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ORIGINAL REPORT

Estrogen Receptor (ER) mRNA and ER-Related Gene Expression in Breast Cancers That Are 1% to 10% ER-Positive by Immunohistochemistry

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ABSTRACT

Purpose
We examined borderline estrogen receptor (ER) –positive cancers, defined as having 1% to 10% positivity by immunohistochemistry (IHC), to determine whether they show the same global gene-expression pattern and high *ESR1* mRNA expression as ER-positive cancers or if they are more similar to ER-negative cancers.

Patients and Methods
ER status was determined by IHC in 465 primary breast cancers and with the Affymetrix U133A gene chip. We compared expressions of *ESR1* mRNA and a 106 probe set ER-associated gene signature score between ER-negative (n = 183), 1% to 9% (n = 25), 10% (n = 6), and more than 10% (n = 251) ER-positive cancers. We also assessed the molecular class by using the PAM50 classifier and plotted survival by ER status.

Results
Among the 1% to 9%, 10%, and more than 10% ER IHC–positive patients, 24%, 67%, and 92% were also positive by *ESR1* mRNA expression. The average *ESR1* expression was significantly higher in the ≥ 10% ER-positive cohorts compared with the 1% to 9% or ER-negative cohort. The average ER gene signature scores were similar for the ER-negative and 1% to 9% IHC-positive patients and were significantly lower than in ≥ 10% ER-positive patients. Among the 1% to 9% ER-positive patients, 8% were luminal B and 48% were basal-like; among the 10% ER-positive patients, 50% were luminal. The overall survival rate of 1% to 9% ER-positive patients with cancer was between those of patients in the ≥ 10% ER-positive and ER-negative groups.

Conclusion
A minority of the 1% to 9% IHC ER–positive tumors show molecular features similar to those of ER-positive, potentially endocrine-sensitive tumors, whereas most show ER-negative, basal-like molecular characteristics. The safest clinical approach may be to use both adjuvant endocrine therapy and chemotherapy in this rare subset of patients.

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INTRODUCTION

How to define estrogen receptor (ER) positivity remains a controversial topic. Recent joint guidelines by the American Society of Clinical Oncology (ASCO) and the American College of Pathologists recommended that ER status should be considered positive if 1% or more of tumor cells demonstrate positive nuclear staining with an immunohistochemistry (IHC) assay.[1] Historically, many investigators and clinicians considered 10% or greater nuclear staining as the threshold for defining ER-positive status and therefore eligibility for endocrine therapy.[2-8] The optimal threshold would ideally be clinically validated against patient outcome in prospectively conducted clinical trials or established from and independently validated in systematically collected archived specimens from randomized clinical trials.[9] No such optimal threshold exists for ER. The ≥ 1% threshold originates from an IHC study that was performed on tissue sections prepared from tumor specimens that were left over after performing an ER ligand-binding assay and were pulverized under ultracold temperatures.[10] This study showed statistically significantly better survival for patients

