Relapse-free survival of statistically standardized continuous RT-PCR estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2): NCIC CTG MA.14

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Abstract Recent ASCO/CAP guidelines focus on decision making associated with the presence/absence of continuous breast biomarkers. Statistical standardization (SS) is demonstrated as a method to evaluate the effects of *continuous* RT-PCR biomarker expression levels on breast cancer outcomes. MA.14 allocated 667 postmenopausal patients to tamoxifen based on locally determined ER/PR. Of 299 available patient tumor samples, 292 passed internal quality control. All tumors were centrally assessed by RT-PCR ER/PR/HER2 with each biomarker's z-scores categorized: $\geq$1.0 standard deviation (SD) below mean; $<$1.0 SD below mean; $\leq$1.0 SD above mean; $>$1.0 SD above mean. Log-rank statistics tested univariate differences in breast cancer relapse-free survival (RFS). Continuous SS-ER/PR/HER2 were assessed in multivariate Cox step-wise forward regression, adding a factor if $p \leq 0.05$. Sensitivity analyses examined an external HER2+ cut-point of 1.32. Patients whose tumors were tested were representative of the MA.14 population (p values = 0.18–0.90). At 9.8 years median follow-up, SS-ER did not univariately impact RFS ($p = 0.31$). SS-PR values above the mean ($z \geq 0.0$) had the best univariate RFS ($p = 0.03$). SS-HER2 also univariately impacted RFS ($p = 0.004$) with lowest (z-scores ≤ -1.0) and highest (z-scores > 1.0) having shortest RFS. Multivariate stratified/unstratified Cox models indicated patients with T1 tumors ($p = 0.02/p = 0.0002$) and higher SS-PR ($p = 0.02/p = 0.01$) had longer RFS; node-negative patients had better RFS (in unstratified analysis, $p < 0.0001$). Local ER/PR status did not impact RFS ($p > 0.05$). Patients with SS HER2+ $\geq$ 1.32 had worse RFS (univariate, $p = 0.05$; multivariate, $p = 0.06$). We demonstrated that higher SS-PR, and SS HER2 levels, measured by RT-PCR impacted breast cancer RFS outcomes. Evaluation in other trials may provide support for this methodology.

Keywords Centrally assessed breast cancer biomarkers · Biomarker standardization · RT-ER · RT-PR · RT-HER2

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Introduction

Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) are commonly assessed breast cancer biomarkers that are clinically relevant in determining appropriate adjuvant therapy. Uncertainties in the accurate assessment of these biomarkers engendered interest in standardization.

At the time of the 2010 American Society of Clinical Oncology and the College of American Pathologists

(ASCO/CAP) guidelines for ER and PR, there was the potential that 20 % of immunohistochemical (IHC) assay results worldwide were either false negatives or false positives [1]. Aspects affecting assays include tumor heterogeneity, acquisition and processing of specimens, antibody choices, laboratory assessment protocols, reproducibility of procedures, external assessment of process, proficiency of laboratory workers, sufficiency of scoring positivity, and cut-points for positivity [1]. The Panel recommended that a cut-off for ER/PR for a specimen to be positive required stain in at least 1 % of tumor cells [1].

The 2007 ASCO/CAP guidelines for HER2 were motivated by a similar concern that approximately 20 % of HER2 testing might be inaccurate [2]. The 2013 update of these guidelines clarified criteria to improve the accuracy of HER2 testing by IHC or in situ hybridization (ISH) [3].

Biomarkers need to be standardized for inter-laboratory comparability of results. The ASCO/CAP guidelines for these three clinical biomarkers focused on the determination that the categorical biomarkers indicate likely responsiveness to respectively endocrine or anti-HER2 therapies, without differentiation of responsiveness related to degree of biomarker expression. Adjunctive SS of continuous biomarker data, akin to bone mineral density (BMD) *z*-scores, permits robust examination of whether quantitative biomarker expression levels affect clinical breast cancer outcomes [4].

