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ARTICLE

E-cadherin clone 36 nuclear staining dictates adverse disease outcome in lobular breast cancer patients

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Abstract

Breast cancer is a heterogeneous disease and additional biomarkers for individually predicting patient outcomes are needed. Aberrant membrane E-cadherin immunoexpression has been demonstrated in lobular breast cancer. Also, E-cadherin nuclear staining has been reported, associating with prognosis in various tumors. Here, we explore whether membrane or nuclear staining of E-cadherin has the potential to dictate prognosis of patients with lobular breast cancer. We selected a cohort of 285 consecutively diagnosed lobular breast cancer patients and performed immunohistochemistry for E-cadherin (clones 36, EP700Y, and NCH38) and P-cadherin (clone 56C1) in representative formalin-fixed paraffin-embedded blocks. All patients were female, HER2-negative and surgically treated in a single institution. Survival curves were computed by Kaplan-Meier analysis. Hazard ratios and respective 95% confidence intervals were estimated using Cox regression models. Statistical significance was set at $p < 0.05$. Nuclear staining for E-cadherin clone 36 was frequent (35%), contrarily to other antibodies tested. Negative correlation was found between nuclear and membrane E-cadherin clone 36 immunostaining ($r_s = -0.30$, $p < 0.001$), whereas positive correlation was found between membrane immunoexpression of E-cadherin clone 36 and P-cadherin ($r_s = 0.31$, $p < 0.001$). Patients with any evidence of E-cadherin clone 36 nuclear immunostaining disclosed significantly worse overall survival, disease-specific-survival and disease/progression-free survival (hazard ratio = 2.059, 95% confidence interval 1.313–3.230; hazard ratio = 1.980, 95% confidence interval 1.121–3.495; and hazard ratio = 2.341, 95% confidence interval 1.403–3.905, respectively). Differences in survival were more remarkable when considering nuclear E-cadherin immunoexpression in ≥50% tumor cells. Poorer survival was maintained in multivariable analysis, after adjusting for age, menopausal and PR status, treatment course, vascular invasion, tumor grade and stage. Our results support the use of antibodies against the cytoplasmic domain of E-cadherin, such as clone 36, which may reveal nuclear immunostaining and indicate more aggressive clinical course in patients with lobular breast cancer. We hypothesize that E-cadherin is cleaved and translocated to nucleus functioning as transcription factor.

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Introduction

Breast cancer patients are categorized into prognostic subgroups (Luminal A-like, Luminal B-like, HER2-positive-like, and triple-negative), based on the expression of classical biomarkers (ER, PR, HER2, and Ki67), which may be assessed by immunohistochemistry, as surrogate for gene expression profiles [1, 2]. Nevertheless, in such heterogeneous disease, there is an unmet need for novel biomarkers that may better predict outcome for each individual patient [3].

The cadherins superfamily includes various transmembrane glycoproteins that play major roles in cell adhesion, proliferation, invasion, migration and differentiation [4, 5]; hence, it is not surprising that they have been found to be deregulated in innumerous malignancies [6–10]. E-cadherin is considered the paradigmatic classical epithelial cadherin and its downregulation is associated with the phenomenon of epithelial-to-mesenchymal transition [11]. In breast cancer, its loss typically occurs in lobular breast carcinoma [12]. P-cadherin is another member of the family; its intracellular portion shares 83% homology with E-cadherin, the two differing mostly in the extracellular portion. It has not been shown useful in discriminating among ductal and lobular breast cancer and its cancer-related function is still a matter of debate, as it might be context-dependent [13]; however, it was reported to be overexpressed in breast cancer with poor prognosis [14, 15].

E-cadherin has been demonstrated to be cleaved and its cytoplasmic domain to be translocated to the nucleus, where it acts as a transcription factor [16, 17]; importantly, this finding has been associated with poor prognostic features in various tumor models [18–20]. Interestingly, E-cadherin nuclear staining has been reported in 21% of fine needle aspirate cytology smears from breast cancer patients [21], and up to 17% of lobular breast cancers have been shown to have perinuclear E-cadherin staining with antibodies that target the extracellular region of E-cadherin [22, 23]. Indeed, differences in staining patterns for the same biomarker are usually due to the types of antibodies used, which may recognize different epitopes [24]. Ventana E-cadherin clone 36 recognizes a region located in the cytoplasmic domain of E-cadherin, whereas other clones, such as EP700Y and NCH38, recognize its extracellular portion. It was demonstrated that clone 36 stains not only the cell membrane, but also the nucleus [18, 19]. This finding has been described in quality reports from European quality scheme organization NordiQC: in NordiQC Run B16 in 2013, nuclear staining of lobular breast cancer with E-cadherin clone 36 resulted in the majority of participants receiving a borderline score [25].

Thus, we sought to determine whether E-cadherin nuclear staining (clone 36) is associated with any change in clinical outcome, namely with patients' survival, in lobular breast cancer. Secondly, we aimed to: compare the frequency of nuclear staining with E-cadherin clones 36, EP700Y, and NCH38, exploring the hypothesis that only clone 36 will stain nuclei in lobular breast cancer; determine the frequency of membrane staining with E-cadherin clone 36 in lobular breast cancer, and compare it with the nuclear staining; evaluate whether P-cadherin shows nuclear staining in lobular breast cancer; and compare E-cadherin and P-cadherin clone staining patterns in lobular breast cancer.

Materials and methods

Patients and samples

Retrospectively, all lobular breast cancer patients were queried from the department of pathology database. From these, only HER2-negative, female patients with at least 5 years of follow-up at the beginning of this study were selected. Consultation cases, cases without enough tumor cells available, and samples not corresponding to breast surgical resections (biopsies, cytology specimens, metastatic samples, etc.) were excluded. In addition, mixed-type carcinomas with lobular components were also disregarded and only pure lobular breast cancers were included in the study. Also, 16 patients receiving neoadjuvant treatment were excluded. Thus, a cohort of 285 consecutively diagnosed lobular breast carcinomas (1996–2011) with available material for analysis was selected for this study. All patients underwent surgery at our institution and were managed by the same multidisciplinary team from the breast cancer clinic.

Clinical charts of each patient were reviewed by a medical oncologist (blinded to the immunoexpression results), in light of the most recent classification / staging systems (American Join Committee on Cancer—AJCC) [26]. Variables collected included: age at diagnosis; menopausal status; tumor grade, multifocality, presence of lympho-vascular invasion and stage; therapies employed (surgery, chemotherapy, radiotherapy, endocrine therapy); and dates of birth, diagnosis, disease-progression/recurrence, death and last follow-up. Follow-up was last updated on May 2018.

