Use of Molecular Tools to Identify Patients With Indolent Breast Cancers With Ultralow Risk Over 2 Decades

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IMPORTANCE The frequency of cancers with indolent behavior has increased with screening. Better tools to identify indolent tumors are needed to avoid overtreatment.

OBJECTIVE To determine if a multigene classifier is associated with indolent behavior of invasive breast cancers in women followed for 2 decades.

DESIGN, SETTING, AND PARTICIPANTS This is a secondary analysis of a randomized clinical trial of tamoxifen vs no systemic therapy, with more than 20-year follow-up. An indolent threshold (ultralow risk) of the US Food and Drug Administration–cleared MammaPrint 70-gene expression score was established above which no breast cancer deaths occurred after 15 years in the absence of systemic therapy. Immunohistochemical markers (n = 727 women) and Agilent microarrays, for MammaPrint risk scoring (n = 652 women), were performed from formalin-fixed paraffin-embedded primary tumor blocks. Participants were postmenopausal women with clinically detected node-negative breast cancers treated with mastectomy or lumpectomy and radiation enrolled in the Stockholm tamoxifen (STO-3) trial, 1976 to 1990.

EXPOSURES After 2 years of tamoxifen vs no systemic therapy, regardless of hormone receptor status, patients without relapse who reconsented were further randomized to 3 additional years or none.

MAIN OUTCOMES AND MEASURES Breast cancer–specific survival assessed by Kaplan-Meier analyses and multivariate Cox proportional hazard modeling, adjusted for treatment, patient age, year of diagnosis, tumor size, grade, hormone receptors, and *ERBB2/HER2* and *Ki67* status.

RESULTS In this secondary analysis of node-negative postmenopausal women, conducted in the era before mammography screening, among the 652 women with MammaPrint scoring available (median age, 62.8 years of age), 377 (58%) and 275 (42%) were MammaPrint low and high risk, respectively, while 98 (15%) were ultralow risk. At 20 years, women with 70-gene high and low tumors but not ultralow tumors had a significantly higher risk of disease-specific death compared with ultralow-risk patients by Cox analysis (hazard ratios, 4.73 [95% CI, 1.38-16.22] and 4.54 [95% CI, 1.40-14.80], respectively). There were no deaths in the ultralow-risk tamoxifen-treated arm at 15 years, and these patients had a 20-year disease-specific survival rate of 97%, whereas for untreated patients the survival rate was 94%. Recursive partitioning identified ultralow risk as the most significant predictor of good outcome. In tumors "not ultralow risk," tumor size greater than 2 cm was the most predictive of outcome.

CONCLUSIONS AND RELEVANCE The ultralow-risk threshold of the 70-gene MammaPrint assay can identify patients whose long-term systemic risk of death from breast cancer after surgery alone is exceedingly low.

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Breast cancer is biologically and molecularly heterogeneous, exhibiting a spectrum of clinical outcomes ranging from very low risk to very high risk for metastatic recurrence. Timing of life-threatening recurrence varies, occurring months to decades after diagnosis and primary therapy.[1] Molecular profiling has spawned the development of signatures that identify tumors associated with early risk for recurrence[2,3] and patients who benefit from systemic chemotherapy to reduce that metastatic risk.[4,5]

With the introduction of screening, the distribution of the biologic subtypes of breast cancer shifted, and overall incidence increased by 10% to 20%. Decades later, this increased age-adjusted incidence of breast cancer has remained high.[6] The bulk of the increase has been in early-stage tumors (stage 0, 1), suggesting that screening contributes disproportionately to the diagnosis of biologically more indolent forms of breast cancer.[7,8] While screening is associated with a relative mortality reduction of 20%,[9,10] it has increased the diagnosis of low-risk lesions and contributes to overtreatment.[6,7,11,12] Scholarly articles reporting the detection of indolent cancers continue to stir controversy.[13] Unfortunately, clinically low-risk invasive cancers can recur very late, even after 10 or 15 years. Tools with the capacity to identify ultralow-risk tumors at the time of diagnosis could prevent overtreatment. Cancer type, histologic grade, proliferative index, and stage are associated with lower early metastatic disease risk but do not reliably identify those with sufficiently low long-term (20 years) risk of recurrence to avoid or further reduce therapy.