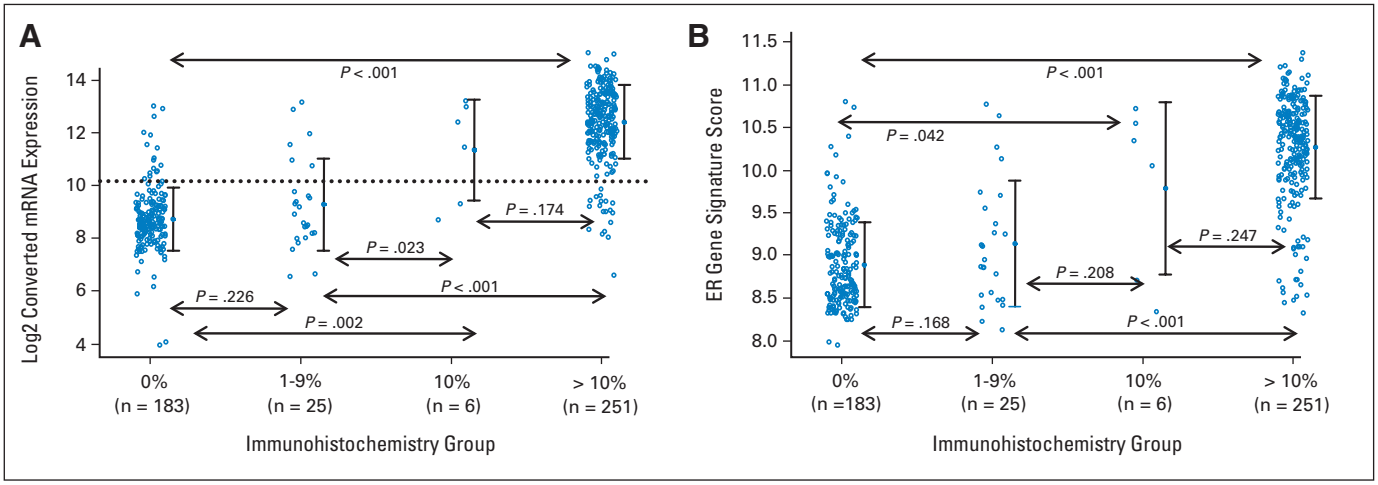

Fig 1. Estrogen receptor (ER) mRNA and ER-associated gene expression in four distinct immunohistochemistry groups. Immunohistochemistry groups were defined by the percentage of cells that were positive for nuclear ER staining. (A) Expression distribution of *ESR1* mRNA. (B) ER-associated gene signature refers to the average expression of 106 probe sets that are highly coexpressed with *ESR1*.[13] *P* values were calculated with the Wilcoxon test.

with ≥ 1% ER-positive cells compared with patients who had completely negative cells, and survival also increased incrementally as the extent of ER positivity increased. Worse survival around the lower limits of the detection threshold of the ER IHC method may reflect biologic differences in these cancers or it could be due to misclassification of ER-negative cancers as borderline weakly ER positive.

The contribution of progesterone receptor expression to endocrine treatment sensitivity and to the definition of potentially hormone-sensitive cancers also remains an important topic of research and controversy but is beyond the scope of this analysis.

ER-positive and ER-negative cancers have large-scale gene-expression differences that extend far beyond variable expression of the ER gene (*ESR1*) itself. A large number of genes are coexpressed with *ESR1*, and they define ER-positive status in a robust manner.[11,12] The aggregate expression of ER-associated genes is also predictive of benefit from adjuvant endocrine therapy.[13] ER protein expression detected by IHC also correlates closely with *ESR1* mRNA levels that provide a more quantitative measure of ER expression.[14,15] These RNA expression-based techniques provide alternative methods for assessing ER status and gauging endocrine sensitivity.

In this study, we examined the expression of *ESR1* mRNA and an ER-associated gene signature from a study by Symmans et al[13] in patients whose tumors showed 1% to 10% ER positivity by IHC and compared these expression values with those seen in clearly ER-negative (0%) and ER-positive (> 10%) tumors. We also assessed the intrinsic molecular class of the borderline ER-positive patients by using the PAM50 classifier.[16] The purpose of these analyses was to determine whether borderline ER-positive cancers show the same global gene-expression patterns and high *ESR1* mRNA expression as ER-positive cancers do or if they are more similar to ER-negative cancers.

PATIENTS AND METHODS

Four hundred sixty-five fine-needle aspiration specimens of newly diagnosed stage I to III breast cancer were studied. These specimens were collected under a prospective biomarker discovery study approved by the institutional review board at The University of Texas MD Anderson Cancer Center (MDACC); biomarker results were reported in a previous series of publications.[11,13,15,17-19] Clinical ER status was determined by IHC using the monoclonal antibody 6F11 (Novocastra/Vector Laboratories, Burlingame, CA) on formalin-fixed paraffin-embedded core biopsies that were obtained to establish the diagnosis of invasive cancer. IHC and visual scoring were performed as part of the routine diagnostic assessment in the clinical pathology laboratory at MDACC.

