [266]. Distributions and associations between variables were evaluated with the Pearson Chi-2 test. Survival analyses were derived using Kaplan-Meyer estimates and compared using the log-rank test. Cox proportional-hazards models were used to estimate hazard ratios. Likelihood ratios, posterior probabilities and 95% CIs were calculated using a Diagnostic Test Calculator [267]. All other calculations were performed using IBM SPSS Statistics v24.0 (SPSS Inc. Chicago, Illinois, USA).

RESULTS

Study I - *Women with breast cancer diagnosed before age 35 have a more advanced disease and receive a more intense treatment, but, still, they have a worse prognosis compared to women aged 50–69*

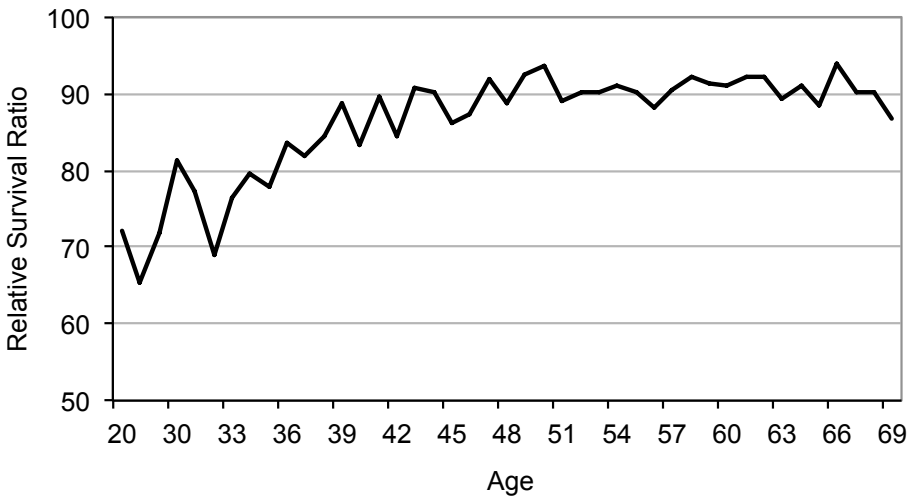
In this large population-based cohort of women aged 20–69, only 2.1% of the women was aged <35 at breast cancer diagnosis and only 6.0% aged <40. Compared to women aged 50–69, women aged <35 had larger tumors and more often with lymph node involvement and stage III–IV at diagnosis (**Figure 4**).

Figure 4. *Stage distribution in 22,017 women diagnosed with primary invasive breast cancer.*



Variables describing tumor biology had a high proportion of missing data, although indicating more aggressive characteristics, more often grade III, hormone receptor negativity and multifocality. Treatment (as by intention to treat) was more intense in women aged <35, with a higher rate of mastectomies and planned chemotherapy more than twice as often, whereas no age-related differences in planned endocrine treatment or radiotherapy were noted.As for survival estimates, the 5-year survival, measured by the relative survival ratio (RSR), was much lower in women aged <35 (74.8%) than in those aged 50–69 (90.7%) (**Figure 5**).

Figure 5. *Relative survival ratio (RSR) by age at diagnosis.*



When translated into risk as expressed by the relative excess ratio (RER) using women aged 50–69 as reference, women aged <35 had 2.8 times as high a risk of dying within 5 years from a breast cancer diagnosis. When stratified by stage at diagnosis, the excess risk of dying for women aged <35, compared to those aged 50–69, was highest in the early stages and most pronounced in those with the smallest tumors, i.e., measuring only 1–10 mm. For women in stage III, there were no age-related differences in survival.

When analysing tumor size, lymph node status and hormonal receptor status, one by one, and adjusting for the other two factors, age <35 was an independent risk for death due to ER+PR+ tumors, regardless of tumor size or lymph node status, but not for ER-PR- tumors. A subgroup analysis of women with stage I, with tumors <10 mm, showed that women aged <35 were scheduled for a very intense treatment with more mastectomies, chemotherapy and endocrine therapy more often than for women aged 50–69. Women aged <35 with 11–20 mm tumors were scheduled more often for chemotherapy. There were no differences in planned radiotherapy for women of different ages with stage I tumors sized 1–20 mm. The combined effect of prognostic factors for women with stage I-IIb tumors was modelled in a Poisson regression where the unadjusted excess risk of death for women aged <35 was 3.6 times as high risk of women aged 50-69. After adding explanatory variables separately (year of diagnosis, stage, grade, hormonal receptor status, multifocality, type of surgery, radiotherapy, chemotherapy and endocrine treatment), age <35 was an independent risk factor for death, conferring an increase that was 1.8 times as high as for women aged 50–69.

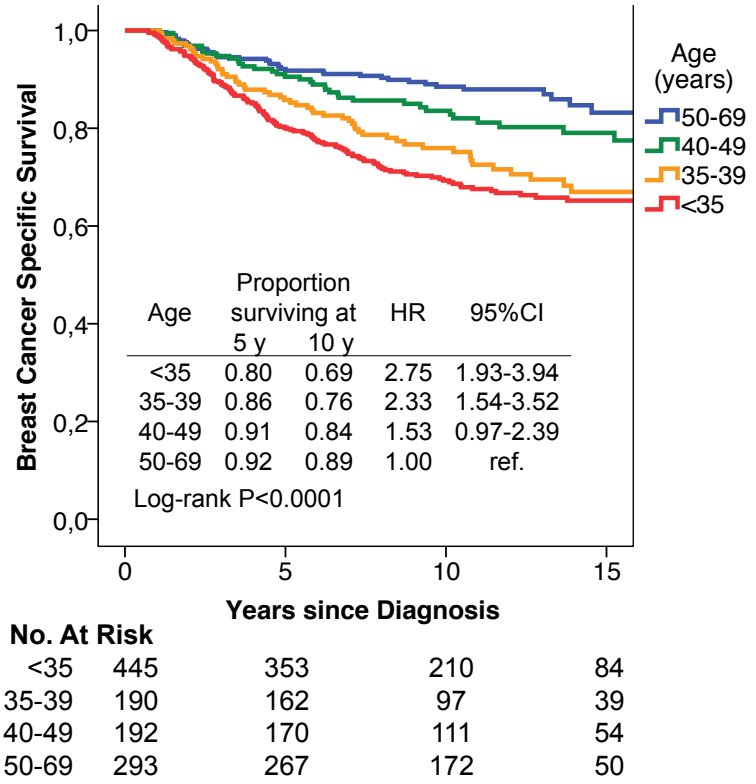
Study II – *In early stages of breast cancer, age <40 is an independent risk factor for distant and locoregional recurrence. Age <40 entails a higher risk of breast cancer death of the Luminal B subtype, but not in the other subtypes.*

At a median follow-up of 10 years, 20.2% of the women aged <35 had had a locoregional recurrence. Corresponding figures for women aged 35–39, 40–49 and 50–69 were 19.5%, 14.1% and 7.5%, and a distant recurrence had occurred in 40.0%, 31.1%, 24.5% and 14.3%, respectively.
Women aged <35 had larger tumors, more often positive lymph nodes, more often tumor grade III, ER-, PR-, Her2+, high Ki-67, presence of LVI, multifocality and EIC. Compared to women aged 50–69, the subtype distribution in women aged <35 showed Luminal A less often and TN and Her2+ subtypes more often. Reflecting differences in stage distribution and tumor biology, breast cancer treatment was more intense in women aged <35 and they had more often mastectomies and more often chemotherapy, which more often included the new drugs of the time, such as taxanes and neoadjuvant chemotherapy. Trastuzumab was introduced during the study period and was given to very few women. Chest wall

radiotherapy after mastectomy was given almost twice as often to women aged <35, whereas endocrine therapy was given as often as to women aged 50-69.

Breast cancer specific survival, taking only age into consideration, showed that women aged <35 had an almost 3-fold risk and women aged 35–39 a more than 2-fold risk of dying from breast cancer, compared to women aged 50–69 (**Figure 6**).

Figure 6. *Breast cancer-specific survival by age for 1120 women stage I-III.*



Among the risk factors for breast cancer-specific deaths among women aged <35, as compared to those aged 50–69, tumor size <20 mm, stage I, grade I–II and Luminal B subtype were identified. For women aged 35–39, the risk pattern was quite similar to that of women aged <35, whereas women aged 40–49 had risks similar to those of women aged 50–69. In the multivariate analysis, adjusting for year, stage, screening detection, grade, subtype and systemic treatment, age <35 and age 35–39 were independent risk factors for locoregional recurrence, but not for breast cancer specific survival (BCSS) or distant disease-free survival (DDFS).

In a subgroup analysis of women in stage I-IIa, split at age 40, age <40 was an independent risk factor after all adjustments for distant (HR, 1.87; 95% CI, 1.03–3.44; p=0.042) and locoregional recurrent disease (HR, 4.10; 95% CI, 2.20–7.66; p <0.001) (**Figure 7**).

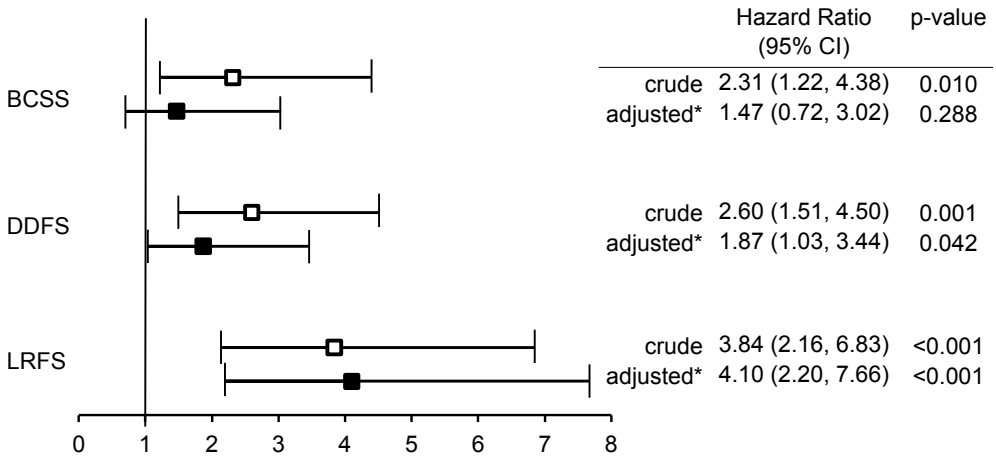


Figure 7. *Forest plot of multivariate Cox model describing risk of event for women with luminal breast cancer stage I-IIa. Women aged <40 (n=152) compared to women aged ≥40 (ref) (n=237).*

Study III –*The higher expression of proliferation markers in young women does not have a strong impact on prognosis. Proliferation markers are less important in young women, and Ki-67 is prognostic only in young women with luminal tumors with PR+. Age <40 years is an independent factor for distant disease only in in the Luminal B PR+ subgroup. The only cyclin adding prognostic value beyond subtype in young women is cyclin E1. A high cyclin E1 is associated with a better prognosis in young women with Luminal B PR– breast cancer.*

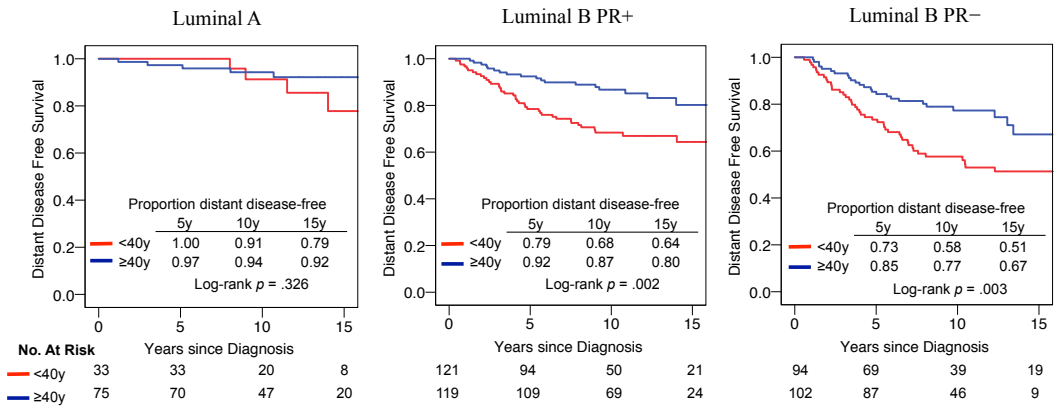
Young women with luminal tumors had a significantly higher expression of all cyclins and Ki-67 than middle-aged women, while in Her2-positive tumors there were no age-related differences in the expression of proliferation markers. In TN tumors, cyclin A2 and E1 were significantly higher expressed in women aged <40.

On studying the association between DDFS and the different proliferation markers, and not taking subtype or age into account, the survival analysis in the whole cohort showed a high expression of Ki-67, cyclin A2 and cyclin E1 to be significantly associated with a worse outcome (Ki-67; $p<0.001$; cyclin A2; $p=0.014$; cyclin E1; $p=0.030$). In young women no proliferation marker was prognostic, not in general, nor in analyses restricted to only luminal tumors. For middle-aged women, a high cyclin D1 expression was associated with a better outcome ($p=0.002$).

A univariate Cox regression analysis of risk factors for distant disease by subtype and age showed age <40 to be associated with a worse DDFS only in luminal tumors (age <40 years; HR 2.37 (1.66–3.37)).

When we stratified luminal tumors by PR status, we found Ki-67 to be prognostic only in luminal PR+ tumors. When analysed with different cut-offs (14%, 20% and 30%), the lower cut-off was shown to have the strongest prognostic value in young women, while the higher cut-off was best in middle-aged women.

In a multivariate analysis, high cyclin E1 remained an independent prognostic factor for a better outcome in young women with Luminal B PR– tumors (HR 0.47 (0.24–0.92); *p*=0.027), while high cyclin D1 remained an independent prognostic factor for a better outcome in women aged ≥40 with Luminal B PR+ tumors (HR 0.19 (0.05–0.74); *p*=0.017). Age <40 years was an independent risk factor for DDFS exclusively in women with Luminal B PR+ tumors (HR 2.35 (1.22–4.50); *p*=0.010) (**Figure 8**).



	Luminal A			Luminal B PR+			Luminal B PR-		
Distant disease free survival	<40y *vs* ≥40y			<40y *vs* ≥40y			<40y *vs* ≥40y		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Unadjusted	1.91	(0.51-7.12)	*.335*	**2.40**	(1.37-4.19)	*.002*	**2.08**	(1.27-3.41)	*.004*
+Year	2.03	(0.55-7.59)	*.290*	**2.38**	(1.36-4.18)	*.002*	**1.83**	(1.10-3.06)	*.020*
+Stage	1.40	(0.33-5.98)	*.651*	**2.25**	(1.28-3.96)	*.005*	1.21	(0.72-2.05)	*.472*
+Grade	1.21	(0.27-5.46)	*.803*	**2.16**	(1.22-3.81)	*.004*	1.09	(0.64-1.87)	*.750*
+LVI	1.22	(0.27-5.56)	*.796*	**2.08**	(1.18-3.68)	*.012*	0.97	(0.56-1.67)	*.903*
+Cyclin A2, B1, D1, E1	0.66	(0.09-4.93)	*.689*	**2.42**	(1.30-4.50)	*.005*	1.15	(0.63-2.09)	*.648*
+Systemic treatment	0.63	(0.08-5.22)	*.671*	**2.35**	(1.22-4.50)	*.010*	1.14	(0.62-2.10)	*.670*

Figure 8. *Prognosis by age in women with breast cancer of the Luminal A, Luminal B PR+ and Luminal B PR– subtypes.*

To put the prognostic importance of cyclin E1 for young women with breast cancer into a clinical context, we performed a survival analysis of DDFS by age and subtype, dividing the luminal tumors into Luminal A, Luminal B PR+, Luminal B PR–/cyclin E1 high and Luminal B PR–/cyclin E1 low (**Figure 9**). Young women with Luminal B PR–/cyclin E1 low tumors had a markedly worse prognosis, with an over 6-fold increased risk of distant disease (HR 6.21 (2.17-17.6)); *p*=0.001) compared to Luminal A tumors.

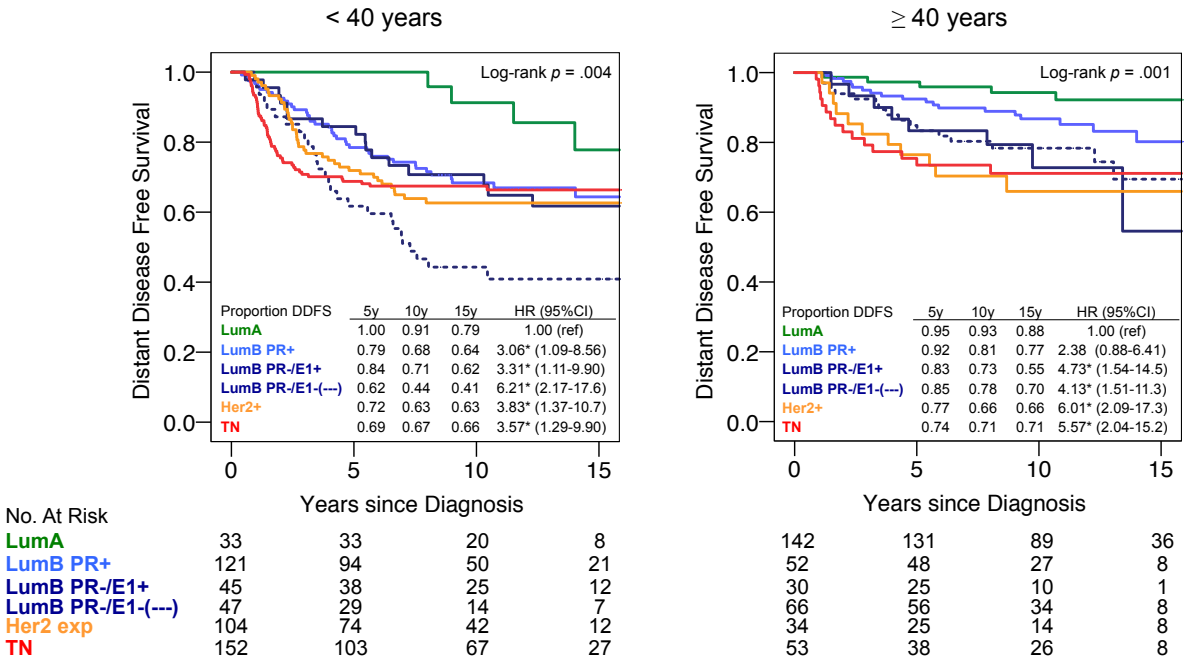


Figure 9. *Kaplan Meyer curves for DDFS by age and subtype. Women with missing data on cyclin E are excluded (n=8).*

Study IV – *Her2-assessment with silver enhanced in situ hybridization (SISH) for all, significantly increases the Her2-positive rate compared to routine Her2-testing, and similarly for women aged <40 and ≥40. Her2 amplification is present in more than a third of cases scored 1+ with IHC. All Her2 amplified cases, both true positive and false negative, had a significantly worse BCSS than the true negative cases.*

Both young and middle-aged women had a significantly higher proportion of Her2-positive tumors when tested by SISH instead of routine testing with IHC plus reflex SISH for equivocal cases (IHC 2+); i.e. from 20.0% to 24.4% (p<0.001). The increase was similar for women aged <40 (25.6% to 30.3%) and ≥40 years (12.8% to 16.1%).

On comparing the two tests, routine testing, with SISH testing, the sensitivity was 81.0% and the specificity 99.4%. Likelihood ratios indicated that a positive routine test considerably upgraded a prior clinical chance of Her2 being positive (to 98%), while a negative test changed the probability of Her2 being positive more modestly (to 6%). Due to a higher prevalence of Her2+ in women aged <40, the risk of a negative Her2 IHC (0-1+) missing a Her2 amplification was 1 in every 14 women and corresponding risk for women aged ≥40 was 1 in every 25 women (**Figure 10**).

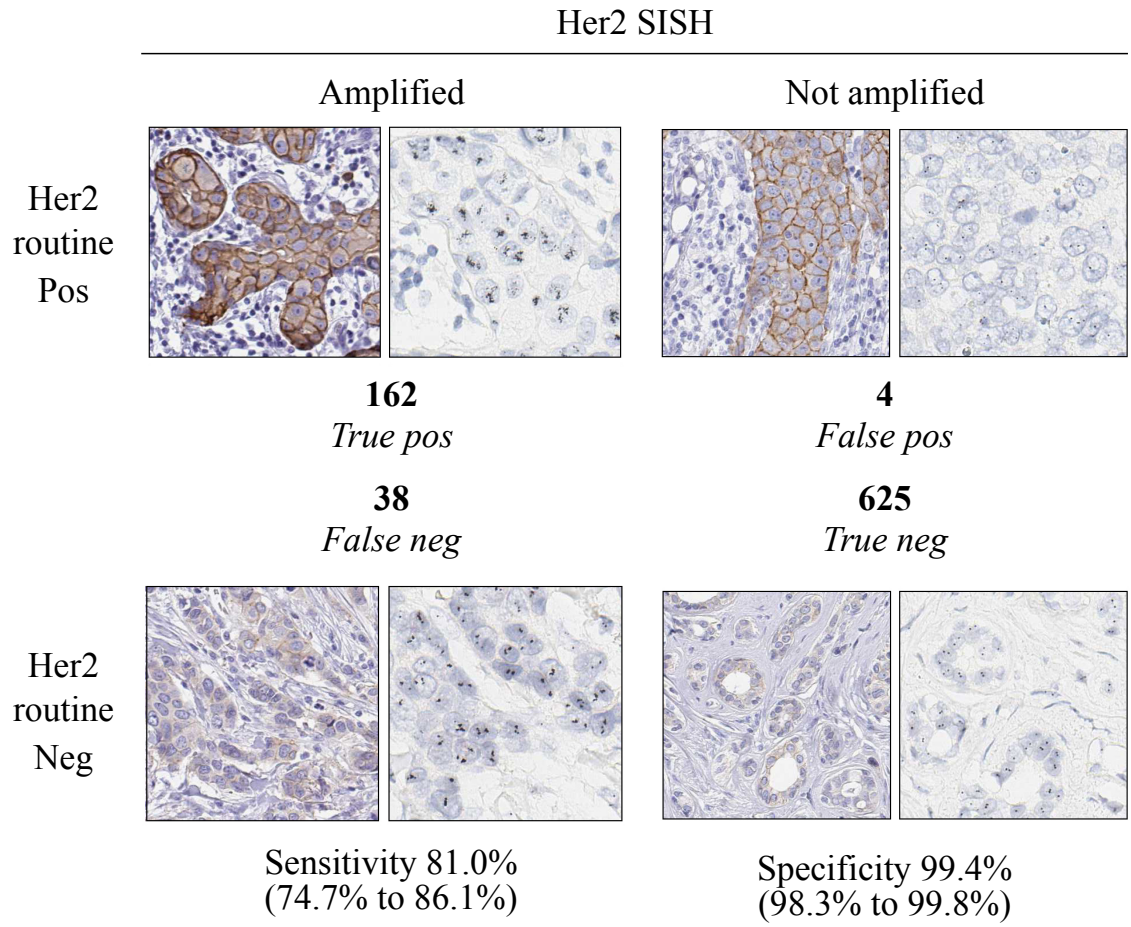
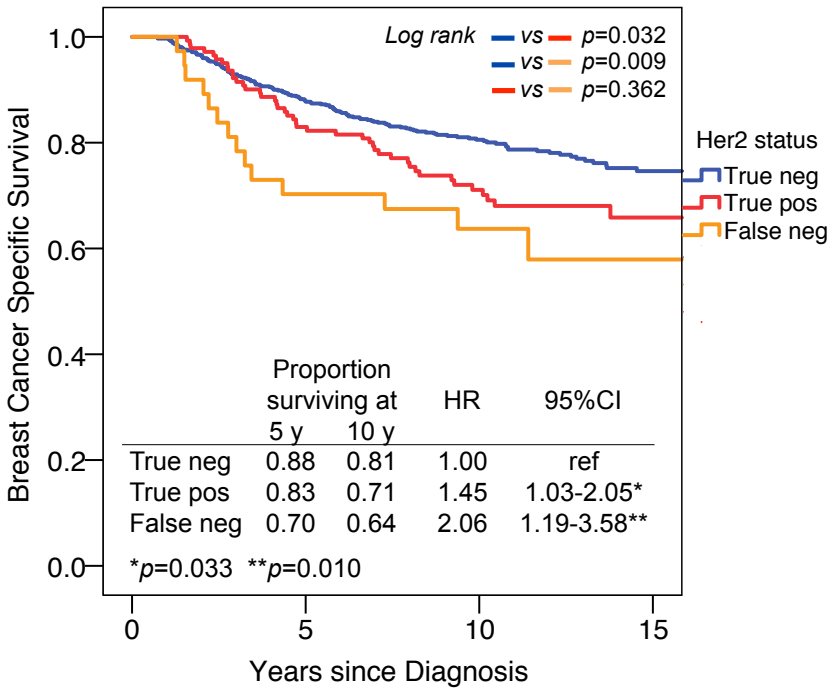


Figure 10. *Likelihood ratios for Her2 routine testing and SISH, respectively, and corresponding histological examples of paired Her2 IHC and SISH stainings.*

Her2 amplification was present in 4.6% of cases scored 0 with IHC, while corresponding proportions for scores 1+, 2+ and 3+ were 36.0%, 83.7% and 96.8%. Her2 had a prognostic value regardless of it was assessed by IHC (3+ *vs* 0-1+, HR 1.51, *p*=0.028), *Her2* gene copy numbers (>6 dots *vs* <4 dots, HR 1.60; *p*=0.005) or *Her2* gene amplification (amplification *vs* no amplification, HR 1.55; *p*=0.008). When the Cox regression analysis were stratified by age, Her2 status was not a risk factor for BCSS in women aged <40, but for women aged ≥40 with Her2 status expressed by SISH copy numbers or amplification (HR 2.69 (1.46-4.97); *p*=0.001 and HR 2.88 (1.54-5.40); *p*=0.001).

