Interaction Between Beta-Catenin and EGFR Expression by Immunohistochemistry Identifies Prognostic Subgroups in Early High-risk Triple-negative Breast Cancer

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Abstract. *Wnt and epidermal growth factor receptor (EGFR) pathway abnormalities and de-stabilization of cell adhesion are all important aspects of the pathogenesis of triple-negative breast cancer (TNBC). Herein we investigated how the expression of related protein markers may affect the outcome of patients bearing TNBC treated in the adjuvant setting. Immunohistochemistry for beta-catenin, Myc (Wnt pathway), E-cadherin, P-cadherin (cell-adhesion), EGFR and cytokeratin 5 (CK5) (identification of basal-like tumors) was carried out in 364 centrally confirmed TNBCs. Survival analysis was performed with Cox-regression models according to dichotomized continuous protein expression data and marker interactions. In 352 evaluable tumors, 81.5% were basal-like TNBC. E-cadherin and P-cadherin were positively associated, with co-expression being present in 68% of tumors. Individual markers did not affect patient outcome. However, a statistically significant interaction was shown such that low expression of beta-catenin in the cell membrane, defined as expression below the median of the H-score distribution, was associated with unfavourable disease-free survival among tumors that expressed EGFR, but not in the absence of EGFR expression (interaction p=0.0085). The interaction persisted after correcting for clinicopathological variables. A considerable number of TNBC co-expresses E-cadherin and P-cadherin, while membranous localization of beta-catenin may predict patient outcome in an EGFR-*

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dependent manner. This novel interaction seems worthy for validating with regards to its biological and clinical relevance.

Triple-negative breast cancer (TNBC) is defined on the basis of negative estrogen receptor-a (ER) and progesterone receptor (PgR) protein expression and lack of human epidermal growth factor receptor 2 (HER2) overexpression or gene amplification. Identifying TNBC is clinically important, because these tumors are highly aggressive, unlikely to benefit from anti-estrogen and anti-HER2 therapy and are thus treated mainly with conventional cytotoxics (1, 2). At the same time, typing by the use of negative selection makes TNBC a rather ambiguous entity with limited value for personalized treatment. In fact, similarly to what has been shown for breast cancer as a whole, TNBC itself is not a single entity at the gene expression as well as at the proteomic level (3-5).

The majority of TNBC correspond to the basal-like intrinsic subtype of breast cancer identified by gene-expression profiling, however 20-30% fall within other subtypes (6). Because basal-like breast cancers are more biologically homogeneous and represent a prognostically uniform group of tumors, their identification among other TNBC is regularly attempted by use of various immunohistochemistry (IHC)-based panels, among which the one combining EGFR and CK5 is considered as the most pragmatic (1). In addition, EGFR constitutes an attractive therapeutic target in TNBC as evidenced by multiple studies currently registered at ClinicalTrials.gov database investigating the efficacy of tyrosine kinase inhibitors and monoclonal antibodies. However, despite overall agreement that most TNBC express EGFR, the prognostic significance of this marker is still controversial (7). Recently, microarray technology identified additional gene-expression profiles of TNBC, that are associated with distinct cancer pathways and thus could be managed with corresponding targeted therapies (4, 6). These subtypes frequently involve gene expression profiles typical of the Wnt signaling pathway and others related to the process of epithelial mesenchymal transition (EMT).

Experimental data have shown that Wnt can induce EMT (8) and enhance the metastatic potential of various cancer cell lines *in vitro* (9). Moreover, the pharmaceutical inhibition of pathway components exerts significant anti-proliferative and pro-apoptotic effects in TNBC pre-clinical models (10). Beta-catenin is a critical component of the canonical Wnt pathway activation by serving as a transcriptional modulator of MYC and other genes that stimulate cell proliferation (11, 12). Beta-catenin expression abnormalities assessed by IHC are more frequent in TNBC than other breast cancer molecular subtypes and may serve as markers of unfavourable outcome (13-15). Beta-catenin also interacts with cadherin-mediated cell adhesion, where it co-localizes with E-cadherin (16). Recently, P-cadherin, classically considered a marker of basal/myepithelial cells, has been shown to interfere with and destabilize adherens junctions (17). For the most part however, adherens junction dynamics are regulated by a complex network of phosphorylation switches including a role for EGFR (18).

Herein, we sought to investigate the patterns of expression and the clinical relevance of selective markers involved in the above described molecular and biochemical processes in a large series of TNBC cases from patients with high-risk operable breast cancer. For this purpose we performed IHC on formalin-fixed paraffin-embedded (FFPE) blocks using antibodies against beta-catenin and Myc (Wnt pathway), E-cadherin and P-cadherin (cell adhesion), while also including two markers (EGFR and CK5) for typing of basal-like TNBC.

Patients and Methods

The translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine (Protocol No: 44/28-6-2013) signed by the Committee Coordinator, professor AD Garyfallos. TNBC cases (N=418) were selected from the HeCOG patient database that stores comprehensive clinical and pathological data for more than 3,500 women who have participated in 6 prospective adjuvant trials investigating the efficacy of taxane-based regimens for the management of high-risk operable breast cancer. All patients provided upon enrolment a written informed consent for research use of their material. Case selection was based on FFPE tissue block availability and on local pathology laboratory reports on ER, PgR and HER2 immunohistochemistry (IHC). Patients were diagnosed and treated from 1997 to 2013. Survival data have been published for the earlier HE 10/97, HE 10/00 and HE 10/05 trials, (19-21) while clinical evaluation of the more recent HE 10/08 and HE 10/10 is ongoing. For some patients, previous central characterization of tumors served as an additional filter for the identification of TNBC (22). The present series was enriched with 43 additional clinical routine cases from HeCOG participating institutes (Table I). All tumors were centrally reviewed by an experienced pathologist (S.L.) at the Laboratory of Molecular Oncology (LMO).

Tissue microarray (TMA) construction was guided by pathologic evaluation of hematoxylin-eosin slides, upon which 17 tumors were excluded due to minimal tumor surface. Twenty low-density TMA blocks comprising 25-27 tumors each represented by duplicate 1.5mm cylinders were assembled on a manual arrayer (Model I, Beecher Instruments, San Prairie, WI, USA). Various benign and reactive tissues were included to serve as positive and negative controls based on antibody provider recommendations and our own experience. Immunohistochemistry was performed on TMAs according to previously published standard protocols of the LMO, including fluorescent in situ hybridization (FISH) in cases with HER2 2+ IHC grading (23). Marker-specific parameters of the staining procedure and information for antibodies are summarized in Table II. Upon central pathology review, 364 tumors were validated as TNBC (90.8%); among the remaining 37 cases (9.2%), 25 were ER/PgR-positive, 9 were HER2-positive and 3 involved lost or folded TMA cores. After accounting for additional loss of TMA cores in consecutive slides and for non-interpretable stains, 352 cases, informative for at least one of the markers, were included in the present study (Figure 1).