Gene-expression profiling was performed on the research fine-needle aspiration biopsy samples by using the Affymetrix HG-U133A gene chips (Affymetrix, Santa Clara, CA).[11,13,15,17-19] Expression data were normalized with the MAS5 algorithm, mean centered to 600 and log2 transformed. Probe set 205225_at was used as the measure of *ESR1* mRNA

Table 1. ER IHC Distribution by *ESR1* mRNA Expression and SET Index

ER IHC		*ESR1* mRNA Expression				SET Index					
		Positive		Negative		High		Intermediate		Low	
IHC Level (%)	No. of Patients	No.	%	No.	%	No.	%	No.	%	No.	%
0	183	16	8.7	167	91.3	2	1.1	2	1.1	179	97.8
1-9	25	6	24.0	19	76.0	0		0		25	100
10	6	4	66.7	2	33.3	0		1	16.7	5	83.3
> 10	251	232	92.4	19	7.6	12	4.8	27	10.8	212	84.5

Abbreviations: ER, estrogen receptor; IHC, immunohistochemistry; SET, sensitivity to endocrine therapy.

Table 2. Molecular Class of Borderline ER-Positive Patients

IHC		Molecular Subtypes by PAM50				
IHC Level (%)	No. of Patients	Luminal A	Luminal B	HER2 Amplified	Basal	Normal
0	183	2	1	51	111	18
1-9	25	0	2	8	12	3
10	6	2	1	1	1	1
> 10	251	120	61	38	16	16

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; PAM50, multigene test used to assign molecular class to individual breast cancers.[16]

expression, and a log2-converted value of ≥ 10.18 was considered as ER positive on the basis of a threshold established and validated in two previous publications.[15,20] The identification of the 106 probe sets with strong positive correlation with *ESR1* was described previously.[13] In this analysis, we used the average of the log2-transformed expression values of these probe sets as the measure of *ESR1*-associated gene expression. The Wilcoxon test was used to determine statistical significance of expression differences in *ESR1* mRNA and ER-associated gene signature scores across the IHC groups. Spearman rank correlation coefficient was used to measure correlation between *ESR1* expression and gene signature score. The PAM50 classifier and sensitivity to endocrine therapy (SET) index were used as previously reported.[13,16] Kaplan-Meier survival curves were plotted for 446 patients to illustrate overall survival by ER expression, and a Cox proportional hazards model was used to test the contribution of clinical variables and ER expression to outcome. Eighteen patients were excluded from the survival analysis because they had metastatic disease diagnosed within 1 month of the research biopsy and therefore represented stage IV disease.

RESULTS

Thirty-one patients (6.7%) had 1% to 10% ER-positive status by routine clinical IHC; of these, 25 (5.4%) had 1% to 9% and six (1.3%) had exactly 10% ER-positive staining. Among the 1% to 9% IHC-positive patients, six (24%) of 25 were also ER positive by *ESR1* mRNA expression. Among those with 10% or more than 10% IHC-positive staining, 67% and 92% were positive by mRNA expression, respectively. There was no significant difference in average *ESR1* mRNA expression between the IHC 0% and the 1% to 9% ER-positive cohorts but *ESR1* mRNA expression levels were significantly higher in the 10% and the more than 10% IHC-positive cohorts compared with the completely negative or 1% to 9% ER-positive cohorts (Fig 1). A similar relationship was observed for the ER-associated gene signature score: the average scores were similar for the ER 0% and the 1% to 9% IHC-positive patients, and they were significantly lower than the scores in ≥ 10% ER-positive patients (Fig 1). The SET index assigned low sensitivity to all 1% to 9% ER-positive patients and to 83% of the 10% ER-positive patients (Table 1). Molecular classification also did not assign luminal A status to any of the 1% to 9% ER-positive patients: two (8%) were luminal B, and twelve (48%) were basal-like; the rest fell into other categories (Table 2). Among the 10% ER-positive patients, two (33%) were luminal A, one (17%) was luminal B, and the rest were other subtypes (Table 2).

Figure 2 illustrates the relationship between ER protein expression by IHC, *ESR1* mRNA level, and ER-associated gene signature. The majority of the 1% to 9% ER-positive patients (76%) showed low *ESR1* mRNA expression and low ER-associated gene signature score and were assigned ER-negative status by these metrics. In contrast, the majority of the 10% ER-positive patients showed *ESR1* mRNA or ER-associated gene signature scores that were consistent with ER-positive status. It should be noted that 16 additional patients who were 0% by IHC (8.7% of all ER-negative patients) also showed *ESR1* mRNA expression and *ESR1*-associated gene signature scores that were consistent with ER-positive status and could be considered false negatives by IHC (Table 2).