This study was approved by the institutional ethics committee of Portuguese Oncology Institute Porto (Comissão de Ética para a Saúde do IPO Porto). Because the study was based on retrospective analysis of archival material, it was exempted from informed consent.

Immunohistochemistry

For each case, the most representative formalin-fixed paraffin-embedded tissue block was chosen and 4μm-thick sections were cut for immunohistochemistry and mounted (all in the same direction) on positively charged glass microscope slides.

Immunohistochemistry was performed using a fully automated BenchMark® ULTRA slide processing system (Ventana, Tucson, AZ) using *ultra*View Universal DAB Detection Kit (760–500). The primary antibodies were: anti-human E-cadherin mouse monoclonal antibody (clone 36, Ventana, ready to use), anti-human E-cadherin rabbit monoclonal antibody (clone EP700Y, Ventana, ready to use); anti-human E-cadherin mouse monoclonal antibody (NCH-38, Dako, Denmark, 1:50 dilution); and anti-human P-cadherin mouse monoclonal antibody (clone 56, BD Biosciences, San Diego, CA, 1:50 dilution). Antigen retrieval was performed with Ultra CC2 (950–223, Ventana) for clone NCH38 and for the remainder of the antibodies with Ultra CC1 (950–224, Ventana).

Appropriate negative (normal thymus) and positive (ductal carcinoma of the breast) controls were used (one sample per run). In addition, one lobular breast carcinoma tissue slide stained with mouse monoclonal negative control antibody and another stained with rabbit monoclonal negative control Ig were added (one sample per run).

Immunostaining was assessed by two independent pathologists, blinded to clinicopathological data. Cases of conflict in assessment were reviewed and a consensus score was annotated. For each of the four antibodies, the percentage of cells with positive staining was estimated, independently, in the membrane (full membrane staining), nucleus, and cytoplasm, including perinuclear/dot-like staining, using 10%-increment intervals.

Statistical analysis

Data was tabulated using Microsoft Excel 2016 and analyzed using IBM SPSS Statistics version 24. Percentages were calculated based on the number of cases with available data. Associations between categorical variables were assessed using chi-square (with continuity correction) and Fischer's exact test, as appropriate. Distribution of continuous variables among groups was compared using the nonparametric Mann-Whitney U test. Correlations between variables were estimated with Spearman's non-parametric test (r_s). Survival analyses were computed with Kaplan-Meier estimator and log-rank test. Hazard ratios and respective 95% confidence intervals were estimated using Cox regression models. A p value equal or inferior to 0.05 was considered statistically significant.

Results

Cohort characterization and validation

Clinicopathologic features of the study cohort are depicted in Table 1. Most patients were post-menopausal (70%) and lobular breast carcinomas were mainly grade 2 (78%), T1/2 (84%), N0 (55%), and stage I/II (72%). The median follow-up time was 110 months. A total of 48 patients died from disease and 59 patients developed disease recurrence/progression during the follow-up period.

Disease stage allowed for accurate discrimination of patients' outcome: patients with higher stage disease experienced significantly worse overall survival, disease-specific survival and disease/progression-free survival ($p <$ 0.001 for all) (Fig. 1).

Post-menopausal patients, PR-negative patients and those with lymphovascular invasion and higher grade, T stage, N stage and stage (TNM) experienced worse overall survival. The same was depicted for disease-specific survival and disease/progression-free survival, except for menopausal status and grade. Patients undergoing adjuvant chemotherapy experienced worse disease-specific survival and disease/progression-free survival when compared to those receiving only endocrine therapy (with or without radiotherapy) (Table 2).

Immunoexpression of E-cadherins and P-cadherins in lobular breast cancer

The frequency and compartment distribution of immunostaining for E-cadherin and P-cadherin are depicted in Table 3.

Nuclear staining for E-cadherin clone 36 was rather frequent (98/284, 35%), with 18 cases showing staining in ≥50% tumor cells. On the contrary, nuclear staining for the other E-cadherin clones was rarely found (2 cases for clone EY700 and 9 cases for clone NCH38) and occurred very focally (in 1% of tumor cells). P-cadherin nuclear staining occurred solely in one case.

Dot-like immunostaining was especially observed for P-cadherin (in 137/280, 49% cases). Membrane staining for all four cadherin antibodies evaluated was not uncommon (26%, 20%, 13%, and 26% for E-cadherin clones 36, EP700Y, NCH38, and P-cadherin, respectively). There was a strong correlation between membrane immunoexpression of all three E-cadherin clones (clones 36 vs. EP700Y: $r_s =$ 0.74, $p < 0.001$; clones 36 vs. NCH38: $r_s = 0.75$, $p < 0.001$; clones EP700Y vs. NCH38: $r_s = 0.76$, $p < 0.001$). Of the discordant tumors (with clone 36 membrane staining but without EP700Y staining [$n = 26$] or NCH38 staining [$n = 36$]), 19 (73%), and 25 (69%) showed P-cadherin membrane staining, respectively.

A negative correlation was found between nuclear and membrane E-cadherin clone 36 immunostaining ($r_s = -0.30$, $p < 0.001$). Of the 74 patients with membrane immunostaining, 64 (87%) disclosed absent immunoexpression in the nucleus; and considering the 98 patients with nuclear immunostaining, 88 (90%) depicted absent immunoexpression in the membrane ($p < 0.001$) (Table 4).