Compared to true positive cases, false negative cases more often had ER-positive, CK5/6-positive, lymph node-negative disease, which was less often multifocal. On comparing BCSS, did the true negative cases have the best survival, followed by the true positive cases (HR 1.45 (1.03-2.05); *p*=0.033), whereas the false negative cases had the worst survival (HR 2.06 (1.19-3.58); *p*=0.010) (**Figure 11**).

Figure 11. *Breast cancer-specific survival by Her2 status in 804 women untreated with trastuzumab.*



DISCUSSION

Young age as a risk factor for poorer survival in breast cancer was suggested as early as in the 1930s [268], with increasing attention to the phenomenon in the 1980s [1,269]. With the development of national registration of diseases, the search for evidence in medicine has been facilitated, making it easier to draw conclusions, especially concerning rare diseases, among which we reckon breast cancer in young women. With highly validated data in many registers, the possibility of identifying differences within a population and then adjusting for them one by one, using epidemiological strategies, there is a prospect of coming closer to the truth. The prognosis in breast cancer is age-dependent since different factors influence survival in different age groups.

The best survival from breast cancer is seen in women aged 40-49 years. These women are included in screening programmes and thereby have their breast cancers detected early. The impact of co-morbidity is low. A large population-based study on elderly women with breast cancer, generated from the same regional breast cancers as in our cohort, showed that women aged >70 (which not are included in screening programs) had a worse survival compared to women aged 50-69 subjected to less diagnostic activity, a later stage diagnosis and less intense treatment [270]. This research arose from the hypothesis that low age could not possibly be a true risk factor, but only a proxy. The aims of this thesis therefore became to identify risk factors for young women with breast cancer by comparing different variables across age groups and thereby to find an explanation for why the young women do worse became the aim of this thesis.

Methodological considerations

Internal validity is crucial to being able to draw conclusions from data. A high internal validity is dependent on the absence of systematic errors such as bias and confounding. Bias is an exposure that influences the risk of the outcome. **Selection bias**, could for example be introduced if certain age groups are registered in the National Breast Cancer Quality Registry less often. The Registry is, however, continuously validated by updates from the Swedish Cancer Registry, as well as the Swedish Causes of Death Registry and is therefore influenced to a low degree by bias. In our study, we included age groups in which the validity is good (not including women aged >75), for whom validity is known to be less good. **Diagnostic bias** might possibly have influenced the results in Study I since women aged <40 were not included in screening programmes. In Study I, the multivariate analysis was adjusted for stage, and in Study II for both stage and detection mode (in Study II data on the detection mode were extracted from the medical records).

Confounders are variables associated with outcome independently from the exposure and should be distributed differentially among the exposed and the un-exposed. In Studies I-IV

we have searched for possible explanatory variables and tried to adjust for them by both stratification and regression analyses. The DAGitty is a graphical tool for drawing causal diagrams [271] (**Figure 12**). The third common way to control for confounding, matching of cases, is not used in these studies.

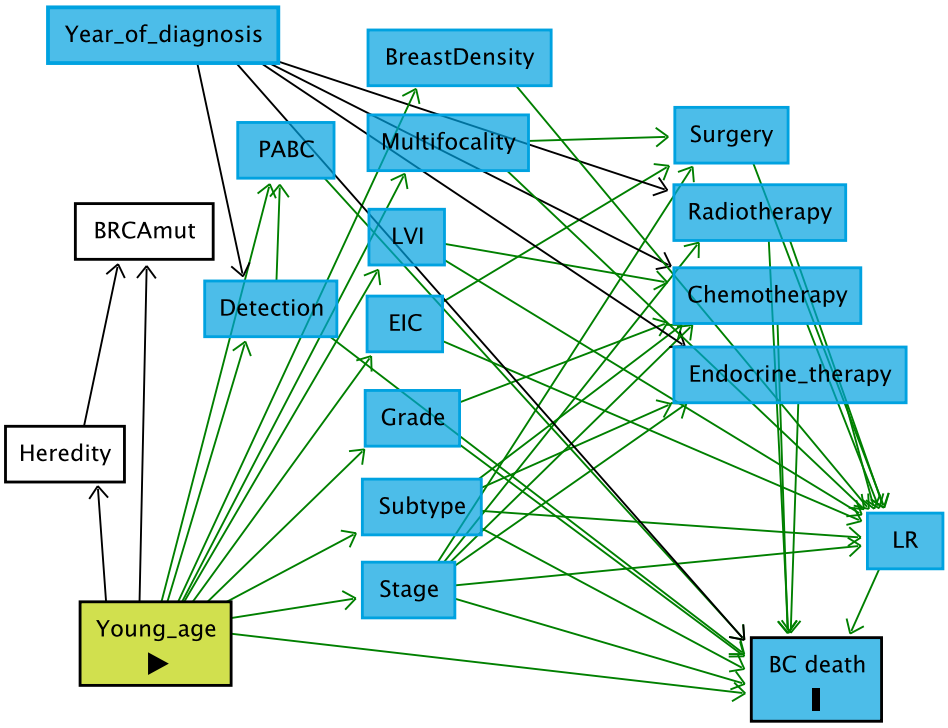


Figure 12. *Causal diagram (DAGitty) illustrating the relation between variables of exposure (young age) and outcome (breast cancer death).*

Loss to follow-up in Study I was minimal due to the mandatory reporting to the Swedish Causes of Death Registry, with which The National Breast Cancer Quality Registry data were matched. In Studies II-IV there were no missing data on cause of death but for 8 of 1171 women lost to follow-up in medical records, there might have been unnotified local recurrences.

Missing data on variables in Study I were evenly distributed between the age groups, except for tumor size, which was missing more often in women aged <35. Young women do more more often have locally advanced disease and thereby a less well-defined tumor size. Stage was missing more often in women aged 50-69. In our cohort, women with undefined stage had a survival resembling that of women in stage I-IIa. In Study II, information collected from the medical records ensured a more complete data set. We found that, despite a 60% missing of stage in Study I, the proportion of grades I, II and III, respectively, was nearly the same as in Study II, indicating that the missing data did not introduce a bias. There was a very high concordance between intention-to-treat data in Study I and the actual treatment given in Study II. The single difference noted was that more intense treatment; especially chemotherapy, had been given to women aged <40 (**Table 5**).

Table 5 *Proportions of planned treatment in Study I compared to that given in Study II.*

Age		BCS	Radio-therapy when BCS	Radio-therapy	Endocrine therapy when ER+	Proportion neoadjuvant	Chemo-therapy
<35	Study I	44.8%	91.9%	71.1%	69.0%	14.0%	65.2%
	Study II	46.3%	95.1%	80.4%	70.4%	17.1%	75.5%
35-39	Study I	48.1%	93.7%	74.2%	65.2%	13.5%	60.8%
	Study II	49.5%	97.9%	78.4%	62.0%	15.3%	74.7%
40-49	Study I	55.5%	93.5%	75.3%	64.8%	8.5%	46.2%
	Study II	60.9%	96.6%	83.3%	62.2%	6.8%	46.4%
50-69	Study I	63.8%	92.4%	75.5%	72.8%	4.3%	26.5%
	Study II	66.2%	96.4%	78.8%	75.0%	2.7%	30.4%

External validity, i.e., the ability to generalize the findings of these studies to another population, we consider high due to the population-based design maintained throughout Studies I-IV.

Study I

We found that age <35 when diagnosed with breast cancer was an independent risk for death in early stages of the disease. The age-related differences in survival were most pronounced in women with small tumors without lymph node involvement. This could have multiple explanations. Women aged 50-69 (and to some extent also women aged 40-49) had a high proportion of early, screening detected tumors. Screening detection *per se* has been shown to be a good independent prognostic factor.

A small tumor size could also mean different things in young *vs* old women. In the group of small tumors we hypothesized that the different age groups had differing proportions of multifocal tumors and tumors with extensive DCIS, but only a small invasive foci. This was addressed in Study II where multifocality and EIC could be eradicated as risk factors. Instead, we found an absence of multifocality and EIC to be a risk, and perhaps the presence of these factors influenced treatment decisions towards more extensive locoregional and systemic treatment.

Furthermore, no differences in survival were seen between tumors in advanced stages or in those with hormone receptor-negative tumors. Based on these findings we hypothesized that women with more advanced stage and hormone receptor-negative disease, regardless of age, reeived intense treatment, thereby reducing the differences between age groups. This hypothesis was strengthened by the study by Kroman et al. suggesting that age-related differences in survival were only present in women not receiving chemotherapy [2].

Tumor biology appeared to be a possible explanation for the findings in Study I, but the tumor biology-related variables available in the registry were few in number and there was a

large amount of missing data on important variables. The missing data on tumor characteristics was evenly distributed across age groups.

Data on the type of surgery was recorded as given treatment with less than 0.5% missing. Systemic treatment was reported as intention-to-treat, and the proportion of missing data was not recorded. Endocrine treatment was planned in only 65-70% of the women with ER+ tumors, irrespective of age, which is considered to be under-treatment by today's standards. Endocrine therapy was not recommended for women with tumors <1 cm or for premenopausal women until the end of the study period.

In the light of the contemporary St Gallen Guidelines, age <35 was an indication for chemotherapy, but was only planned for 34% of the women aged <35 in stage I within our study. The proportion of women scheduled for chemotherapy was, however, much higher for women aged <35 and 35-39 than for middle-aged women, indicating that tumor biological characteristics or low age in itself was indications for the more intense systemic therapy planned in this early stage.

To conclude, we found that stage at diagnosis could partly explain the worse survival in young women, but that the remaining independent prognostic impact of age was most likely only a proxy for aggressive tumor biology.

Study II

Study II was designed for further in-depth studies of age-related differences in prognosis based on the results from Study I. We aimed to study the same research question but now including several variables on tumor characteristics not available in the registers, e.g., data on parity, pregnancies and heredity, as well as data on treatment with a complete long-term follow-up. The design also enabled a validation of all variables from the registry previously studied, but with a large proportion of missing data. By collecting detailed clinical data and enabling new analyses of archival tumor tissue, we hoped to dismiss low age as a risk for breast cancer death and instead get insight into the biological explanation behind the association between age and outcome.

The finding that women aged <35 and women aged 35-39 share the distribution of tumor characteristics, risk profiles and survival outcomes is interesting from a biological point of view. One factor that can influence the prognosis in young women, but not in middle-aged ones, is whether the tumor arises during, or whithin, a year of pregnancy (PABC). On reanalysing data from Study II, PABC was found to be an increased risk for BCSS in women aged <35 (60 PABC of 440 women aged <35; 13.6%) (HR 1.70 (1.12-2.59); p=0.013) but not in women aged 35-39 (12 of 182; 6.6%) or women aged 40-49 (2 of 189; 1.1%). Including PABC in the multivariate model did not change the main findings. Another factor

that only influences women aged <40 is that they are not included in screening mammography, which we adjusted for in the multivariate model.

The finding of similarities between women aged <35 and 35-39, and the fact that women aged 40-49 had only one risk factor that differed between them and those aged 50-69 (PR-negativity was a risk for women aged 40-49), reassured us that combining the two youngest and the two oldest age groups would not introduce any substantial bias. This strategy was later also used for Studies III and IV.

One finding in Study II was the increased risk of locoregional recurrence (LR) in young women. Women aged <40, stage I-III, had twice as high a risk of LR as women aged ≥40, which is in line with earlier publications [212,218]. We know that women with advanced stage, Her2+ and TN breast cancer are at higher risk of LR, but also when the analysis is restricted to women with luminal Her2-negative early disease, the risk for women aged <40 having a LR as first event was 4-fold higher compared to those aged ≥40.

We have chosen not to use disease-free survival, but instead locoregional recurrence-free and distant recurrence-free survival in our studies, knowing the comparably higher risk of LR in young women than in middle-aged women, so as not to mix the two different events. Absolute figures of locoregional and distant recurrences are shown in **Figure 13**.

On analysing all women, regardless of stage or subtype, women aged <40 had LR as the first separate event twice as often as women aged ≥40 (20% *vs* 10%). The corresponding figures for distant disease or breast cancer death, as the first event was 24% *vs* 14%. Women aged <40 had distant disease or breast cancer death twice as often as women aged ≥40 (36% *vs* 18%). The proportion of women remaining disease-free after a LR was 40% *vs* 55%. Possibly, the LR was a chance to receive a second-try adjuvant round of treatments, which substantially decreased the risk of a later distant recurrence.

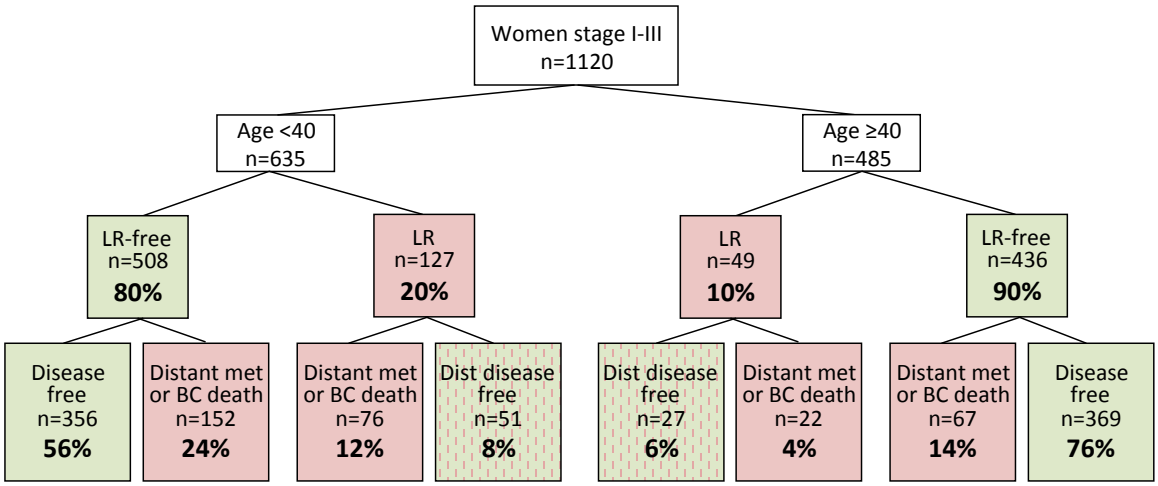


Figure 13. *LRFS and DDFS in women stage I-III.*

In Study II, we still found young age to be an independent risk factor for distant and locoregional recurrence in early stages of disease, but now restricted to tumors with luminal (Her2-negative) biology.

Study III

In the design of the cohort for Studies II-IV, one of the aims was to find new prognostic markers for the young population. With the findings from Study II regarding age-related differences in prognosis being restricted to luminal tumors, we decided that the first group of prognostic markers to be addressed were proliferation markers since they constitute the main water-shed in the division of the Luminal A *vs* Luminal B subtypes.

Cyclins were discovered in the mid-1990s and showed promising results as prognostic markers in breast cancer and with an age-specific expression. Since subtypes made their entrance in the prognostic arena, the value of cyclins has been investigated only by a few authors [122-124] and never in a population-based material.

We extended our earlier analyses of IHC markers (ER, PR, Ki-67, Her2, CK5/6) from Study II with analyses of cyclins A2, B1, D1 and E1. To mimic the clinical situation and reveal the prognostic impact of proliferation markers within different subtypes, we stratified our analyses by subtype (Luminal Her2-negative, Her2+ and TN).

The main findings were: Proliferation markers are significantly more highly expressed in women aged <40, but they do not have a strong impact on prognosis. Ki-67 was prognostic only in young women with Luminal PR+ tumors. The optimal cut-off for Ki-67 as a prognostic marker in luminal PR+ tumors was lower for younger women than for middle-aged ones. Age <40 was an independent risk factor for a worse DDFS in women with Luminal B PR+ tumors. Cyclins added only limited prognostic value, except in young women with Luminal B PR– breast cancer, a subgroup with a sinister prognosis, where a high expression of cyclin E1 was associated with a statistically significant better prognosis.

On considering the results from our Study III, the absence of standardized methods for measuring Ki-67 and the question of reproducibility of the results are crucial. One of the strengths of this study is the central re-evaluation of Ki-67 which was performed in this population-based material with tumor tissue from such a large proportion of the women included. While it has been shown that protein expression of ER, PR and Her2 on archival TMA cores highly correlates with the analysis of whole sections [272], the analysis of Ki-67 on tissue cores has been shown to generate a generally lower Ki-67 score than analyses of whole sections [273]. Also, the way Ki-67 is assessed matters. Focke et al. showed that the higher the number of cells counted in luminal tumors, the lower where Ki-67 scores and thus

the higher were the proportion of Luminal A tumors [274]. We analysed Ki-67 on duplicate cores from each patient, read in hotspots. Since the main goal of this study is a comparison between age groups, a potential bias derived from a low Ki-67 count on TMA cores, should not in any major way affect our results. On the contrary, Ki-67 in our cohort was comparably high, corresponding to the age profile in the cohort. A rule of thumb is that in a 'normal breast cancer population' 1 out of 3 tumors should be considered to be highly proliferating. On applying this rule to our cohort in Study III and defining women aged 50-69 as normal, a Ki-67 cut-off of 30% will produce a proportion of 69% slowly proliferating tumors. This strengthens the assumption that the optimal cut-off of Ki-67 for prognostic use is 30% for women aged ≥40 years.

Age remains an independent negative prognostic factor in Luminal B PR+ breast cancer, with a more than two-fold risk of distant disease compared to middle-aged women with corresponding stage and biology. This is a clinically highly relevant finding which has to be considered in treatment decisions. One explanation for this finding may be endocrine resistance, which is noted more often in young women in studies on gene expression signatures related to endocrine resistance [222]. Young women are less adherent to endocrine therapy which might be another explanation [191,192].

With these results, we draw the following conclusions concerning women aged <40 with luminal tumors; (1) PR-negativity is an important marker of a worse survival; (2) the optimal cut-off for defining a Luminal A tumor in PR+ tumors is sooner 14% than 20% or 30% and (3) the Luminal A subtype is very uncommon in young women, and the separation from the Luminal B subtype is even more difficult than in middle-aged ones, and perhaps gene-based subtyping should be used for these women. The question is raised as to whether a classic Luminal A subtype accompanied by a good prognosis actually exists in women aged <40 since the 15-year distant disease-free survival in the young women with Luminal A tumors (79%) is about the same as that seen in middle-aged women with the Luminal B subtype (80%). 4) Further studies are needed to clarify the explanation behind the divergent prognosis in young and middle-aged women with Luminal B PR+ tumors despite more intense treatment in young women. With current biomarkers and tumor characteristics used in clinical routine, we found no significant differences between the age groups.

Study IV

The main findings were: 1) A significantly higher proportion of Her2-positivity was generated by SISH-analysis in all women, compared to routine testing with IHC followed by SISH only for those equivocal (IHC 2+). Her2 routine testing led to a missed Her2-positivity in every 14th woman aged <40 and every 25th in those aged ≥40. We deem the clinical utility of this finding to be high since in real-life breast cancer care, every case of early Her2-positive

breast cancer nowadays has a good chance of cure due to targeted therapies developed against the Her2 receptor. 2) All Her2 amplified cases, both true positive and false negative, had a significantly worse BCSS than the true negative cases.

A Her2 IHC score of 3+ had a very high correlation to Her2 amplification by SISH (96.8%), a statement also true for those who scored IHC 2+ (83.7%). The unexpected finding was that, among the cases scoring IHC 1+, 36.0% were, in fact, Her2-amplified. In the pathological routine assessment of Her2 status, the judgment as to whether a case scores 1+ or 2+ by IHC can be very difficult, and often involves a second opinion by another pathologist.

With the results from Study IV it seems obvious to suggest a changed routine to perform Her2 SISH for both cases with Her2 IHC score 1+ and 2+. It can also be added that the proportion of cases scoring 1+ using Her2 IHC is small, especially after the new assessment guidelines by ASCO/CAP from 2013 [275,276], and would accordingly not be very expensive. *In situ* hybridization is however, in comparison to IHC, more expensive, has higher failure rates, takes a longer time both to test and interpret [277]. We also observed a high failure rate of SISH (SISH-analysis ending uninformative). Of 939 cases eligible for the study (all having information on Her2 IHC), 110 (12%) failed the SISH test and were excluded. The failure rate in our material is in line with Dybdal et al. having a 15% failure rate [278]. Of the 110 cases uninformative with SISH, 100 had IHC score 0, seven had score 1+, one had score 2+ and two score 3+. Assuming these cases were amplified to the same extent as those included in the analyses, the Her2 positive rates would have been 17.7% with routine test and 22.4% with SISH; thus the difference between the methods would have been the same as when we restricted analyses to only informative cases (20.0% vs 24.1%).

The false negative rate in our study was relatively low (38 of 829; 4.6%); however, for these 38 women, hypothetically, a positive Her2 test could have changed their prognosis to the better thanks to the targeted anti-Her2 treatments offered to women with Her2 positive tumors today.

The false negative cases were more often ER+, which is in concordance with findings by Lee et al. indicating tumors with high intratumoral heterogeneity and inaccurate Her2 assessment are more frequent in the Luminal-Her2+ subtype [279]. Multifocality can also increase the risk of intratumoral heterogeneity and is more often present in Luminal-Her2+ tumors [280,281]. We tried to overcome intratumoral heterogeneity by viewing duplicate cores from each case and performing both IHC and SISH at the same laboratory and on consecutive sections of the TMA tumor block. According to the current evidence analyzing Her2 status only on the largest tumor foci is safe as long as the smaller concomitant foci do not differ in histological type or tumor grade [282].