Table I. *Patient demographics, clinicopathological data and frequencies of marker binary categories.*

			N (%)
Adjuvant HeCOG trial	N=352	Clinical routine	43 (12.2)
		HE 1000	85 (24.2)
		HE 1004	21 (6.0)
		HE 1005	91 (25.8)
		HE 1008	78 (22.2)
		HE 1010	11 (3.1)
		HE 1097	23 (6.5)
Age (median)	N=352	<52.8	174 (49.4)
		≥52.8	178 (50.6)
Menopausal status	N=352	post	197 (56.0)
		pre	155 (44.0)
Multifocality	N=352	No	330 (93.8)
		Yes	22 (6.2)
pT	N=351	<=2cm	128 (36.5)
		2-5cm	190 (54.1)
		>5cm	32 (9.0)
		Tx	1 (0.2)
pN	N=350	0	117 (33.4)
		1-3	121 (34.5)
		≥4	111 (31.8)
		Nx	1 (0.3)
Histological type	N=352	Ductal	273 (77.6)
		Apocrine	12 (3.4)
		Lobular	17 (4.8)
		Medullary	13 (3.6)
		"Atypical Medullary"	9 (2.6)
		Metaplastic	17 (4.8)
		Other	11 (3.2)
Histological grade	N=352	I-II	66 (18.8)
		III	286 (81.2)
Adjuvant chemotherapy	N=352	Yes	341 (96.8)
		No	11 (3.2)
Adjuvant hormonal therapy	N=352	Yes	71 (20.2)
		No	281 (79.8)
Taxanes	N=348	Yes	311 (89.3)
		No	37 (10.6)
CK5	N=330	Neg	94 (28.5)
		Pos	237 (71.5)
EGFR	N=346	Neg	160 (46.2)
		Pos	186 (53.8)
m/beta-catenin (median=55)	N=278	High	140 (50.3)
		Low	138 (49.7)
n/beta-catenin	N=278	Neg	267 (96)
		Pos	11 (4)
Myc	N=278	Neg	248 (79.2)
		Pos	30 (10.8)
E-cadherin	N=296	Neg	85 (28.7)
		Pos	211 (71.3)
P-cadherin	N=309	Neg	52 (16.8)
		Pos	257 (73.2)

IHC interpretation and scoring. After careful evaluation of in-slide controls, the percentage of positive tumor cells was recorded at three intensity levels (low, medium, high) and separately for each cellular compartment (membrane, cytoplasm and nucleus). A minimum of 100 cells or an area of tumor equal to or greater than 5% of the core surface were required for a case to be considered eligible for evaluation. Staining interpretation was performed by four experienced pathologists (S.L., T.K. I.K. and M.B.). The pattern of staining was membranous and cytoplasmic for HER2, CK5, EGFR, E-cadherin and P-cadherin, cytoplasmic and nuclear for Myc, nuclear for ER and PgR and in all three cellular compartments for beta-catenin (Figure 2). Previously published cut-off thresholds for assigning negative versus positive categories were applied on the average core value when both cores were available or on the remaining core if one was lost or folded (Table II) (24-28). Regarding beta-catenin, the median of expression range by H-score was used to distinguish between low and high membrane expression (m/b-catenin), whereas a tumor was considered positive for nuclear beta-catenin (n/beta-catenin) when staining of any intensity or extent was present in tumor cell nuclei. Basal-like TNBC were defined as per Nielsen *et al*. (29).

Statistical analysis. Categorical variables were presented as frequencies and percentages, with continuous variables categorized appropriately, while associations were examined using the Chi-square test. Disease-free survival (DFS) was measured from the time of diagnosis until verified disease progression, death or last contact, and overall survival (OS) from diagnosis until death from any cause or date of last contact. Time-to-event distributions were estimated using Kaplan-Meier curves, while log-rank tests and univariate Cox analysis were used for evaluating DFS and OS differences and reporting hazard ratios. Interaction analysis was performed in order to capture the differentiation in terms of DFS and OS of one marker's effect among the different levels of another. In univariate analysis, using Cox regression, significance was determined at the level of 5% and in multivariate at 10% (two-sided). All tests were two-sided. The SAS software was used for statistical analysis (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA), while no adjustment for multiple comparisons is reported. The study was designed and performed in accordance with reporting recommendations for tumor marker prognostic studies (REMARK) criteria (30).

Results

Patients' demographics, clinicopathological characteristics and frequency of marker positivity are presented in Table I. The vast majority of women had received anthracycline-based chemotherapy in combination with taxanes. There were 283 (81.5%) basal-like and 64 (18.5%) non-basal-like cases based on the combined CK5 and/or EGFR profile. The number of examined cases varied between markers due to progressive loss of tissue cores in consecutive TMA sections (Table I). Associations of protein marker expression with various patient and tumor characteristics are presented in groups according to their contribution in the basal-like phenotype (Table III), cell adhesion (Table IV) and the Wnt pathway (Table V). Basal-like tumors were more frequently linked to higher histological grade (p=0.001) and fewer metastatic lymph nodes (p=0.006) and were slightly more prevalent among patients who had received adjuvant hormonal therapy or taxanes (p=0.035 and p=0.047 respectively), but not with younger age. The expression of

Table II. *Antibodies, protocols and interpretation of IHC stains.*

Marker	Clone	Source	AR	Incubation	Localization	Cut-off
ER	6F11	Leica Biosystems	pH6	1:70, 20min	n	≥1%
PgR	1A6	Leica Biosystems	pH6	1:70, 20min	n	≥1%
HER2	polyclonal	DAKO	pH6	1:500, 30min	m	10%
CK5	XM 26	Leica Biosystems	pH9	RU, 20min	c and/or m	>0
EGFR	31G7	Invitrogen Corp	Pepsin	1:50, 20min	m	>0
P-cadherin	56C1	Thermo Scientific	pH9	1:200 o/n	m	≥10%
E-cadherin	36B5	Leica Biosystems	pH9	RU, 20min	m	≥10%
beta-catenin	17C2	Leica Biosystems	pH6	1:350 o/n	m and/or n	≥H-score median
Myc	9E10	BD Pharmingen	pH9	1:300, 20'	n	≥H-score 100

ER: Estrogen receptor, PgR: progesterone receptor, HER2: human epidermal growth factor receptor 2, CK5: cytokeratin 5, EGFR: epidermal growth factor receptor, AR: antigen retrieval, RU: ready to use, n: nucleus, c: cytoplasm, m: membrane, o/n: overnight.