Table 1 Clinicopathological features of lobular breast cancer patients

Variables	Lobular breast cancer cohort ($n = 285$)
Age [years (median, interquartile range)]	59 (50–69)
Laterality (n, %)	
Right	131 (46)
Left	152 (53)
Bilateral	2 (1)
Menopausal status (n, %)	
Pre-menopause	83/273 (30)
Post-menopause	190/273 (70)
Multifocality (n, %)	
Yes	75/265 (28)
No	190/265 (72)
Lymphovascular invasion (n, %)	
Yes	44 (15)
No	241 (85)
Grade (n, %)	
G1	51 (18)
G2	223 (78)
G3	11 (4)
Molecular subtype (n, %)	
Luminal, HER2-negative	266/271 (98)
Triple-negative	5/271 (2)
T stage (n, %)	
T1	120/280 (43)
T2	115/280 (41)
T3	34/280 (12)
T4	11/280 (4)
N stage (n, %)	
N0	152/278 (55)
N1	66/278 (24)
N2	19/278 (6)
N3	41/278 (15)
Stage, TNM (n, %)	
I	94/281 (34)
II	108/281 (38)
III	69/281 (25)
IV	10/281 (3)
Topography of metastatic events at diagnosis (n, %)	
Non-regional lymph-nodes	1/10 (10)
Bone	5/10 (50)
Bone + Liver	1/10 (10)
Bone marrow	2/10 (20)
Skin	1/10 (10)
Therapies (n, %)	
Adjuvant chemotherapy	140/285 (49)
Radiotherapy	178/285 (63)
Endocrine therapy	263/285 (92)

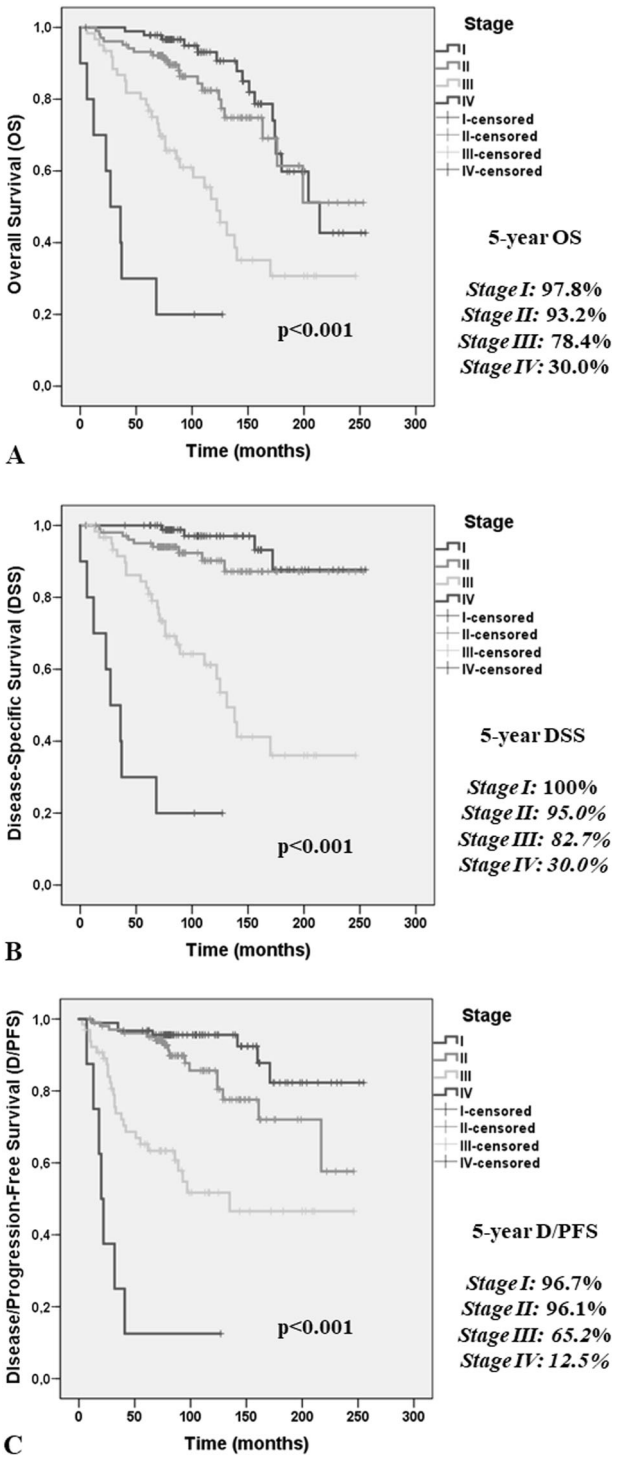

Fig. 1 Overall survival (**a**), disease-specific survival (**b**), and disease/progression-free survival (**c**) of lobular breast cancer patients according to disease stage. p-value refers to overall comparisons (test of equality of survival distributions for the different levels of Stage—log-rank)

On the other hand, a positive correlation was found between membrane immunoexpression of both E-cadherin clone 36 and P-cadherin ($r_s = 0.31$, $p < 0.001$). Of the 72

Table 2 Impact of nuclear E-Cadherin clone 36 immunoexpression in overall survival, disease-specific survival and disease/progression-free survival in univariable and multivariable analyses