The 70-gene MammaPrint assay (US Food and Drug Administration–cleared) was initially developed as a prognostic tool for women with breast cancer who did not receive adjuvant systemic therapy. The low- vs high-risk threshold was initially used to differentiate recurrence at 5 years after diagnosis because this predicts benefit of systemic chemotherapy. In the recently reported 6693-person randomized clinical trial MINDACT,[5] patients with clinically high but molecularly low-risk disease without chemotherapy were found to have a 94.7% distant metastasis–free survival at 5 years, even with up to 3 positive nodes and tumors up to 5 cm in size.

We previously demonstrated that screening has led to an increase in the proportion of 70-gene low-risk tumors relative to the era prior to the advent of screening. We had set an ultralow-risk threshold (where there were no metastatic events at 5 years, in the original 70-gene cohort) and found that ultralow-risk disease increased from 10% in an unscreened population to 30% in a screened population.[14] However, hormone-positive cancers can recur decades after diagnosis, and two-thirds of recurrences are after 5 years. To truly identify cancers with indolent lesions of epithelial origin behavior (extremely low risk for systemic recurrence), we set a threshold where there were no breast cancer deaths for node-negative patients at 15 years, using 25-year follow-up of the NKI295 series,[1,15] and this was confirmed in the original European validation set for the 70-gene assay[3] (eFigure 1 and eReference in the Supplement).[16]

To independently and rigorously validate the new ultralow-risk threshold, we used a unique resource, the Stockholm randomized clinical trial, STO-3, of postmenopausal women with clinically detected node-negative tumors 3 cm or smaller assigned to tamoxifen vs no adjuvant therapy, which had tissue blocks available and meticulous follow-up.[17,18]

Key Points

Question Can a molecular signature—an ultralow-risk threshold of the US Food and Drug Administration–cleared MammaPrint 70-gene expression score—be used to identify tumors with indolent behavior?

Findings In this secondary analysis of a trial of node-negative postmenopausal women randomized to tamoxifen vs no systemic therapy that included 652 patients with MammaPrint risk scoring, 15% met the ultralow-risk threshold. Ultralow-risk patients have a 20-year disease-specific survival rates of 97% and 94% in the tamoxifen arm and control arm, respectively.

Meaning The ultralow-risk threshold can identify patients whose long-term risk of dying from breast cancer is exceedingly low.

Methods

Study Population

The STO-3 study group conducted a randomized clinical trial of tamoxifen from 1976 until 1990 in postmenopausal women.[17] The STO-3 low-risk trial included 1780 lymph node-negative patients with tumors smaller than or equal to 3 cm in diameter, randomized to 2 years of adjuvant tamoxifen (40 mg daily) vs no adjuvant treatment. In 1983, patients who reconsented and were recurrence-free after 2 years of tamoxifen treatment were randomized to 3 additional years of tamoxifen or no further therapy. The STO-3 trial was approved by the ethical committee at Karolinska Institutet, and participants provided oral consent. Ethics approval was also obtained for the secondary analysis presented in this study. .

There were 808 patients with formalin-fixed paraffin-embedded (FFPE) tissue blocks available for molecular analysis, with 81 patients excluded because of an insufficient amount of invasive tumor tissue.[18] The patient subset with available FFPE material was well balanced relative to the original cohort with regards to tumor characteristics (tumor size, estrogen receptor status and treatment arm assignment).[19] Immunohistochemical analysis (estrogen receptor, progesterone receptor, *ERBB2/HER2*, and *Ki67*) was performed on 727 specimens at a single laboratory. RNA was extracted, and 652 patients had 70-gene signature classification passing the quality check (**Figure 1**): 339 had received tamoxifen, and 313 had not received adjuvant systemic therapy.