In our study were the false negative cases more often CK5/6 positive. Cytokeratins indicate a basal phenotype, and CK5/6-positive, Her2+ tumors are associated with worse prognosis and a high proportion of non-responders to targeted therapy [260,283,284].

When comparing the prognosis between the true negative, the true positive and the false negative cases, the true positive cases had, as expected, a worse BCSS than the true negative cases (HR 1.45 (1.03-2.05)). The false negative cases did also have a worse prognosis than the true negative ones (HR 2.06 (1.19-3.58)), however, not statistically significantly different from the true positive cases (p=0.362).

As expected, we found Her2 status not to be a prognostic factor in young women. Young women have an over-representation of the aggressive subtypes Luminal B and TN, which have about the same outcome as seen in Her2+ subtypes. On the contrary, Her2-status was clearly prognostic in women aged ≥40 with a subtype distribution dominated by Luminal A and Luminal B, both associated with a better survival.

CONCLUSIONS

- Women with breast cancer diagnosed before age 35 have a more advanced disease at diagnosis. They receive a more intense treatment, but still have a worse prognosis than women aged 50-69.

- In luminal (ER-positive, Her2-negative) breast cancer there is a significantly different distribution of both tumor characteristics and prognosis between young and middle-aged women.

- Tumor characteristics as well as prognosis are very similar for young and middle aged women with the Her2 positive and triple-negative subtypes.

- In early luminal breast cancer, age <40 is an independent risk factor for distant and locoregional recurrence. The risk for young women compared to middle-aged women to have a distant recurrence is 2-fold, and to have a locoregional recurrence 4-fold. Age <40 confers a higher risk of breast cancer mortality in women with tumors of the Luminal B subtype, but not in other subtypes.

- Proliferation markers are important only in luminal tumors, where they are significantly higher expressed in women aged <40. They do not have a strong impact on prognosis. Ki-67 has a prognostic value in luminal tumors, restricted to the luminal PR+ group. Age <40 is an independent risk factor for distant disease only in women with tumors of the Luminal B subtype being PR+.

- The prognostic value of cyclins are restricted to luminal tumors. For women aged <40 with luminal PR– tumors (18% of the young population), a high cyclin E1 was an independent marker for decreased risk of distant recurrence.

- Her2 status examined by silver in situ hybridization (SISH) generates a higher proportion of Her2 positive cases than routine testing with immunohistochemistry. In trastuzumab-untreated women Her2 was a strong prognostic marker for women aged ≥40, but had no value as a prognosticator in women aged <40.

FUTURE PERSPECTIVES

The observations made, and conclusions drawn in this thesis are based on historical data, which is a prerequisite to obtain a long follow-up. During the period from 1992 and until 2005, diagnostics and treatment of breast cancer has undergone dramatic changes. Breast cancer screening became fully implemented and treatment regimes changed according to evidence-based studies. The survival rates increased for all women, but most for middle-aged women, which concomitantly increased the differences in outcome between young and middle-aged women.

In the 1940s young age was in itself identified as a risk factor for lower survival in breast cancer. Later were age-related differences in survival were restricted to early stages of the disease, and more recently the age-related differences have been limited to women with tumors of the luminal (ER+/Her2–) subtypes. Gene expression profiling has identified age-related differences in genes regulating tumor growth, immune response, mammary stem cells and apoptosis. With an increased insight into the biology of breast cancer in young women, we will most certainly discover that age is just a proxy for a combination of other factors. Until then some crucial questions need to be answered:

1. Why are young women more prone to Luminal B, Her2-positive and triple-negative subtypes? Is the answer in the genes?

2. Why are the age-related differences in survival restricted to women with Luminal B tumors expressing PR?

3. Is the explanation for age-related differences in prognosis found in either of; stroma-related factors, stem-cell properties, angiogenetic factors, immune response markers, or hormonal receptors?

4. Can we find new biomarkers selecting young women at low risk of recurrent disease and thereby reduce the use of systemic therapy?

5. Do age-related differences in survival persist in an era of modern therapies?

6. Can young women with non-genetic risk factors for breast cancer (like breast density) be identified and benefit from screening?

We have constructed our young breast cancer cohort with the aim to elucidate some of these unanswered questions, whereas other questions need new material, new cohorts and new collaborations to be answered.

SVENSK SAMMANFATTNING

Målet med denna avhandling var att få en ökad kunskap om långtidsprognosen hos unga kvinnor med bröstcancer.

I en populationsbaserad kohort bestående av 22 017 kvinnor med bröstcancer, studerade vi prognosen i olika åldersgrupper. Kvinnor under 35 år vid diagnos (471 st), kvinnor 35-39 år (858 st) och kvinnor 40-49 år (4789 st) jämfördes med kvinnor i åldern 50-69 år (15 899 st) med avseende på överlevnad uttryckt som relativ överrisk att dö jämfört med normalbefolkningen i samma åldersgrupp. Kvinnorna under 35 år hade den sämsta överlevnaden vilket delvis kunde förklaras av en högre andel unga med diagnos i ett senare skede av sjukdomen. När man kontrollerade statistiskt för skillnaden i stadium fann man att en sämre överlevnad för de unga endast kan ses vid diagnos i tidigt stadium.

I nästa studie gick vi vidare och studerade alla kvinnorna under 35 år vid diagnos, men jämförde dem nu med en mindre, slumpmässigt utvald grupp om 700 kvinnor från den stora kohorten. För dessa kvinnor insamlades journaluppgifter om deras bröstcancer, men även uppgifter om barnafödande, ärftlighet, behandling och uppföljning. Arkiverat tumörmaterial insamlades till en tumörbank, och på detta gjordes nya tumörbiologiska analyser. Vi studerade risken för bröstcancerspecifik död, risken för lokalt återfall (i bröstet eller på bröstkorgsväggen) samt risken för återfall av bröstcancern i andra organ. När man i analyserna korrigerade för bl a skillnader i stadium, tumörbiologi och behandling fann vi att låg ålder i sig fortfarande var förenat med en högre risk att få lokalt återfall än hos kvinnor i åldern 50-69. Unga kvinnor med tidigt stadium av bröstcancer av östrogenkänslig typ och utan överuttryck av Her2-genen hade även en ökad risk för återfall i andra organ. Dessa kvinnor hade en nästan dubbelt så stor risk att få återfall i andra organ än en kvinna som var 50-69 år.

Fyndet att unga kvinnor med östrogen-positiva, Her2-negativa tumörer var de som hade en sämre prognos fick oss att gå vidare med analyser av tumörernas tillväxttakt, proliferation, en markör som generellt är viktig för att skilja tumörer med en bra respektive sämre prognos. På tumörmaterialet gjordes analyser av Ki-67 och cykliner, proteiner som indikerar hög tillväxt. Resultaten från föregående studie stod sig, men vi kunde nu ytterligare specificera gruppen med åldersskillnader i prognos till tumörer av Luminal B subtyp med känslighet för ett annat könshormon, progesteron. Dessa kvinnor under 40 år hade en drygt dubblerad risk för återfall i andra organ. Detta fynd hjälper oss att förstå vilka unga kvinnor som kan behöva mer eller annan behandling än vad som ges till en medelålders kvinna med samma tumörtyp.

I avhandlingens sista studie studerades Her2-positiva tumörer. Dessa har en sämre prognos än många andra subtyper av bröstcancer, men å andra sidan finns sedan ca 10 år en

målinriktad behandling för dessa tumörer vilket lett till att prognosen förbättrats drastiskt. Her2-positiva tumörer är dubbelt så vanligt hos unga som hos äldre kvinnor. Vi jämförde två testmetoder som används för att diagnosticera Her2-positiv bröstcancer och fann att metoden som påvisar Her2-positivitet på gennivå gav en högre andel av Her2-positiva fall än rutinmetoden där Her2 undersöks på proteinnivå. Detta är ett viktigt fynd eftersom alla kvinnor som går med oupptäckt Her2-positiv bröstcancer skulle kunna få en avsevärt förbättrad behandling med större chans till bot.

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Paper I

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Breast Cancer in Young Women: Poor Survival Despite Intensive Treatment

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Abstract

Background: Breast cancer is uncommon in young women and correlates with a less favourable prognosis; still it is the most frequent cancer in women under 40, accounting for 30–40% of all incident female cancer. The aim of this study was to study prognosis in young women, quantifying how much stage at diagnosis and management on the one hand, and tumour biology on the other; each contribute to the worse prognosis seen in this age group.

Methodology/Principal Findings: In a registry based cohort of women aged 20–69 (n = 22 017) with a primary diagnosis of invasive breast cancer (1992–2005), women aged 20–34 (n = 471), 35–39 (n = 858) and 40–49 (n = 4789) were compared with women aged 50–69 years (n = 15 899). The cumulative 5-year relative survival ratio and the relative excess mortality (RER) were calculated. The cumulative 5-year relative survival ratio was lowest in women aged 20–34. The RER was 2.84 for women aged 20–34 and decreased with increasing age (RER 1.76 and 1.17 for women aged 35–39 and 40–49, respectively). The excess risk was, however, present only in disease stages I and II. For women aged 20–34 with stage I disease RER was 4.63, and 6.70 in the subgroup with tumour size 1–10 mm. The absolute difference in stage I between the youngest and the reference groups amounted to nearly 8%, with a 90% 5-year survival in women aged 20–34. In stages IIa and IIb, the relative excess risk was not as dramatic, but the absolute differences approached 15%. The youngest women with small tumours generally received more aggressive treatment than women in older age groups.

Conclusions: After correction for stage, tumour characteristics and treatment, age remained an independent risk factor for breast cancer death in women <35 years of age. The excess risk for young women was only seen in early stages of disease and was most pronounced in women with small tumours. Young women affected by breast cancer have a high risk of dying compared to their middle-aged counterparts even if diagnosed early and receiving an intense treatment.

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Introduction

Approximately 7% of all breast cancers are diagnosed in women <40 years of age and less than 4% in women below the age of 35 [1,2,3]. Although breast cancer is uncommon in young women, it is the most frequent cancer in women <40, accounting for 30–40% of all incident female cancer [4,5].

Young age at diagnosis influences prognosis negatively [1,6,7,8,9,10]. This could partly be explained by young women more often being diagnosed at advanced stages [1,11] and by unfavourable tumour characteristics more often being present [8,11,12,13,14,15]. It has not been quantified how much stage at diagnosis and management on one hand, and tumour biology on the other, each contribute to the poor prognosis.

At the other end of the age spectrum, breast cancer in elderly women is also associated with an inferior prognosis when compared to that of middle-aged women [6,16]. In a prior study we focused on the elderly and found that less diagnostic activity and less intensive treatment were major explanations for their relatively low survival [17]. We interpreted our findings to be partly due to less rigorous guidelines for the treatment of women aged >70 and were alarmed that unclear guidelines might also contribute to the worse prognosis seen in young women, since guidelines for treating younger women have been less structured than for middle-aged women.

The aim of the present study was to investigate to what degree a worse prognosis in young women can be explained by stage, tumour characteristics and treatment procedures.

Methods

2.1. Participants

Data were collected from the regional breast cancer registers in two of Sweden's six health-care regions (Stockholm/Gotland and

Uppsala/Örebro), which currently serve a population of almost 3.9 million inhabitants, thus covering about 43% of the Swedish population. The registers contain prospectively collected data on patient and tumour characteristics, types of treatment and follow-up. The registers are updated continuously by matching with the National Population Register and the mandatory Swedish Cancer Register to ensure a high coverage. The validity of the Swedish Cancer Register has been tested previously and found to be very high for the purpose of breast cancer studies [18,19].

A cohort of all women aged 20–69 with a primary diagnosis of invasive breast cancer between 1992 and 2005 was followed until the end of 2006. Of the original cohort, 22 017 women were eligible after exclusion of 52 women with less than one month follow-up. Women with bilateral disease at diagnosis were included only once, with the most advanced cancer as the index tumour. For women offered neoadjuvant treatment and those not operated on, staging was based on clinical and preoperative biopsy data. Data on tumour stage were based on the UICC criteria for histopathological TNM stage [20]. The age interval was chosen to allow comparison of young women with middle-aged women, as the latter group is known to have the best survival [10,21,22,23]. The time interval was chosen to represent recent clinical practice and registration procedures, i.e. with homogeneous treatment guidelines and full coverage by regional breast cancer registers.

We chose the cut off at 50 years to define young women and further divided them into three groups (20–34 years, 35–39 years and 40–49 years) in accordance with earlier studies and current treatment recommendations for women <35 years [24]. Women aged 50–69 years served as a comparison group.

All women aged 50–69 years were invited to screening mammography every second year. For women aged 40–49 the routines for screening mammography differed between the two regions, implying that approximately 50% of women aged 40–49 were invited for mammography with an interval of 1.5 years, while the other 50% were not.

2.2. Treatment

During the study period treatment for breast cancer was performed according to regional and national guidelines that closely follow international practice. Surgery involved either modified radical mastectomy or breast conserving surgery [25], combined with either a level I and II axillary clearance [26] or a sentinel node biopsy [27].

Radiotherapy to the remaining breast parenchyma up to a total dose of 50–54 Gy (Grey) was recommended as standard treatment after breast conserving surgery. Since 2003 a boost of 10–16 Gy to the tumour bed has been offered to women of 40–45 years. Radiotherapy to the axilla in a dose of 46–54 Gy has been given to women with involved axillary nodes (to all with $\geq$4 involved nodes and to a majority with 1–3 involved nodes). After mastectomy a total dose of 50–54 Gy has been given to the chest wall if the tumour primarily invaded the pectoral muscle, if extensive multifocality, when tumour size $\geq$50 mm or smaller if involved axillary nodes.

During the early years of the study period, tamoxifen was not routinely given to premenopausal women [28]. After 1994 tamoxifen was recommended to all women with hormone receptor positive disease but in one of the two regions, only to women with tumours larger than 10 mm. Since 2005 aromatase inhibitors have been recommended postmenopausal women with node positive disease.

Chemotherapy, mainly 5-fluorouracil, epirubicin, cyclophosphamide (FEC), was recommended to all women with node-positive disease or node-negative, hormone receptor negative disease. For node-positive breast cancer docetaxel in sequence with anthracyclines was introduced in 2004.

2.3. Ethics

The regional breast cancer data bases used in this study have a general ethic committee admittance from the regional ethics committee at Karolinska Institutet (diary number 03–630) for studies assessing given treatment and outcome based on the retrospective data collected. The data are to be handled and analyzed without possibility to identify individual patients, and no written consents are thus requested. This retrospective study has fully met the stated criteria and has thus been performed under the above mentioned general admission.

2.4. Statistical Methods

The studied end-point was 5-year relative survival ratio (RSR). The observation time was defined as the time between the date of diagnosis and death. In the absence of event, the observation time was censored at the date of end of follow up (31 December 2006). The relative survival ratio was calculated by comparing the observed survival of the women in the study population with the expected survival of the general population matched with age, sex, calendar period and county of residency [29]. The general population in this study was represented by all females in the Uppsala/Örebro and Stockholm/Gotland health-care regions stratified by county (8 counties). Data for calculating county-specific life-tables were collected from Statistics Sweden [30]. SAS 9.1 software was used for all statistical analyses.

The Fisher's exact test was used to test the independence between age and tumour and treatment variables (dichotomized into extremes).

To study differences in survival between age groups while adjusting for the confounding factors available in the dataset, i.e., stage (I, IIa, IIb, III, IV, and undefined owing to unknown lymph node status or tumour size) and calendar period of diagnosis (1992–93, 1994–95, 1996–97, 1998–99, 2000–01, 2002–03, 2004–05), we modelled excess mortality using Poisson regression [31]. In addition, information was retrieved on tumour size (1–10 mm, 11–20 mm, 21–50 mm, $\geq$51 mm, missing), lymph node involvement (positive, negative, missing, 1–3 engaged lymph nodes, $\geq$4 engaged lymph nodes), tumour grade (I, II, III, missing), hormonal receptor status [estrogen and progesterone (ER/PR); positive, negative, missing], multifocality (yes, missing), surgical treatment [mastectomy, breast conservation (BCS), other operation, none, missing] and prescribed oncological treatment [neoadjuvant chemotherapy, chemotherapy, radiotherapy, endocrine therapy (ET)]. Stratification according to stage was also performed in order to study whether the differences in survival between age groups were consistent across levels of this variable.

We performed a multivariate analysis adjusted by year of diagnosis, stage at diagnosis and oncological treatment (radiotherapy, chemotherapy, ET), stratified on tumour characteristics to evaluate the independent effect of age on survival. Furthermore, we studied differences in survival between the age groups in stage I-IIb while adjusting for the potential determinants by modeling the excess mortality (RER) using Poisson regression. To assess the effect of the different variables separately, as well as in addition to each other, five separate models were made.

Results

3.1. Patient Characteristics

Tumour and treatment characteristics of the 22 017 women included in the study are shown in Table 1. Compared with

Table 1. Distribution of patient, tumour and treatment characteristics.

	Age at diagnosis (years)			
	20–34	**35–39**	**40–49**	**50–69**
	n = 471 (2.1)	**n = 858 (3.9)**	**n = 4789 (21.8)**	**n = 15899 (72.2)**
Tumour size (mm)				
1–10	64 (13.6)	129 (15.0)	876 (18.3)	4317 (27.2)
11–20	153 (32.5)	332 (38.7)	1965 (41.0)	6746 (42.4)
21–50	172 (36.5)	288 (33.6)	1441 (30.1)	3698 (23.3)
≥51	35 (7.4)	57 (6.6)	206 (4.3)	470 (3.0)
Missing	47 (10.0)	52 (6.1)	301 (6.3)	668 (4.2)
Axillary lymph node status				
Negative	234 (49.7)	410 (47.8)	2624 (54.8)	9661 (60.8)
Positive	205 (43.5)	412 (48.0)	1908 (39.8)	4818 (30.3)
1–3 pos nodes	118 (25.1)	263 (30.7)	1279 (26.7)	3279 (20.6)
≥4 pos nodes	87 (18.5)	149 (17.4)	629 (13.1)	1539 (9.7)
Missing	32 (6.8)	36 (4.2)	257 (5.3)	1420 (8.9)
Tumour stage				
I (T1+N0)	126 (26.8)	264 (30.8)	1836 (38.3)	7430 (46.7)
IIa (T1+N1 or T2+N0)	146 (31.0)	262 (30.5)	1395 (29.1)	4179 (26.3)
IIb (T2+N1 or T3+N0)	106 (22.5)	184 (21.5)	826 (17.2)	1877 (11.8)
III (T3+N1 or T4)	55 (11.7)	87 (10.1)	382 (8.0)	678 (4.3)
IV (M1)	15 (3.2)	25 (2.9)	95 (2.0)	271 (1.7)
Undefined	23 (4.9)	36 (4.2)	255 (5.3)	1464 (9.2)
Tumour grade				
I	14 (3.0)	42 (4.9)	364 (7.6)	1850 (11.6)
II	38 (8.1)	134 (15.6)	862 (18.0)	3248 (20.4)
III	115 (24.4)	176 (20.5)	575 (12.0)	1610 (10.1)
Missing	304 (64.5)	506 (59.0)	2988 (62.4)	9191 (57.8)
Hormone receptor status				
Positive*	219 (46.5)	517 (60.3)	3050 (63.7)	10148 (63.8)
Negative**	156 (33.1)	194 (22.6)	778 (16.2)	2565 (16.1)
Missing	96 (20.4)	147 (17.1)	961 (20.1)	3186 (20.0)
Multifocal tumour				
Yes	81 (17.2)	196 (22.8)	788 (16.4)	2006 (12.6)
Missing	156 (33.1)	279 (32.5)	1706 (35.6)	5659 (35.6)
Surgical treatment				
Breast Conservation (BCS)	211 (44.8)	413 (48.1)	2658 (55.5)	10151 (63.8)
Mastectomy	224 (47.6)	411 (47.9)	1914 (40.0)	5229 (32.9)
Other operation	3 (0.6)	8 (0.9)	42 (0.9)	81 (0.5)
None	31 (6.6)	26 (3.0)	157 (3.3)	382 (2.4)
Missing	2 (0.4)	0	18 (0.4)	56 (0.4)
Oncological therapy				
Neoadjuvant treatment	61 (14.0)	111 (13.5)	387 (8.5)	662 (4.3)
Chemotherapy	307 (65.2)	522 (60.8)	2212 (46.2)	4209 (26.5)
Radiotherapy	335 (71.1)	637 (74.2)	3608 (75.3)	11999 (75.5)
If BCS also radiation	194 (91.9)	387 (93.7)	2486 (93.5)	9382 (92.4)
Endocrine therapy (ET)	192 (40.8)	416 (48.5)	2436 (50.9)	9659 (60.8)
If hormonal positive also ET	151 (69.0)	337 (65.2)	1975 (64.8)	7391 (72.8)

Distribution of patient-, tumour- and treatment characteristics of women aged 20–69 years diagnosed with primary breast cancer of all stages between 1992 and 2005 (22 017 women). Values are numbers (percentages). *ER positive, PR positive or negative, **ER and PR negative.

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women aged 50–69 years, women <35 years had larger tumours: (49% were ≥21 mm vs. 27% in the older age group). Their tumours were also more often multifocal (26% vs 20%), high grade (69% vs 24% for grade III) and hormone receptor- negative (33% vs 16%). Fewer women <35 years presented with stage I disease (27% vs 47%). Lymphatic involvement was more common in women <35 years (49% vs 34%) and they displayed a more advanced lymph node status (42% vs 32% having ≥4 involved lymph nodes).

3.2. Treatment

When comparing treatment regimes between women <35 years and women of 50–69 years, there were differences as expected from the stage distribution: a larger proportion of women <35 years were treated with mastectomy (48% vs 33%), chemotherapy was more common in women <35 years (65% vs 26%), and use of endocrine therapy was not as common (41% vs 61%) as in the older age group. There were no differences between the age groups in either use of radiotherapy (71% vs 76%) or use of endocrine therapy (in subjects with endocrine responsive tumours 69% vs 73%).

3.3. Survival

As of December 31 2006, 3723 (17%) women in our study population had died, of whom 125 were in the age group 20–34 years (26% of all women aged 20–34), 173 aged 35–39 years (20%), 757 aged 40–49 years (16%) and 2668 aged 50–69 years (17%).

The cumulative 5-year relative survival ratio (RSR) was lowest in women <35 years and increased with age. There was a marked distinction in relative excess mortality (RER) in women <35 years compared with older age groups in a model unadjusted for stage (RER 2.84 in comparison with women aged 50–69). In the crude analysis women of 35–49 years also had a worse prognosis compared with the oldest group (Table 2). As expected from the stage distribution in the different age groups, stage at diagnosis was a major explanatory factor for these differences. The adjusted analysis no longer showed a worse survival for the women aged 35–39, but the adjustment did not remove a higher RER for the youngest women.

We further stratified the survival analyses by stage (I, IIa, IIb, III+IV and undefined) to look at stage specific differences (Table 3). Women <35 years with stage I disease had a 4.63-fold excess risk of dying within 5 years. When dividing stage I into tumour sizes 1–10 mm and 11–20 mm, the highest relative excess mortality was seen in the two youngest age groups with the smallest tumours, i.e. 1–10 mm. The absolute difference in stage I between the youngest and the reference groups amounted to nearly 8%, with a 90% 5-year survival in women aged 20–34. In stages IIa and IIb, the relative excess risk was not as dramatic, but the absolute differences approached 15%. In stage III and IV, the RER for the younger women were actually lower than 1.0, but these subgroups are small with limited statistical precision. Figure 1 illustrates the relationship further: the prognosis is generally good in stage I, but there are clear differences between the age groups. Prognosis rapidly becomes worse with stage, with more pronounced absolute differences between the age groups up to and including stage IIb.