Overall survival		
Variables[a]	Univariable	Multivariable (CadE36, adjusted for…)
CadE36 nuclear expression	Hazard ratio = 2.059, 95% confidence interval 1.313–3.230[b]	–
Age	Hazard ratio = 1.068, 95% confidence interval 1.047–1.089[b]	Hazard ratio = 1.557, 95% confidence interval 0.978–2.480
Menopausal status	Hazard ratio = 3.253, 95% confidence interval 1.667–6.348[b]	Hazard ratio = 2.130, 95% confidence interval 1.342–3.379[b]
Grade	G3/G1: hazard ratio = 3.198, 95% confidence interval 1.331–7.683[b]	Hazard ratio = 1.977, 95% confidence interval 1.247–3.136[b]
Lymphovascular invasion	Hazard ratio = 2.040, 95% confidence interval 1.188–3.502[b]	Hazard ratio = 2.051, 95% confidence interval 1.307–3.218[b]
PR (positive vs. negative)	Hazard ratio = 1.980, 95% confidence interval 1.162–3.375[b]	Hazard ratio = 2.099, 95% confidence interval 1.338–3.293[b]
Treatment course (chemotherapy +/− radiotherapy/ endocrine therapy vs. endocrine therapy +/− radiotherapy)	Hazard ratio = 1.036, 95% confidence interval 0.651–1.649	Hazard ratio = 2.164, 95% confidence interval 1.352–3.465[b]
T stage	T2/T1: hazard ratio = 1.734, 95% confidence interval 1.010–2.976[b] T3/T1: hazard ratio = 3.535, 95% confidence interval 1.840–6.790[b] T4/T1: hazard ratio = 6.427, 95% confidence interval 2.430–17.002[b]	Hazard ratio = 2.097, 95% confidence interval 1.295–3.396[b]
N stage	N3/N0: hazard ratio = 8.674, 95% confidence interval 5.029–14.961[b]	Hazard ratio = 2.332, 95% confidence interval 1.442–3.771[b]
Stage (TNM)	III/I: hazard ratio = 4.109, 95% confidence interval 2.242–7.530[b] IV/I: hazard ratio = 17.898, 95% confidence interval 7.465–42.909[b]	Hazard ratio = 1.655, 95% confidence interval 1.012–2.707[b]
Stage groups (I/II vs. III/IV)	Hazard ratio = 4.122, 95% confidence interval 2.614–6.500[b]	Hazard ratio = 1.949, 95% confidence interval 1.234–3.078[b]
Disease-specific survival		
Variables[a]	Univariable	Multivariable (CadE36, adjusted for…)
CadE36 nuclear expression	Hazard ratio = 1.980, 95% confidence interval 1.121–3.495[b]	–
Age	Hazard ratio = 1.021, 95% confidence interval 0.997–1.046	Hazard ratio = 1.858, 95% confidence interval 1.044–3.309[b]
Menopausal status	Hazard ratio = 1.738, 95% confidence interval 0.860–3.512	Hazard ratio = 2.146, 95% confidence interval 1.192–3.862[b]
Grade	G3/G1: hazard ratio = 3.026, 95% confidence interval 0.908–10.083	Hazard ratio = 1.954, 95% confidence interval 1.094–3.491[b]
Lymphovascular invasion	Hazard ratio = 2.874, 95% confidence interval 1.539–5.367[b]	Hazard ratio = 1.943, 95% confidence interval 1.100–3.433[b]
PR (positive vs. negative)	Hazard ratio = 2.260, 95% confidence interval 1.194–4.278[b]	Hazard ratio = 2.039, 95% confidence interval 1.154–3.602[b]
Treatment course (chemotherapy +/− radiotherapy/ endocrine therapy vs. endocrine therapy +/− radiotherapy)	Hazard ratio = 2.958, 95% confidence interval 1.458–5.999[b]	Hazard ratio = 1.846, 95% confidence interval 1.017–3.349[b]
T stage	T2/T1: hazard ratio = 2.759, 95% confidence interval 1.255–6.065[b] T3/T1: hazard ratio = 8.121, 95% confidence interval 3.512–18.780[b]	Hazard ratio = 1.874, 95% confidence interval 1.020–3.443[b]

Table 2 (continued)

Disease-specific survival		
Variables[a]	Univariable	Multivariable (CadE36, adjusted for…)
N stage	T4/T1: hazard ratio = 10.228, 95% confidence interval 2.762–37.869[b] N2/N0: hazard ratio = 3.698, 95% confidence interval 1.157–11.818[b] N3/N0: hazard ratio = 20.061, 95% confidence interval 9.617–41.849[b]	Hazard ratio = 2.257, 95% confidence interval 1.225–4.161[b]
Stage (TNM)	III/I: hazard ratio = 13.167, 95% confidence interval 4.588–37.784[b] IV/I: hazard ratio = 62.706, 95% confidence interval 18.472–212.865[b]	Hazard ratio = 1.276, 95% confidence interval 0.676–2.408
Stage groups (I/II vs. III/IV)	hazard ratio = 9.653, 95% confidence interval 5.086–18.320[b]	Hazard ratio = 1.748, 95% confidence interval 0.983–3.110
Disease/progression-free survival		
Variables[a]	Univariable	Multivariable (CadE36, adjusted for…)
CadE36 nuclear expression	Hazard ratio = 2.341, 95% confidence interval 1.403–3.905[b]	–
Age	Hazard ratio = 1.010, 95% confidence interval 0.988–1.032	Hazard ratio = 2.305, 95% confidence interval 1.372–3.873[b]
Menopausal status	Hazard ratio = 1.768, 95% confidence interval 0.932–3.354	Hazard ratio = 2.378, 95% confidence interval 1.396–4.053[b]
Grade	G3/G1: hazard ratio = 1.888, 95% confidence interval 0.612–5.827	Hazard ratio = 2.260, 95% confidence interval 1.344–3.800[b]
Lymphovascular invasion	Hazard ratio = 2.701, 95% confidence interval 1.516–4.810[b]	Hazard ratio = 2.340, 95% confidence interval 1.401–3.908[b]
PR (positive vs. negative)	Hazard ratio = 2.745, 95% confidence interval 1.557–4.842[b]	Hazard ratio = 2.396, 95% confidence interval 1.436–3.998[b]
Treatment course (chemotherapy +/− radiotherapy/ endocrine therapy vs. endocrine therapy +/− radiotherapy)	Hazard ratio = 2.382, 95% confidence interval 1.334–4.251[b]	Hazard ratio = 2.280, 95% confidence interval 1.352–3.844[b]
T stage	T2/T1: hazard ratio = 2.201, 95% confidence interval 1.165–4.161[b] T3/T1: hazard ratio = 5.150, 95% confidence interval 2.477–10.707[b]	Hazard ratio = 2.090, 95% confidence interval 1.230–3.551[b]
N stage	N3/N0: hazard ratio = 13.614, 95% confidence interval 7.359–25.186[b]	Hazard ratio = 2.525, 95% confidence interval 1.478–4.312[b]
Stage (TNM)	III/I: hazard ratio = 8.465, 95% confidence interval 3.675–19.500[b] IV/I: hazard ratio = 37.945, 95% confidence interval 12.993–110.810[b]	Hazard ratio = 1.666, 95% confidence interval 0.952–2.916
Stage groups (I/II vs. III/IV)	Hazard ratio = 6.043, 95% confidence interval 3.545–10.302[b]	Hazard ratio = 2.097, 95% confidence interval 1.243–3.538[b]

[a]Significant values

[b]Reference categories: CadE negative nuclear expression; pre-menopausal patients; grade 1 tumors; absent lymphovascular invasion; positive PR; endocrine therapy +/− radiotherapy; T1 tumors; N0 tumors; stage I tumors; stage I/II tumors

patients with evidence of membrane P-cadherin immunostaining, 37 (51%) also showed membrane staining for E-cadherin clone 36 ($p < 0.001$).

An illustrative example of the immunoexpression of E-cadherins and P-cadherin is shown in Fig. 2.