We previously examined ER and PR for tumors of patients in the premenopausal placebo-controlled tamoxifen trial, NCIC CTG MA.12. MA.12 tumors were determined locally for both hormone receptor positive and negative status, and we showed that SS hormone receptors assayed with real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) or by IHC had similar multivariate prognostic effects [4]. Here, we investigate the continuous prognostic effects of SS for centrally assessed RT-PCR ER, PR, and HER2 in the postmenopausal NCIC CTG MA.14 trial where all patients were allocated tamoxifen.

Patients and methods

Study design

NCIC CTG MA.14 [ClinicalTrials.gov Identifier: NCT00002864; protocol available online as supplemental material at journal website] enrolled 667 postmenopausal women between 1996 and 2000 [5]. Patients were randomly assigned to tamoxifen, (TAM) 20 mg orally once daily for 5 years, or to TAM 20 mg orally once daily for 5 years plus octreotide long-acting release (OCT) 90 mg intramuscularly monthly for 5 years (TAM-OCT). Patients were stratified by adjuvant chemotherapy (none, concurrent, or sequential), nodal status (none, one to three, four or more, or unknown), and locally determined receptor status (ER and/or PR positive, ER and PR negative, or ER and PR unknown). Ethics approval was obtained by all participating centers. All patients provided written informed consent. In July 2000, the duration of OCT on study was reduced from 5 to 2 years because of a greater incidence of gallbladder toxicity in the OCT arm of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-29 trial. MA.14 conduct was overseen by NCIC CTG (now, Canadian Cancer Trials Group; CCTG), and Novartis Canada (which provided the OCT) and the independent CCTG Data Safety Monitoring Committee.

Study population

Patients had histologically proven adenocarcinoma of the breast with adequately treated primary surgery [5]. No previous or concurrent malignancies were allowed except adequately treated carcinoma of the skin (basal cell), cervix, endometrium, colon, or thyroid, treated more than 5 years before study entry, and all patients had an estimated life expectancy of at least 5 years. Tumors could be ER and/or PR positive (biochemical value $\geq$10 fmol/mg, or positive by immuno-histochemistry), negative, or unknown. MA.14 patient tumors were not assessed for HER2, nor did any patient receive adjuvant trastuzumab. Baseline serum IGF-1, IGFBP-3, and C-peptide were centrally assessed for 646 MA.14 patients (96.9 %), and 25-hydroxy (OH) vitamin D for 607 of the MA.14 patients (91 %).

Study end points

The primary end point of MA.14 was event-free survival (EFS); events included disease recurrence, second malignancy, or death from any cause. Overall survival (OS) was a secondary end point. Relapse-free survival (RFS) was an additional secondary endpoint of MA.14, and is the primary end point for this investigation. RFS was defined as time from randomization to the time of recurrence of primary disease, it includes locoregional and distant relapse, excludes contralateral disease.

MA.14 trial analysis

At the final analysis, at a median follow-up of 7.9 years, the EFS stratified hazard ratio (HR) for (TAM-OCT to TAM) was 0.93 (95 % confidence interval (CI) 0.71–1.22; $p = 0.62$) [5]. OS showed a HR of 0.97 (95 % CI 0.69–1.37; $p = 0.86$). The RFS HR was 0.84 (95 % CI 0.59–1.18; $p = 0.31$). Patients allocated to Tamoxifen had

an absolute 2.7 % higher rate of RFS. At a median 9.8 years of follow-up, the RFS HR was 0.87 (95 % CI 0.63–1.21; $p = 0.40$) [6]. The median patient follow-up of 9.8 years was used in these investigations.

Study objectives

Our primary objective was to examine the continuous prognostic effects of SS-ER and SS-PR on RFS, using pooled data across both MA.14 treatment arms. In the absence of adjuvant anti-HER2 testing or anti-HER2 treatment, a secondary objective was to examine the continuous prognostic effect of SS-HER2 on RFS. Other secondary objectives included to examine the effect of categorizing SS-ER, SS-PR, and SS-HER2 by multiples of standard deviations (SD) below and above the SS-means. The approach of defining effects at categorical z-score SDs is similar to that used in the clinic for bone mineral density (BMD), and is described further below.