CK5 was positively associated with multifocality and tumor grade (p=0.005 and p<0.001 respectively) and negatively with pN stage (p=0.004). Positivity for CK5 was slightly more frequent among patients that had received taxanes (p=0.017). Several associations were observed between the basal-like phenotype and tumor histology (p<0.001). Ductal and apocrine carcinomas were evenly distributed across basal-like and non-basal-like profiles. By contrast, all but one medullary (including atypical) and metaplastic tumors were basal-like, whereas the opposite was shown for lobular carcinomas. P-cadherin expression was more often observed in younger patients (p=0.015), in tumors of higher grade and basal-like (p<0.001) and also in metaplastic carcinomas as opposed to lobular tumors (p<0.001). By contrast, E-cadherin was only rarely positive in lobular carcinomas, as expected (p<0.001). Similar to P-cadherin, a strong association was found between E-cadherin and basal-like tumors (p<0.001). Decreased membrane staining of beta-catenin, including cases with nuclear positivity were more common in non-basal-like than basal-like TNBC. Increased Myc expression was more often observed in tumors of lower histological grade than high-grade tumors (p=0.017). Several associations were observed between n/beta-catenin and various clinicopathological parameters but these are considered of limited significance due to the small number of positive cases (Table V).

Associations between markers of the Wnt pathway and other markers are depicted in Table VI. Decreased m/beta-catenin as well as nuclear localization were both frequently associated with absence of E-cadherin (p<0.001 and p=0.009 respectively) and P-cadherin (p<0.001 and p=0.003 respectively). Similarly, tumors expressing lower than the median m/beta-catenin were more frequently negative for CK5 and EGFR (p=0.016 and p<0.001 respectively). From the 11 tumors with nuclear localization of beta-catenin, 10 also harboured a reduction in the membranous staining (p=0.010). The pattern of expression was significantly coordinated within and between cadherins and basal markers. As shown in Table VII, tumors frequently expressed both basal markers (42.2%) and more often both cadherins (68.8%), while any basal marker expression was also more frequent upon the presence of any cadherin.

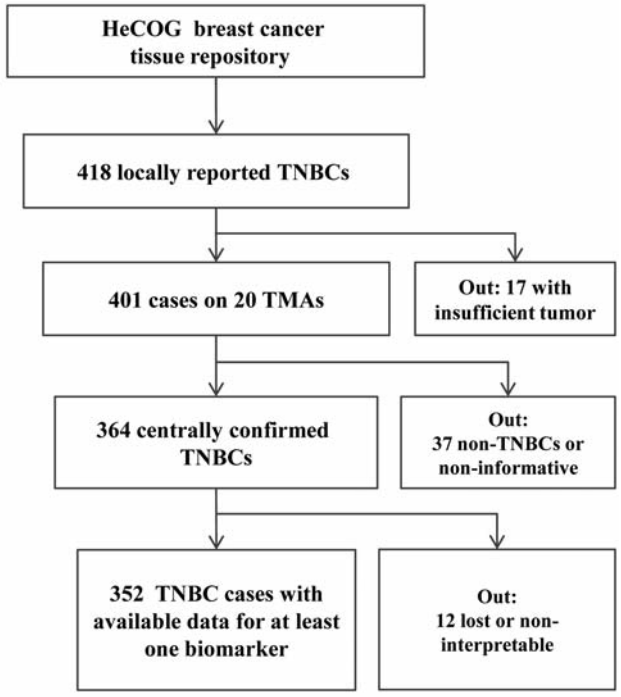

Figure 1. *REMARK flowchart. After accounting for inadequate material and cases lost during processing 352 TNBC tumors were valid for statistical analysis.*

The results of univariate analysis are described in Table VIII. Small tumor size, the presence of less than 4 infiltrated

Table III. *Associations between basal markers and clinicopathological variables.*

	CK5			EGFR			Basal		
	Neg (%)	Pos (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value
Age									
<52.8	41 (44.6)	118 (50.4)	0.389	82 (51.9)	86 (47.3)	0.447	28 (44.4)	140 (50.4)	0.407
≥52.8	51 (55.4)	116 (49.6)		76 (48.1)	96 (52.7)		35 (55.6)	138 (49.6)	
Menopausal status									
post	59 (62.8)	128 (54.0)	0.147	91 (56.9)	104 (55.9)	0.857	41 (64.1)	154 (54.4)	0.160
pre	35 (37.2)	109 (46.0)		69 (43.1)	82 (44.1)		23 (35.9)	129 (45.6)	
Multifocality									
No	82 (87.2)	228 (96.2)	**0.005**	151 (94.4)	174 (93.5)	0.824	57 (89.1)	269 (95.1)	0.082
Yes	12 (12.8)	9 (3.8)		9 (5.6)	12 (6.5)		7 (10.9)	14 (4.9)	
pT									
2-5cm	50 (53.8)	133 (57.1)	0.357	82 (52.2)	104 (56.8)	0.685	34 (54.0)	153 (55.0)	0.435
≤2cm	31 (33.3)	82 (35.2)		60 (38.2)	64 (35.0)		21 (33.3)	103 (37.1)	
>5cm	12 (12.9)	18 (7.7)		15 (9.6)	15 (8.2)		8 (12.7)	22 (7.9)	
pN									
0	22 (24.2)	85 (36.8)	**0.004**	52 (33.5)	61 (33.7)	0.132	14 (22.6)	100 (36.4)	**0.006**
1-3	28 (30.8)	86 (37.2)		47 (30.3)	71 (39.2)		18 (29.0)	100 (36.4)	
≥4	41 (45.1)	60 (26.0)		56 (36.1)	49 (27.1)		30 (48.4)	75 (27.3)	
Histological type									
Ductal	67 (71.3)	188 (79.3)	**<0.001**	128 (80.0)	140 (75.3)	**0.004**	45 (70.3)	224 (79.2)	**<0.001**
Apocrine	6 (6.4)	4 (1.7)		2 (1.3)	10 (5.4)		2 (3.1)	10 (3.5)	
Lobular	13 (13.8)	3 (1.3)		13 (8.1)	3 (1.6)		12 (18.8)	4 (1.4)	
Medullary	2 (2.1)	11 (4.6)		6 (3.8)	7 (3.8)		1 (1.6)	12 (4.2)	
"Atypical Medullary"	2 (2.1)	7 (3.0)		4 (2.5)	5 (2.7)		1 (1.6)	8 (2.8)	
Metaplastic	1 (1.1)	16 (6.8)		3 (1.9)	14 (7.5)		1 (1.6)	16 (5.7)	
Other	3 (3.2)	8 (3.4)		4 (2.5)	7 (3.8)		2 (3.1)	9 (3.2)	
Histological grade									
I-II	28 (30.4)	30 (12.7)	**<0.001**	38 (24.1)	25 (13.5)	0.017	21 (33.9)	42 (14.9)	**0.001**
III	64 (69.6)	206 (87.3)		120 (75.9)	160 (86.5)		41 (66.1)	240 (85.1)	
Adjuvant chemotherapy									
No	5 (5.3)	6 (2.5)	0.304	3 (1.9)	8 (4.3)	0.234	1 (1.6)	10 (3.5)	0.697
Yes	89 (94.7)	231 (97.5)		157 (98.1)	178 (95.7)		63 (98.4)	273 (96.5)	
Adjuvant hormonal therapy									
No	71 (75.5)	198 (83.5)	0.117	129 (80.6)	149 (80.1)	1	45 (70.3)	234 (82.7)	**0.035**
Yes	23 (24.5)	39 (16.5)		31 (19.4)	37 (19.9)		19 (29.7)	49 (17.3)	
Taxanes									
No	14 (15.6)	15 (6.6)	**0.017**	14 (9.3)	17 (9.5)	1	10 (16.7)	21 (7.7)	**0.047**
Yes	76 (84.4)	212 (93.4)		137 (90.7)	162 (90.5)		50 (83.3)	250 (92.3)	