E-cadherin clone 36 nuclear expression—association with clinicopathologic features

E-cadherin clone 36 nuclear immunostaining associated with patients' age (as a continuous variable, $p = 0.005$). An

Table 3 Frequency and compartment distribution of immunostaining for E-Cadherin and P-Cadherin antibody clones

Compartment	ECAD, clone 36 (*n*, %)	ECAD, clone EY700 (*n*, %)	ECAD, clone NCH38 (*n*, %)	PCAD (*n*, %)
Membrane				
Negative	210/284 (74)	225/283 (80)	246/284 (87)	208/280 (74)
Positive, ≥1%	74/284 (26)	58/283 (20)	38/284 (13)	72/280 (26)
Positive, ≥50%	38/284 (13)	39/283 (14)	25/284 (9)	14/280 (5)
Perinuclear/dot-like				
Negative	227/284 (80)	257/283 (91)	269/284 (95)	143/280 (51)
Positive, ≥1%	57/284 (20)	26/283 (9)	15/284 (5)	137/280 (49)
Positive, ≥50%	2/284 (1)	1/284 (<1)	1/284 (<1)	17/280 (6)
Nuclear				
Negative	186/284 (65)	281/283 (99)	275/284 (97)	279/280 (~100)
Positive, ≥1%	98/284 (35)	2/283 (1)[a]	9/284 (3.3)[a]	1/280 (<1)
Positive, ≥50%	18/284 (6)	0 (0)	0 (0)	1/280 (<1)
Cytoplasmic				
Absent	258/284 (91)	258/283 (91)	253/284 (89)	254/280 (91)
Present	26/284 (9)	25/283 (9)	31/284 (11)	26/280 (9)

ECAD E-Cadherin, *PCAD* P-Cadherin

[a]in only 1% of tumor cells

Table 4 Association between nuclear and membrane immunostaining for E-Cadherin clone 36

	Membrane negative (*n*)	Membrane positive (*n*)	Total (*n*)
Nuclear negative (*n*)	122	64	186
Nuclear positive (*n*)	88	10	98
Total (*n*)	210	74	284

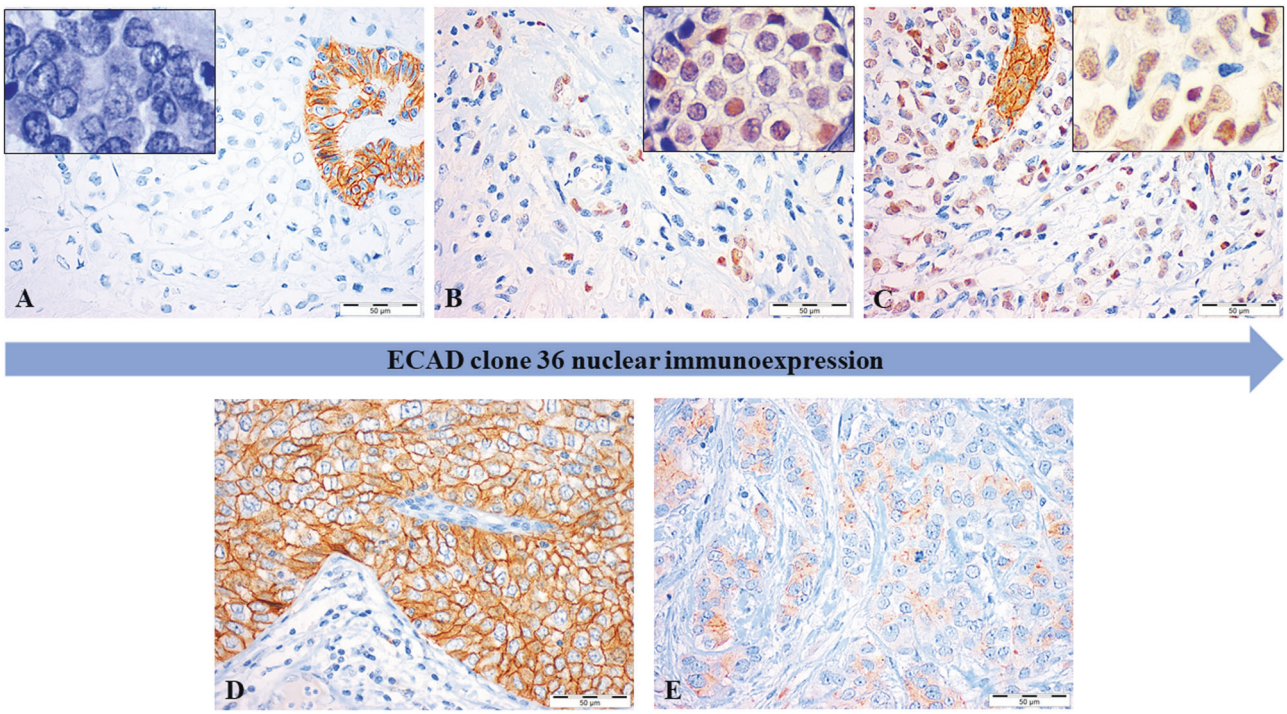

Fig. 2 Immunoexpression of E-cadherins and P-cadherins in lobular breast cancer. **a–c** E-cadherin clone 36 nuclear immunoexpression in lobular breast carcinomas—negative, 30–40% and 80–90%, respectively (insets: detail of the nuclei with negative and positive immunostaining); **d** – E-cadherin clone 36 membrane immunoexpression in ductal carcinoma; **e** – P-cadherin immunoexpression in lobular breast carcinoma, in the cytoplasm and in dot-like fashion

association was also found with T stage ($p = 0.002$), lymph-node metastases ($p = 0.049$) and stage/TNM ($p = 0.001$). A trend for association with tumor grade was depicted ($p = 0.05$). There were no significant associations with lymphovascular invasion or PR and menopausal status (Table 5).

E-cadherin clone 36 membrane immunostaining did not associate significantly with any of the aforementioned clinicopathological variables.

E-cadherin clone 36 nuclear expression—impact on patient survival

Univariable analysis

Nuclear immunostaining for E-cadherin clone 36 allowed for discrimination of patients with different survival outcome (Fig. 3). Patients with positive E-cadherin clone 36 nuclear immunostaining endured significantly worse overall survival, disease-specific survival and disease/progression-free survival (hazard ratio = 2.059, 95% confidence interval 1.313–3.230; hazard ratio = 1.980, 95% confidence interval 1.121–3.495; and hazard ratio = 2.341, 95% confidence interval 1.403–3.905, respectively) (Table 2). On the contrary, no significant impact on overall survival, disease-specific survival or disease/progression-free survival was depicted for membrane immunoexpression of any E-cadherin clone (clone 36: hazard ratio = 1.014, 95% confidence interval 0.620–1.657; hazard ratio = 1.065, 95% confidence interval 0.570–1.988; hazard ratio = 0.745, 95% confidence interval 0.402–1.382; clone EP700Y: hazard ratio = 0.689, 95% confidence interval 0.388–1.223; hazard ratio = 0.742, 95% confidence interval 0.358–1.537; hazard ratio = 0.674, 95% confidence interval 0.340–1.337; clone NCH38: 0.981, 95% confidence interval 0.526–1.829; hazard ratio = 1.193, 95% confidence interval 0.557–2.557; hazard ratio = 0.803; 95% confidence interval 0.363–1.775), respectively.