Gene expression analysis by real-time (RT)-PCR

For each formalin-fixed, paraffin-embedded tumor sample, three 8-μm tissue sections were subjected to gross macrodissection to enrich for tumor content. RNA extraction, amplification, and real-time time quantitative polymerase chain reaction (RT-qPCR) were performed at bioTheranostics Inc. (San Diego, CA, USA), a Clinical Laboratory Improvements Amendments–certified laboratory, with researchers blinded to clinical outcome. The procedure used for assessing ER/PR/HER2 was for research purposes only and not intended for patient testing under CLIA. Cases were excluded if there was insufficient RNA: average cycle threshold for normalizing genes was >28.5. Determination of ER was with ESR1 and determination of PR with PGR.

Statistical analyses

We utilized Fisher exact test to examine whether there were significant imbalances by treatment arm and stratification factors in who was, or was not, assessable for ER, PR, and HER2. Continuous gene expressions for ER, PR, and HER2 were histogrammed to examine whether a Box–Cox transformation should be considered to reduce asymmetry and stabilize variances. Statistically standardized distributions were also plot.

ER/PR/HER2 z-scores were assigned and categorized for univariate investigations: Group 1, ≥1.0 standard deviation (SD) below mean; Group 2: <1.0 SD below mean; Group 3, ≤1.0 SD above mean; and Group 4, >1.0 SD above mean. The log-rank test statistics examined univariate differences in relapse-free survival (RFS) between these groups. Kaplan–Meier plots are shown for graphical depiction.

Continuous SS biomarkers were utilized in multivariate investigations of the effects of biomarkers on RFS. Exploratory stratified and unstratified step-wise forward Cox regressions were performed, where baseline patient and tumor characteristics were added if two-sided p was ≤0.05, with a likelihood ratio criterion test statistic ($\sim x^2_{(1)}$). Unstratified Cox regression assessed the effect of locally determined hormone receptor status. Sensitivity analyses examined an external HER2+ cut-point of 1.32. This cut-point was determined from previous Breast Cancer Index (BCI) investigations with the MA.17 trial as the cut-point that produced the greatest accuracy (97 % concordance) in HER2 status between RT-PCR and centrally assessed IHC/FISH test results [7].

Results

Of the 667 MA.14 patients, 299 patients had banked tumor tissue (Fig. 1). We did not find significant imbalances by treatment arm or by stratification factors in who was, or was not: assessable for RT-PCR ER, PR, and HER2 by lymph node status ($p = 0.90$); hormone receptor status ($p = 0.19$); adjuvant chemotherapy ($p = 0.90$); and tumor size ($p = 0.18$). Median follow-up of patients by trial arm was 10.01 years on TAM and 10.12 years on TAM-OCT, compared with the 9.8 years in the overall trial.