Kaplan-Meier survival analysis showed significantly better overall survival for patients who were ≥ 10% ER-positive compared with ER-negative patients; the 1% to 9% ER-positive patients had intermediate survival outcome (Table 3 and Fig 3). Better overall survival was also associated with *ESR1* mRNA expression levels, and in stepwise multivariate analysis, tumor size and *ESR1* expression level remained statistically significant. Since the use of adjuvant endocrine therapy was closely associated with ER status, this variable was not selected for the final model.

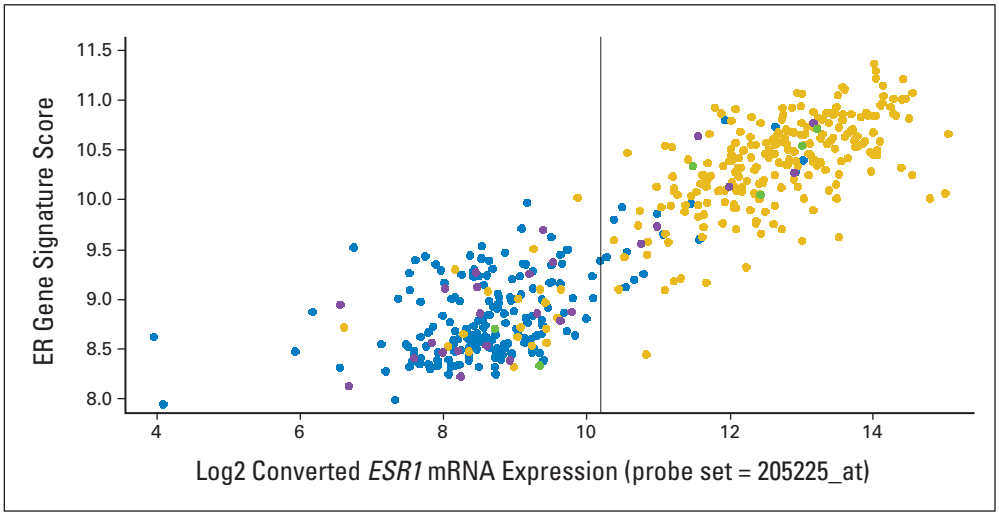

Fig 2. Scatter plot showing the relationship between estrogen receptor (ER) immunohistochemistry status, *ESR1* mRNA expression, and ER-associated gene signature expression (n = 465). ER-associated gene signature refers to the average expression of 106 genes that are highly coexpressed with *ESR1*.[13] *ESR1* positivity was defined as log2-converted *ESR1* mRNA expression value ≥ 10.18. Spearman's correlation coefficients (ρ) between *ESR1* expression and gene signature scores were calculated separately for the *ESR1*-negative and *ESR1*-positive patients. ER immunohistochemistry categories are color coded; blue, 0%; green, 1% to 9%; purple, 10%; gold, greater than 10%.

Table 3. Characteristics of Patients Included in Survival Analysis (n = 446)

Characteristic	ER IHC Level (%) 0 No.	0 %	1-9 No.	1-9 %	≥ 10 No.	≥ 10 %	*P* (0% *v* 1%-9%)	*P* (≥ 10% *v* 1%-9%)
No. of patients	179	40.1	24	5.4	243	54.5	—	—
Tumor size at diagnosis (TNM)							.513	1.000
T1/T2	105	23.5	16	3.6	164	36.8		
T3/T4	74	16.6	8	1.8	79	17.7		
Lymph node							.812	.273
Positive	126	28.3	18	4.0	153	34.3		
Negative	53	11.9	6	1.3	90	20.2		
ER mRNA level							.029	< .001
Positive	16	3.6	6	1.3	222	49.8		
Negative	163	36.5	18	4.0	21	4.7		
HER2 status							.645	.040
Amplified*	59	13.2	8	1.8	37	8.3		
Normal	119	26.7	16	3.6	206	46.2		
N/A	1	0.2	0	0.0	0	0.0		
Modified Black's nuclear grade							1.000	< .001
1	1	0.2	0	0.0	21	4.7		
2	27	6.1	3	0.7	135	30.3		
3	150	33.6	21	4.7	87	19.5		
N/A	1	0.2	0	0.0	0	0.0		
Adjuvant chemotherapy							.795	.541
Paclitaxel-FAC	164	36.8	22	4.9	212	47.5		
FAC	11	2.5	2	0.4	16	3.6		
None	4	0.9	0	0.0	15	3.4		
Adjuvant endocrine therapy							< .001	< .001
Yes	0	0.0	4	0.9	243	54.5		
None	179	40.1	20	4.5	0	0.0		
Adjuvant HER2-targeted therapy among patients with HER2-amplified status							.453	.121
Yes	33	31.7	6	5.8	15	14.4		
None	26	25.0	2	1.9	22	21.2		