Estrogen-Receptor, Progesterone Receptor, *ERBB2/HER2*, and *Ki67* Immunohistochemical Analysis

The FFPE tissue sections were sectioned at 4 µm and mounted on plus-coated glass slides, shipped to University of California, Davis, and immunohistochemically stained in the CLIA laboratory using a DAKO Autostainer Link 48. Antibodies used were estrogen receptor (SP1; Spring Bioscience M301), progesterone receptor (PgR 636; DAKO IR068), *ERBB2/HER2* (HercepTest;

DAKO SK001), and *Ki67* (MIB-1; DAKO M7240), with EnVision plus detection, following standard recommended procedures.

70-Gene Prognosis and 80-Gene Subtype Assignments

MammaPrint and BluePrint assays were performed according to standard protocols and have been previously described.[20,21] These tests are based on microarray gene expression analysis of RNA extracted from FFPE breast tumor tissue and use custom-designed array chips manufactured by Agilent Technologies. The Agilent oligonucleotide microarray platform assesses the messenger RNA expression of the 70-gene MammaPrint or 80-gene BluePrint subtype signatures, 465 normalization genes, and more than 250 probes for hybridization and printing quality control. Seventy-gene signature tumors were classified into risk categories as either ultralow (≥0.355), low but not ultra-low (>0, <0.355), and high risk (<0) using thresholds previously developed.[15] BluePrint subtype assigns to luminal, basal, or *ERBB2/HER2*-type.[21]

PAM50 Intrinsic Subtype Assignment

In addition to Blueprint subtype assignment, tumors were assigned to 1 of 5 intrinsic subtypes (luminal A, luminal B, *ERBB2/HER2*, basal, normal-like) using the PAM50 classifier as described in Parker et al[22] and eMethods in the Supplement.

Statistical Analysis

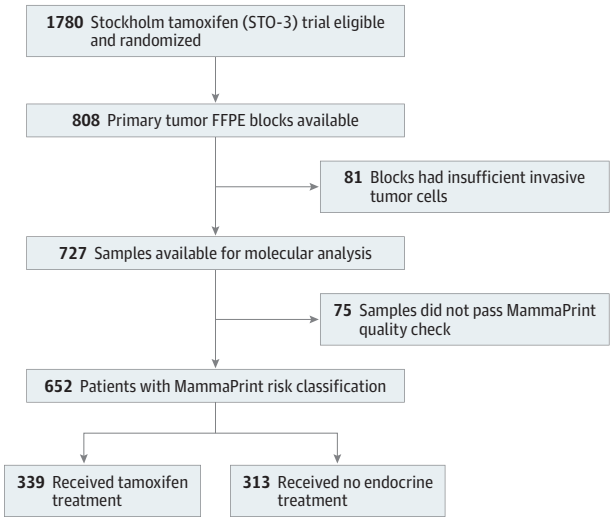
Kaplan-Meier analysis of 20-year breast cancer-specific survival by MammaPrint risk categories (high- vs low-risk) was performed, and significance assessed using the log-rank test. Similarly, Kaplan-Meier analysis of 20-year breast cancer-specific survival according to 70-gene risk categories were conducted for all patients, as well as within each STO-3 trial treatment arm (tamoxifen or untreated) separately. In addition, analyses of 20-year breast cancer-specific survival by the ultralow-risk threshold, used as the reference category, were performed by multivariate Cox proportional hazard modeling adjusting for age and year of breast cancer diagnosis, estrogen receptor, progesterone receptor, *ERBB2/HER2*, *Ki67*, tumor grade, tumor size, and STO-3 trial treatment arm (tamoxifen or untreated).

Recursive partitioning was performed using the *rpart* package in R software (R Foundation) to construct a survival tree that best predicts 20-year breast cancer-specific survival. Input variables to the model includes 70-gene risk categories, BluePrint intrinsic subtype, age and year of breast cancer diagnosis, estrogen receptor, progesterone receptor, *ERBB2/HER2*, and *Ki67* status, tumor grade (1, 2, or 3), tumor size, and treatment arm. The final tree was selected by minimizing the 10-fold cross validation error.