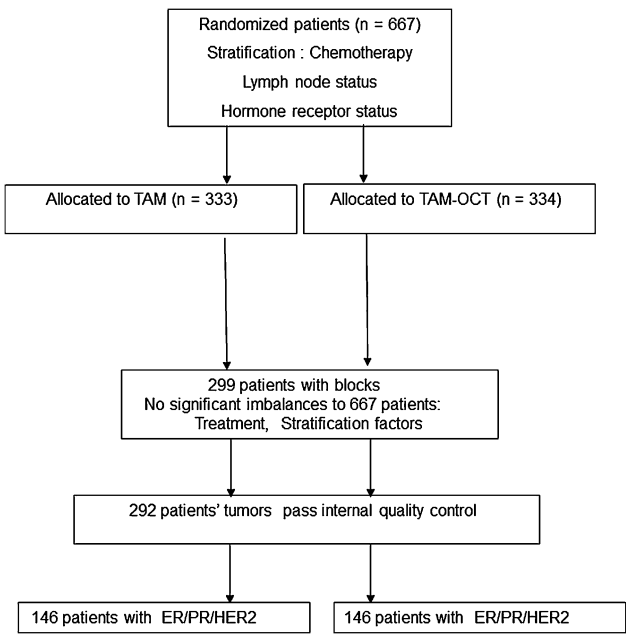

Fig. 1 CONSORT diagram

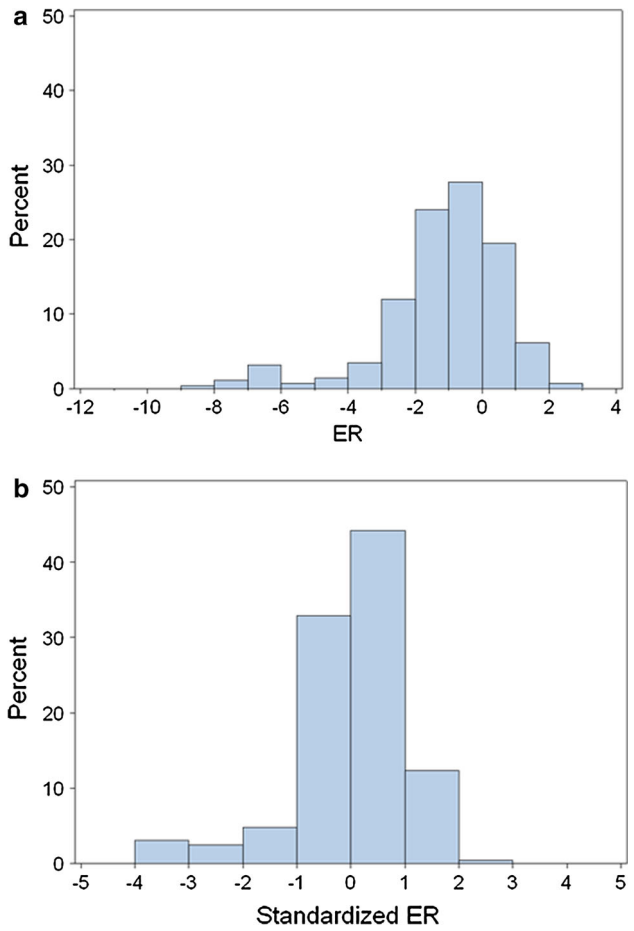

Fig. 2 Histogram of RT-ER, **a** laboratory values, **b** *z*-scores

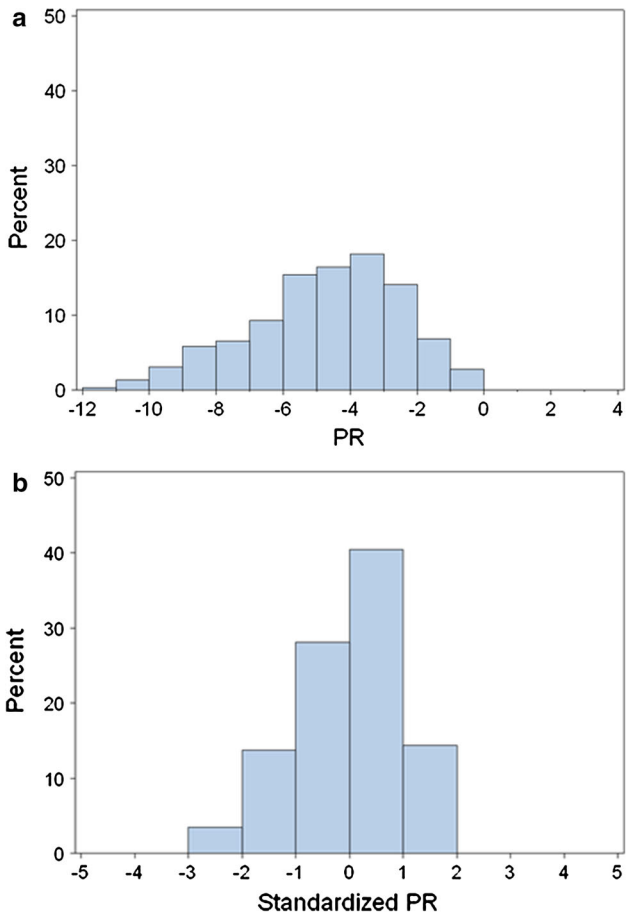

Fig. 3 Histogram of RT-PR, **a** laboratory values, **b** *z*-scores

From the 299 patients with tumor blocks, 292 samples passed internal quality control. The RT-PCR histograms for patient ER (Fig. 2a), PR (Fig. 3a), and HER2 (Fig. 4a) suggested unimodal continuous distributions which under SS were centered at a *z*-score of 0: ER (Fig. 2b), PR (Fig. 3b), and HER2 (Fig. 4b).