Statistically significant *p*-values are indicated in bold.

lymph nodes and taxane treatment were all strong predictors of favourable outcome. By contrast, significantly lower survival rates were shown for a subset of patients that had received adjuvant hormonal therapy. These patients had been enrolled in the earlier HE10/97 and 10/00 trials and approximately half reflected discrepancies between local IHC and previous central assessments at LMO. The other half involved mainly tumors with unfavourable pathological features. Of note, at the time of enrolment of the HE10/97 trial, hormonal manipulation, mainly ovarian ablation alone was favoured by some HeCOG participating centres for aggressive tumors irrespective of hormone receptor status, a practice that has since been abandoned.

The aim of distinguishing patients into prognostic subgroups on the basis of single marker expression was not successful. The only exception was a numerical survival disadvantage for DFS observed for tumors displaying a reduction in m/beta-catenin expression (Figure 3A). In order to exclude a confounding role of tumor histological types with distinctly favorable or unfavorable outcomes, such as medullary or metaplastic carcinomas, univariate analysis was repeated in the more homogeneous subgroup of NST ductal carcinomas; however, results did not differ (data not shown). To further address the biological heterogeneity of TNBC and identify tumor phenotypes with a different clinical course we explored the interactions between markers of the basal-like

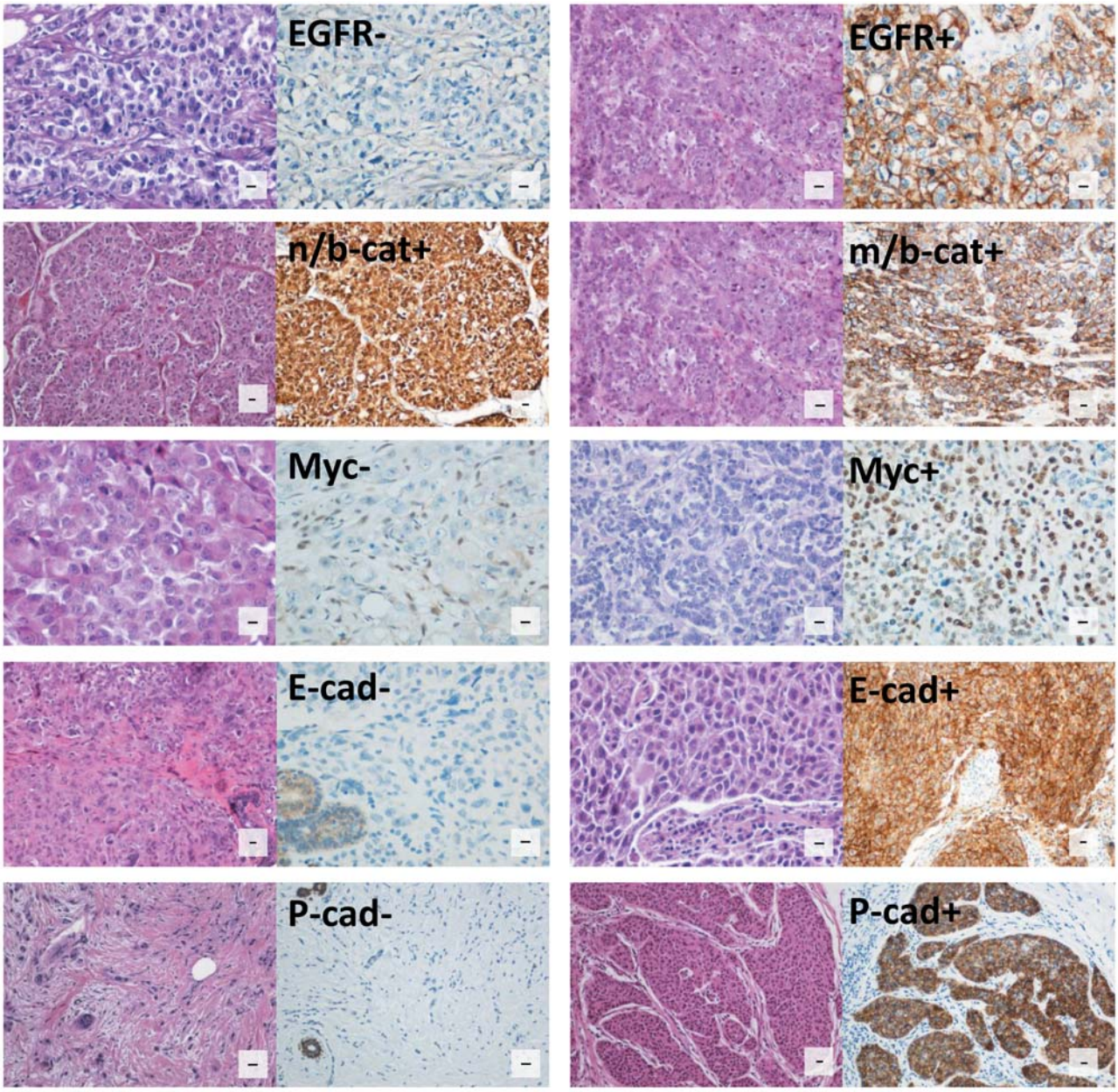

Figure 2. *Representative negative and positive staining examples. Pairs of Hematoxylin-Eosin and IHC microphotographs of tumors included in the study are shown with scale bars set at 10 μm. E-cad: E-cadherin, P-cad: P-cadherin, m/b-cat: membranous beta-catenin, n/b-cat: nuclear beta-catenin.*

phenotype, cell-adhesion proteins (E-cadherin and P-cadherin) and those involved in the Wnt pathway. This approach resulted in one significant interaction between m/beta-catenin and EGFR expression affecting DFS. In the presence of EGFR, low m/beta-catenin was associated with inferior DFS and, marginally, OS, whereas in the absence of EGFR, Kaplan-Meier curves were indicative of a numerical trend to the opposite direction (Figure 3B and C). The prognostic interaction was statistically significant for DFS, revealing an increased risk of relapse associated with low m/beta-catenin in EGFR-positive patients (HR=2.44, 95%CI=1.36-4.40; p=0.0085). The same interaction appeared as a trend for OS in the same patient group (HR=2.03, 95%CI=1.06-3.89; p=0.058). In addition, as illustrated in Figure 4, significance for DFS persisted in a multivariate Cox-regression model including tumor lymph node status and treatment with or without taxanes. In order to exclude potential bias from lobular carcinomas, which are