For P-cadherin, membrane immunoexpression resulted in a tendency for poorer overall survival, disease-specific survival and disease/progression-free survival, despite not reaching statistical significance (hazard ratio = 1.422, 95% confidence interval 0.877–2.306; hazard ratio = 1.429, 95% confidence interval 0.775–2.634; hazard ratio = 1.477, 95% confidence interval 0.853–2.557). The same tendency was seen for P-cadherin perinuclear/dot-like immunoexpression concerning overall survival and disease-specific survival, but not for disease/progression-free survival (hazard ratio = 1.375, 95% confidence interval 0.873–2.165; hazard ratio = 1.345, 95% confidence interval 0.761–2.375; hazard ratio = 1.068, 95% confidence interval 0.640–1.781).

When applying the 50% cutoff for E-cadherin clone 36 nuclear immunoexpression, differences in survival became more remarkable. Patients with nuclear immunoexpression in ≥50% tumor cells disclosed significantly worse overall survival, disease-specific survival and disease/progression-free survival (hazard ratio = 2.787, 95% confidence interval 1.430–5.429; hazard ratio = 4.565, 95% confidence interval 2.210–9.428; hazard ratio = 4.179, 95% confidence interval 2.113–8.266, respectively) (Fig. 3).

Table 5 Association between nuclear immunostaining for E-Cadherin clone 36 and clinicopathological features

Variables	ECAD clone 36 nuclear: negative	ECAD clone 36 nuclear: positive	p*
Age [years (median, interquartile range)]	58 (50–66)	64 (50–75)	**0.005**
Menopausal status (n, % within ECAD nuclear)			
Pre-menopause	60/176 (34)	22/96 (23)	0.075
Post-menopause	116/176 (66)	74/96 (77)	
Lymphovascular invasion (n, % within ECAD nuclear)			
Yes	26/186 (14)	18/98 (18)	0.424
No	160/186 (86)	80/98 (82)	
PR positivity (n, % within ECAD nuclear)			
Positive	156/186 (84)	85/98 (87)	0.641
Negative	30/186 (16)	13/98 (13)	
Grade (n, % within ECAD nuclear)			
G1	30/186 (16)	21/98 (22)	0.05
G2	152/186 (82)	70/98 (71)	
G3	4/186 (2)	7/98 (7)	
T stage (n, % within ECAD nuclear)			
T1	93/183 (51)	26/96 (27)	**0.002**
T2	63/183 (34)	52/96 (54)	
T3	21/183 (12)	13/96 (14)	
T4	6/183 (3)	5/96 (5)	
N stage (n, % within ECAD nuclear)			
N0	108/183 (59)	43/94 (46)	**0.049**
N+	75/183 (41)	51/94 (54)	
Stage, TNM (n, % within ECAD nuclear)			
I	74/183 (40)	19/97 (20)	**0.001**
II	64/183 (35)	44/97 (45)	
III	42/183 (23)	27/97 (28)	
IV	3/183 (2)	7/97 (7)	

ECAD E-Cadherin

*Significant results are given as bold values

Multivariable analysis

Patients with positive E-cadherin clone 36 nuclear immunostaining showed poorer overall survival when adjusted, individually, for menopausal status, grade, lymphovascular invasion, PR status, treatment course, T stage, N stage and stage (TNM); poorer disease-specific survival when adjusted for age, menopausal status, grade, lymphovascular invasion, PR status, treatment course, T stage and N Stage;

and also poorer disease/progression-free survival when adjusted for age, menopausal status, grade, lymphovascular invasion, PR status, treatment course, T stage, N stage, and stage (TNM) (Table 2).

Discussion

Breast cancer is a heterogeneous disease, with some cases disclosing slow growth and excellent prognosis, whereas other patients exhibit more rapid disease progression, with metastasis and, despite all recent improvements in therapies, eventually die from the disease. The 5-year survival rate following metastatic diagnosis is of ~15%, and it is predicted that both incidence and mortality rates will keep rising. Because breast cancer represents a major public health issue [27, 28], it is imperative to uncover novel prognostic and predictive biomarkers that might allow for better patient stratification. Herein, we performed a comparative immunohistochemistry study using three different E-cadherin clones and additionally evaluated P-cadherin immunoexpression in a series of 285 consecutively diagnosed HER2-negative lobular breast cancers in female patients with long follow-up, treated in a single cancer center by the same multidisciplinary team, seeking novel prognostic parameters in this specific breast cancer subtype.

The loss of membranous immunoexpression of E-cadherin allows tumor cells to infiltrate and spread, accounting for the radiological and histopathological infiltrative and dyscohesive pattern of invasion [12]. Pathologists take advantage of this intrinsic tumorigenic mechanism for diagnostic purposes: in difficult-to-assess cases and in mixed tumors, ductal carcinoma subtype exhibits E-cadherin membrane staining whereas it is mostly absent in lobular breast carcinoma [29]. Nevertheless, discordances between morphology and immunohistochemistry have been reported, and lobular breast cancer cases were documented to have "aberrant" E-cadherin immunoexpression, in the membrane and/or cytoplasm [30]. The prevalence of membrane immunostaining in lobular breast cancer is difficult to establish but has been reported as 16% in one study assessing 239 tumors [31]. Interestingly, we found more frequent E-cadherin membrane immunoexpression (in 26%, 20% and 13% of lobular breast carcinomas, with clones 36, EP700Y, and NCH38, respectively), which underlines the relevance of the antibody chosen to assess E-cadherin expression. In the aforementioned study, no associations between E-cadherin membrane immunoexpression and major clinicopathological features, except for lymphovascular invasion, were found [31], paralleling our observations. It is hypothesized that these "aberrant" immunoexpression patterns might reflect non-functional E-cadherin accumulation and, importantly, misinterpretation may hinder the accurate diagnosis of lobular breast cancer, which impacts on patient management and treatment [29]. It should be emphasized that our series is one of the largest reported for lobular breast cancer and the significant prognostic value found for standard clinicopathologic parameters (such as age, menopausal and PR status, treatment course, lymphovascular invasion, tumor grade and staging) further validates our results.