Survival outcomes of patients in this cohort were followed through December 31, 2012. The 20-year, breast cancer-specific survival analysis is presented owing to concerns for model stability stemming from the small number of patients still alive and at risk within the ultralow-risk group after 20 years. Patients with contralateral primary breast cancers were censored at the time that the contralateral cancer was diagnosed to avoid confusion about ascribing breast cancer mortality to the contralateral cancer rather than the initial cancer event. Altogether, 61 patients with contralateral cancers (29 were tamoxifen treated [8.6%], 32 were untreated [10.2%]) were censored for the survival analysis.

All data preparation and analysis were done using SAS statistical software (version 9.4; SAS Institute Inc) and R, version 3.1.2.

Figure 1. CONSORT Diagram



FFPE indicates formalin-fixed paraffin-embedded.

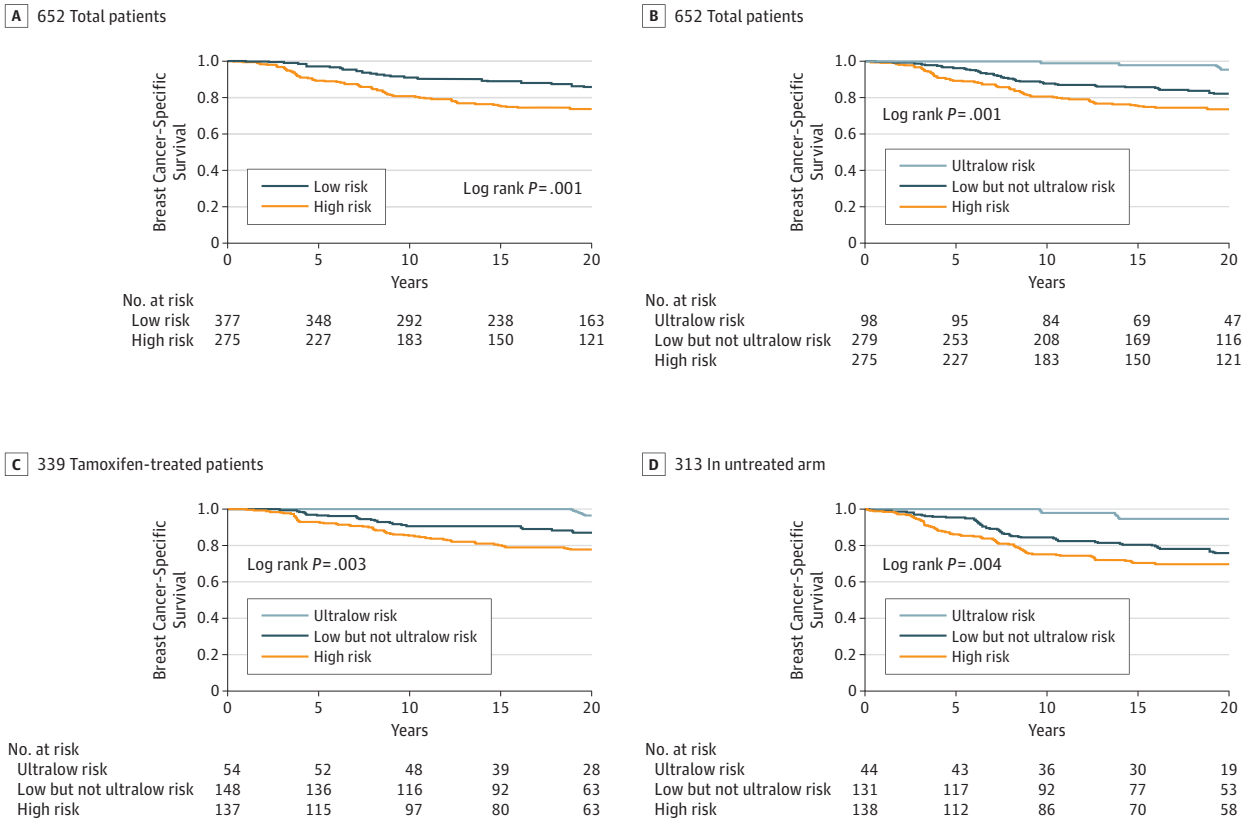
Results

Patient and tumor characteristics of the postmenopausal women with available microarray data and within each treatment arm (n = 652) are in the eTable in the Supplement. No significant differences in age, type of surgery received, receptor status, tumor grade, and size were observed between the treatment arms. Most patients (516 [79%]) received mastectomy and axillary dissection. Of the tumors reassessed for estrogen receptor, progesterone receptor, *ERBB2/HER2*, and *Ki67* status, 538 (83%) were estrogen receptor-positive; 369 (58%) were progesterone receptor-positive; 53 (8%) were *ERBB2/HER2*-positive, and 178 (29%) had *Ki67* greater than or equal to 15%; 121 (19%), 375 (58%), and 147 (23%) were grade 1, 2, and 3, respectively; and 499 patients (78%) had tumor size smaller than 2 cm and were not different from the original cohort (n = 1780).[23]

Long-term Survival Analyses of Cause-Specific Breast Cancer

The 70-gene signature scored 42% of patients as high-risk and 58% as low-risk. Kaplan-Meier breast cancer survival graphs of high- and low-risk patients censored at contralateral breast cancer diagnosis are shown in **Figure 2**A. There is a statistically significant difference in outcome between the groups (log rank $P < .001$). In particular, low-risk patients have an excel-

Figure 2. Kaplan-Meier Plots of Breast Cancer–Specific Survival



Kaplan-Meier plots of breast cancer–specific survival of the STO-3 trial cohort stratified based on the 70-gene score into (A) MammaPrint low- and high-risk groups and (B-D) ultralow, low but not ultralow, and high-risk categories for (B) all patients, and patients (C) with and (D) without tamoxifen treatment. Tamoxifen was given to all patients in the tamoxifen treatment arm for 2 years; approximately 35% of patients received tamoxifen for an additional 3 years. COD BC indicates cause of death, breast cancer.