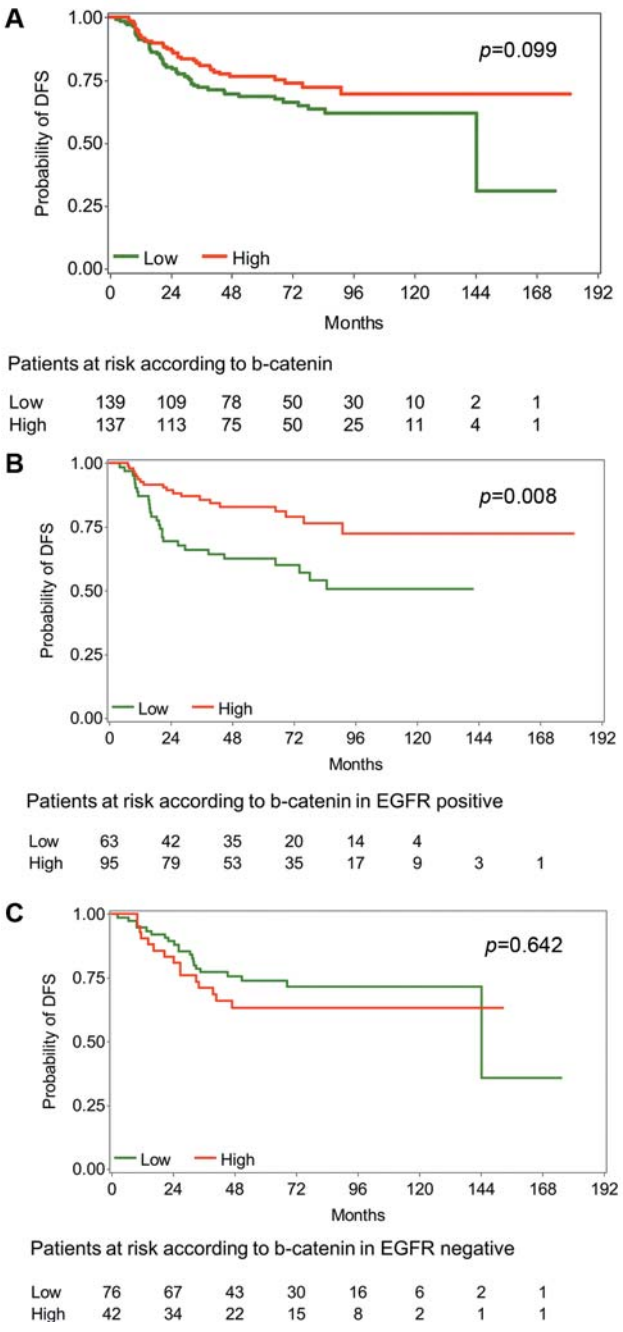

Figure 3. *Kaplan-Meier curves for disease-free survival according to m/beta-catenin expression at 10 years follow-up. (A) Entire cohort. (B) Patients whose tumors were EGFR-positive. (C) Patients with EGFR-negative tumors.*

known to harbour beta-catenin-related abnormalities due to well-defined molecular mechanisms, statistical analyses were repeated after excluding these cases, but results did not differ from those in the entire dataset (data not shown).

Discussion

Most TNBC are tumors carrying the unfavourable molecular fingerprint of the basal-like intrinsic subtype of breast cancer and can be readily distinguished on FFPE tissue using Nielsen's definition (29). In line with previous data from population-based cohorts we confirm that this IHC-based basal-like TNBC phenotype (termed "core basal" elsewhere) is of higher histological grade compared to non-basal-like TNBC and metastasizes to regional lymph nodes less frequently (28, 31). However, contrary to what has been previously shown for patients uniformly treated with anthracyclines, there was no prognostic disadvantage for basal-like TNBC in our series, in which all but 37 patients had additionally received taxanes (31). Given the small size of the non-taxane group we did not consider it statistically sound to investigate the interaction between treatment type and the basal-like phenotype. Therefore, we cannot conclude whether the comparable outcome of basal-like and non-basal-like TNBC is a cohort-specific characteristic or an effect related to anthacycline-taxane combination treatment.

In recent years, EGFR has emerged as a promising target for the treatment of TNBC due to its widespread expression in these tumors, although its biological role has not been fully elucidated (32). Some studies, including ours, do not support an adverse effect of EGFR expression or overexpression on patient outcome (7, 33), whereas others have shown this association to be of significance (34-36). As is often the case with outcome prediction based on IHC, discordant findings between studies may come as a result of methodological variations, which, for EGFR, mainly involve modes of interpretation (recording of membranous only or membranous and cytoplasmic reaction) and cut-offs (0%, 10% or HER2-like systems). Methodological issues aside, recent data from a phase II trial of the monoclonal antibody cetuximab in metastatic TNBC underscore the potential significance of ligand-independent activation of the EGFR pathway in these tumors (37). This suggests that EGFR protein expression alone may not be able to adequately approximate the status of EGFR signaling in TNBC, thus offering an explanation for the inconsistency of data regarding the prognostic effect of this marker.

Following evidence of the tumorigenic role of Wnt in mouse mammary tumor models, Wnt pathway deregulation has become the issue of many IHC-based studies in breast cancer including TNBC (13-15, 38, 39). Nuclear localization of beta-catenin by IHC has been reported in up to 50% of TNBCs (13). The much lower frequency in our series (4.3%) is yet another example of the high variability of beta-catenin IHC positivity rates across studies, even when using the same clone and similar analytical processing (14, 15, 38). When present, nuclear beta-catenin expression is often described as spotty and weak, and this IHC pattern, also encountered frequently herein,