Patient baseline characteristics are shown in Table 1. Each arm had 146 patients assessed, and they were similar in the two arms. 53 % of the investigative group were at least 60 years of age, 92 % had hormone receptor positive tumors by local determination, 51 % had lymph node-negative disease, and 35 % had received adjuvant chemotherapy.

SS-ER did not univariately impact RFS (Fig. 5a; $p = 0.31$). SS-PR values above the mean ($z \geq 0.0$) had the best univariate RFS (Fig. 5b; $p = 0.03$). SS-HER2 also univariately impacted RFS (Fig. 5c; $p = 0.004$) with lowest (z-scores ≤ -1.0) and highest (z-scores > 1.0) having shortest RFS. Patients with SS-HER2 ≥ 1.32 SD had worse RFS than those <1.32 SD [HR 2.09 (95 % CI 0.99–4.41); $p = 0.05$; Supplemental figure A1, online only].

Multivariate stratified/unstratified Cox models indicated patients with T1 tumors ($p = 0.02/p < 0.001$) and higher SS-PR ($p = 0.02/p = 0.01$) had longer RFS; N-ve patients had better RFS (unstratified $p < 0.001$). Local ER/PR status did not impact RFS ($p > 0.05$). Patients with SS HER2+ ≥ 1.32 SD had worse RFS in multivariate analyses ($p = 0.06$).

Discussion

The ASCO/CAP guidelines for ER, PR, and HER2 increased awareness about uncertainties affecting accurate assessment of these pivotal breast cancer biomarkers and identified procedural elements that can improve laboratory assessments [1–3]. Good quality assurance methods are essential to producing reliable clinical test results. The focus of the guidelines was on the determination of presence or absence of the biomarkers with a view to likely responsiveness to endocrine or anti-HER2 therapies.

We previously showed that biomarker expression level, rather than just an indication of presence or absence of

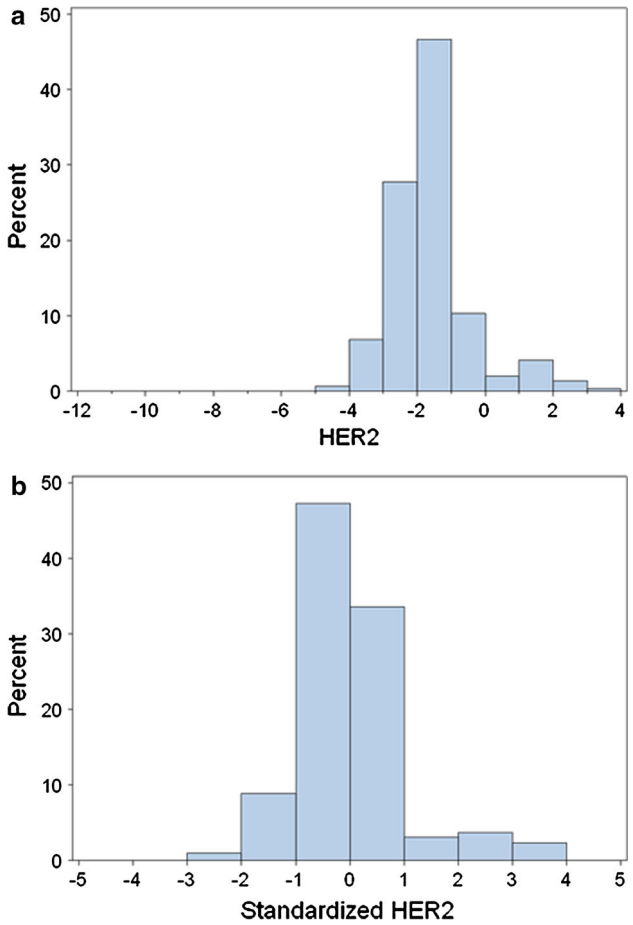

Fig. 4 Histogram of RT-HER2, **a** laboratory values, **b** *z*-scores

biomarkers, can affect clinical outcome [4, 8, 9]. After good laboratory measures, adjunctive SS of continuous biomarker data permits robust examination of whether biomarker expression level affects outcome, with a potential application being informed decision making about alternative therapeutic strategies based on clinical outcome [4]. Additionally, biomarkers need to be standardized across different assessment platforms for inter-laboratory comparability of clinical test results. Gene-based assessment methodologies generally have proprietary applications that impede direct cross platform comparisons. Results expressed by SS would overcome this proprietary obstacle.