As the excess risk was noted only in young women with the earliest stages of disease (stages I, IIa and IIb), further analyses were restricted to women in these stages. To elucidate the effect of age vs treatment variables on survival, we performed a multivariate analysis stratified on tumour characteristics, correcting for year, stage at diagnosis and oncological treatment (Table 4). We found a significant excess risk of mortality in the women <35 years, compared with the reference group of 50–69 years, in strata with traditionally good prognostic signs such as small tumour size, absence of lymphatic involvement and hormone receptor-positive tumours. The worst relative survival was observed in the youngest women with the smallest tumours. The general pattern was a worse prognosis for women <40 years in nearly all subgroups, but a tendency for women aged 40–49 to do better than those aged 50–69 with the exception of the strata with tumours less than 20 mm.

We further addressed the question if the youngest women with small tumours were under-treated, but found, as shown in Table 5, that they generally received more aggressive treatment than women in older age groups.

Finally, we undertook a series of multivariate analyses to investigate the combined effect of all potential determinants available in the dataset and to try to elucidate which of the covariates contributed most to the differences (Table 6). In the simplest model, women aged 20–34 years with stage I, IIa and IIb disease had a markedly worse relative survival (RER 3.62) than women aged 50–69 years. Inclusion of year of diagnosis (model 2)

Table 2. Cumulative 5-year relative survival ratio (RSR) and the estimated relative excess risks of mortality (RER) by age.

	Total	5-years survival				Crude[2,4]		Adjusted[3,5]	
	No	Expected	Observed	RSR	95% CI	RER	95% CI	RER	95% CI
Age									
20–34	471	99.8	74.7	74.8	70.1–78.9	2.84	2.31–3.49	1.63	1.32–2.01
35–39	858	99.7	83.8	84.1	81.2–86.6	1.76	1.45–2.14	1.08	0.89–1.32
40–49	4789	99.1	88.3	89.0	88.0–90.0	1.17	1.04–1.31	0.84	0.75–0.94
50–69	15899	96.8	87.8	90.7	90.1–91.2	1.00	(ref.)	1.00	(ref.)

The deviance is a measure of the models goodness-of-fit. Under the hypothesis that the model fits, the deviance should follow a chi-square distribution with the specified degrees of freedom).

[2]Likelihood ratio test of the effect of age in the model; df = 3, chi-square = 96.7, p<0.0001.

[3]Model adjusted for year (1992–93, 1994–95, 1996–97, 1998–99, 2000–01, 2002–03, 2004–05) and stage (I, IIa, IIb, III, IV, undefined). Likelihood ratio test of the effect of age in the model; df = 3, chi-square = 33.5, p<0.0001.

[4]Deviance 26, Residual df 12.

[5]Deviance 945, Residual df 760.

Cumulative 5-year relative survival ratio (RSR) and the estimated relative excess risks of mortality (RER) by age with 95% confidence intervals (CI) of women 20–69 years, diagnosed with primary breast cancer of all stages between 1992 and 2005 (22 017 women).

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Table 3. Breast cancer survival by age and stage at diagnosis.

			5-years survival		Crude	
Stage	**Age**	**No.**	**RSR**	**95% CI**	**RER**	**95% CI**
I						
	20–34	126	90.1	88.4–94.8	4.63	2.26–9.50
	35–39	264	93.1	88.4–94.8	3.37	1.87–6.09
	40–49	1836	97.1	96.0–98.0	1.40	0.92–2.14
	50–69	7430	97.9	97.3–98.5	1.00	(ref.)
T = 1–10 mm						
	20–34	36	90.9	67.4–97.9	6.70	1.49–30.2
	35–39	78	95.1	84.8–98.6	3.48	0.93–13.0
	40–49	623	99.2	97.6–100.0	0.61	0.18–2.13
	50–69	2881	98.6	97.7–99.5	1.00	(ref.)
T = 11–20 mm						
	20–34	90	90.1	80.0–95.2	3.88	1.73–8.72
	35–39	186	92.3	86.6–95.7	3.11	1.62–6.01
	40–49	1212	96.0	94.4–97.1	1.61	1.03–2.52
	50–69	4542	97.5	96.6–98.2	1.00	(ref.)
IIA						
	20–34	146	77.0	68.4–83.5	3.04	2.05–4.50
	35–39	262	92.1	87.6–95.1	1.05	0.64–1.74
	40–49	1395	89.5	87.5–91.2	1.30	1.03–1.63
	50–69	4179	91.9	90.8–92.9	1.00	(ref.)
IIB						
	20–34	106	61.7	50.7–71.0	1.78	1.25–2.52
	35–39	184	78.0	70.7–83.8	0.97	0.68–1.37
	40–49	826	84.6	81.7–87.2	0.64	0.51–0.81
	50–69	1877	77.4	75.1–79.5	1.00	(ref.)
III						
	20–34	55	72.4	57.5–82.8	0.81	0.47–1.40
	35–39	87	68.6	56.4–78.0	0.86	0.56–1.32
	40–49	382	70.8	65.2–75.7	0.73	0.57–0.93
	50–69	678	63.2	58.8–67.3	1.00	(ref.)
IV						
	20–34	15	00.0	-	0.67	0.33–1-37
	35–39	25	26.8	9.8–47.4	0.80	0.48–1.33
	40–49	95	27.1	16.8–38.3	0.74	0.55–1.00
	50–69	271	23.7	17.8–30.1	1.00	(ref.)
Undefined						
	20–34	23	77.8	54.4–90.2	2.81	1.13–6-96
	35–39	36	74.3	56.1–85.9	3.04	1.52–6.09
	40–49	255	88.6	83.8–92.1	1.33	0.86–2.06
	50–69	1464	91.3	89.4–92.9	1.00	(ref.)

Cumulative 5-year RSR and the estimated RER and 95% CI by stage at diagnosis of women aged 20–69 years, diagnosed with primary breast cancer of all stages between 1992 and 2005 (22 017 women).
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changed this estimate little, but introduction of stage (model 3) lowered the RER to 2.35, indicating that stage at diagnosis is an important explanatory variable. After the introduction of all tumour characteristics, the RER dropped to 1.83 (model 4). The final introduction of treatment (model 5) led to a minor shift in the RER estimate to 1.76, thus indicating that tumour characteristics rather that treatment activity is the most important explanatory variable.

Discussion

Prognosis in breast cancer has improved dramatically over the past decades, and this study underlines the very good prognosis especially in early stages. As expected from earlier studies women <35 years have a distinctly worse survival than middle-aged women. This study also confirms that women aged 40–49 years have the best survival [6,16,22,23]. Younger women present at a later stage of disease, but that alone does not explain their worse survival since they also have a worse prognosis stage by stage. The distribution of tumour characteristics shown in this study strengthens the assumption that tumour biology is involved. We found no evidence that treatment is less active in the younger women; rather we noticed a higher intensity of treatment corresponding to treatment guidelines. The finding that the age differences in survival are present primarily in stage I and II breast cancer is thought-provoking.

The study population comprises a cohort of consecutive women with primary breast cancer treated according to national guidelines and international practice. The study base is large including a considerable number of young women, conferring a high statistical power for a study in this field. Data were collected from well validated databases in two mainly urban regions of Sweden, and very few women have been lost to follow-up.

The major advantage of using relative survival in this type of analysis is that information on cause of death is not required and that it provides a measure of the excess mortality experienced by patients diagnosed with cancer, irrespective of whether the excess mortality is directly or indirectly attributable to the cancer.

When studying survival by age group in relative terms, one can expect to see large differences as young women with clinically detected tumours with generally more aggressive characteristics stage by stage are being compared with a large group of women with tumours mainly detected by screening mammography with less aggressive characteristics. Still, this reflects that women <35 years, with a normally long life expectancy, will have an absolute risk of dying from their cancer of 25% in such a short follow-up period as 5-year survival. Studies of long-term survival in young women have also shown an increased mortality continuously for up to 40 years after diagnosis [6,32]. This applies even when breast cancer is diagnosed in a localized stage and in the absence of a second primary breast cancer.

It is remarkable that there are such pronounced age-dependent differences in survival in early breast cancer, which theoretically should be curable. The most striking finding in this study is the high relative excess risk in women <35 years in stage I. Other authors have also found the survival difference by age to be more pronounced in early stages of disease [33,34]. Kroman et al found the negative effect of young age to be significant only in women with low risk disease who received no adjuvant chemotherapy. It seems reasonable to search for the explanation for these differences in tumour biology.

In our analyses of classical prognostic factors we found the same pattern of more aggressive tumour characteristics in the youngest women as previously published [8,11,12,13,14,15]. We lack data on other reported important adverse prognostic factors in the young such as high proliferation index [13,15,35], lymphovascular invasion [8,15], and amplification of the Her-2 gene [14], as well as on preoperative mammography findings with implications for

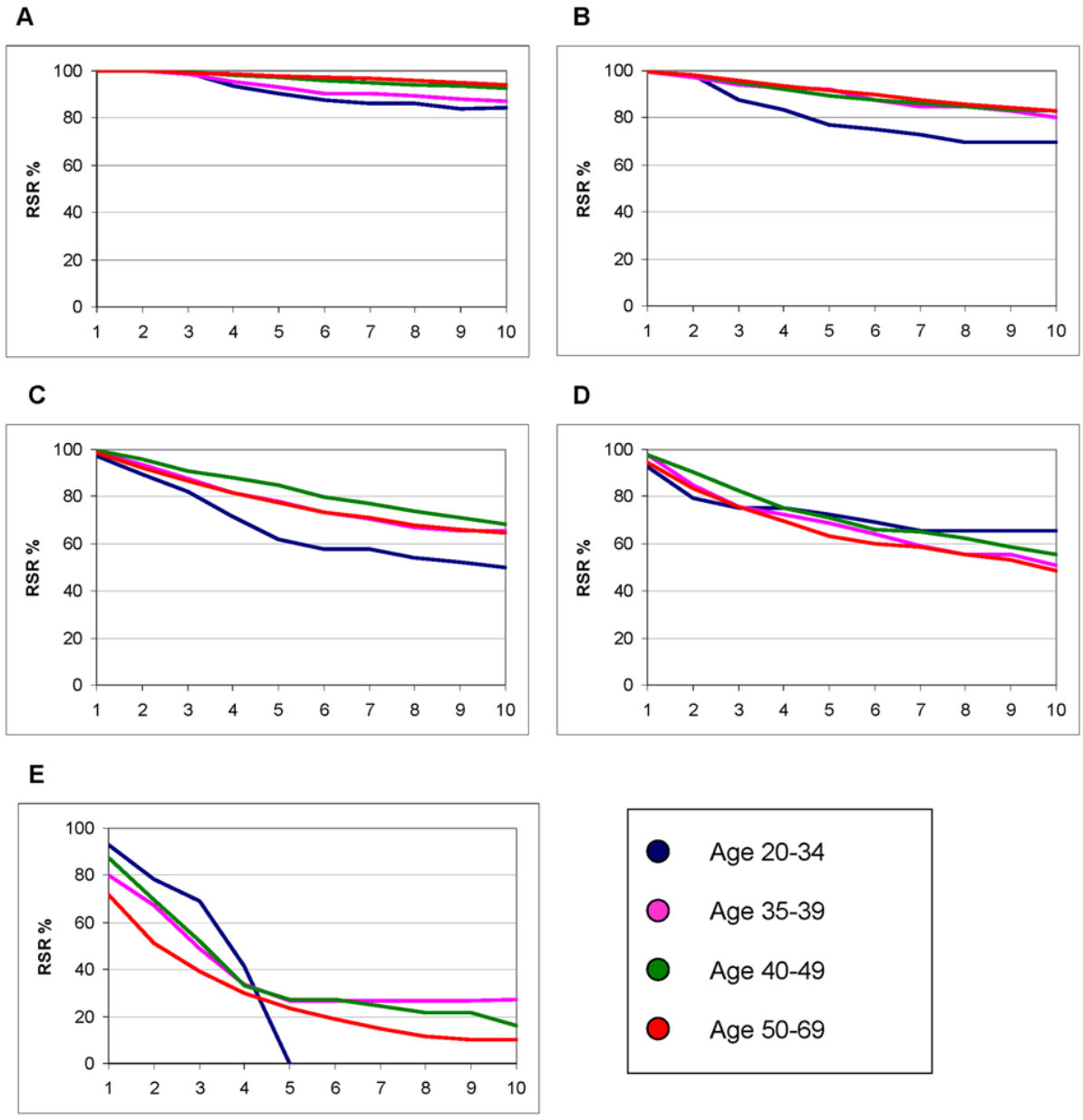

Figure 1. Ten year cumulative survival in relation to expected survival (RSR) according to age and stage of women aged 20–69 years, diagnosed with primary breast cancer between 1992 and 2005 (22 017 women). Size of the groups as in Table 3. A: Stage I, B: Stage IIa, C: Stage IIb, D: Stage III, E: Stage IV.
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histopathology and prognosis. Tabar et al have reported findings of small tumours, 1–14 mm, showing a mammography pattern with casting-type calcifications being more often present in young women and independently predicting a poor survival [36].

After controlling for different histopathological features most studies have shown young age to remain as a powerful predictor of poor survival [8,37]. A possible development is that gene expression profiling will be able to differentiate otherwise similar breast cancers at the molecular level to find clues for the explanation of this age effect [38,39]. Hereditary breast cancer (e.g. BRCA1 and 2 mutations) is more frequent in young women with breast cancer but this has not implied a worse survival in most studies [40]. Other hypotheses to explain the remaining difference in survival between age groups after corrections for tumour characteristics are that the increased risk of local recurrence associated with low age [41,42,43] leads to an increased risk of breast cancer death [44] and that young women may differ from older with respect to the treatment they are given and their responsiveness to it, or presumably a combination of both [45,46,47].

The young women in the study had been given more intensive treatment than the older women. However, judged against current treatment guidelines, women in all age groups received somewhat suboptimal treatment. Of the women operated with breast conservation, 92–94% were given radiotherapy, while 65–73% of those with hormone receptor-positive tumours

Table 4. The effect of prognostic factors on early breast cancer survival.

	Age at diagnosis (years)						
	20–34		35–39		40–49		50–69
	RER	95% CI	RER	95% CI	RER	95% CI	
Total	1.76	1.37–2.26	0.98	0.75–1.27	0.76	0.65–0.89	
Tumour size, 1–10 mm[1]	6.20	2.19–17.53	2.67	0.97–7.33	1.48	0.66–3.29	
Tumour size, 10–20 mm[1]	2.93	1.83–4.69	1.27	0.79–2.04	1.19	0.91–1.54	
Tumour size, 21+ mm[1]	1.41	1.01–1.95	0.77	0.55–1.09	0.58	0.48–0.72	Reference
Lymph nodes, no[2]	2.34	1.49–3.68	1.76	1.13–2.73	0.99	0.75–1.31	
Lymph nodes, yes[2]	1.66	1.22–2.26	0.78	0.56–1.09	0.71	0.59–0.86	
ER+/PR+[3]	2.27	1.47–3.50	1.15	0.77–1.70	0.80	0.62–1.02	
ER−/PR−[3]	1.32	0.91–1.92	1.00	0.67–1.49	0.92	0.73–1.16	

[1]Adjusted for year at diagnosis, lymph node status and oncological treatment.
[2]Adjusted for year at diagnosis, tumour size and oncological treatment.
[3]Adjusted for year at diagnosis, stage and oncological treatment.
Effect on survival of women aged 20–69 years, diagnosed with primary breast cancer stage I-IIb between 1992 and 2005 (18 631 women), adjusted by year of diagnosis, stage at diagnosis and oncological treatment (radiotherapy, chemotherapy, and endocrine therapy) and stratified on tumour characteristics.
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received endocrine therapy, which is in line with results from other population-based series [48,49]. The young women should - according to the 1998 St Gallen guidelines [24] - have received chemotherapy, but only 22% of the women <35 years with stage I disease with tumour size 1–10 mm and 39% with tumour size 11–20 mm did so. The start of our study period several years before the publication of the guidelines might explain the low frequency of chemotherapy. Consequently, there is room for further intensification of the treatment given to all women.

With the results from this study based on a large, well-validated data set, we can conclude that there are two major factors explaining the worse prognosis in young women: late presentation and a smaller, but highly significant component of more aggressive tumour biology. The former underlines the need for a raised awareness of breast cancer in society and among doctors seeing younger patients for breast complaints. The latter triggers several questions: can this aggressiveness be counteracted by even more active treatment with modalities available today, or are new modalities needed? Can we understand the tumour behaviour in the younger women better in order to aid management, e.g. by defining new therapeutic targets? Will such knowledge further improve our understanding of breast cancer biology overall? It would seem that young women are a target group for intensified research of the same importance as e.g. women with triple-negative breast cancer.

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Table 5. Treatments given to women with stage I breast cancer by age and tumour size.

Tumour size	Total	Mastectomy	Chemotherapy	Total no. with BCS	Radiotherapy if BCS	Total no. with hormone positive tumour	Endocrine therapy if hormone positive tumour
	No.	No. (%)	No. (%)	No.	No. (%)	No.	No. (%)
1–10 mm							
20–34 years	36	16 (44.4)	8 (22.2)	20	18 (90.0)	22	15 (68.2)
35–39 years	78	22 (28.2)	9 (11.5)	56	49 (87.5)	48	24 (50.0)
40–49 years	623	115 (18.5)	26 (4.2)	503	473 (94.0)	365	161 (44.1)
50–69 years	2881	498 (17.3)	60 (2.1)	2363	2160 (91.4)	1744	951 (54.5)
11–20 mm							
20–34 years	90	23 (25.8)	35 (38.9)	66	61 (92.4)	53	39 (73.6)
35–39 years	186	53 (28.7)	70 (37.6)	132	130 (98.5)	123	70 (56.9)
40–49 years	1212	277 (22.9)	212 (17.5)	926	879 (94.5)	859	538 (62.6)
50–69 years	4542	976 (21.5)	422 (9.3)	3542	3351 (94.6)	3296	2344 (71.1)

Proportions of women aged 20–69 years, diagnosed with primary breast cancer stage I between 1992 and 2005 (9656 women), receiving specific treatments, by tumour size and age at diagnosis.
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Table 6. The combined effect of prognostic factors and treatment on breast cancer stage I-IIb.

			Model 1		Model 2		Model 3		Model 4		Model 5	
Age	**No.**	**RSR**	**RER**	**95% CI**	**RER**	**95% CI**	**RER**	**95% CI**	**RER**	**95% CI**	**RER**	**95% CI**
20–34	378	76.7	3.62	2.83–4.63	3.63	2.84–4.66	2.35	1.83–3.01	1.83	1.42–2.35	1.76	1.36–2.28
35–39	710	88.6	1.73	1.34–2.24	1.71	1.32–2.22	1.13	0.87–1.47	1.05	0.81–1.36	1.05	0.80–1.37
40–49	4057	92.0	1.18	1.01–1.37	1.13	0.97–1.32	0.87	0.75–1.01	0.89	0.77–1.04	0.88	0.75–1.03
50–69	13486	93.2	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)	1.00	(ref.)

Model 1: Crude.
Model 2: Adjusted for year of diagnosis (1992–93, 1994–95, 1996–97, 1998–99, 2001–01, 2002–03, 2004–05).
Model 3: Adjusted for year of diagnosis, tumour stage (tumour size, lymph node status).
Model 4: Adjusted for year of diagnosis, all tumour characteristics (tumour size, lymph node status, tumour grade, hormone receptor status, multifocality).
Model 5: Adjusted for year of diagnosis, all tumour characteristics and treatment (preoperative treatment, type of surgery, radiotherapy, chemotherapy and endocrine therapy).
Estimated RER with 95% CI for women aged 20–69 years, diagnosed with primary breast cancer stage I-IIb between 1992 and 2005 (18 631 women), by age at diagnosis, adjusted for year of diagnosis, tumour stage, tumour characteristics and treatment.
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Author Contributions

Conceived and designed the experiments: HF SE JF LH IF HL. Performed the experiments: HF SE JF LH IF HL. Analyzed the data: HF SE JF LH IF HL. Contributed reagents/materials/analysis tools: HF SE JF LH IF HL. Wrote the paper: HF SE JF LH IF HL.

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Paper II

Breast Cancer Res Treat
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EPIDEMIOLOGY

Long-term outcome in young women with breast cancer: a population-based study

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Abstract
Purpose Whether young age at diagnosis of breast cancer is an independent risk factor for death remains controversial, and the question whether young age should be considered in treatment decisions is still to be answered.
Methods From a population-based cohort of 22,017 women with breast cancer, all women <35 years ($n = 471$) were compared to a random sample of 700 women aged 35–69 years from the same cohort. Information on patient and tumor characteristics, treatment, and follow-up was collected from the medical records. Tissue microarrays were produced for analysis of classical biomarkers. Breast cancer-specific survival (BCSS), distant disease-free survival (DDFS), and locoregional recurrence-free survival (LRFS) by age were compared using women 50–69 years as reference.
Results At 10 years follow-up, women <35 years and 35–39 years had a worse BCSS [age <35 years 69 % (HR 2.75, 95 % CI 1.93–3.94), age 35–39 years 76 % (HR 2.33, 95 % CI 1.54–3.52), age 40–49 years 84 % (HR 1.53, 95 % CI 0.97–2.39), and age 50–69 years 89 % (reference)]. The worse BCSS was statistically significant in stages I–IIa and Luminal B tumors. At multivariate analysis age <35 years and 35–39 years confined a risk in LRFS (HR 2.13, 95 % CI 1.21–3.76 and HR 1.97, 95 % CI 1.06–3.68) but not in DDFS and BCSS. In the subgroup of women <40 years with luminal tumors stage I–IIa, low age remained an independent risk factor also in DDFS (HR 1.87, 95 % CI 1.03–3.44).
Conclusion Young women have a high risk of systemic disease even when diagnosed in an early stage. The excess risk of relapse is most pronounced in Luminal B tumors, where low age is an independent prognostic factor of DDFS and LRFS.

Keywords Breast cancer · Young age · Subtype · Luminal B · Early stage · Prognosis · Population-based

Introduction

Young women with breast cancer have a worse prognosis than middle-aged women [1–7], partly explained by diagnosis at a later stage [2–4, 6, 8] and by a higher proportion

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of unfavorable tumor characteristics. Young women more often have high grade, hormone receptor-negative, Her2-positive tumors, and also more often multifocality, high proliferation, and lymphovascular invasion (LVI) [2, 3, 6, 9–13]. Young women have a higher proportion of intrinsic breast cancer subtypes [14] associated with a worse prognosis: the triple-negative, Her2-positive, and Luminal B subtypes [5, 13, 15–18]. Recently, the prognostic significance of young age has been shown to differ between the different subtypes. Whether young age is an independent prognostic marker for poorer survival even when taking subtype into account remains controversial [5, 10, 15, 18].

In a previous large registry-based cohort study, we found women <35 years to have a worse survival than middle-aged women [3]. Stage at diagnosis was a major explanatory factor; however, the excess risk of breast cancer death seen in younger women was only present in early disease, most pronounced in women with small tumors. After correction for stage and tumor characteristics, young age remained an independent risk factor for death.

As it is not likely that young age in itself confers a worse prognosis, but rather this reflects other associations we had not been able to correct for in our registry-based study, we continued with in-depth studies on a large subpopulation from the original cohort. We collected detailed data from the medical records (tumor characteristics, heredity, parity, and treatment), re-evaluated slides (grade, LVI), and collected tumor tissue for TMA providing us with an immunohistochemical (IHC) surrogate of the intrinsic breast subtypes for this population-based cohort study with almost complete and long-term follow-up to study the independent effect of young age on breast cancer-specific survival (BCSS), distant disease-free survival (DDFS), and locoregional recurrence-free survival (LRFS).