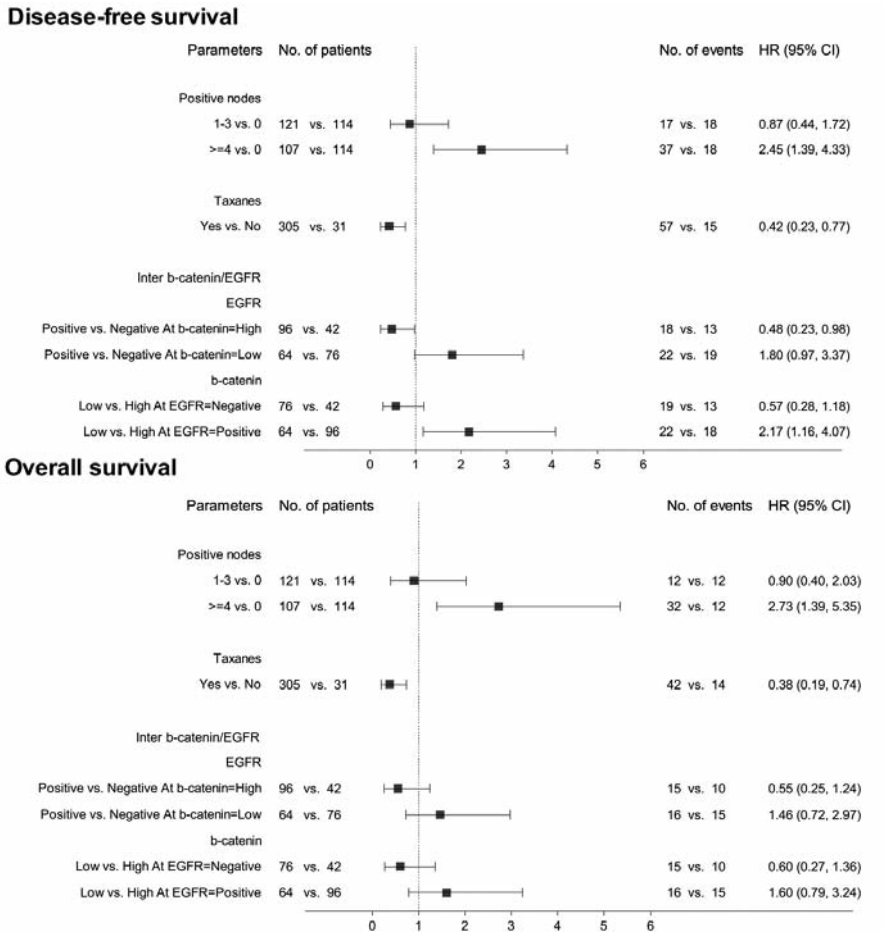

Figure 4. *Forest plots of hazard ratios for disease-free survival and overall survival.*

could account for poor reproducibility and reduced sensitivity. Despite the fact that our study is among the largest to have analyzed beta-catenin expression in TNBC, we could show only a trend towards reduced survival for tumors with nuclear expression, due to the small number of positive cases recorded in this cellular compartment. This trend is in line with previous findings in a smaller patient cohort and consistent with the detrimental effects exerted on tumor biology by the nuclear translocation of beta-catenin (15, 16).

All but one case harbouring beta-catenin nuclear expression simultaneously displayed membrane expression levels below the median of the H-score distribution. Of note, this threshold was chosen arbitrarily, due to lack of consensus on the evaluation of beta-catenin IHC and the absence of a validated cut-off for continuous measurements. By using this approach we showed that among tumors expressing EGFR, those with lower than the median expression of beta-catenin in the tumor cell membrane carry a higher risk of relapse and death, whereas in EGFR-negative tumors, outcome is unrelated to the expression of membrane beta-catenin. The biological basis of this herein identified EGFR/beta-catenin interaction is difficult to interpret. Given the fact that beta-catenin localizes in adherens junctions and these structures are subject to regulation by kinases, reduction of membrane beta-catenin in EGFR-positive tumors might be a manifestation of EGFR-induced destabilization of cell-to-cell adhesion (18). In addition, there is adequate evidence from tumor cell lines that EGFR can directly phosphorylate beta-catenin and promote EMT by disrupting the association of beta-catenin with E-cadherin (9, 40, 41) thus promoting tumor spread. Nevertheless, although explanation of the biology behind the EGFR/beta-catenin interaction remains hypothetical, this novel finding seems a significant predictor of TNBC patient outcome and merits investigation in larger independent studies.

The reduction of membrane beta-catenin in our series was closely associated with loss of E-cadherin, that may be seen as further evidence of dissociation of the E-cadherin/beta-catenin complex in these tumors. However, the loss of E-

Table IV. *Associations between EMT-related markers and clinicopathological variables.*

	E-cadherin			P-cadherin		
	Neg (%)	Pos (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value
Age						
<52.8	36 (42.9)	108 (51.7)	0.197	17 (33.3)	132 (52.2)	**0.015**
≥52.8	48 (57.1)	101 (48.3)		34 (66.7)	121 (47.8)	
Menopausal status						
post	50 (58.8)	116 (55.0)	0.546	34 (65.4)	139 (54.1)	0.134
pre	35 (41.2)	95 (45.0)		18 (32.7)	118 (45.9)	
Multifocality						
No	81 (95.3)	199 (94.3)	1	50 (96.2)	240 (93.4)	0.751
Yes	4 (4.7)	12 (5.7)		2 (3.8)	17 (6.6)	
pT						
2-5cm	55 (66.3)	114 (54.3)	0.186	30 (60.0)	142 (55.9)	0.804
≤2cm	23 (27.7)	78 (37.1)		16 (32.0)	94 (37.0)	
>5cm	5 (6.0)	18 (8.6)		4 (8.0)	18 (7.1)	
pN						
0	23 (27.4)	74 (35.7)	0.219	17 (33.3)	86 (34.3)	0.408
1-3	29 (34.5)	74 (35.7)		15 (29.4)	94 (37.4)	
≥4	32 (38.1)	59 (28.6)		19 (37.3)	71 (28.3)	
Histological type						
Ductal	59 (69.4)	171 (81.0)	**0.008**	33 (63.5)	207 (80.5)	**<0.001**
Apocrine	2 (2.4)	6 (2.8)		2 (3.8)	7 (2.7)	
Lobular	9 (10.6)	2 (0.9)		10 (19.2)	3 (1.2)	
Medullary	5 (5.9)	7 (3.3)		3 (5.8)	10 (3.9)	
"Atypical Medullary"	3 (3.5)	5 (2.4)		1 (1.9)	7 (2.7)	
Metaplastic	5 (5.9)	12 (5.7)		1 (1.9)	14 (5.4)	
Other	2 (2.4)	8 (3.8)		2 (3.8)	9 (3.5)	
Histological grade						
I-II	18 (21.4)	33 (15.7)	0.239	20 (39.2)	36 (14.1)	**<0.001**
III	66 (78.6)	177 (84.3)		31 (60.8)	220 (85.9)	
Basal						
Yes	57 (68.7)	192 (91.9)	**<0.001**	28 (54.9)	228 (90.1)	**<0.001**
No	26 (31.3)	17 (8.1)		23 (45.1)	25 (9.0)	
Adjuvant chemotherapy						
No		9 (4.3)	0.064		10 (3.9)	0.222
Yes	85 (100.0)	202 (95.7)		52 (100.0)	247 (96.1)	
Adjuvant hormonal therapy						
No	66 (77.6)	176 (83.4)	0.249	38 (73.1)	214 (83.3)	0.115
Yes	19 (22.4)	35 (16.6)		14 (26.9)	43 (16.7)	
Taxanes						
No	6 (7.4)	19 (9.4)	0.817	5 (10.0)	22 (8.9)	0.789
Yes	75 (92.6)	184 (90.6)		45 (90.0)	225 (91.1)	

cadherin was not a very common phenomenon, while, surprisingly, the majority of E-cadherin positive carcinomas also expressed P-cadherin, which is a marker of basal/myoepithelial cells (34, 42). This mixed E/P-cadherin phenotype has been recently proposed as a sign of cadherin-catenin complex destabilization present in cells that undergo a transient state of EMT (17). Such cells would be characterized by increased plasticity and metastastability and could be candidates for therapy with P-cadherin antagonists (43). Our findings confirm the existence of this co-expressor phenotype in two thirds of the TNBC cases analyzed.