We showed in the premenopausal placebo-controlled tamoxifen trial, NCIC CTG MA.12, that SS hormone receptors assayed by qPCR or by IHC had similar multivariate prognostic effects; the MA.12 tumors were by local determination both hormone receptor positive and negative [4]. We investigated here the continuous prognostic effects of SS for centrally assessed RT-PCR ER, PR, and HER2 in the postmenopausal NCIC CTG MA.14 trial where patients in both trial arms were given tamoxifen.

Table 1 Baseline patient characteristics

	Tamoxifen *N* (%)	Tamoxifen + octreotide LAR *N* (%)	Total *N* (%)
Total	146 (100)	146 (100)	292 (100)
Age at allocation			
Median age (years)	60.4	61.0	60.6
0: age < 60	71 (49)	65 (45)	136 (47)
1: age ≥ 60	75 (51)	81 (55)	156 (53)
Race			
0: Caucasian	142 (97)	137 (94)	279 (96)
1: Not Caucasian	4 (3)	9 (6)	13 (4)
Performance status (ECOG)			
0: 0, unknown	106 (73)	118 (81)	224 (77)
1: 1, 2	40 (27)	28 (19)	68 (23)
T pathologic classification			
0: 0, 1	87 (60)	91 (62)	178 (61)
1: 2, 3A, 4, unknown	59 (40)	55 (38)	114 (39)
N pathologic classification			
0: 0	74 (51)	75 (51)	149 (51)
1: 1, 2, unknown	72 (49)	71 (49)	143 (49)
Breast surgery type			
0: total mastectomy	50 (34)	58 (40)	108 (37)
1: other			
Segmental mastectomy	96 (66)	88 (60)	184 (63)
Number of positive axillary nodes			
0: 0	74 (51)	75 (51)	149 (51)
1: 1–3, 4+, unknown	72 (49)	71 (49)	143 (49)
Estrogen/progesterone receptor status			
0: negative, unknown	14 (10)	10 (7)	24 (8)
1: positive	132 (90)	136 (93)	268 (92)
Adjuvant chemotherapy			
0: none	96 (66)	94 (64)	190 (65)
1: concurrent, sequential	50 (34)	52 (36)	102 (35)

We hypothesized that hormone receptor level would impact RFS in this tamoxifen-treated postmenopausal population. Locally determined ER and PR status were not associated with RFS although it is recognized that 92 % of the patients in this investigation had locally determined ER and/or PR tumor positivity. In MA.14, there was no indication that SS-ER level affected RFS since it was not associated with RFS in either univariate or multivariate analyses. However, patients with higher SS-PR, in particular those with SS-PR above the mean, had significantly better univariate RFS ($p = 0.03$). Further, higher continuous SS-PR had better RFS in stratified ($p = 0.02$) and unstratified ($p = 0.01$) multivariate analyses. The observation of ER