Methods

Study design

Through the regional breast cancer registries in two of Sweden's six health-care regions, a population-based cohort of 22,017 women with a primary invasive breast cancer diagnosed from 1992 to 2005 at 69 years of age or younger were identified. All women <35 years at diagnosis ($n = 471$) were compared to random sampled groups of women aged 35–39 years ($n = 200$), 40–49 years ($n = 200$), and 50–69 years ($n = 300$) (Flow chart Fig. S1). The sample size was set after power calculations based on the effect sizes from the registry-based study [3]. To reach a power of 80 % at a 95 % significance level, we needed 326 individuals to detect a difference in BCSS and 262 individuals to detect a difference in LRFS.

Information on patient and tumor characteristics, including the treatments given and follow-up until the end of 2012 or until death was collected from the medical records. For women with synchronous bilateral breast cancer, the largest tumor was chosen as the index cancer. Staging was performed using the American Joint Committee on Cancer, 7th edition [19]. The study conforms to the STROBE and REMARK guidelines [20, 21].

Tumor material

Archival haematoxylin and eosin stained sections and corresponding formalin-fixed and paraffin-embedded tumor blocks were retrieved and histologically reviewed for grade [22] and LVI. Re-sectioning and re-staining were carried out when archival sections were missing. When histological review was not possible, data on grade and LVI were extracted from pathology reports. The presence of multifocality (defined as two or more invasive tumor foci separated by at least 1 cm) and ductal carcinoma in situ (DCIS) with extensive growth (defined as >25 % of the tumor consisting of DCIS with intraductal component also beyond the edge of the invasive tumor) was extracted from the original pathology reports.

TMAs were generated for protein expression profiling using IHC. TMA production, IHC staining, slide scanning, and evaluation of outcome were performed in accordance with strategies and standards used in the Human Protein Atlas project [23, 24]. All patients with tumor material available, 983/1120 (88 %), were included in the set of TMAs. For IHC, the following primary antibodies were used: ER (estrogen receptor) 1:150 (M7047, Dako, Glostrup, Denmark), PR (progesterone receptor) 1:1000 (M3569, Dako), Ki67 1:200 (M7240, Dako), and Her2 1:1000 (A0485, Dako). IHC was performed as previously described [25]. In brief, 4 μm sections of the TMA blocks were cut and automated IHC was done using a Lab Vision Autostainer 480 (Thermo Fisher Scientific). The IHC-stained and mounted TMA slides were scanned at ×20 magnification with a ScanScope XT system (Aperio Technologies, Vista, USA). The high-resolution digital images of each tissue core were annotated with respect to the outcome of IHC staining. ER was defined as positive when >1 % of the tumor cell nuclei were positive and PR as positive when >25 % of the tumor cell nuclei were positive. Ki67 was considered high when >20 % of the tumor cell nuclei were positive [26, 27]. Her2 was annotated using Her2 ASCO guidelines [28]. Membrane staining intensity of 3+ was considered positive, while 2+ was further verified through chromogenic in situ hybridization (CISH) to determine Her2-gene amplification [28–30].

CISH was performed on an automated Ventana BenchMark ULTRA IHC/ISH Staining Module (Ventana Medical Systems, Inc Tuscon, AZ, USA) using the INFORM HER2 Dual ISH DNA Probe Cocktail. CISH-stained slides were examined under the microscope and the amount of positive Her2 signals scored in tumor cell nuclei. The outcome was scored as Her2 amplified (>6 dots or clusters of positive signal) or non-Her2 amplified (≤6 dots per nuclei).

To define the intrinsic breast cancer subtypes, we used surrogate definitions based on central IHC re-evaluation of ER, PR, Ki67, and Her2 according to the St Gallen consensus statement [27]. Luminal A was defined as ER+, PR+, Her2−, and Ki67 low, Luminal B as ER+, PR+, Her2−, and Ki67 high or ER+, PR−, Her2−, and any Ki67, Luminal-Her2 as ER+ and Her2+, any PR or Ki67, Her2-positive (non-luminal) as ER−, PR− and Her2+, any Ki67 and triple-negative as ER−, PR− and Her2−, any Ki67.

Statistical analysis

Endpoints were BCSS, DDFS, and LRFS. BCSS was calculated using time from diagnosis to death from breast cancer censoring for end of follow-up. DDFS was estimated using time from diagnosis to distant recurrence or death from breast cancer, whichever came first censoring for the end of follow-up. LRFS was calculated using time from diagnosis to locoregional recurrence as first event. Kaplan–Meier curves were used to estimate survival time [31] as death from other causes than breast cancer was uncommon in this population. Survival curves were compared using log-rank test [32]. Cox proportional-hazards models were used to estimate the univariate and multivariate hazard ratios (HR) and 95 % confidence intervals (95 % CI) [33]. All statistical tests were two-sided and p values < 0.05 were deemed significant. All calculations were performed using IBM SPSS Statistics v22.0 (SPSS Inc. Illinois, USA).

Results

Population characteristics

Data on patient and tumor characteristics divided by age group are shown in Table 1. Women <35 years had larger tumors and more often involved lymph nodes than women aged 50–69 years. Fewer women <35 years presented with stage I disease. Women <35 years more often had tumors that were grade III, hormone receptor negative, Her2-positive, and high Ki67. This translates to a lower proportion of the luminal subtypes and a higher proportion of the triple-negative and Her2-positive subtypes among younger women. Multifocal disease, LVI, and the presence of extensive DCIS were more common in women <35 years. Altogether, characteristics in women 35–39 years were similar to those in women <35 years, whereas the characteristics in women 40–49 years group together well with those aged 50–69 years.

Treatment was performed according to the national guidelines for each time period, closely following international practice. Data on treatment by age group are shown in Table 2 and time trends of systemic treatment in relation to age, tumor size, lymph node status, grade, and subtype are presented in Fig. S2.

Median follow-up time was 10 years (range 0–20). In the group aged <35 years, 90 of 445 had a locoregional recurrence as first event. The corresponding figures were for women 35–39 years 37 of 190, 40–49 years 27 of 192, and 50–69 years 22 of 293. Distant disease occurred in 169 of 445 women <35 years, in 59 of 190 women aged 35–39 years, in 47 of 192 women aged 40–49 years, and in 42 of 293 women aged 50–69 years.

Univariate analysis

Univariate analyses of risk factors for breast cancer death stratified by age are shown in Table 3. The increased risk of breast cancer death in young versus middle-aged women was significant during the earlier part of the studied period and mainly noted in tumors with favorable characteristics, namely: small tumor size, low grade, Her2-negativity, and no LVI.

At 10-year follow-up, the BCSS was for women <35 years 69 % (HR 2.75, 95 % CI 1.93–3.94), for women 35–39 years 76 % (HR 2.33, 95 % CI 1.54–3.52), for women 40–49 years 84 % (HR 1.53, 95 % CI 0.97–2.39), and women 50–69 years 89 % (HR = 1.00 reference) (Fig. 1).

Figure 2 shows BCSS by tumor characteristics and age. Women aged <40 years had a statistically significantly worse survival than women ≥40 years in stages I and IIa (HR 3.03, 95 % CI 1.65–5.57 and HR 2.08, 95 % CI 1.16–3.74), irrespective of tumor grade (grade I; HR 12.25, 95 % CI 1.35–111.17, grade II; HR 1.82, 95 % CI 1.15–2.87 and grade III; HR 1.50, 95 % CI 1.01–2.23), and in the Luminal B subtype (HR = 1.79, 95 % CI = 1.15-2.78). In women <40 years, the best survival was seen in those with Luminal A tumors (10-year BCSS 92 %) while it was markedly worse in the other subtypes (Luminal B 75 %, Her2-positive 68 % (in this analysis Luminal-Her2 and Her2-positive combined), and triple-negative 67 %).

Multivariate analysis

In the multivariate analysis (Table 4), successively correcting for year of diagnosis, stage at diagnosis, detection

Table 1 Patient- and tumor characteristics for women with primary breast cancer stage I–III diagnosed 1992–2005, by age at diagnosis ($N = 1120$)

	<35 years		35–39 years		40–49 years		50–69 years	
	$n = 445$		$n = 190$		$n = 192$		$n = 293$	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Year of diagnosis								
1992–1997	169	(38.0)	82	(43.2)	86	(44.8)	89	(30.4)
1998–2002	175	(39.3)	61	(32.1)	62	(32.3)	132	(45.1)
2003–2005	101	(22.7)	47	(24.7)	44	(22.9)	72	(24.6)
Detection by screening	6	(1.3)	5	(2.6)	45	(23.4)	167	(57.0)
Heredity[a]								
Any heredity	187	(42.0)	73	(38.4)	59	(30.7)	80	(27.3)
≥1 first grade relative	81	(18.2)	37	(19.5)	25	(13.0)	50	(17.1)
Tumor size								
1–10 mm	67	(15.1)	27	(14.2)	35	(18.2)	77	(26.3)
11–20 mm	148	(33.3)	72	(37.9)	80	(41.7)	136	(46.4)
21–50 mm	189	(42.5)	72	(37.9)	70	(36.5)	67	(22.9)
>51 mm	38	(8.5)	16	(8.4)	6	(3.1)	11	(3.8)
Missing	3	(0.7)	3	(1.6)	1	(0.5)	2	(0.7)
Lymph node status								
Node neg	227	(51.0)	90	(47.4)	116	(60.4)	214	(73.0)
1–3 nodes pos	126	(28.3)	68	(35.8)	47	(24.5)	57	(19.5)
>4 nodes pos	92	(20.7)	32	(16.8)	29	(15.1)	22	(7.5)
Stage								
I	14	(32.4)	63	(33.2)	87	(45.3)	172	(58.7)
IIa	126	(28.3)	50	(26.3)	44	(22.9)	74	(25.3)
IIb	71	(16.0)	35	(18.4)	28	(14.6)	21	(7.2)
III	103	(23.1)	40	(21.1)	33	(17.2)	26	(8.9)
Unstaged	1	(0.2)	2	(1.1)	0		0	
Grade (Elston)								
I	21	(5.3)	21	(12.7)	31	(17.9)	75	(27.3)
II	140	(35.6)	63	(38.2)	79	(45.7)	131	(47.6)
III	232	(59.0)	81	(49.1)	63	(36.4)	69	(25.1)
Missing	52		25		19		18	
Estrogen receptor[b]								
Pos	208	(47.2)	122	(64.9)	146	(77.7)	225	(78.1)
Neg	233	(52.8)	66	(35.1)	42	(22.3)	63	(21.9)
Missing	4		2		4		5	
Progesterone receptor[b]								
Pos	155	(35.4)	85	(45.2)	114	(60.6)	145	(51.1)
Neg	283	(64.6)	103	(54.8)	74	(39.4)	139	(48.9)
Missing	7		2		4		9	
Ki-67 (%)								
Low ≤20	70	(18.8)	42	(26.9)	67	(40.1)	127	(51.2)
High >20	302	(81.2)	114	(73.1)	100	(59.9)	121	(48.8)
Missing	73		34		25		45	
Her2								
Neg	296	(79.6)	127	(81.4)	150	(90.4)	225	(91.8)
Pos	76	(20.4)	29	(18.6)	16	(9.6)	20	(8.2)
Missing	73		34		26		48	

Table 1 continued

	<35 years		35–39 years		40–49 years		50–69 years	
	$n = 445$		$n = 190$		$n = 192$		$n = 293$	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Subtype								
Luminal A	27	(7.7)	23	(15.1)	40	(25.8)	59	(25.9)
Luminal B	132	(37.5)	66	(43.4)	80	(51.6)	117	(51.3)
Luminal-Her2	35	(9.9)	16	(10.5)	7	(4.5)	10	(4.4)
Her2-positive	40	(11.4)	13	(8.6)	8	(5.2)	9	(3.9)
Triple-negative	118	(33.5)	34	(22.4)	20	(12.9)	33	(14.5)
Unclassified	93		38		37		65	
Presence of:								
LVI[b]	139	(31.2)	43	(22.6)	39	(20.3)	32	(10.9)
Invasive multifocality	96	(21.6)	39	(20.5)	35	(18.2)	46	(15.7)
Extensive DCIS	92	(20.7)	43	(22.6)	30	(15.6)	37	(12.6)

[a] Any family history of breast or ovarian cancer

[b] Data retrieved by re-evaluation with IHC (ER and PR) or reviewed by a pathologist (LVI). If missing data, information was retrieved from medical records

mode, grade, subtype, and systemic treatment, young age (<35 years and 35–39 years) was an independent risk factor in LRFS (HR 2.13, 95 % CI 1.21–3.76 and HR 1.97, 95 % CI 1.06–3.68) but not in DDFS or BCSS.

To focus on the subpopulation of women where the survival analyses indicated substantial differences between women aged <40 and ≥40 years (Luminal Her-2 negative breast cancer stage I-IIa), we performed a separate multivariate analysis (Fig. 3). Age <40 years was a statistically significant independent risk factor in DDFS (HR 1.87, 95 % CI 1.03–3.44) and in LRFS (HR 4.10, 95 % CI 2.20–7.66), but not in BCSS (HR 1.47, 95 % CI 0.72–3.02).

Discussion

This population-based cohort study included 1120 women with breast cancer stage I–III of which 445 were <35 years at diagnosis with a median follow-up of 10 years. Women aged <35 years and 35–39 years had more advanced stage at diagnosis and a higher proportion of Her2-positive and triple-negative subtypes and less common Luminal A subtype. Women <35 years and 35–39 years received more intense treatment reflecting their stage and subtype distribution. Women <40 years had a worse BCSS compared to women ≥40 years in stage I and IIa, in all tumor grades and in the Luminal B subtype. At multivariate analysis, age remained an independent risk factor in LRFS but not significantly in BCSS or DDFS. In women with luminal early-stage disease, young age was an independent risk factor also of DDFS.

Treatment was given according to national guidelines and best international practice at that time. The number of women <35 years and 35–39 years is large, with detailed data on patient, tumor, and treatment characteristics and follow-up extracted from medical records. The long-term follow-up is nearing completion.

In a central pathology review, we re-evaluated grade and LVI and re-analyzed prognostic markers with modern methods at one single laboratory. Using IHC methods to separate Luminal A from Luminal B tumors has limitations [34]. In this study, we performed new IHC-analyses on archival material to avoid the effects of low intra- and inter-laboratory reproducibility and different antibodies for testing. To validate our results with regard to the arbitrarily set cutoffs, we performed a sensitivity analysis using alternative subtype definitions; grade instead of Ki67, Ki67 cutoff 14 %, ER-positive cutoff >10 % stained nuclei, which did not change the results.

During the study period of 14 years, treatment regimes have changed, and the time trends have not been the same in the compared age groups. More intense treatment was offered to young women and modern regimes were introduced earlier, which might have led to an underestimation of age-related survival differences in the multivariate analyses.

Many studies have shown young women with breast cancer to have a worse prognosis compared to their older counterparts. Our findings demonstrate that the differences in BCSS between age groups diminished over time, and lost significance during the last part of the studied period. In a recent Canadian study, outcomes for young breast cancer patients across two time periods were compared to

Table 2 Given treatment for women with primary breast cancer stage I–III diagnosed 1992–2005, by age at diagnosis ($N = 1120$)

	<35 years		35–39 years		40–49 years		50–69 years	
	$n = 445$		$n = 190$		$n = 192$		$n = 293$	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Breast surgery								
BCS	206	(46.3)	94	(49.5)	117	(60.9)	194	(66.2)
Mastectomy	239	(53.7)	94	(49.5)	74	(38.5)	99	(33.8)
No surgery	0		2	(1.1)	1	(0.5)	0	
Chemotherapy								
No	109	(24.5)	48	(25.3)	103	(53.6)	204	(69.6)
Yes	336	(75.5)	142	(74.7)	89	(46.4)	89	(30.4)
CMF	78	(23.2)	45	(31.7)	27	(30.3)	24	(27.0)
FEC	208	(61.9)	82	(57.7)	54	(60.7)	60	(67.4)
Taxanes	47	(14.0)	15	(10.6)	6	(6.7)	5	(5.6)
Other	3	(0.9)	0		2	(2.2)	0	
Proportion neoadjuvant	76	(17.1)	29	(15.3)	13	(6.8)	8	(2.7)
Chemotherapy when N+	214	(98.2)	95	(95.0)	72	(94.7)	61	(77.2)
Chemotherapy when hormone rec pos[a]	169	(67.6)	85	(65.9)	65	(41.7)	59	(25.0)
Trastuzumab								
Yes	18	(4.0)	5	(2.6)	3	(1.6)	4	(1.4)
No	427	(96.0)	185	(97.4)	189	(98.4)	289	(98.6)
Radiotherapy								
Yes	358	(80.4)	149	(78.4)	160	(83.3)	231	(78.8)
No	87	(19.6)	41	(21.6)	32	(16.7)	62	(21.2)
Breast radiation when BCS	196	(95.1)	92	(97.9)	113	(96.6)	187	(96.4)
Chest wall radiation when mastectomy	162	(67.8)	57	(60.6)	46	(62.2)	43	(43.4)
Axillary radiation when N+	168	(77.1)	68	(68.0)	49	(64.5)	58	(73.4)
Endocrine therapy								
Yes	208	(46.7)	90	(47.4)	109	(56.8)	190	(64.8)
No	237	(53.3)	100	(52.6)	82	(42.7)	102	(34.8)
Missing	0		0		1	(0.5)	1	(0.3)
Endocrine therapy[b] when hormone rec pos[a]	176	(70.4)	80	(62.0)	97	(62.2)	177	(75.0)
Ovarian suppression when hormone rec pos[a]	79	(31.9)	28	(22.0)	17	(11.1)	4	(1.7)

BCS breast conserving surgery, *CMF* cyclophosphamide, methotrexate, 5-fluorouracil, *FEC* 5-fluorouracil, epirubicin, cyclophosphamide, *N+* lymph node positive

[a] Hormone receptor positive defined as either ER pos or PR pos

[b] Endocrine therapy including ovarian suppression

determine whether the poor prognosis persists in the context of modern adjuvant therapies. There was an improvement in breast cancer outcome over time for all subgroups, but age <40 continued to predict inferior survival despite modern therapies [18]. Published data from The Surveillance, Epidemiology, and End Results program showed improved outcomes for young women with breast cancer over time, however restricted only to women with ER-positive disease [35].

The difference in prognosis between age groups has consistently been reported to be particularly evident in young women with ER-positive tumors [36–40]. More recently, the prognostic significance of young age has been shown to be most prominent in the Luminal B subtype [5, 12, 15, 18, 41] even though some reports have indicated an increased risk compared with older women also among young with triple-negative [15, 16] and Her2-positive subtypes [42, 43]. In the present study, women aged <40 had a significantly worse survival only in the Luminal B subtype. Thus, the effect of age seems to vary within tumor subtypes.

Morrison et al. found Luminal B tumors among young women to demonstrate more aggressive features, with significantly lower ER and PR levels, higher Ki67, and p53 overexpression, than in older women with the same subtype. The high proliferation and p53 level, coupled with

Table 3 Univariate analysis of risk factors for breast cancer death by age for women with stage I–III breast cancer diagnosed 1992–2005 ($N = 1120$)

	<35 years		35–39 years		40–49 years		50–69 years
	$n = 445$		$n = 190$		$n = 192$		$n = 293$
Unadjusted	**2.75**	**(1.93–3.94)**	**2.33**	**(1.54–3.52)**	1.53	(0.97–2.39)	
Year of diagnosis							
1992–1997	**2.18**	**(1.31–3.63)**	**2.04**	**(1.16–3.59)**	1.28	(0.70–2.35)	
1998–2002	**4.02**	**(2.16–7.50)**	**2.93**	**(1.37–6.27)**	1.80	(0.78–4.16)	
2003–2005	1.90	(0.79–4.54)	1.33	(0.45–3.95)	1.17	(0.37–3.68)	
Non screening detection	**1.80**	**(1.17–2.78)**	1.54	(0.95–2.49)	1.16	(0.69–1.96)	
Positive heredity	1.47	(0.84–2.58)	1.41	(0.73–2.73)	1.15	(0.56–2.35)	
Tumor size (mm)							
≤20	**3.42**	**(1.91–6.12)**	**3.19**	**(1.65–6.20)**	1.75	(0.85–3.63)	
21–50	1.60	(0.93–2.76)	1.49	(0.79–2.78)	1.24	(0.65–2.37)	
≥51	1.26	(0.51–3.11)	0.55	(0.18–1.70)	0.23	(0.03–1.93)	
Lymph node status							
Negative	**2.10**	**(1.22–3.62)**	**2.30**	**(1.21–4.39)**	0.63	(0.26–1.49)	
1–3 nodes positive	**2.63**	**(1.33–5.19)**	1.90	(0.90–4.00)	1.20	(0.50–2.89)	
≥4 nodes positive	1.41	(0.70–2.87)	1.05	(0.45–2.41)	2.05	(0.94–4.47)	
Stage							
I	**3.07**	**(1.41–6.68)**	**3.75**	**(1.58–8.90)**	1.24	(0.44–3.48)	
IIa	1.55	(0.82–2.94)	1.24	(0.56–2.78)	0.24	(0.05–1.06)	
IIb	1.71	(0.71–4.08)	1.20	(0.45–3.15)	0.83	(0.29–2.39)	
III	1.42	(0.73–2.80)	1.04	(0.48–2.27)	1.94	(0.92–4.10)	
Grade							
I–II	**3.25**	**(1.81–5.81)**	**2.38**	**(1.18–4.81)**	1.64	(0.80–3.36)	
III	1.46	(0.87–2.47)	1.37	(0.75–2.51)	0.91	(0.46–1.83)	
Estrogen receptor							
Positive	**2.89**	**(1.85–4.53)**	**2.28**	**(1.36–3.85)**	1.36	(0.78–2.39)	
Negative	**1.91**	**(1.03–3.52)**	1.85	(0.91–3.73)	1.59	(0.73–3.49)	
Progesterone receptor							1.00 (ref.)
Positive	**2.77**	**(1.53–5.01)**	**2.45**	**(1.26–4.79)**	1.10	(0.52–2.31)	
Negative	**2.37**	**(1.51–3.73)**	**2.04**	**(1.20–3.48)**	**1.91**	**(1.07–3.41)**	
Ki67 (%)							
Low ≤20	**3.15**	**(1.49–6.67)**	1.33	(0.46–3.82)	1.72	(0.73–4.06)	
High ≥21	**1.70**	**(1.08–2.68)**	1.65	(0.98–2.78)	1.09	(0.61–1.96)	
Her2							
Negative	**2.36**	**(1.55–3.60)**	**2.15**	**(1.31–3.53)**	1.27	(0.74–2.17)	
Positive	1.45	(0.56–3.74)	0.78	(0.25–2.46)	1.38	(0.42–4.52)	
Subtype							
Luminal A	1.84	(0.49–6.86)	0.55	(0.06–4.67)	0.29	(0.03–2.44)	
Luminal B	**2.30**	**(1.27–4.19)**	**2.30**	**(1.18–4.49)**	1.64	(0.82–3.28)	
Luminal-Her2	0.84	(0.23–3.02)	0.75	(0.18–3.16)	0.75	(0.13–4.50)	
Her2-positive	1.77	(0.41–7.67)	0.56	(0.08–3.98)	2.20	(0.40–12.03)	
Triple-negative	1.26	(0.61–2.61)	1.35	(0.57–3.21)	1.09	(0.39–3.07)	
Lymphovascular invasion							
No	**2.66**	**(1.74–4.06)**	**2.12**	**(1.29–3.49)**	1.14	(0.64–2.01)	
Yes	1.56	(0.77–3.15)	1.57	(0.71–3.47)	1.60	(0.71–3.60)	
Invasive multifocality							
No	**3.08**	**(2.04–4.63)**	**2.18**	**(1.33–3.55)**	1.62	(0.97–2.71)	

Table 3 continued

	<35 years		35–39 years		40–49 years		50–69 years
	$n = 445$		$n = 190$		$n = 192$		$n = 293$
Yes	1.76	(0.84–3.71)	**2.26**	**(1.00–5.12)**	1.12	(0.43–2.91)	
Extensive DCIS							
No	**3.82**	**(1.93–7.54)**	**4.03**	**(1.89–8.62)**	1.93	(0.85–4.41)	
Yes	**5.11**	**(1.20–21.74)**	**5.30**	**(1.19–23.68)**	4.63	(0.96–22.27)	
Locoregional recurrence[a]							
No	**2.75**	**(1.82–4.17)**	**2.11**	**(1.28–3.48)**	1.53	(0.91–2.59)	
Yes	1.44	(0.71–2.94)	1.50	(0.69–3.27)	0.96	(0.40–2.31)	

Hazard ratio (95 % confidence interval) for risk of breast cancer death according to age and one additional risk factor

Bold values indicate statistical significance at the $p < 0.05$ level

[a] Locoregional recurrence as first event

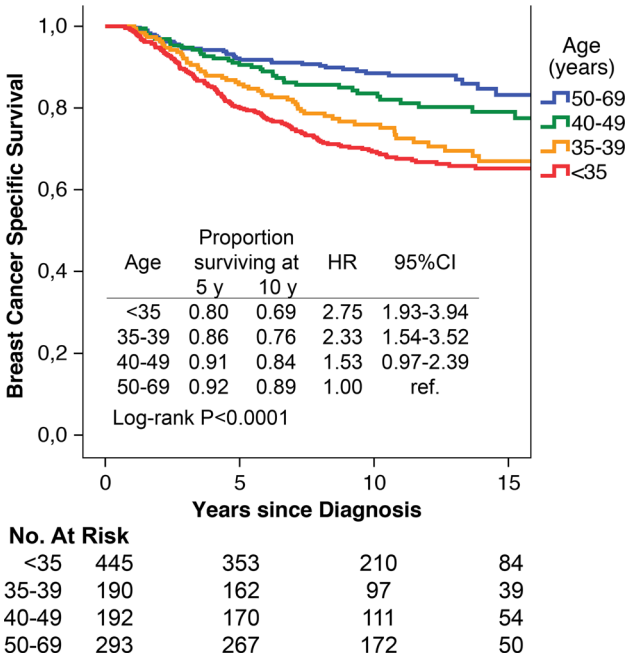

Fig. 1 Breast cancer-specific survival by age in a population-based cohort of 1120 women with primary breast cancer stage I–III diagnosed 1992–2005 divided by age <35 years, 35–39 years, 40–49 years, and 50–69 years. Proportion of women surviving at 5, 10, and 15 years from diagnosis. Hazard ratios (HR) of breast cancer death are given with their 95 % confidence intervals (95 % CI). Survival curves are compared by log-rank test

low ER and PR expression in young women, suggests that these tumors may originate from less-differentiated luminal cells [13].