NOTE. *P* was calculated by Fisher's exact test.
Abbreviations: ER, estrogen receptor; FAC, fluorouracil, doxorubicin, cyclophosphamide; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; N/A, not available.
*HER2-amplified status, IHC 3+, or > 2.0 by fluorescent in situ hybridization.

DISCUSSION

Borderline ER-positive status defined as 1% to 9% ER positivity by IHC is rare. This study and previous reports indicate that approximately 6% of all breast cancers fall into this category.[21] Because of the rarity of borderline ER-positive status, it is unlikely that a prospective clinical trial would ever be conducted to define the optimal treatment strategy for this disease subset. Low ER-positive IHC status may arise from testing artifacts, including borderline false-positive IHC results in an ER-negative tumor or erroneously low ER results in a truly ER-positive tumor. It may also indicate the presence of a small ER-positive subpopulation of cells within a larger ER-negative tumor. mRNA-based methods to assess ER status may help resolve some of the uncertainties.[14,22]

We assessed gene-expression profile–based ER status in 465 primary breast cancers to find out how often weak ER-positive (1% to 10% by IHC) patients showed molecular features of ER-positive disease. The majority (67%) of the exactly 10% ER-positive cancers and a minority (24%) of the 1% to 9% ER-positive cancers showed ER-positive status by *ESR1* mRNA expression and by ER-associated gene signature. Ten of the 31 patients with weakly positive status by IHC were clearly ER positive by mRNA-based methods, indicating that in these patients, IHC may have underestimated ER positivity. The interpretation of low *ESR1* mRNA and low ER gene signature expression in weakly ER-positive cancers by IHC is less straightforward. Such a result can be equally attributed either to a false-positive weak ER immunostaining or to the presence of a truly ER-positive small subpopulation of cells within a larger ER-negative tumor. In the latter, mRNA signals from the ER-positive cells could be drowned out by the bulk of the ER-negative tumor cell population. Since these two scenarios cannot be distinguished by current analytic methods, inclusion of these tumors among ER-positive tumors is not unreasonable since some of these tumors may contain at least some potentially endocrine-sensitive tumor cells.[2,6,7,10] Overall, however, the expected benefit from endocrine therapy is small in 1% to 9% IHC-positive tumors because these tumors tend to be ER-negative by *ESR1* mRNA (76%), show low predicted endocrine sensitivity by the SET gene signature (100%), and are predominantly nonluminal class (92%). Considering these molecular characteristics, the most expeditious therapeutic approach may be to use both adjuvant endocrine therapy and chemotherapy in this subset of patients.