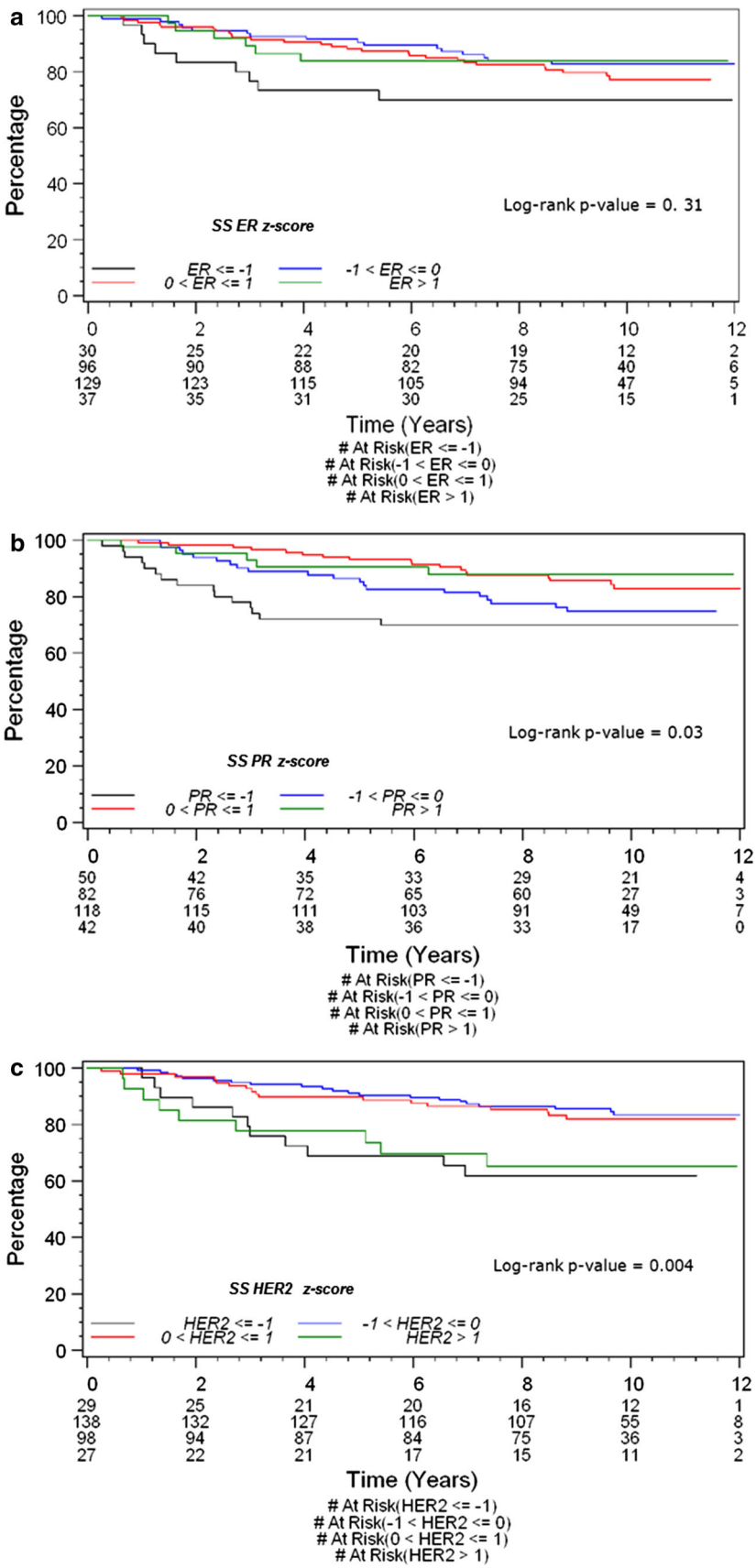

Fig. 5 **a** The univariate effects of categorized statistically standardized (SS) ER z-scores on RFS are compared relative to lowest z-scores, ≤ -1, i.e., relative to SS-ER ≥ 1 standard deviation (SD) below the mean: ER > 1 (>1 SD above mean) compared to ER ≤ -1 (≥ 1 SD below mean), hazard ratio (HR) of 0.49 (95 % CI 0.17–1.37); $0 <$ ER ≤ 1 (≤ 1 SD above mean) compared to ER ≤ -1 (≥ 1 SD below mean): HR of 0.61 (95 % CI 0.29–1.31); $-1 <$ ER ≤ 0 (<1 SD below mean) compared to ER ≤ -1 (≥ 1 SD below mean): HR of 0.48 (95 % CI 0.21–1.08). **b** The univariate effects of categorized statistically standardized (SS) PR z-scores on RFS are compared relative to lowest z-scores, ≤ -1, i.e., relative to SS-PR ≥ 1 standard deviation (SD) below the mean: PR > 1 (>1 SD above mean) compared to PR ≤ -1 (≥ 1.0 SD below mean): hazard ratio (HR) of 0.33 (95 % CI 0.12–0.90); $0 <$ PR ≤ 1 (≤ 1 SD above mean) compared to PR ≤ -1 (≥ 1 SD below mean): HR of 0.42 (95 % CI 0.21–0.83); $-1 <$ PR ≤ 0 (<1 SD below mean) compared to PR ≤ -1 (≥ 1 SD below mean): HR of 0.70 (95 % CI 0.36–1.37). **c** The univariate effects of categorized statistically standardized (SS) HER2 z-scores on RFS are compared relative to lowest z-scores, ≤ -1, i.e., relative to SS HER2 ≥ 1 standard deviation (SD) below the mean: HER2 > 1 (>1 SD above mean) compared to HER2 ≤ -1 (≥ 1.0 SD below mean): hazard ratio (HR) of 0.90 (95 % CI 0.37–2.16); $0 <$ HER2 ≤ 1 (≤ 1 SD above mean) compared to HER2 ≤ -1 (≥ 1 SD below mean): HR of 0.39 (95 % CI 0.18–0.84); $-1 <$ HER2 ≤ 0 (<1 SD below mean) compared to HER2 ≤ -1 (≥ 1 SD below mean): HR of 0.34 (95 % CI 0.16–0.70)