lent (>95%) breast cancer-specific survival at 5 years. However, with extended follow-up, patients continue to die from their disease.

Survival curves (Figure 2B) are shown for the 652 patients based on ultralow-, low but not ultralow, and high-risk assignments, 15% (98) of which were ultralow-risk. The difference in outcome based on the 3 designations is significant with a log rank $P < .001$. When stratified by treatment, in patients who received tamoxifen, there were no breast cancer-specific deaths at 15 years and breast cancer-specific survival was 97% at 20 years (Figure 2C); patients without any systemic therapy had a 97% and 94% breast cancer-specific survival at 10 and 20 years, respectively (Figure 2D). Results without censoring for second cancers are shown in eFigure 2 in the Supplement.

Multivariate proportional hazards analyses of 20-year breast cancer-specific survival according to 70-gene risk groups adjusting for age and year of primary breast cancer diagnosis, estrogen receptor, progesterone receptor, *ERBB2/HER2,* and *Ki67* status, tumor grade, and tumor size, and treatment arm was performed. After adjustment, patients with high-risk and low-risk assignments had a statistically significant increased long-term risk of 4.73 (95% CI, 1.38-16.22) and 4.54 (95% CI, 1.40-14.80) of breast cancer-specific death, respectively, compared with patients with MammaPrint ultralow tumors.

Characteristics of MammaPrint Ultralow-Risk Tumors

Figure 3A shows the intrinsic and biological characteristics of tumors that meet the MammaPrint ultralow-risk threshold. All ultralow tumors were hormone receptor-positive *ERBB2/HER2*-negative and of the luminal subtype by BluePrint. Using a 15% cutoff for *Ki67* "low," 96% of ultralow-risk tumors with known *Ki67* assessments were *Ki67*-low. When the PAM50 algorithm was used to assign intrinsic subtype, 89% of ultralow-risk tumors were designated as luminal A. Interestingly, only 19% of hormone receptor-positive *ERBB2/HER2*-negative tumors and 20% of *Ki67*-low tumors are ultralow risk (Figure 3B). Similarly, only 25% and 26% of tumors characterized as luminal A by PAM50, and Blueprint, respectively, meet the ultralow-risk threshold. On review of pathologic features of the ultralow-risk tumors, invasive ductal (no special type) carcinomas were the most frequent, but lobular, tubular, invasive papillary, and invasive cribriform subtypes were enriched, and mucinous types were absent.