Nuclear expression of E-cadherin has been reported in various neoplasms, including renal cell clear cell carcinoma, bladder, colorectal, gastric, esophageal and lung carcinoma, Merkel cell carcinoma, pituitary adenoma, pancreatic neuroendocrine tumors and solid pseudo-papillary tumors of the pancreas, in some cases being associated with aggressive features (poor survival and plasmacytoid differentiation in bladder urothelial carcinoma; peritoneal dissemination in colorectal cancer) [18–20, 32–40]. E-cadherin nuclear immunoexpression, however, is highly dependent on the antibody used as this feature was mostly observed with antibodies directed towards the cytoplasmic domain of E-cadherin [24]. Indeed, our study corroborates this finding as E-cadherin nuclear immunoexpression was observed in only two and nine tumors using the antibodies targeting the extracellular domain, EP700Y and NCH38, respectively, and it was very focal (<1% of tumor cells). Conversely, using clone 36, which targets E-cadherin cytoplasmic domain, a total of 98 lobular breast carcinomas (35%) disclosed nuclear immunoexpression, emphasizing the relevance of choosing the adequate antibody to search for specific immunostaining patterns. Importantly, we found that nuclear immunoexpression using E-cadherin clone 36 associated with patients' age and major clinicopathologic features such as staging, along with a trend for association with tumor grade. Furthermore, it also predicted poorer overall survival, disease-specific survival, and disease/progression-free survival, both in univariable and multivariable analysis, when adjusting for other classic variables. Remarkably, differences in survival were further highlighted when a 50% cutoff for E-cadherin expression was used. This finding, which to best of our knowledge has not been reported thus far, might be of clinical relevance. Indeed, assessment of E-cadherin clone 36 nuclear immunoexpression in breast biopsy or surgical specimens might perfect patient stratification, improving therapeutic strategies.

Considerable effort has been put in recent years to fully understand the role of loss of E-cadherin membrane expression and its impact in downstream pathways. Two of the major players explored thus far have been β-catenin and p120. The cleavage of E-cadherin in the cell membrane, which leads to an accumulation of E-cadherin in the cytoplasm, results in the nuclear shift of β-catenin. This way, it

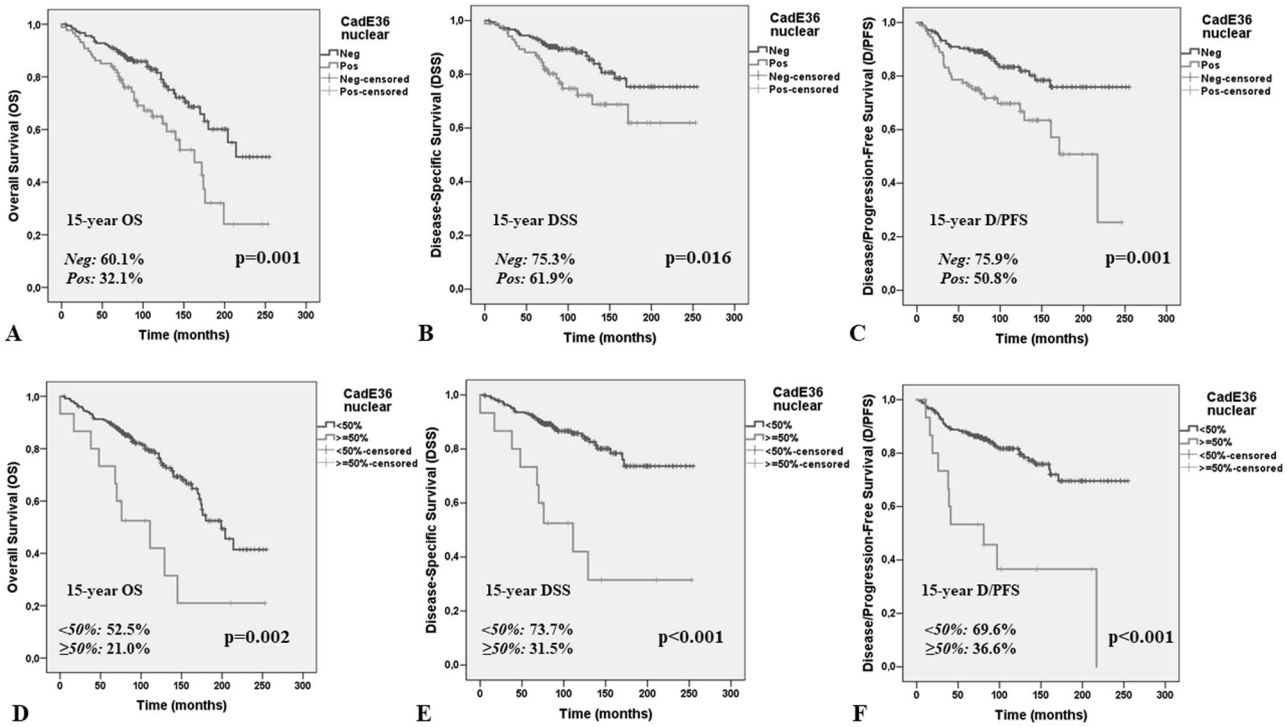

Fig. 3 Overall-survival, disease-specific survival and disease/progression-free survival of lobular breast cancer patients according to E-cadherin clone 36 nuclear immunoexpression. **a–c** any percentage of nuclear immunoexpression; **d–f** 50%-cutoff of nuclear immunoexpression

was suggested that E-cadherin might translocate to the nucleus in a similar way as β-catenin, either taking advantage of it ("β-catenin piggyback") or independently from it, facilitated by p120 protein [16, 24]. However, very little is known about the precise mechanism of nuclear translocation of E-cadherin. Interestingly, even in the scenario of complete loss of E-cadherin membrane staining, we still found heterogeneity in the percentage of nuclear staining. The positively stained nuclei do not seem to derive from a clonal event as they are not all clustered together; instead it appears that each cell develops the ability to incorporate E-cadherin in the nucleus independently, which might give clues to the mechanism of this translocation. While in many cases E-cadherin is inactivated by mutation [41], which could potentially result in losing the ability to translocate to the nucleus, other mechanisms of inactivation such as the various dynamic epigenetic (de)regulation mechanisms might be involved that hinder E-cadherin and perhaps facilitate its translocation to the nucleus in certain tumor cells [42–44].