Regarding Myc, we used the cut-off from a previous retrospective study to define overexpression, because it closely represented the average expression of this marker in adjacent non-neoplastic cells in our series (34). Overexpression was observed in a minority of tumors and was unrelated to patient outcome. Moreover, we could not establish an association between Myc and beta-catenin protein expression. The exact mode of MYC regulation by Wnt pathway members in breast cancer requires further investigation and observational studies are perhaps of limited value in this regard, due to the complexity arising from the

Table V. *Associations between Wnt-related markers and clinicopathological variables.*

	m/beta-catenin			n/beta-catenin			Myc		
	High (%)	Low (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value
Age									
<52.8	67 (49.3)	70 (50.7)	0.904	130 (49.4)	7 (63.6)	0.54	120 (49.2)	14 (46.7)	0.848
≥52.8	69 (50.7)	68 (49.3)		133 (50.6)	4 (36.4)		124 (50.8)	16 (53.3)	
Menopausal status									
post	78 (55.7)	76 (55.1)	0.914	149 (55.8)	5 (45.5)	0.499	134 (54.0)	19 (63.3)	0.333
pre	62 (44.3)	62 (44.9)		118 (44.2)	6 (54.5)		114 (46.0)	11 (36.7)	
Multifocality									
No	129 (93.5)	131 (93.6)	1	249 (93.3)	11 (100.0)	1	234 (94.4)	27 (90.0)	0.408
Yes	9 (6.5)	9 (6.4)		18 (6.7)			14 (5.6)	3 (10.0)	
pT									
≤2cm	46 (33.6)	47 (34.3)	0.083	92 (34.8)	1 (10.0)	0.192	82 (33.5)	14 (48.3)	0.058
2-5cm	84 (61.3)	73 (53.3)		149 (56.4)	8 (80.0)		145 (59.2)	11 (37.9)	
>5cm	7 (5.1)	17 (12.4)		23 (8.7)	1 (10.0)		18 (7.3)	4 (13.8)	
pN									
0	45 (33.3)	46 (33.6)	0.298	85 (32.6)	6 (54.5)	0.34	84 (34.6)	13 (44.8)	0.481
1-3	53 (39.3)	43 (31.4)		93 (35.6)	3 (27.3)		90 (37.0)	8 (27.6)	
≥4	37 (27.4)	48 (35.0)		83 (31.8)	2 (18.2)		69 (28.4)	8 (27.6)	
Histological type									
Ductal	110 (79.7)	104 (74.3)	0.107	209 (78.3)	5 (45.5)	**0.006**	189 (76.2)	25 (83.3)	0.237
Apocrine	6 (4.3)	2 (1.4)		8 (3.0)			8 (3.2)		
Lobular	1 (0.7)	8 (5.7)		6 (2.2)	3 (27.3)		7 (2.8)	2 (6.7)	
Medullary	5 (3.6)	8 (5.7)		13 (4.9)			12 (4.8)	1 (3.3)	
"Atypical Medullary"	4 (2.9)	3 (2.1)		6 (2.2)	1 (9.1)		5 (2.0)	2 (6.7)	
Metaplastic	6 (4.3)	11 (7.9)		16 (6.0)	1 (9.1)		17 (6.9)		
Other	6 (4.3)	4 (2.9)		9 (3.4)	1 (9.1)		10 (4.0)		
Histological grade									
I-II	22 (16.1)	23 (16.7)	1	39 (14.7)	6 (60.0)	**0.002**	36 (14.6)	10 (33.3)	**0.017**
III	115 (83.9)	115 (83.3)		226 (85.3)	4 (40.0)		210 (85.4)	20 (66.7)	
Basal									
Yes	127 (92.0)	109 (77.8)	**<0.001**	230 (86.1)	6 (55.0)	**0.014**	217 (87.5)	26 (86.7)	0.778
No	11 (8.0)	31 (22.2)		37 (13.9)	5 (45.0)		31 (12.5)	4 (13.3)	
Adjuvant chemotherapy									
No	6 (4.3)	4 (2.9)	0.539	10 (3.7)		1	6 (2.4)	4 (13.3)	**0.015**
Yes	132 (95.7)	136 (97.1)		257 (96.3)	11 (100.0)		242 (97.6)	26 (86.7)	
Adjuvant hormonal therapy									
No	112 (81.2)	118 (84.3)	0.528	221 (82.8)	9 (81.8)	1	201 (81.0)	29 (96.7)	**0.038**
Yes	26 (18.8)	22 (15.7)		46 (17.2)	2 (18.2)		47 (19.0)	1 (3.3)	
Taxanes									
No	11 (8.2)	15 (11.4)	0.416	21 (8.2)	5 (50.0)	**0.001**	19 (7.9)	5 (19.2)	0.068
Yes	123 (91.8)	117 (88.6)		235 (91.8)	5 (50.0)		222 (92.1)	21 (80.8)	

biological role of Myc as a downstream effector of diverse signaling cascades (44).

The present work carries the advantage of using material from adjuvant clinical trials in which patients received uniform treatment. However, tumors themselves were of diverse histological types some of which, such as metaplastic and medullary carcinomas, are known to predict for distinct outcomes. In the present dataset though, a statistically significant difference in patient outcome between tumors of different histologies could not been shown, while the prognostic effect of protein markers was not different when comparing the entire dataset with the more homogeneous ductal NST carcinomas. We chose not to investigate the distribution of histological types within the four EGFR/beta-catenin combined phenotypes, because the size of the resulting groups with the exception of carcinomas of NST would have been too small to allow for meaningful statistical considerations. Therefore, the nature of potential associations between metaplastic or medullary carcinomas and the herein described EGFR-positive and beta-catenin-low phenotype remains elusive and may be worth investigating in studies with sample sizes large enough to sustain multi-level subgroup

Table VI. *Associations between Wnt-related and other phenotypical markers.*

	n/beta-catenin			m/beta-catenin			Myc		
	Neg (%)	Pos (%)	*p*-Value	High (%)	Low (%)	*p*-Value	Neg (%)	Pos (%)	*p*-Value
CK5									
Neg	72 (27.1)	5 (45.5)	0.19	29 (21.2)	48 (34.3)	**0.016**	65 (26.2)	7 (23.3)	0.83
Pos	194 (72.9)	6 (54.5)		108 (78.8)	92 (65.7)		183 (73.8)	23 (76.7)	
EGFR									
Neg	110 (41.2)	8 (72.7)	0.06	42 (30.4)	76 (54.3)	**<0.001**	97 (39.3)	15 (50.0)	0.33
Pos	157 (58.8)	3 (27.3)		96 (69.6)	64 (45.7)		150 (60.7)	15 (50.0)	
E-cadherin									
Neg	62 (24.6)	7 (63.6)	**0.009**	21 (15.6)	48 (37.5)	**<0.001**	61 (25.6)	9 (31.0)	0.51
Pos	190 (75.4)	4 (36.4)		114 (84.4)	80 (62.5)		177 (74.4)	20 (69.0)	
P-cadherin									
Neg	36 (14.1)	6 (54.5)	**0.003**	9 (6.7)	33 (25.0)	**<0.001**	35 (14.5)	8 (27.6)	0.1
Pos	220 (85.9)	5 (45.5)		126 (93.3)	99 (75.0)		207 (85.5)	21 (72.4)	