Recursive Partitioning

We used *rpart*, a recursive partitioning tool, with cross-validation to integrate molecular and clinical variables and construct a survival tree that best predicts 20-year breast cancer-specific survival. The resulting model (**Figure 4**A) first divides patients by the ultralow-risk classification. No further subdivision of ultralow-risk patients is observed. In contrast, for tumors that do not meet the ultralow-risk classification, size is selected as the next most predictive factor, where patients with tumors greater than 2 cm have the worst outcome, with only about 70% breast cancer-specific survival at 10 years (Figure 4B).

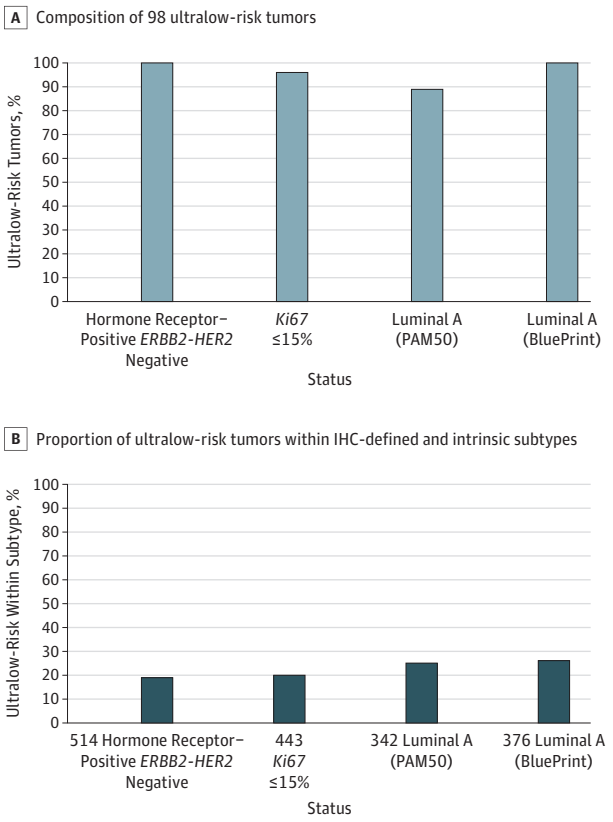
Discussion

The ultralow-risk threshold of the 70-gene signature, in postmenopausal women with node-negative tumors 3 cm or smaller, reliably identifies women with minimal risk of death from breast cancer out to 20 years in the absence of any systemic therapy and negligible risk of death with 2 or more years of tamoxifen therapy alone. The ultralow-risk tumors are hormone-positive, mostly luminal A with low proliferation (*Ki67* < 15%); however, they are only a small subset of cancers with those characteristics.

How is this relevant today? Women who have a tumor that is an ultralow-risk tumor by 70-gene signature can be reassured that their long-term outcome is expected to be excellent, with or without endocrine therapy. In the tamoxifen-treated arm, most had only 2 years of therapy and still had excellent long-term survival. Many women are unable to tolerate 5 years of endocrine therapy, and fewer than 60% complete 5 years of treatment.[24] With the updated American Society of Clinical Oncology guidelines of 10 years of adjuvant endocrine therapy for patients with hormone receptor-positive breast cancers,[25] a test that accurately identifies a population of women who have very little risk to begin with should be welcomed by patients and clinicians alike. Importantly, this data set allowed us to evaluate the outcome of women with surgical therapy alone, and no systemic therapy, demonstrating an extremely low risk of recurrence. Furthermore, elderly women (>75 years) with comorbidities and a life expectancy of less than 10 years who present with an ultralow-risk breast tumor can be offered excision alone, with confidence that the treatment will be sufficient.