Not much is known also about the pathways affected by this movement. A regulatory role of cancer stem cell phenotype and apoptosis has been suggested [16, 39]. Given the documentation of an E-cadherin fragment bound to DNA, it is suggested that E-cadherin might indeed function as a transcription factor [17]. Interestingly, we found a significant negative correlation between E-cadherin clone 36 membrane and nuclear immunoexpression. This finding suggests that, in lobular breast cancer, membrane-anchored E-cadherin is disassembled and only then migrates to the nucleus, so that membrane and nuclear E-cadherin are mostly mutually exclusive. Indeed, only 10 cases depicted simultaneous expression in the membrane and nucleus, and this might be occurring in different cells, as co-localization studies were not performed. Hence, we hypothesize that in lobular breast cancer E-cadherin is inactivated, resulting in loss of membrane staining, and only then some of the neoplastic cells end up developing the ability to translocate the cytoplasmic domain of E-cadherin to the nucleus. The latter acts as a transcription factor, stimulating expression of genes involved in cancer progression and metastasis, which is then reflected in poorer overall survival, disease-specific survival and disease/progression-free survival (Fig. 4).

P-cadherin membrane immunoexpression was observed in 26% of lobular breast cancer and a tendency for resulting in poorer overall survival, disease-specific survival and disease/progression-free survival was depicted; still, and contrarily to other studies [45], it did not reach statistical significance. However, P-cadherin expression seems to be common in triple-negative breast cancer, and only five tumors of our cohort corresponded to this molecular breast cancer subtype, the remaining corresponding to luminal-like

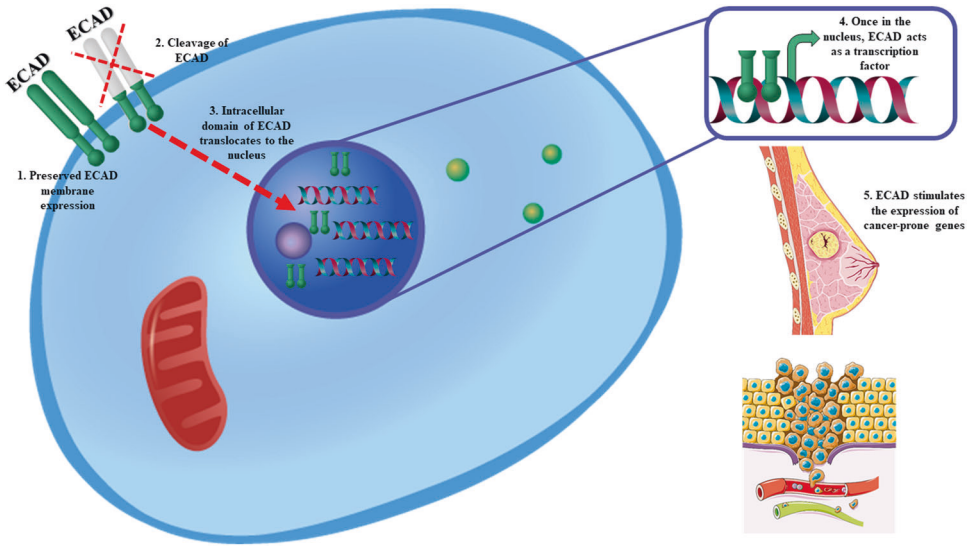

Fig. 4 Proposed model of nuclear translocation of E-cadherin in lobular breast cancer. E-cadherin is inactivated and translocated to the nucleus, acting as transcription factor, stimulating expression of genes involved in cancer progression and metastasis

breast cancer. Furthermore, the aforementioned study included 150 "invasive breast carcinomas", while we restricted our analysis to 285 lobular breast cancers. In addition, our findings must be interpreted in the context of frequent E-cadherin loss of expression, as P-cadherin-induced phenotype is highly dependent on E-cadherin expression status. Indeed, we found a significant positive correlation between E-cadherin and P-cadherin membrane immunoexpression, which is in line with the hypothesized cooperation of both proteins in establishing tumor-promoter phenotypes [14]. Despite not showing significant impact on survival, the finding of frequent P-cadherin perinuclear/dot-like immunostaining (49% of lobular breast cancers) is of interest, and its biological meaning should be further explored. Nuclear immunoexpression of P-cadherin, on the other hand, was found in only one case, indicating that translocation to the nucleus is a very uncommon phenomenon, contrarily to E-cadherin.

The main limitations of our work are its retrospective nature and the extended time frame for recruiting patients. Indeed, diagnostic and treatment strategies have changed over time, creating bias. Nevertheless, the extended time frame allowed us to gather a large cohort of pure lobular breast cancer cases, with long-term follow-up, which is key to evaluating the prognostic-impact of a biomarker. Furthermore, we have restricted the study to female patients not receiving neoadjuvant treatments and HER2-negative tumors, and all women were managed at the same institution by the same multidisciplinary team, contributing to cohort homogeneity. Clinical charts were also reviewed according to most recent staging systems and, importantly, classical major prognostic parameters allowed for discrimination of patients with distinct disease outcome, further validating our series. Despite some subjectivity inherent to immunohistochemistry assessment, two pathologists did the interpretation (blinded to clinicopathological features) on full histological slides (no tissue microarray was used so that to minimize possible staining heterogeneity), and several controls were used throughout the fully automated immunostaining process, minimizing technical issues. To the best of our knowledge, this is the first study assessing the frequency and prognostic value of E-cadherin nuclear immunoexpression in lobular breast cancer. Immunostaining for E-cadherin is a routine and practical procedure in most pathology labs, which might be performed in breast cancer biopsies or resections. Our results support the use of E-cadherin clone 36 which recognizes the cytoplasmic domain thus providing the extra ability to observe nuclear expression, which in turn is useful for discriminating patients with different disease aggressiveness and prognosis. This might allow clinicians to better tailor and individualize therapies and adjust follow-up strategies for women with lobular breast cancer.

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Compliance with ethical standards

Conflict of interest AHN, JC, GH, EW, and IB are employees of Ventana Medical Systems. The remaining authors declare that they have no conflict of interest.

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