and/or PR being significant in different studies has been observed previously in the literature [8, 10].

Patients with SS-HER2 categorized by SDs above and below the mean exhibited significantly different univariate RFS ($p = 0.004$). Patients with SS-HER2 within 1 SD of the mean, i.e., (Groups 2 and 3: -1.0 SD $<$ SS-HER2 $\leq$ 1.0 SD) had better RFS than those with lowest SS-HER2 (Group 1: HER2 $\geq$ 1 SD below the mean). The similar univariate RFS experience seen by the lowest SS-HER2 (Group 1) and the highest SS-HER2 (Group 4) may reflect that in the absence of anti-HER2 therapy, high expression of HER2 may sensitize tumor cells to therapy. We recognize that the number of patients in Group 1 ($N = 29$) and Group 4 ($N = 27$) are limited. However, in the N9831 trial of adjuvant chemotherapy with and without trastuzumab, the benefit from anti-HER2 therapy was greatest for both the lowest (RT-PCR negative) and highest (RT-PCR positive) centrally reviewed RT-PCR HER2 expression levels; there was no significant benefit for patients with RT-PCR equivocal tumors [11]. Further, patients with normal gene copy numbers in the NSABP B-31 trial also derived significant benefit from trastuzumab [12]. Here, the external cut-point at 1.32 SS-HER2 led to delineation of patients with better RFS (SS-HER2 $<$ 1.32 SD), and multivariate association of SS-HER2 with RFS ($p = 0.06$).

Limitations of our study include MA.14 had relatively few postmenopausal patients whose tumors were assessed by RT-PCR ($N = 292$), although they adequately represented the full trial population of 667 patients; this may limit the applicability to larger series of patients treated with current adjuvant chemotherapy and aromatase inhibitor adjuvant treatment. We note, though, that both MA.14 and our much larger aromatase inhibitor trial MA.27 ($N = 7576$) had a similar proportion of about 30 percent of patients administered adjuvant chemotherapy [5, 13]. Also, the median 10-year follow-up with these MA.14 patients robustly reports experience for the particular patients investigated. Another limitation is that the MA.14 trial pre-dated the introduction of anti-HER2 therapy. Further, the small number of patients in this investigation precludes more detailed analyses to specifically examine here the continuous results for ER and PR with outcome separately for patients with hormone receptor positive/HER2- and triple negative disease. We wait for the results of such analyses from others.

Clinical trials have tended to stratify hormone receptor status and HER2 status as positive or negative, frequently on local determinations. The current movement toward routine central laboratory assessment of these pivotal biomarkers in completed breast cancer trials raises the prospect that outcome could be quantified as a function of SS-ER, SS-PR, and SS-HER2. Evaluation of, and observation that, continuous ER, PR, and HER2 are associated with outcome would motivate going beyond the simple categorization of these biomarkers as present or absent. SS holds the potential of robust assessment both within and between assessment platforms for better reporting and eventually clinical application. Evaluation in other trials may provide support for this methodology.

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Compliance with ethical standards

Conflicts of Interest YZ and CAS were paid and have stock ownership in bioTheranostics, Inc.; ME had a consultant/advisory role in bioTheranostics, Inc.; TB-C has been paid by Roche. No other authors have conflicts to disclose.

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