In the STO-3 randomized clinical trial, all women received either mastectomy or lumpectomy and radiation, so there is no direct evidence to support reducing local therapy. However, the ultralow-risk tumors were a subset of luminal A tumors, and there is evidence that postmenopausal women with luminal A tumors have only a 5% chance of local recurrence with or without radiation.[26] Other randomized clinical trials also document similar groups of women who do not benefit from radiation,[26-28] and where the small fraction of women who recur can be successfully treated at the time of recurrence. However, despite level 1 evidence in 3 trials, the use of radiation for these subtypes has changed very little. The ultralow-risk classification, a smaller, more restricted subset of

Figure 3. Association Among MammaPrint Ultralow-Risk Tumors and Other Low-Risk Biological Features

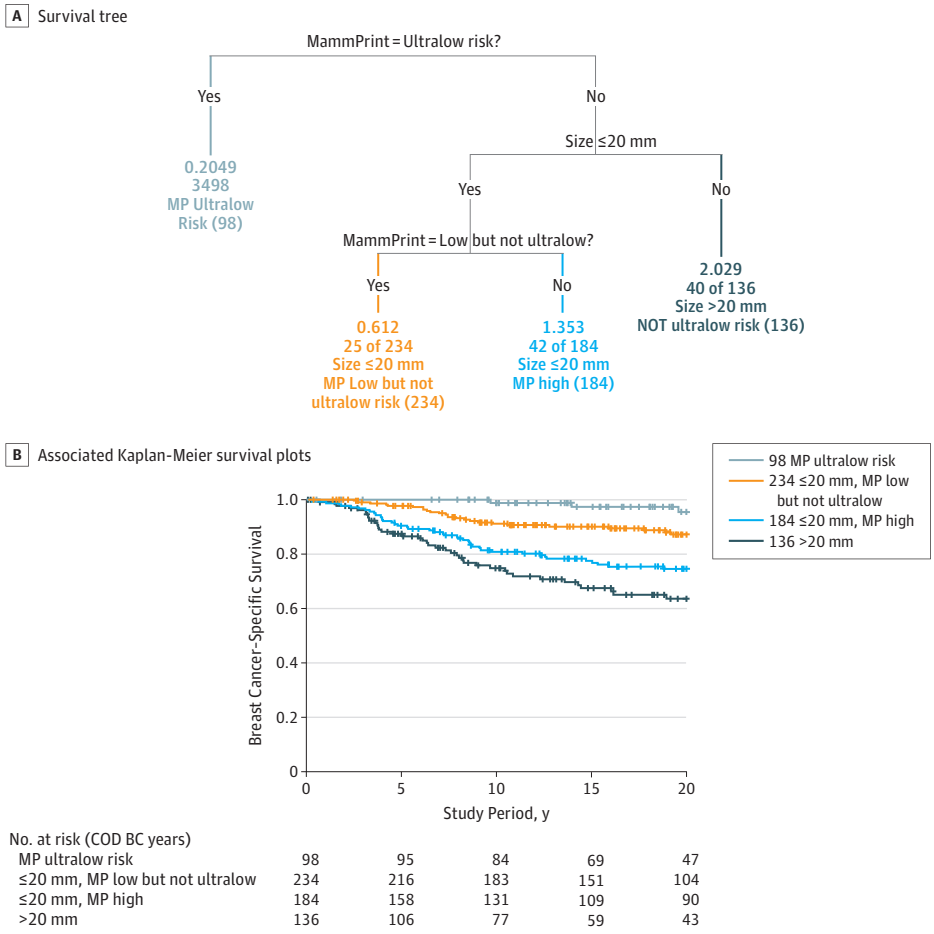


the *Ki67* < 15% and luminal A cases, should surely qualify for "no radiation." The indolent behavior of these tumors and lack of systemic risk over 2 decades support a less aggressive approach to treatment.

Almost all women in the STO-3 randomized clinical trial presented with palpable primary tumors, since breast cancer screening was not initiated in the Stockholm health care region until 1989. Importantly, 15% of these symptomatic (clinically detected) tumors were ultralow, showing that these tumors are an inherent part of the spectrum of breast cancers detected, regardless of screening. Women with ultralow-risk tumors who did not receive adjuvant therapy had excellent survival (94%), with the rare recurrence appearing 10 to 20 years after diagnosis. The group that had 2 or more years of tamoxifen had a 3% chance of death from breast cancer at 20 years. It is therefore unlikely that early detection of these types of lesions, before they come to clinical attention, would result in further clinical benefit. Even in the absence of screening, there are breast cancers that pose little or no systemic risk. The opportunity now is to recognize and properly classify these indolent tumors to avoid overtreatment.