Using genomic expression analysis, Azim and colleagues could, even after adjustment for subtype, observe remaining genetic differences by age with enrichment of processes related to immature mammary epithelial cells, growth factor signaling, and down-regulation of apoptosis-related genes [5]. Johnson et al. studied age-related gene expression differences within and across breast cancer subtypes. After adjustment for subtype, four key genes for proliferation, invasion, and metastasis persisted, some of which predicted inferior disease-free survival in younger women [43]. Also Liao et al. demonstrated unique genomic signatures differentiating premenopausal breast cancer from postmenopausal breast cancer, with the differences being limited to ER-positive tumors [44].

Whether the age-related biological differences within subtypes fully can explain the worse outcome for young women, or if treatment also plays a major role here, remains unclear. Except for age-related differences in the given treatment, one must also consider age-related differences in compliance to and effect of treatment. In the present study, all women were undertreated by today's standards, with chemotherapy given to only 76 % of women <35 years and endocrine treatment to those with hormone receptor-positive disease in only 70 %. Ovarian suppression was offered to one-third of the youngest women with hormone receptor-positive tumors. Some authors have found young women to be less compliant with endocrine treatment [45–47]. Women with Luminal B breast cancer derive less benefit from endocrine therapy compared to those with Luminal A breast cancer [48], and likewise less benefit from paclitaxel and doxorubicin-containing preoperative chemotherapy compared with HER2-enriched and basal-like breast cancers [49–51]. Studies on neoadjuvant chemotherapy in women with luminal tumors have shown women <40 years to have a higher rate of pathological complete response than women >50 years also with positive effect on survival [52]. However, survival differences between young and older women with luminal tumors have been demonstrated also in untreated cohorts [5, 39].

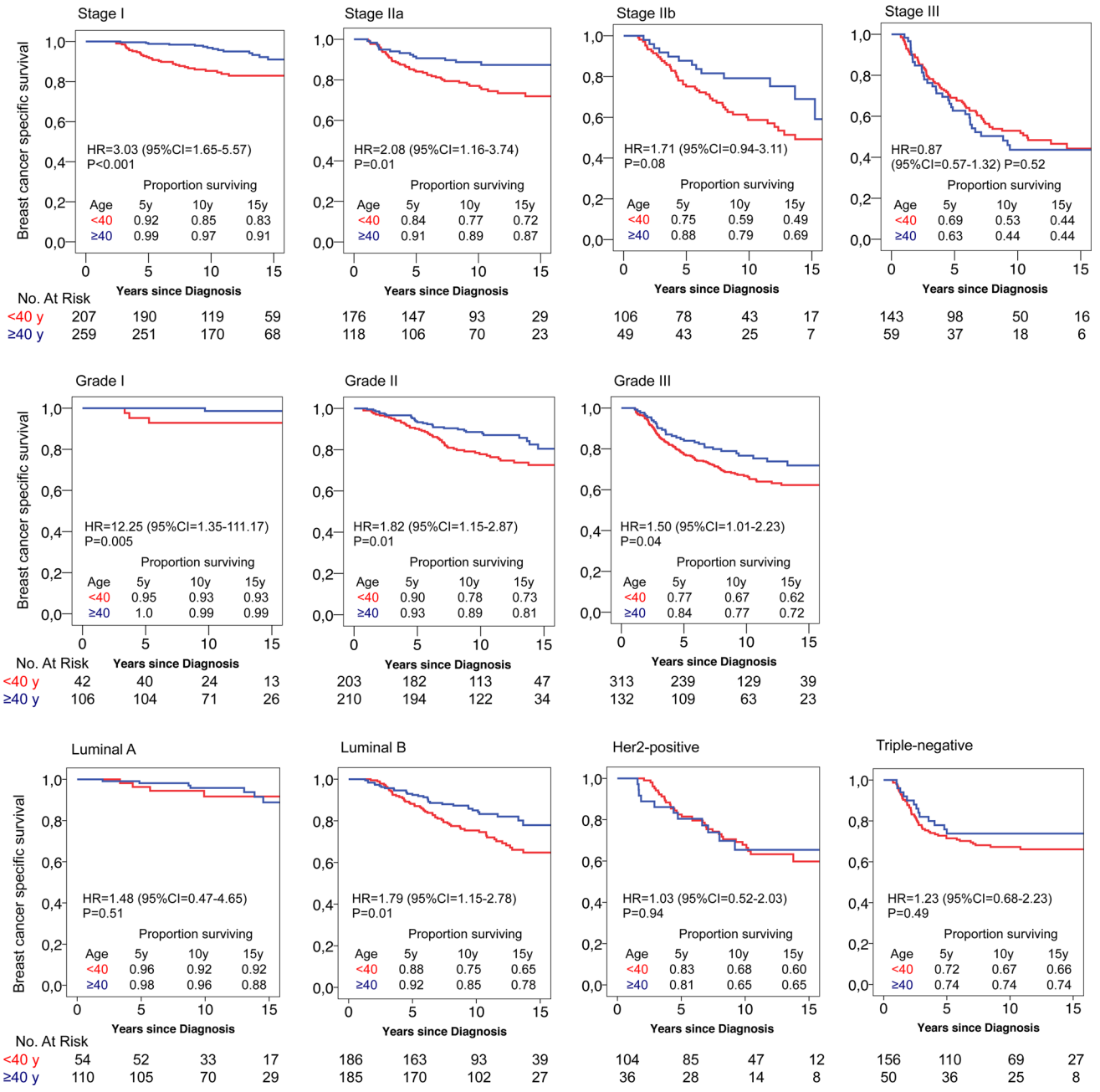

Fig. 2 Breast cancer-specific survival by age, stage, grade, and subtype for women with primary breast cancer stage I-III diagnosed 1992–2005 ($N = 1120$) divided by age <40 years and ≥40 years. Hazard ratios (HR) are given with their 95 % confidence intervals (95 % CI). Survival curves are compared by log-rank test. Proportion of women surviving at 5, 10, and 15 years from diagnosis

To conclude, the effect of age is modified by tumor subtype. Despite correction for biology and more intense treatment in the young, young age is an independent risk factor for systemic disease in women with early-stage luminal tumors, with a two-fold risk of distant disease. However, current prognostic markers cannot reliably discriminate the young women benefitting from more intense systemic therapy and studies on prognostic markers relevant in the young population, and especially for the Luminal B subtype, are urgently needed. Age remains an important variable in treatment decisions until new relevant predictive markers are found.

Authors' Contributions *Conception and design* H. Fredholm, S. Eaker Fält, H. Lindman, L. Holmberg, J. Frisell, I. Fredriksson.

Table 4 Multivariate analysis of prognostic factors[a] affecting breast cancer death, distant disease, and locoregional recurrence by age

	<35 years		35–39 years		40–49 years		50–69 years
	$N = 445$		$N = 190$		$N = 192$		$N = 293$
Breast cancer death							
Unadjusted	**2.75**	**(1.93–3.94)**	**2.33**	**(1.54–3.52)**	1.53	(0.97–2.39)	1.00 (ref)
+Year	**2.69**	**(1.88–3.85)**	**2.23**	**(1.48–3.38)**	1.45	(0.92–2.28)	
+Stage	**1.80**	**(1.25–2.60)**	1.42	(0.93–2.17)	1.13	(0.72–1.78)	
+Detection mode	1.39	(0.93–2.07)	1.10	(0.70–1.72)	0.94	(0.59–1.50)	
+Grade	1.16	(0.77–1.73)	0.94	(0.59–1.49)	0.85	(0.53–1.36)	
+Subtype	1.10	(0.73–1.64)	0.93	(0.59–1.47)	0.86	(0.53–1.38)	
+Systemic treatment	1.04	(0.68–1.58)	0.88	(0.55–1.41)	0.84	(0.52–1.36)	
Distant disease							
Unadjusted	**3.11**	**(2.22–4.36)**	**2.37**	**(1.60–3.53)**	**1.74**	**(1.15–2.64)**	1.00 (ref)
+Year	**3.04**	**(2.17–4.26)**	**2.28**	**(1.53–3.39)**	**1.65**	**(1.09–2.50)**	
+Stage	**2.09**	**(1.48–2.96)**	1.46	(0.97–2.18)	1.29	(0.85–1.96)	
+Detection mode	**1.61**	**(1.10–2.35)**	1.13	(0.73–1.73)	1.07	(0.70–1.66)	
+Grade	1.41	(0.96–2.06)	1.02	(0.66–1.57)	1.00	(0.65–1.55)	
+Subtype	1.40	(0.96–2.05)	1.01	(0.65–1.55)	1.00	(0.65–1.55)	
+Systemic treatment	1.36	(0.91–2.02)	0.97	(0.62–1.52)	0.99	(0.64–1.54)	
Locoregional recurrence							
Unadjusted	**3.16**	**(1.98–5.04)**	**2.88**	**(1.70–4.89)**	**1.94**	**(1.11–3.41)**	1.00 (ref)
+Year	**3.09**	**(1.94–4.94)**	**2.80**	**(1.65–4.78)**	**1.85**	**(1.05–3.25)**	
+Stage	**2.88**	**(1.79–4.64)**	**2.60**	**(1.52–4.45)**	**1.78**	**(1.01–3.15)**	
+Detection mode	**2.38**	**(1.37–4.12)**	**2.15**	**(1.18–3.93)**	1.58	(0.87–2.86)	
+Grade	**2.11**	**(1.21–3.67)**	**1.96**	**(1.07–3.59)**	1.50	(0.82–2.72)	
+Subtype	**2.09**	**(1.20–3.65)**	**1.94**	**(1.06–3.57)**	1.51	(0.83–2.74)	
+Systemic treatment	**2.13**	**(1.21–3.76)**	**1.97**	**(1.06–3.68)**	1.51	(0.83–2.75)	

Women with stage I–III breast cancer diagnosed 1992–2005 ($N = 1120$). Women age 50–69 serves as reference category. Hazard ratio (95 % confidence interval)

Bold values indicate statistical significance at the $p < 0.05$ level

[a] Adjusted for year of diagnosis (1992–1997, 1998–2002, 2003–2005), stage (*tumor size* 1–10, 11–20, ≥20 mm, missing and *lymph node status*; node neg, node pos), detection mode (screening or clinically detected), grade (Elston I, II, III, missing), subtype (Lum A, Lum B, Lum-Her2, Her2-pos, Triple-neg, unclassified), systemic treatment (chemotherapy and endocrine treatment including ovarian suppression)

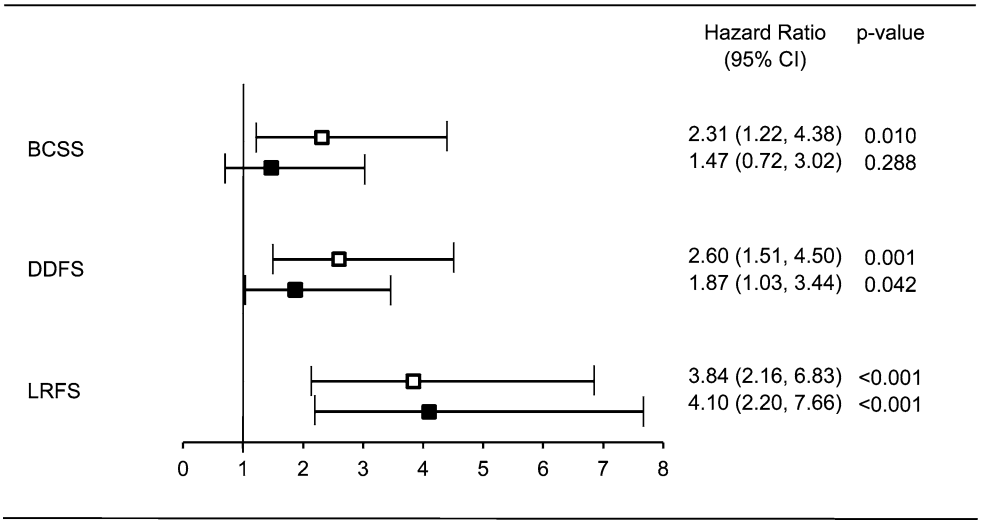

Fig. 3 Forest plot of multivariate Cox regression of risk of event for women with stage I–IIa, estrogen receptor positive, and Her2-negative breast cancer ($N = 389$) by age <40 years ($n = 152$) versus reference ≥40 years ($n = 237$). *BCSS* Breast cancer-specific survival, *DDFS* distant disease-free survival, *LRFS* locoregional recurrence-free survival. *Open square* Crude, *filled square* adjusted for diagnostic period, tumor size, lymph node status, grade, subtype (Luminal A or Luminal B), endocrine therapy, and chemotherapy

Development of methodology H. Fredholm, K. Magnusson, L. S. Lindström, H. Garmo, S. Eaker Fält, H. Lindman, J. Bergh, L. Holmberg, F. Pontén, J. Frisell, I. Fredriksson. *Acquisition of data (provided animals, acquired and managed patients, provided facilities,* etc.*)* H. Fredholm, K. Magnusson, L. S Lindström, H. Garmo, S. Eaker Fält, H. Lindman, J. Bergh, L. Holmberg, F. Pontén, J. Frisell, I. Fredriksson. *Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis)* H. Fredholm, K. Magnusson, L. S. Lindström, H. Garmo, S. Eaker Fält, H. Lindman, J. Bergh, L. Holmberg, F. Pontén, J. Frisell, I. Fredriksson. *Writing, review, and/or revision of the manuscript* H. Fredholm, K. Magnusson, L. S. Lindström, H. Garmo, S. Eaker Fält, H. Lindman, J. Bergh, L. Holmberg, F. Pontén, J. Frisell, I. Fredriksson. *Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases)* H. Fredholm, K. Magnusson, L. S. Lindström, H. Garmo, S. Eaker Fält, H. Lindman, J. Bergh, L. Holmberg, F. Pontén, J. Frisell, I. Fredriksson. *Study supervision* I. Fredriksson.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the Research Ethics Committee at Karolinska Institutet, Stockholm, Sweden (approval diary number 2009/1174-31/1 and 2010/586-32) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent The Research Ethics Committee at Karolinska Institutet, Stockholm, Sweden, approved an informed consent waiver for the retrospective medical record review (approval diary number 2009/1174-31/1 and 2010/586-32).

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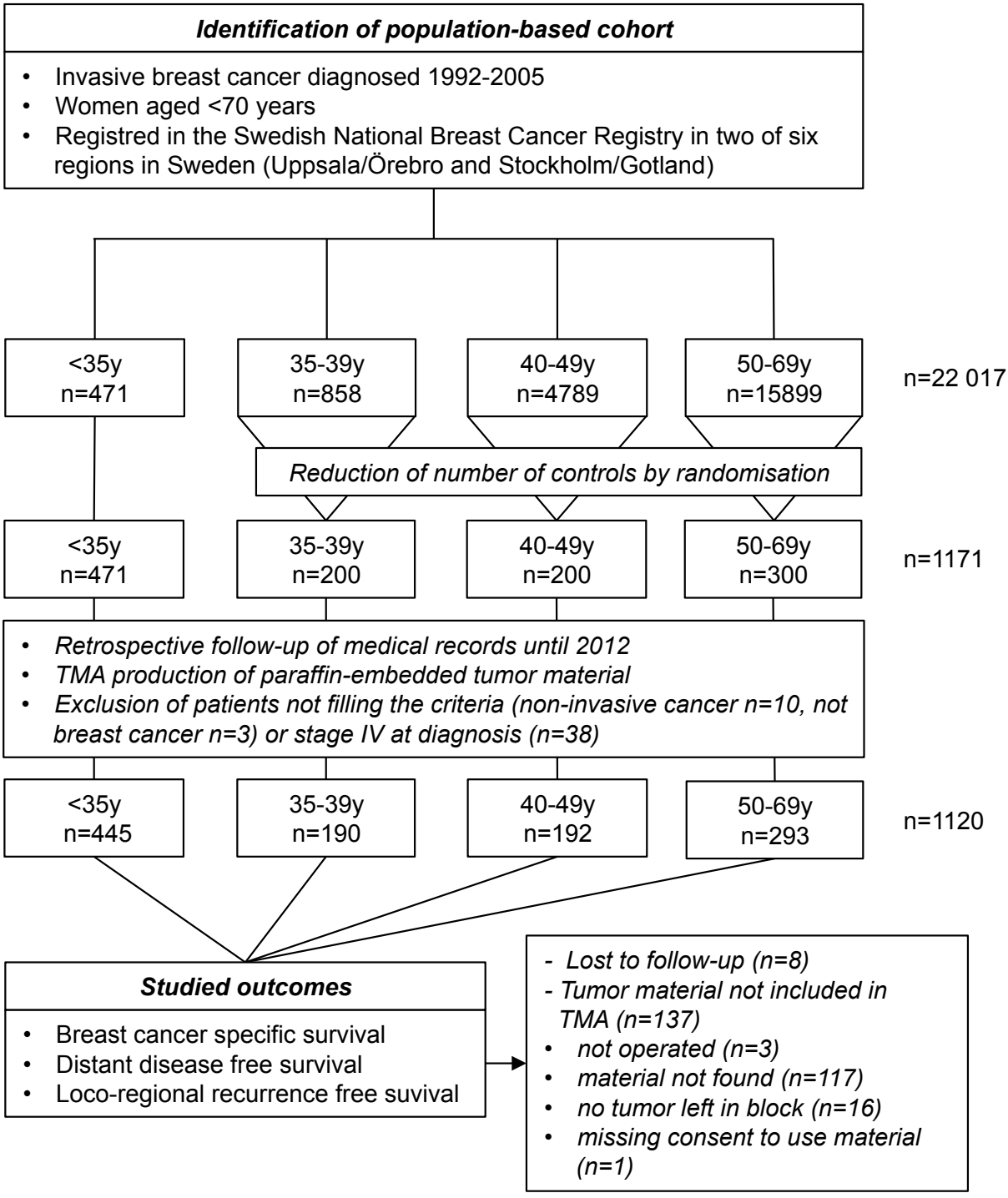
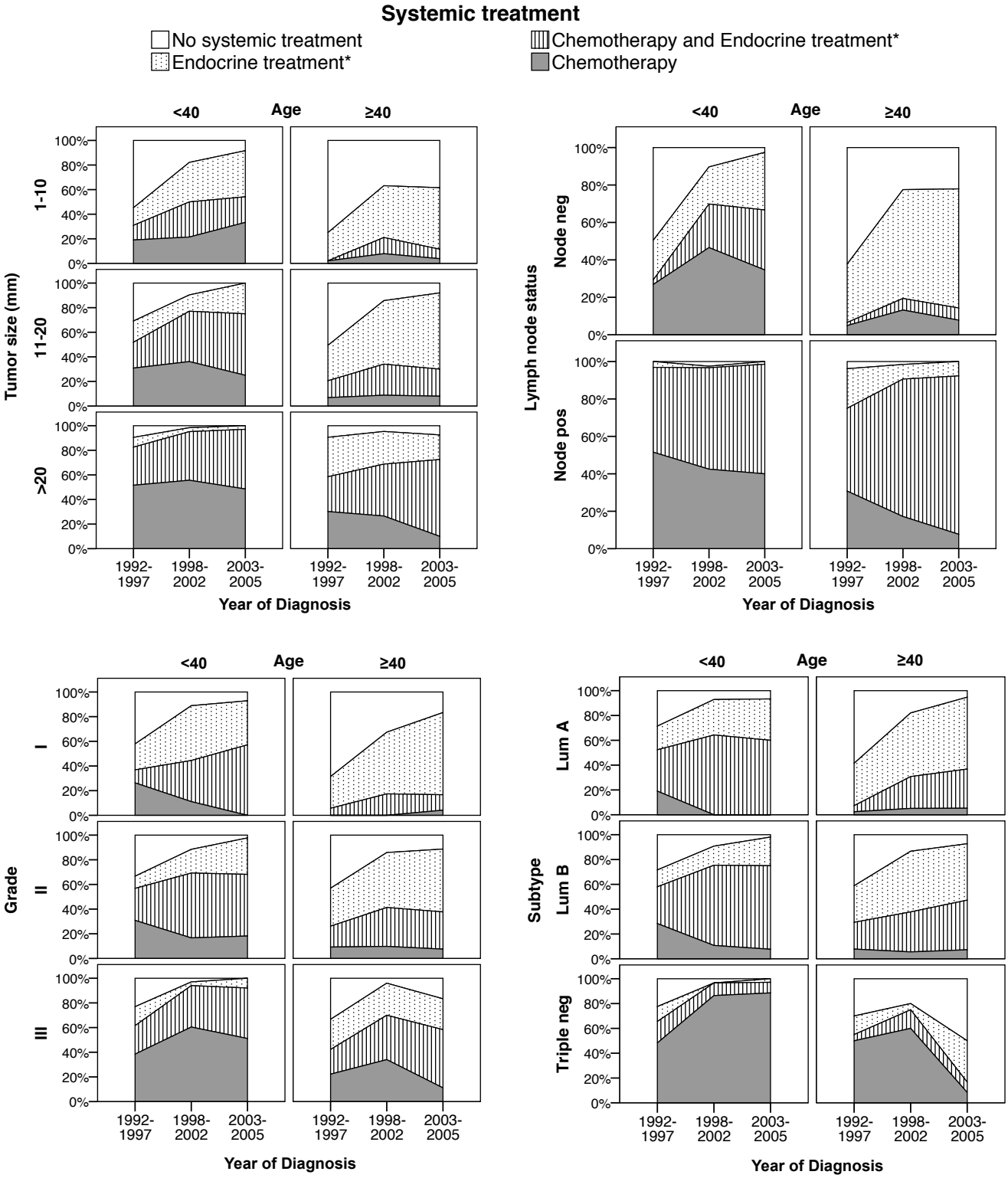
Supplementary Figure S1. Patient flow diagram



Supplementary Figure S2. Time trends of systemic treatment in 1120 women with stage I-III breast cancer by tumor size, lymph node status, grade and subtype according to age (<40, n=635, ≥40, n=485)



Un-informative cases are excluded from the analysis (systemic treatment; n=2, tumor size; n=3, grade; n=79). Only cases classified as subtype Luminal A, Luminal B and Triple-negative are included in the subtype panel (n=747). *Endocrine treatment including ovarian suppression.