Table VII. *Associations between basal and EMT-related markers.*

	CK5			EGFR			E-cadherin			P-cadherin		
	Neg	Pos	*p*-Value	Neg	Pos	*p*-Value	Neg	Pos	*p*-Value	Neg	Pos	*p*-Value
CK5												
Neg				52 (34.9)	41 (22.7)	**0.019**	29 (35.4)	49 (23.4)	0.055	29 (56.9)	55 (21.9)	**<0.001**
Pos				97 (65.1)	140 (77.3)		53 (64.6)	160 (76.6)		22 (43.1)	196 (78.1)	
EGFR												
Neg	52 (55.9)	97 (40.9)	**0.019**				52 (63.4)	74 (35.4)	**<0.001**	35 (70.0)	97 (38.3)	**<0.001**
Pos	41 (44.1)	140 (59.1)					30 (36.6)	135 (64.6)		15 (30.0)	156 (61.7)	
E-cadherin												
Neg	29 (37.2)	53 (24.9)	0.055	52 (41.3)	30 (18.2)	**<0.001**				42 (85.7)	40 (16.9)	**<0.001**
Pos	49 (62.8)	160 (75.1)		74 (58.7)	135 (81.8)					7 (14.3)	197 (83.1)	
P-cadherin												
Neg	29 (34.5)	22 (10.1)	**<0.001**	35 (26.5)	15 (8.8)	**<0.001**	42 (51.2)	7 (3.4)	**<0.001**			
Pos	55 (65.5)	196 (89.9)		97 (73.5)	156 (91.2)		40 (48.8)	197 (96.6)				

analyses. Another limitation of our study may come from the use of TMAs, which may have generated lower frequencies than in corresponding whole sections for markers with a focal and heterogeneous expression pattern such as nuclear beta-catenin. Of note, we did not specifically sample the tumor invasive front or the mesenchymal component of metaplastic carcinomas, which may be enriched with cells with nuclear expression. In addition, the use of a cohort-specific cut-off such as the median of the H-score for membrane beta-catenin represents a limitation for the reproducibility of our findings. Finally, we chose not to evaluate cytoplasmic beta-catenin, due to previously addressed technical limitations (13). However, cytoplasmic expression could have biological implications for patient outcome, because it may be a sign of compromised protein degradation (16).

Conclusion

The present work investigated the expression of six markers by IHC, namely those used for the definition of the basal-like phenotype of TNBC (EGFR and CK5), two with important roles in the Wnt pathway (beta-catenin and Myc) and two related to cell adhesion (E- and P-cadherin) in a large series of TNBC from patients enrolled in adjuvant clinical trials. We confirmed recent observations that a subset of TNBC may be characterized by a unique pattern of E- and P-cadherin co-expression. Although no marker was individually associated with patient outcome, we identified an interesting prognostic interaction between EGFR and beta-catenin, which may deserve validation in independent datasets. The molecular heterogeneity of TNBC is also

Table VIII. *Univariate analysis*.

		DFS			OS		
		HR	95%CI	Wald's p	HR	95%CI	Wald's p
Age (median)	>=52.8 *vs*. <52.8	0.820	0.561-1.200	0.307	0.966	0.634-1.473	0.874
Menopausal status	post *vs*. pre	1.044	0.713-1.529	0.824	0.852	0.556-1.306	0.462
Multifocality	Yes *vs*. No	1.139	0.529-2.453	0.739	1.620	0.747-3.514	0.222
pT	2-5cm *vs*. <=2cm	1.419	0.913-2.206	0.119	1.718	1.030-2.866	0.038
	>5cm *vs*. <=2cm	3.041	1.690-5.472	0.001	3.733	1.944-7.168	<0.001
pN	1-3 *vs*. 0	0.980	0.549-1.750	0.946	1.117	0.555-2.249	0.757
	>=4 *vs*. 0	3.316	2.032-5.410	<0.001	4.318	2.395-7.785	<0.001
Histological type	Ductal *vs*. Apocrine	0.660	0.268-1.627	0.367	0.649	0.236-1.780	0.401
	Lobular *vs*. Apocrine	0.969	0.316-2.969	0.956	1.218	0.366-4.056	0.748
	Medullary *vs*. Apocrine	<0.001	<0.001-.	0.977	<0.001	<0.001-.	0.980
	"Atypical Medullary" *vs*. Apocrine	0.198	0.023-1.700	0.140	0.257	0.029-2.297	0.224
	Metaplastic *vs*. Apocrine	0.491	0.132-1.828	0.289	0.664	0.166-2.656	0.563
	Other *vs*. Apocrine	0.465	0.090-2.396	0.360	0.331	0.037-2.963	0.323
Histological grade	III *vs*. I-II	1.152	0.699-1.898	0.580	0.997	0.586-1.698	0.993
Adjuvant chemotherapy	Yes *vs*. No	0.275	0.128-0.593	0.001	0.191	0.088-0.416	<0.001
Adjuvant hormonal therapy	Yes *vs*. No	1.550	1.016-2.365	0.042	1.648	1.044-2.599	0.032
Taxanes	Yes *vs*. No	0.311	0.197-0.492	<0.001	0.301	0.182-0.497	<0.001
CK5	Pos *vs*. Neg	1.028	0.663-1.594	0.901	0.915	0.571-1.468	0.714
EGFR	Pos *vs*. Neg	0.966	0.660-1.415	0.859	1.056	0.691-1.615	0.800
Basal	Yes *vs*. No	1.186	0.718-1.958	0.506	1.194	0.687-2.077	0.529
m/beta-catenin (median=55)	Low *vs*. High	1.443	0.930-2.240	0.102	1.328	0.814-2.165	0.255
n/beta-catenin	Pos *vs*. Neg	1.992	0.864-4.591	0.106	1.402	0.508-3.866	0.514
Myc	Pos *vs*. Neg	1.025	0.513-2.049	0.945	1.396	0.690-2.823	0.354
E-cadherin	Pos *vs*. Neg	1.045	0.658-1.661	0.851	1.068	0.637-1.789	0.804
P-cadherin	Pos *vs*. Neg	1.194	0.676-2.110	0.541	1.144	0.617-2.122	0.669

evident at the level of protein expression and may explain failure to identify markers with undisputed prognostic significance.

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Conflicts of Interest

The Authors declare that they have no conflict of interest.

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