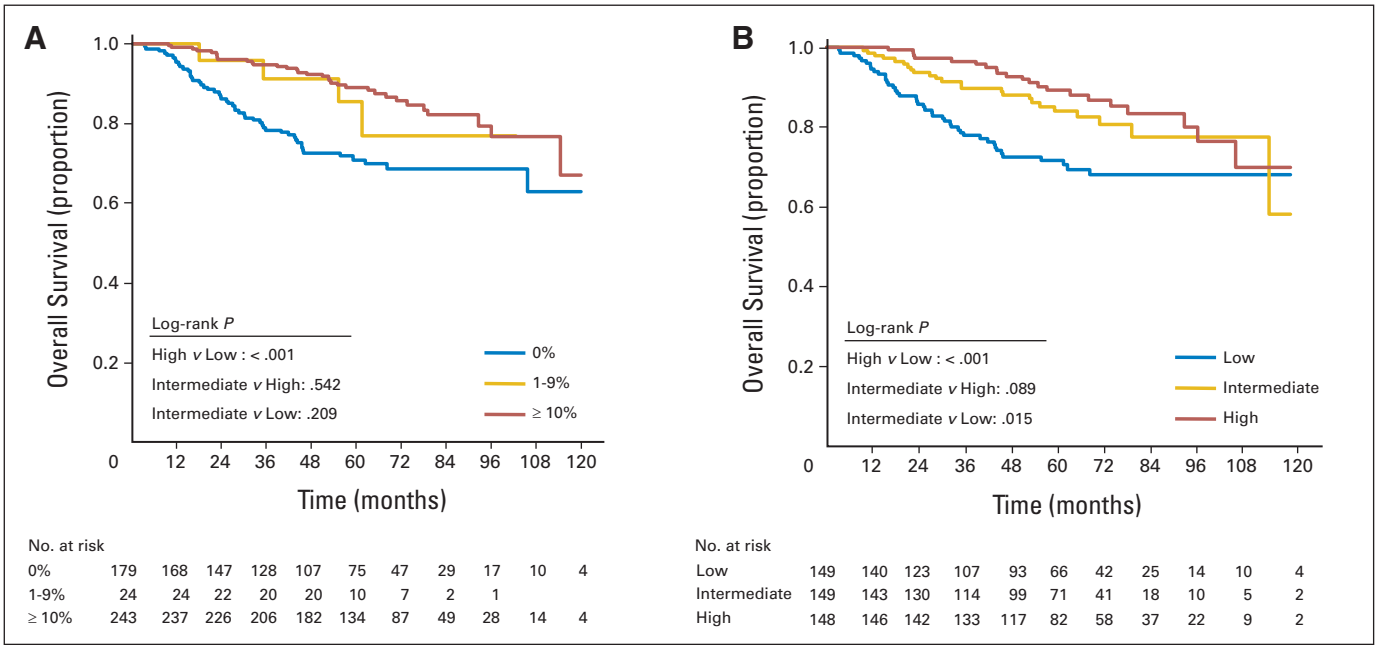

Fig 3. Kaplan-Meier overall survival curves by estrogen receptor immunohistochemistry status and by *ESR1* mRNA expression (n = 446). (A) Immunohistochemistry groups were defined by the percentage of estrogen receptor–positive cells. (B) Cohorts represent tertiles of *ESR1* mRNA expression. Log-rank *P* values for pairwise comparisons are provided.

This study has limitations. The number of borderline ER-positive patients is low, which is inherent in any analysis because of the rarity of this entity. Nevertheless, this study is the largest in the literature and has the advantages of using centrally reviewed IHC results and a uniformly performed gene-expression analysis that yielded generally consistent results for four different RNA-based methods to assess ER status and endocrine sensitivity. The uneven sample sizes for the ER IHC cohorts, various adjuvant therapies, and different TNM stages across cohorts limit the power of survival analysis. Nevertheless, we could show the expected significant survival difference between ER-positive and ER-negative patients, and ER mRNA expression was one of the most predictive variables for survival in multivariate analysis. The survival outcome of the borderline patients fell between the strongly ER-positive and ER-negative groups, which is consistent with the molecular observations.

Overall, about one quarter of the 1% to 9% ER-positive tumors show *ESR1* mRNA levels and gene signatures that are consistent with ER-positive, potentially endocrine-sensitive tumors. A second RNA-based ER assessment may help identify these *ESR1* mRNA-positive IHC borderline patients. There is likely to be a small, if any, clinical benefit from endocrine therapy among the remaining IHC borderline patients with low *ESR1* mRNA. To truly establish the outcome of borderline ER-positive patients after stratification by mRNA-based ER status would require a large study to first identify the 6% with borderline IHC results and then to have sufficient numbers of such patients to compare the outcome by *ESR1* mRNA expression. Such data are unlikely to be generated in the near future. In the meantime, since potentially life-saving benefit from empirical adjuvant endocrine therapy cannot be completely excluded, inclusion of this small subpopulation among the ER-positive cancers is not unreasonable. However, the safest clinical approach may be to use both adjuvant endocrine therapy and chemotherapy in this rare subset of patients.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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