Paper III

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Original Research

Breast cancer in young women and prognosis: How important are proliferation markers?

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Cyclin;
Prognosis;
Population-based

Abstract ***Aim:*** Compared to middle-aged women, young women with breast cancer have a higher risk of systemic disease. We studied expression of proliferation markers in relation to age and subtype and their association with long-term prognosis.
Methods: Distant disease-free survival (DDFS) was studied in 504 women aged <40 years and 383 women aged ≥40 years from a population-based cohort. Information on patient characteristics, treatment and follow-up was collected from medical records. Tissue microarrays were produced for analysis of oestrogen receptor, progesterone receptor (PR), Her2, Ki-67 and cyclins.
Results: Young women with luminal tumours had significantly higher expression of Ki-67 and cyclins. Proliferation markers were prognostic only within this subtype. Ki-67 was a prognostic indicator only in young women with luminal PR+ tumours. The optimal cut-off for Ki-67 varied by age. High expression of cyclin E1 conferred a better DDFS in women aged <40 years with luminal PR− tumours (hazard ratio [HR] 0.47 [0.24–0.92]). Age <40 years

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was an independent risk factor of DDFS exclusively in women with luminal B PR+ tumours (HR 2.35 [1.22–4.50]). Young women with luminal B PR− tumours expressing low cyclin E1 had a six-fold risk of distant disease compared with luminal A (HR 6.21 [2.17–17.6]).
Conclusions: The higher expression of proliferation markers in young women does not have a strong impact on prognosis. Ki-67 is only prognostic in the subgroup of young women with luminal PR+ tumours. The only cyclin adding prognostic value beyond subtype is cyclin E1. Age is an independent prognostic factor only in women with luminal B PR+ tumours.

1. Introduction

Although early breast cancer generally has an excellent prognosis, breast cancer in young women is associated with a high risk of systemic disease at long-term follow-up [1–6]. Young women tend to be diagnosed at a later stage with highly proliferative, high-grade tumours with the presence of lymphovascular invasion (LVI) [2–4,7–11]. The breast cancer subtypes associated with a worse prognosis; luminal B, human epidermal growth factor receptor 2 (Her2)–positive and triple-negative (TN), are more common in young relative to middle-aged women [3,10]. The prognostic importance of age seems to differ between subtypes, being independently significant in oestrogen receptor positive (ER+) breast cancer [12,13] and specifically in the luminal B subtype [1,3,13,14].

Proliferation markers are highly expressed in young women with breast cancer [1,3,5,10,11,13,15] and have proven to be particularly prognostic in luminal tumours [16,17]. In the original description of molecular subtypes by Sorlie and Perou, proliferation genes are highly important [18] and have also been shown to play a central role in the prognostic capacity of commercially available gene-assays such as MammaPrint and Oncotype DX [19]. In clinical treatment decision-making, proliferation markers such as Ki-67 are heavily relied upon, particularly when gene expression analyses are not available or cost prohibitive. Furthermore, the level of Ki-67 is used to separate the luminal A and B subtypes by immunohistochemistry (IHC) [20,21].

For tumour cells to sustain proliferative signalling, a hallmark of cancer, they must first circumvent the tightly regulated cell division cycle. Central to the cell cycle are the cyclin family of proteins that regulate cellular growth and division in both normal and malignant cells. Cyclins display subtype-specific expression in breast cancer [22,23] and are generally expressed in higher levels in young women [24–26], potentially contributing to age-related differences in disease-specific survival. Despite their central role in breast cancer oncogenesis, it is currently unclear whether cyclins retain their prognostic value in young women when taking other strong prognostic indicators such as age and subtype into account.

To get further insight into the biology behind the age-related differences in prognosis in breast cancer, we performed IHC analysis of Ki-67, cyclin A2, B1, D1 and E1 in a population-based cohort of women with stage I–III breast cancer and related the expression to prognosis in different age groups and subtypes. As a secondary aim, we determined whether any of the cyclins add prognostic information to standard biomarkers in young women with breast cancer.

2. Patients and methods

2.1. Study design

From a Swedish population-based registry cohort of 22,017 women diagnosed with primary invasive breast cancer from 1992 to 2005 at age 69 or younger [2], a smaller cohort including all women aged <35 years (n = 471) and random sampled groups of women aged 35–39 years (n = 200), 40–49 years (n = 200) and 50–69 years (n = 300) was constructed. The sample size was decided after power calculations based on effect sizes in the full cohort [2], with the aim to over-sample young women but still with a reasonable possibility to collect detailed clinical data from the medical records as well as tumour tissue for both cases and comparison group. This smaller cohort consisted of 1120 women with full information on patient and tumour characteristics, including treatments given and follow-up until the end of 2012 or until death. Tumour tissues were retrieved from 88% of the women (983/1120) and protein expression centrally evaluated on tissue microarrays (TMAs) [3]. Women with full information on ER, progesterone receptor (PR), Ki-67 and Her2 at central re-analyses were selected for the present study, which thereby consisted of 887 women stage I–III in ages <35 (n = 352), 35–39 (n = 152), 40–49 (n = 155) and 50–69 years (n = 228) (Supplementary Fig. 1). Analyses were performed stratified by age <40 and ≥40. The study conforms to the STROBE and REMARK guidelines [27,28].

2.2. Tumour material

Archival haematoxylin and eosin–stained sections and corresponding formalin-fixed and paraffin-embedded

tumour blocks were retrieved and histologically reviewed for grade and LVI. Cores were taken from two representative peripheral parts of each tumour for protein expression profiling using IHC. TMA production, IHC staining, slide scanning and annotation were performed in accordance with strategies and standards used in the Human Protein Atlas project [29]. IHC of ER, PR, Ki-67 and Her2 were performed as previously described [3]. The following primary antibodies were used for cyclin expression analysis: cyclin A2 1:200 (CAB000114, Novocastra, Germany), cyclin B1 1:1000 (CAB000115, Transduction laboratories, USA), cyclin D1 1:20 (CAB000024, Novocastra, Germany) and cyclin E1 1:200 (CAB000308, Pharmingen, USA). ER, PR, and cyclins were annotated as follows: 0–1%, 2–10%, 11–25%, 26–50%, 51–75%, >75%. Ki-67 was annotated in levels 0–1%, 2–10%, 11–14%, 15–20%, 21–30%, 31–40%, 41–50%, 51–75%, >75%.

ER was defined as positive when >1% of the tumour-cell nuclei were positive [30]. PR was defined as positive when >25% of the tumour-cell nuclei were positive, and this cut-off was chosen because recent studies [31–33] suggest luminal A and B tumours to be better distinguished with a higher PR cut-off than the 1% cut-off recommended in the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines [30]. As the optimal cut-off for Ki-67 depends on local laboratory reference, type of specimen (whole section or TMA) and possibly also age, we chose to analyse and present data for all commonly used cut-offs; 14%, 20% and 30% [20,21,34]. Ki-67 is known to have a heterogeneous intratumoural expression and has been shown to have the strongest prognostic impact when counted in hot-spot areas [35] in the tumour periphery [36]. Hot spots might be missed when using cores for analysis of Ki-67, which implies a risk for underestimation of the true proliferation rate [37]. Therefore, when not especially specified, Ki-67 was considered high when >14% of the tumour-cell nuclei were positive. Protein expression of Her2 was deemed positive in case of membrane-staining intensity of 3+ or 2+ when further verification through silver *in situ* hybridisation showed Her2-gene amplification (>6 dots) [38]. Cyclin A2, D1 and E1 were defined as positive when >10% of the tumour-cell nuclei were positive and cyclin B1 when >10% of the tumour cell cytoplasm were positive. There are no internationally established cut-offs for cyclins why the 10% level was arbitrarily chosen, being the most commonly selected level in the literature (cyclin A2 [39–41], cyclin B1 [42], cyclin D1 [43] and cyclin E1 [44]).

2.3. Subtypes

To define the intrinsic breast cancer subtypes we used surrogate definitions based on central IHC re-evaluation of ER, PR, Ki-67 and Her2. Luminal A was defined as ER + PR + Her2 negative (−), Ki-67≤14%; luminal B as ER + PR + Her2−, Ki-67>14% or ER + PR−Her2−, any Ki-67; luminal-Her2 as Her2+ER+, any PR and Ki-67; Her2 expressing (non-luminal) as ER−PR−Her2+ and TN as ER−PR−Her2−.

2.4. Statistical analysis

The end-point was distant disease-free survival (DDFS), calculated using time from diagnosis to distant recurrence or death from breast cancer, whichever came first, censoring for last day of follow-up. Associations between variables were evaluated with the X^2-test. Survival curves were derived from Kaplan–Meier estimates compared by log-rank test. Cox proportional hazards models were used to estimate the univariate and multivariate hazard ratios (HRs) and 95% confidence intervals. All statistical tests were two-sided and p-values <0.05 were deemed significant. All calculations were performed using IBM SPSS Statistics, version 24.0 (SPSS Inc. Illinois, USA).

3. Results

3.1. Population characteristics

Median follow-up time was 9.6 years (women aged <40; 8.7 years, women aged ≥40; 10.3 years). Significant age-related differences in tumour characteristics were present only in the luminal and TN subtypes (Table 1). In luminal tumours, women aged <40 had a more advanced stage at diagnosis, higher grade and proliferation and more often the presence of LVI and multifocality. Young women more often had luminal B tumours and less often luminal A tumours. Within the TN subtype, women aged <40 had a more advanced stage at diagnosis and a significantly higher proportion of grade III tumours compared with middle-aged women. There were no age-related differences in characteristics within the Her2+ subtype except that young women had a significantly higher proportion of LVI (44.2% vs 14.7%, $p = .002$). Across all subtypes young women aged <40 were given chemotherapy more often than those aged ≥40. Chemotherapy was used more often in the young women both in node-positive and hormone receptor–positive disease (Supplementary Table 1).

3.2. Expression of cyclins and Ki-67 by age and subtype

Young women with luminal tumours had significantly higher expression of all cyclins and Ki-67 (Table 1). In Her2-positive tumours there were no age-related differences in expression of proliferation markers. In TN

Table 1
Tumour characteristics in women with primary breast cancer stage I–III diagnosed 1992–2005 by age at diagnosis and subtype (N = 887).

Variable	Luminal			Her2 positive			Triple-negative		
	<40 years	≥40 years	p	<40 years	≥40 years	p	<40 years	≥40 years	p
	n = 248	n = 296		n = 104	n = 34		n = 152	n = 53	
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Year of diagnosis									
1992–1997	102 (41.1)	93 (31.4)	**.013**	33 (31.7)	16 (47.1)	.116	58 (38.2)	20 (37.7)	.995
1998–2002	79 (31.9)	129 (43.6)		48 (46.2)	9 (26.5)		59 (38.8)	21 (39.6)	
2003–2005	67 (27.0)	74 (25.0)		23 (22.1)	9 (26.5)		35 (23.0)	12 (22.6)	
Tumour size									
1–10 mm	43 (17.3)	60 (20.3)	**.015**	16 (15.4)	5 (14.7)	.808	10 (6.6)	11 (20.8)	**.004**
11–20 mm	100 (40.3)	150 (50.7)		33 (31.7)	9 (26.5)		42 (27.6)	21 (39.6)	
21–50 mm	82 (33.1)	73 (24.7)		50 (48.1)	17 (50.0)		90 (59.2)	17 (32.1)	
≥51 mm	21 (8.5)	11 (3.7)		5 (4.8)	3 (8.8)		8 (5.3)	3 (5.7)	
Missing	2 (0.8)	2 (0.7)		0	0		2 (1.3)	1 (1.9)	
Lymph node status									
Node negative	120 (48.4)	200 (67.6)	**.000**	42 (40.4)	15 (44.1)	.929	85 (55.9)	36 (67.9)	.276
1–3 nodes pos	79 (31.9)	63 (21.3)		36 (34.6)	11 (32.4)		44 (28.9)	10 (18.9)	
>4 nodes positive	49 (19.8)	33 (11.1)		26 (25.0)	8 (23.5)		23 (15.1)	7 (13.2)	
Stage									
Stage I	95 (38.3)	161 (54.4)	**.000**	34 (32.7)	10 (29.4)	.838	31 (20.4)	25 (47.2)	**.003**
Stage IIA	57 (23.0)	76 (25.7)		19 (18.3)	5 (14.7)		68 (44.7)	12 (22.6)	
Stage IIB	39 (15.7)	20 (6.8)		23 (22.1)	10 (29.4)		27 (17.8)	8 (15.1)	
Stage III	56 (22.6)	39 (13.2)		28 (26.9)	9 (26.5)		25 (16.4)	8 (15.1)	
Stage missing	1 (0.4)	0		0	0		1 (0.7)	0	
Grade (Elston)									
I	33 (14.1)	79 (27.0)	**.000**	2 (2.1)	1 (3.3)	.921	0	2 (4.0)	**.001**
II	123 (52.6)	151 (51.5)		30 (30.9)	9 (30.0)		15 (10.3)	13 (26.0)	
III	78 (33.3)	63 (21.5)		65 (67.0)	20 (66.7)		130 (89.7)	35 (70.0)	
Missing	14	3		7	4		7	3	
Oestrogen receptor									
Positive	248 (100)	296 (100)		51 (49.0)	17 (50.0)	.922	0	0	
Negative	0	0		53 (51.0)	17 (50.0)		152 (100)	53 (100)	
Progesterone receptor									
Positive	154 (62.1)	194 (65.5)	.405	22 (21.2)	5 (14.7)	.411	0	0	
Negative	94 (37.9)	102 (34.5)		82 (78.8)	29 (85.3)		152 (100)	53 (100)	
Ki-67 (%)									
Low ≤14	52 (21.0)	123 (41.6)	**.000**	12 (11.5)	6 (17.6)	.277	9 (5.9)	7 (13.2)	.059
High >14	196 (79.0)	172 (58.1)		92 (88.5)	28 (82.4)		143 (94.1)	46 (86.8)	
Missing	0	1		0	0		0	0	
Cyclin A2 (%)									
Negative ≤10	163 (66.3)	236 (80.3)	**.000**	52 (50.0)	19 (55.9)	.551	29 (19.2)	18 (35.3)	**.019**
Positive >10	83 (33.7)	58 (19.7)		52 (50.0)	15 (44.1)		122 (80.8)	33 (64.7)	
Missing	2	2		0	0		1	2	
Cyclin B1 (%)									
Negative ≤10	198 (82.5)	268 (92.1)	**.001**	64 (62.1)	26 (76.5)	.127	69 (45.7)	29 (56.9)	.168
Positive >10	42 (17.5)	23 (7.9)		39 (37.9)	8 (23.5)		82 (54.3)	22 (43.1)	
Missing	8	5		1	0		1	2	
Cyclin D1 (%)									
Negative ≤10	12 (4.9)	31 (10.6)	**.014**	12 (11.5)	2 (5.9)	.343	90 (59.2)	25 (49.0)	.204
Positive >10	235 (95.1)	262 (89.4)		92 (88.5)	32 (94.1)		62 (40.8)	26 (51.0)	
Missing	1	3		0	0		0	2	
Cyclin E1 (%)									
Negative ≤10	131 (55.0)	218 (76.8)	**.000**	30 (28.8)	14 (41.2)	.180	16 (11.0)	13 (25.5)	**.012**
Positive >10	107 (45.0)	66 (23.2)		74 (71.2)	20 (58.8)		130 (89.0)	38 (74.5)	
Missing	10	12		0	0		6	2	
Subtype									
Luminal A	33 (13.3)	75 (25.3)	**.002**	–	–		–	–	
Luminal B	215 (86.7)	221 (74.7)		–	–		–	–	
Luminal Her2	–	–		51 (49.0)	17 (50.0)	.922	–	–	
Her2 expressing	–	–		53 (51.0)	17 (50.0)		–	–	
Triple-negative	–	–		–	–		152 (100)	53 (100)	
Presence of:									
LVI	67 (27.0)	48 (16.2)	**.002**	46 (44.2)	5 (14.7)	**.002**	37 (24.3)	13 (24.5)	.978
Multifocality	58 (23.4)	43 (14.5)	**.008**	37 (35.6)	11 (32.4)	.732	20 (13.2)	8 (15.1)	.724

LVI, lymphovascular invasion. Bold figures indicate $p < .05$.

tumours, cyclin A2 and E1 were significantly higher expressed in women aged <40.

3.3. Cyclins, Ki-67 and prognosis

Survival curves describing the association between DDFS and proliferation markers, not taking subtype or age into account, are shown in Fig. 1A. High expression of Ki-67, cyclin A2 and cyclin E1 were all significantly associated with a worse outcome (Ki-67, $p < .000$; cyclin A2, $p = .014$; cyclin E1, $p = .030$), whereas the expression of cyclin B1 and D1 did not significantly affect prognosis (cyclin B1, $p = .281$ and cyclin D1, $p = .074$). When stratifying the analysis on age (small graphs in Fig. 1A), we found neither Ki-67 nor any of the cyclins to be prognostic for women aged <40 (all tumours). In women aged ≥40, on the contrary, cyclins and Ki-67 were prognostic, with the exception of cyclin B1 that reached only borderline significance ($p = .058$). As we found age-related differences in expression of proliferation markers exclusively in luminal tumours, we also studied their expression and their association with DDFS separately (Fig. 1B). None of the markers were found to be prognostic in young women, whereas in women aged ≥40 high cyclin D1 expression was associated with a better outcome ($p = .002$).

In univariate analysis (Table 2), young women with luminal tumours had an increased risk of distant disease compared with women aged ≥40 (HR 2.37 [1.66–3.37]), whereas no age-related differences were present in women with Her2-positive or TN tumours. Stage was the major prognostic determinant across subtypes and age groups. Grade was prognostic exclusively in luminal tumours. PR-negativity indicated a worse prognosis in luminal tumours irrespective of age (<40 years; HR 1.83 [1.20–2.80] and ≥40 years; HR 2.34 [1.33–4.14]). Ki-67 was prognostic with all commonly used cut-off levels (14%, 20% and 30%) but only in luminal tumours. Generally, high expression of cyclins did not confer an increased risk of distant disease in any subtype or age group with the exception of high cyclin D1, which indicated a decreased risk of distant disease in women aged ≥40 with luminal tumours (HR 0.34 [0.17–0.69]; Table 2).

3.4. Proliferation markers by age in the luminal subtypes

As Ki-67 and cyclins did not demonstrate prognostic significance in univariate analysis of Her2-positive and TN tumours, we subsequently focussed on luminal breast cancer. A univariate analysis restricted to women with luminal tumours was performed ($n = 544$), split by luminal PR+ and luminal PR− tumours (Table 3). Ki-67 was only prognostic in luminal PR+ tumours. A Ki-67 cut-off of 14% yielded the best prognostic information for women aged <40 (HR 3.15 [1.12–8.81]), whereas the 30% level was optimal for women aged ≥40 (HR 2.30 [1.01–5.25]).

High cyclin E1 in young women with luminal PR− tumours indicated a lower risk of distant disease (HR 0.51 [0.27–0.95], $p = .033$; Table 3). In a multivariate analysis, high cyclin E1 remained an independent prognostic factor for better outcome in young women with luminal B PR− tumours (HR 0.47 [0.24–0.92], $p = .027$; Supplementary Fig. 2A).

In luminal PR+ tumours, a high cyclin D1 in women aged ≥40 was associated with a better outcome (HR 0.28 [0.08–0.97], $p = .044$; Table 3). In the multivariate analysis, high cyclin D1 remained an independent prognostic factor for better outcome in women aged ≥40 with luminal B PR+ tumours (HR 0.19 [0.05–0.74], $p = .017$; Supplementary Fig. 2B). However, the cyclin D1 finding should be interpreted with caution as very few of the studied women had low cyclin D1 expression ($n = 11$).

To test whether cyclin expression could explain the worse DDFS in young women with luminal tumours, cyclin expression as well as year of diagnosis, stage, grade, LVI and systemic treatment were included in a multivariate model with associated survival plots, stratified by luminal A, luminal B PR+ and Luminal B PR− tumours (Fig. 2). Cyclin expression could not explain differences in outcome between young and middle-aged women. Age <40 years was an independent risk factor for DDFS exclusively in women with luminal B PR+ tumours (HR 2.35 [1.22–4.50], $p = .010$).

To put the prognostic importance of cyclin E1 in young women with breast cancer into a clinical context, we analysed DDFS by age and subtype dividing the luminal tumours into luminal A, luminal B PR+, luminal B PR−/cyclin E1 high and luminal B PR−/cyclin E1 low (Fig. 3). Young women with luminal B PR−/cyclin E1 low tumours had a markedly worse prognosis, with an over six-fold increased risk of distant disease (HR 6.21 [2.17–17.6], $p = .001$) compared with luminal A tumours.