The frequency of ultralow-risk cancers in a screened population of postmenopausal women is likely in the range of 25%.[29] In the United States, over a 10-year period, 2 million cancers will be diagnosed, of which 500 000 might be indolent. Tools

Figure 4. Recursive Partitioning Survival Tree on 20-Year Survival With Kaplan-Meier Plots



Risk prediction and clinical strategies that integrate molecular and clinical information. A and B, The recursive partitioning algorithm *rpart* was used to integrate molecular and clinical variables for risk prediction. A, Survival tree that best predicts 20-year breast cancer–specific survival. B, Associated Kaplan Meier survival plots. MP indicates MammaPrint.

such as the ultralow-risk threshold of the 70-gene test can enable these tumors to be classified at diagnosis and provide reassurance that, once removed, the condition is rarely associated with distant recurrence or death (Figure 4C). This will provide support for patients and their physicians to choose less aggressive therapy.

The overall objective of screening is to identify breast cancers at an earlier stage and thereby reduce the mortality that is associated with more clinically advanced disease. Mitigating the harms of screening[30,31] requires both the recognition that ultralow risk tumors exist, and the ability to reliably identify them with a diagnostic tool. Recursive partitioning (Figure 4A) demonstrates that the ultralow-risk classification is the most predictive factor at 20 years of follow-up.[8] Interestingly, once the ultralow-risk tumors are removed from the population, the next most predictive factor that drives prognosis is tumor size greater than 2 cm, suggesting that early detection is important for those tumors that are not ultralow risk (although some of this risk will be addressed with other systemic treatments not available at the time this study was conducted). The ability to identify an ultralow-risk category of tumors represents another critical advance in how molecular tools enable care to be personalized.[1,4,32-36]

Since the outcome of tumors with indolent behavior is excellent, even when detected as a palpable mass, detection of their precursors would not deliver benefit. These data provide the impetus to explore whether we can identify the types of ductal carcinoma in situ that precede indolent lesions and refine our targets for screening. We are also investigating the genes that characterize ultralow-risk lesions and will explore commonalities across cancers that originate in other organ sites.[37] In prostate cancer screening, Gleason 3 + 3 is a marker of indolent disease. Men treated with active surveillance have now been shown to have excellent outcomes without excision, with 10-year disease-free survival rates of 97%.[38] Common biologic features of indolent behavior could inform a change in nomenclature.

Interestingly, tamoxifen reduces the risk of contralateral cancers, but not until 15 years, possibly reflecting the long term preventive benefit of tamoxifen.

Limitations

Limitations of this study include the fact that we did not address whether breast cancer–specific survival was significantly affected by any additional endocrine therapy given at the time of first recurrence. Also, all women had either

mastectomy or lumpectomy and radiation; there are no data on outcomes with less aggressive local therapy. The STO-3 trial population is racially homogeneous; however, this should not affect our findings because aggressive behavior associated with African and African American race is explained by a higher preponderance of high-risk basal genotypes.[39] Finally, there were 98 patients in the ultralow-risk group. However, with only 15% of patients classified as ultralow risk, a very large trial would be needed to have a larger number of patients with ultralow tumors. The long follow-up, however, is unique and enables studies in Sweden, with their detailed and complete registries, to address questions for which long follow-up is essential. Their cancer registries are a model for the field.

Conclusions

We have used a molecular classifier to demonstrate long-term indolent tumor behavior. Such tools, if integrated into screening, treatment, and trials, can prevent inadvertent overtreatment and enable excellent outcomes with less toxic effects.

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Author Contributions: Dr Esserman had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Esserman, Borowsky, Benz, Lindström.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Esserman, Yau, Borowsky, Benz, Lindström.
Critical revision of the manuscript for important intellectual content: Esserman, Thompson, van't Veer, Borowsky, Hoadley, Tobin, Nordenskjöld, Fornander, Stål, Benz, Lindström.
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