4. Discussion

In this comparably large population-based cohort of young women with breast cancer and long-term follow-up, we performed central TMA-based analyses of Ki-67 and cyclins to reveal their prognostic impact per subtype and age. We found age-related differences in expression of cyclins and Ki-67 predominantly in women with luminal tumours and age-related differences in survival restricted to women with luminal B PR+ tumours. Young women with luminal B PR+ tumours had a more than doubled risk of distant disease compared with middle-aged women. Associations between proliferation markers and prognosis that were found when considering tumours in women with all ages, remained

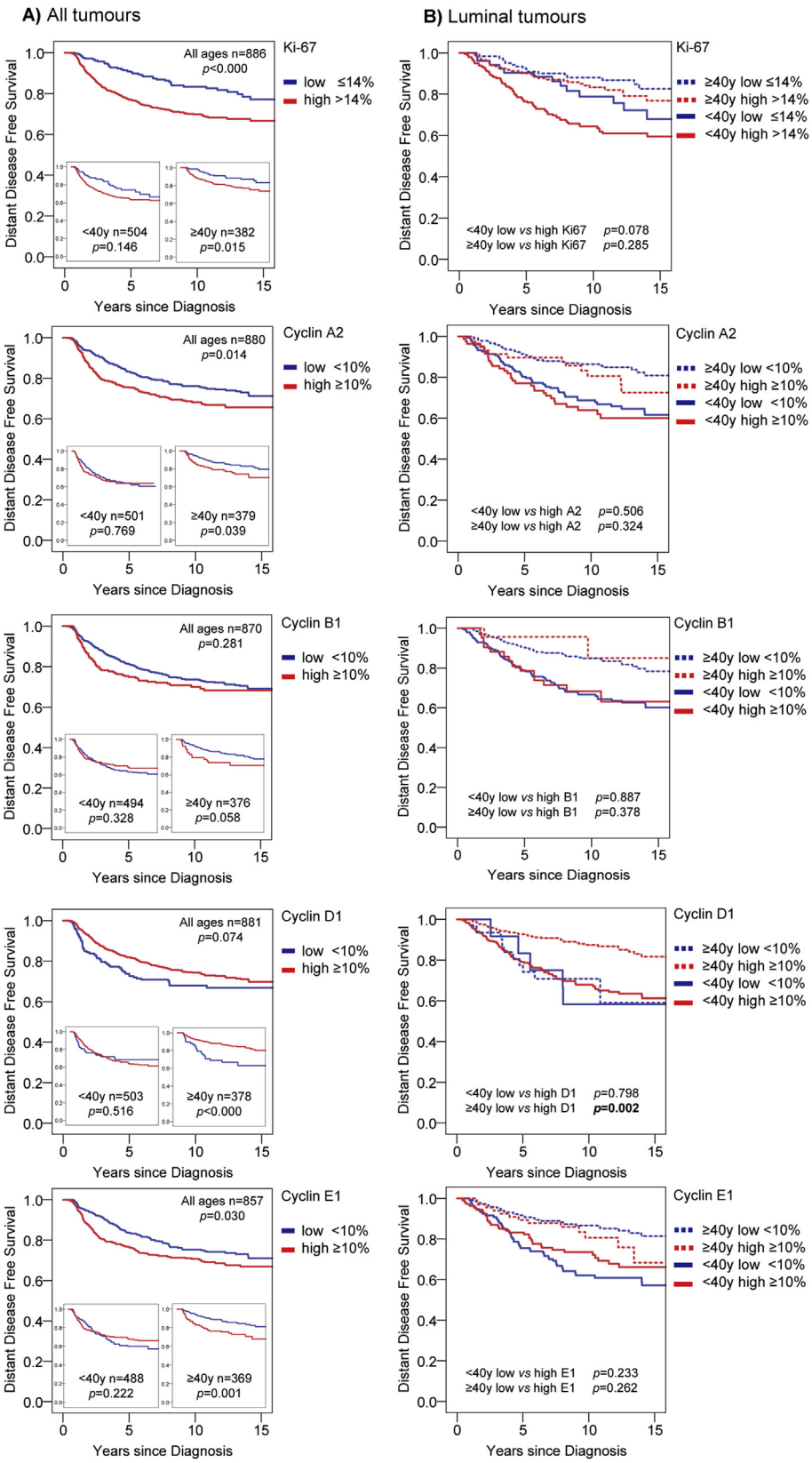


Fig. 1. Survival curves illustrating distant disease-free survival in women with breast cancer stage I–III, by age and expression of Ki-67, cyclin A2, B1, D1 and E1. A) For all tumours, B) for luminal tumours. High and low expressions were compared by log-rank test.

Table 2
Univariate analysis of risk factors for distant disease or death by breast cancer in women with breast cancer stage I–III (n = 887), by age and subtype. HR (95% confidence interval).

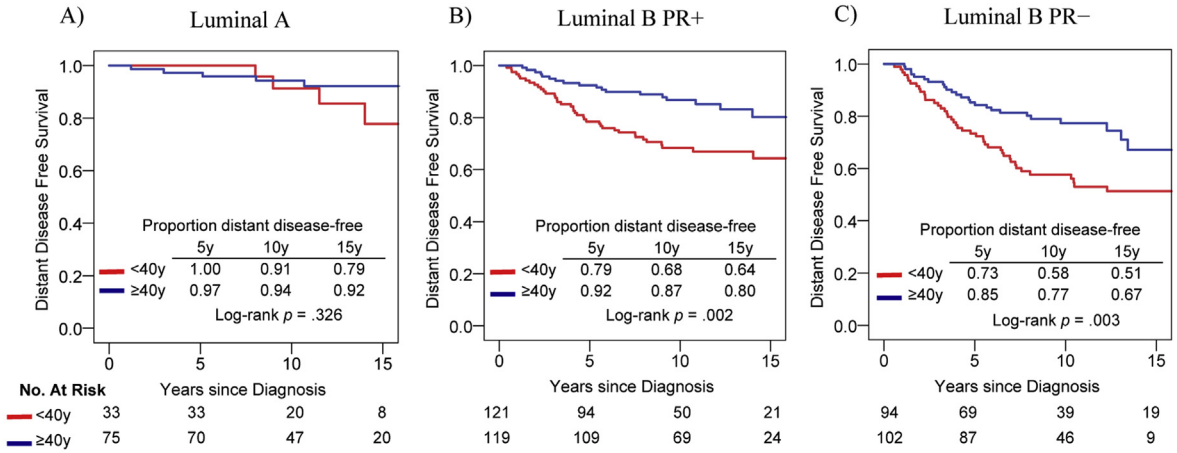
Variable	Luminal			Her2 positive			Triple-negative		
	All	<40 years	≥40 years	All	<40 years	≥40 years	All	<40 years	≥40 years
	n = 544	n = 248	n = 296	n = 138	n = 104	n = 34	n = 205	n = 152	n = 53
Age <40 versus ≥40 years		**2.37** (1.66–3.37)	1.00 (ref)		1.19 (0.61–2.33)	1.00 (ref)		1.17 (0.67–2.05)	1.00 (ref)
Year of diagnosis									
1992–1997 versus 2003–2005	**1.92** (1.17–3.13)	**1.88** (1.04–3.40)	1.93 (0.81–4.59)	1.89 (0.84–4.26)	1.82 (0.70–4.69)	2.18 (0.45–10.6)	**2.59** (1.18–5.66)	**2.79** (1.13–6.86)	1.99 (0.40–9.89)
1998–2002 versus 2003–2005	1.08 (0.64–1.82)	1.25 (0.66–2.39)	1.10 (0.45–2.67)	1.26 (0.55–2.89)	1.30 (0.51–3.29)	0.92 (0.13–6.57)	**2.22** (1.01–4.88)	2.30 (0.93–5.70)	2.01 (0.42–9.73)
Tumour size									
21–50 versus 1–20 mm	**3.34** (2.32–4.82)	**2.44** (1.56–3.84)	**4.87** (2.59–9.18)	**3.15** (1.62–6.09)	**3.07** (1.47–6.40)	3.69 (0.78–17.4)	1.43 (0.83–2.45)	1.24 (0.66–2.35)	1.95 (0.68–5.58)
≥51 mm versus 1–20 mm	**4.83** (2.75–8.46)	**2.14** (1.03–4.44)	**15.0** (6.15–36.8)	**5.17** (1.82–14.7)	**8.10** (2.51–26.2)	2.76 (0.25–30.5)	**3.48** (1.40–8.63)	**4.11** (1.48–11.4)	1.87 (0.23–15.3)
Lymph node status									
1–3 N+ versus N0	**2.59** (1.70–3.95)	**2.70** (1.59–4.57)	1.52 (0.69–3.33)	1.37 (0.66–2.83)	1.12 (0.51–2.45)	4.32 (0.45–41.6)	**4.18** (2.32–7.53)	**4.00** (2.04–7.83)	**3.84** (1.06–13.9)
≥4 N+ versus N0	**6.20** (4.09–9.39)	**4.11** (2.36–7.15)	**8.89** (4.72–16.7)	**3.68** (1.86–7.28)	**2.30** (1.08–4.91)	**25.4** (3.05–211)	**7.24** (3.85–13.6)	**6.02** (2.86–12.7)	**13.7** (4.06–46.5)
Stage									
Stage IIA versus I	1.51 (0.88–2.59)	1.13 (0.56–2.27)	2.08 (0.88–4.89)	1.94 (0.73–5.17)	1.70 (0.61–4.71)	NS	**2.86** (1.16–7.05)	2.24 (0.76–6.63)	4.63 (0.85–25.3)
Stage IIB versus I	**5.48** (3.30–9.11)	**4.16** (2.30–7.55)	**4.55** (1.57–13.2)	2.23 (0.91–5.45)	1.85 (0.71–4.81)	NS	**4.99** (1.94–12.9)	**4.40** (1.42–13.7)	4.27 (0.69–26.5)
Stage III versus I	**6.33** (4.04–9.91)	**3.24** (1.83–5.73)	**13.1** (6.33–27.2)	**4.82** (2.14–10.9)	**3.15** (1.33–7.46)	NS	**11.4** (4.62–28.3)	**7.99** (2.65–24.1)	**29.1** (5.86–144)
Grade (Elston)									
III versus I–II	**2.32** (1.61–3.33)	**2.33** (1.48–3.68)	1.79 (0.95–3.35)	1.45 (0.73–2.89)	1.52 (0.70–3.29)	1.04 (0.28–6.95)	0.83 (0.42–1.63)	0.48 (0.21–1.07)	1.53 (0.42–5.58)
PR									
Negative versus positive	**2.04** (1.45–2.87)	**1.83** (1.20–2.80)	**2.34** (1.33–4.14)		–	–		–	–
Ki-67									
High versus low 14%	**1.82** (1.21–2.73)	1.69 (0.94–3.05)	1.38 (0.76–2.49)	1.22 (0.52–2.85)	0.75 (0.31–1.79)	28.7 (0.05–15800)	1.96 (0.62–6.25)	1.51 (0.37–6.21)	2.57 (0.34–19.6)
High versus low 20%	**1.58** (1.11–2.27)	1.42 (0.87–2.32)	1.20 (0.68–2.12)	1.22 (0.57–2.60)	0.89 (0.39–2.01)	3.44 (0.44–26.9)	1.58 (0.57–4.34)	1.00 (0.31–3.22)	3.23 (0.43–24.5)
High versus low 30%	**1.73** (1.24–2.43)	1.46 (0.95–2.42)	1.42 (0.78–2.58)	1.13 (0.63–2.01)	1.13 (0.58–2.20)	1.13 (0.34–3.71)	1.70 (0.74–3.95)	1.30 (0.40–4.17)	2.25 (0.64–7.95)
Cyclin									
A2 high versus low 10%	1.41 (0.98–2.02)	1.16 (0.75–1.80)	1.39 (0.72–2.67)	0.89 (0.51–1.55)	0.76 (0.40–1.43)	1.55 (0.47–5.09)	1.02 (0.57–1.81)	1.03 (0.50–2.11)	0.93 (0.34–2.55)
B1 high versus low 10%	1.04 (0.62–1.76)	0.96 (0.54–1.70)	0.53 (0.13–2.20)	0.84 (0.46–1.54)	0.66 (0.33–1.34)	1.91 (0.56–6.55)	1.01 (0.63–1.64)	0.80 (0.46–1.39)	2.12 (0.78–5.76)
D1 high versus low 10%	0.61 (0.36–1.05)	0.89 (0.36–2.19)	**0.34** (0.17–0.69)	2.06 (0.64–6.63)	3.02 (0.73–12.5)	0.44 (0.06–3.48)	0.92 (0.56–1.51)	1.14 (0.65–1.99)	0.47 (0.16–1.37)
E1 high versus low 10%	1.15 (0.80–1.65)	0.77 (0.50–1.19)	1.44 (0.76–2.74)	1.00 (0.55–1.81)	0.76 (0.39–1.49)	2.18 (0.58–8.24)	1.16 (0.55–2.44)	0.70 (0.30–1.66)	2.74 (0.62–12.1)
Subtype									
Luminal B versus luminal A	**3.85** (1.96–7.57)	**1.89** (1.00–3.55)	**2.78** (1.30–5.95)		–	–		–	–
Luminal Her2 versus luminal A		–	–	**5.09** (2.37–11.0)	1.92 (0.91–4.04)	**4.44** (1.45–13.6)		–	–
Her2 expressing versus luminal A		–	–	**5.64** (2.64–12.0)	2.03 (0.97–4.23)	**5.51** (1.91–15.9)		–	–
Triple-negative versus luminal A		–	–		–	–	**4.95** (2.47–9.94)	1.84 (0.96–3.54)	**4.61** (1.97–10.8)
LVI yes versus no	**2.59** (1.82–3.68)	**2.05** (1.33–3.15)	**2.89** (1.57–5.32)	**2.25** (1.29–3.94)	**2.25** (1.19–4.27)	2.70 (0.69–10.5)	**2.31** (1.40–3.83)	**2.29** (1.28–4.09)	2.38 (0.86–6.58)
Multifocality yes versus no	1.40 (0.94–2.08)	1.30 (0.81–2.08)	1.17 (0.55–2.49)	1.71 (0.98–2.98)	1.47 (0.78–2.78)	2.60 (0.79–8.53)	1.59 (0.85–2.98)	1.69 (0.82–3.49)	1.41 (0.40–4.97)

LVI, lymphovascular invasion. Bold figures indicate HR with significance level <0.05. NS, not significant.

Table 3
Univariate analysis of risk factors for distant disease or death by breast cancer in women with luminal tumours by PR-expression and age (n = 544).

Cut-off	Luminal PR+			Luminal PR−		
	All ages	<40 y	≥40 y	All ages	<40 y	≥40 y
	n = 348	n = 154	n = 194	n = 196	n = 94	n = 102
Ki-67						
>14% versus ≤14%	**3.19 (1.58–6.44)**	**3.15 (1.12–8.81)**	2.41 (0.89–6.49)	1.27 (0.76–2.12)	0.98 (0.47–2.05)	1.05 (0.48–2.29)
>20% versus ≤20%	**2.02 (1.18–3.44)**	1.49 (0.75–2.96)	2.12 (0.90–5.01)	1.25 (0.77–2.04)	1.19 (0.59–2.42)	0.77 (0.34–1.74)
>30% versus ≤30%	**2.19 (1.35–3.54)**	1.65 (0.91–3.02)	**2.30 (1.01–5.25)**	1.22 (0.76–1.97)	1.07 (0.58–1.96)	0.78 (0.31–1.96)
Cyclin						
A2 >10% versus ≤10%	**1.67** (1.00–2.79)	1.36 (0.73–2.55)	1.84 (0.76–4.82)	1.09 (0.65–1.82)	0.87 (0.47–1.61)	1.00 (0.38–2.67)
B1 >10% versus ≤10%	1.40 (0.69–2.83)	1.27 (0.59–2.74)	0.64 (0.09–4.78)	0.71 (0.33–1.55)	0.69 (0.29–1.63)	0.39 (0.05–2.88)
D1 >10% versus ≤10%	**0.42 (0.18–0.98)**	0.51 (0.16–1.64)	**0.28 (0.08–0.97)**	1.00 (0.50–2.02)	1.79 (0.43–7.39)	0.54 (0.22–1.30)
E1 >10% versus ≤10%	1.41 (0.84–2.37)	1.02 (0.55–1.89)	1.37 (0.50–3.71)	0.79 (0.48–1.30)	**0.51 (0.27–0.95)**	1.15 (0.49–2.69)

Bold figures indicate p < .05.



Distant disease free survival	Luminal A <40y *vs* ≥40y			Luminal B PR+ <40y *vs* ≥40y			Luminal B PR- <40y *vs* ≥40y		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Unadjusted	1.91	(0.51-7.12)	*.335*	**2.40**	(1.37-4.19)	*.002*	**2.08**	(1.27-3.41)	*.004*
+Year	2.03	(0.55-7.59)	*.290*	**2.38**	(1.36-4.18)	*.002*	**1.83**	(1.10-3.06)	*.020*
+Stage	1.40	(0.33-5.98)	*.651*	**2.25**	(1.28-3.96)	*.005*	1.21	(0.72-2.05)	*.472*
+Grade	1.21	(0.27-5.46)	*.803*	**2.16**	(1.22-3.81)	*.004*	1.09	(0.64-1.87)	*.750*
+LVI	1.22	(0.27-5.56)	*.796*	**2.08**	(1.18-3.68)	*.012*	0.97	(0.56-1.67)	*.903*
+Cyclin A2, B1, D1, E1	0.66	(0.09-4.93)	*.689*	**2.42**	(1.30-4.50)	*.005*	1.15	(0.63-2.09)	*.648*
+Systemic treatment	0.63	(0.08-5.22)	*.671*	**2.35**	(1.22-4.50)	*.010*	1.14	(0.62-2.10)	*.670*

Fig. 2. Kaplan–Meier curves of distant disease-free survival by age and luminal subtype; A) luminal A, B) luminal B PR+, C) luminal B PR−, combined with multivariate Cox regression of risk for distant disease adjusted for year of diagnosis, stage, grade, LVI, cyclin expression and systemic treatment (chemotherapy and endocrine therapy including ovarian suppression). Women aged <40, n = 248, women aged ≥40, n = 296. Bold figures indicate statistically significant result, p < .05. CI, confidence interval; HR, hazard ratio; LVI, lymphovascular invasion.

significantly important only in middle-aged women after stratifying for age. Ki-67 was associated with a worse prognosis in young women with luminal PR+ breast cancer, where the optimal cut-off for Ki-67 to predict DDFS was significantly lower in young women (Ki-67 14%) than in middle-aged women (Ki-67 30%).

A weakness of this study is the limited amount of tumour tissue examined. TMA enables multiple testing of tissue samples in a standardised, tissue-sparing manner; however, it requires careful sampling and careful interpretation of the results, especially concerning Ki-67. Previous studies have shown good statistical correlation between evaluation of Ki-67 through TMA and

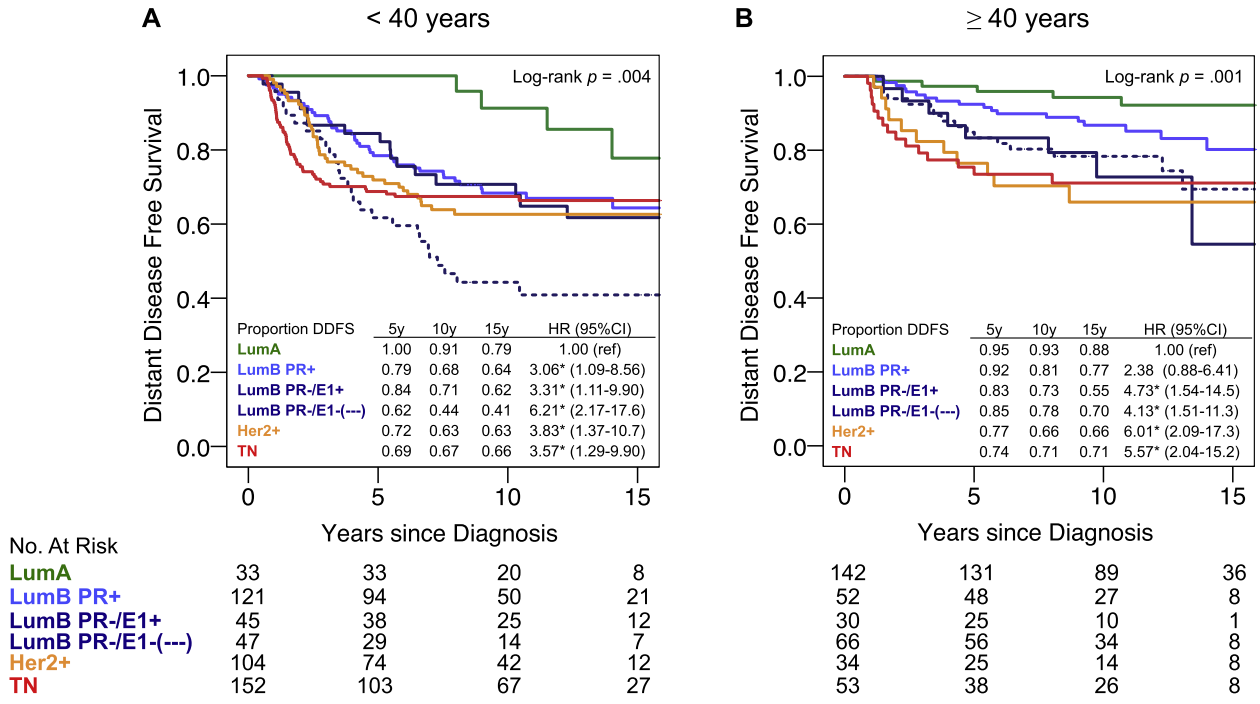


Fig. 3. Kaplan–Meier curves of distant disease-free survival by age and subtype. Women aged <40, n = 502 (A), women aged ≥40, n = 377 (B). Women with missing information on cyclin E were excluded (n = 8). CI, confidence interval; HR, hazard ratio; TN, triple-negative.

through full sections [45,46], although Ki-67 results derived from TMA sections are lower [37]. We tried to diminish the risk of underestimation of Ki-67 using duplicate cores from representative peripheral parts of the tumours and by choosing the 14% cut-off mainly based on the study from Cheang *et al.* [16] performed on TMA cores that found this cut-off to best match the gene expression–profiled subtypes.

As both young age and subtype are strong prognostic factors, there is a need to clarify whether the higher proliferation associated with tumours arising in young women can explain their poorer outcome. Our results show that Ki-67 and cyclins are overexpressed in young women with luminal tumours, which is in line with earlier publications [22,25,40]. Our findings suggest that cyclin expression adds very little prognostic information in spite of being significantly differentially expressed within subtypes as well as age groups. The only cyclin that added prognostic value beyond subtype was cyclin E1.

A high cyclin E1 was associated with a better prognosis in young women with luminal B PR– breast cancer. Young women with luminal B PR– breast cancer with a low cyclin E1 had the worst prognosis, with a more than six-fold higher risk of distant disease compared to young women with luminal A tumours.

In most studies, a high expression of cyclin E, rather than a low expression, has been significantly associated with a poor prognosis [44,47–52]. Also in our study a high cyclin E1 was associated with poor prognosis, but with stratification by age, that association remained only in women aged ≥40. Cyclin E1 and the association with a lower risk of distant disease in the young women is thought provoking and may be explained by the benefit of chemotherapy being larger in tumours with a high proliferation rate [53,54]. In clinical practice, young women with luminal B PR– tumours will be offered chemotherapy no matter the expression of cyclin E1.

Similarly, we found high cyclin D1 to be an indicator of good prognosis in luminal tumours, in line with other publications [22,23,55]. The protective role of a high cyclin D1 was only seen in women ≥40 years. As the proportion of tumours with low cyclin D1 was small in our study, these findings need further validation.

Our finding of low age being an independent prognostic factor only in luminal B PR+ breast cancer is clinically important. We have delimited the prognostic importance of low age to one-fourth of the young women and thereby lined out in what subpopulation of young women further research on age-related differences in prognosis should be focussed. The worse prognosis in these young women is noted despite more intense treatment than in their middle-aged counterparts.

We conclude that in this unique cohort of young women, the prognostic value of proliferation markers is limited after adjusting for breast cancer subtype. For young women with luminal tumours, low age in itself and PR-status seems to be more important than proliferation, which should be considered in treatment decisions. The prognostic association between Ki-67 and DDFS was only seen in young women with luminal

PR+ tumours. The optimal cut-off for Ki-67 seems to vary by age and was most significant in young women at a cut-off of 14%. The added prognostic value of cyclins in the young population is minor. However, the new data indicating a high cyclin E1 to be prognostically beneficial in luminal B PR− tumours, are biologically interesting and hypothesis generating but not clinically relevant without further studies. The findings imply the possibility that proliferation markers could serve differing functions in different age groups, which in turn may be related to the composition of the hormonal milieu or tumour genotype.

Authors' contributions

HF, HL, LH, JF and IF contributed to the study concepts. HF, KM, LSL, HL, JB, LH, FP, JF and IF contributed in data acquisition. Data analysis and interpretation were performed by HF, NT, JB, LH, FP, JF and IF. Statistical analysis was performed by HF, KM, LSL and IF. Manuscript preparation was performed by HF, KM, NT and IF. Manuscript editing was performed HF, KM, LSL, NT, HL, JB, LH, FP, JF and IF.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee at Karolinska Institutet, Stockholm, Sweden (approval diary number 2009/1174-31/1 and 2010/586-32) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent

The Research Ethics Committee at Karolinska Institutet, Stockholm, Sweden, approved an informed consent waiver for the retrospective medical record review (approval diary number 2009/1174-31/1 and 2010/586-32).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2017.07.044.

Conflict of interest statement

None declared.

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