Immunoexpression of claudins 4 and 7 among invasive breast carcinoma subtypes: A large diagnostic study using tissue microarray

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Abstract. Molecular phenotyping and tissue microarray (TMA) studies have identified distinct invasive breast carcinoma subtypes: Luminal A, luminal B, enriched with overexpressed human epidermal growth factor receptor 2 (HER-2) and triple-negative, i.e., negative for HER-2, as well as for estrogen and progesterone receptor (ER and PR, respectively) expression. These subtypes are useful in clinical management, since they bear distinct prognoses and predictive responses to targeted therapy. However, although molecular profiling provides important prognostic indicators, breast cancer risk stratification remains a challenge in triple-negative cases. What is referred to as claudin-low subtype was identified as a triple-negative subset that is associated with more aggressive tumor behavior and worse prognosis. However, the immunohistochemical expression of claudins has not yet been standardized. Our objective was to verify whether the immunoexpression of claudins 4 and 7 (the main claudins specifically expressed in human breast tissue) in TMA is associated with survival and prognosis in luminal A, HER-2 and triple-negative molecular subtypes. In this diagnostic study, we investigated ER/PR receptor status, HER-2, claudin 4 and 7 expression and stem cell CD44/24 profiles, and verified the association with prognosis and survival outcomes in 803 invasive breast carcinoma cases arranged in four TMAs. Among these, 503 (62.6%) were positive for claudin 4 and 369 (46.0%) for claudin 7. Claudin 4 exhibited the lowest expression in luminal A and triple-negative subtypes, and the highest frequency of expression in HER-2-enriched subtypes, whereas claudin 7 staining was not associated with any subtype. The stem cell phenotype was not associated with subgroups or claudins 4 and 7. Claudin immunoexpression profile was not able to distinguish between patients with better or worse prognosis, and it was not correlated to triple-negative cases. Therefore, it may be concluded that the immunoexpression of claudins 4 and 7, individually or within the usual immunohistochemical context (ER, PR and HER-2), does not provide additional prognostic information on breast cancer subtypes.

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Introduction

The possibility of applying targeted therapies in oncology introduced a new era of tailor-made treatments for breast cancer. The currently available therapies are indicated based on the morphological and genetic characteristics of the tumor (1,2). Novel molecular techniques of gene expression profiling and microarray analysis enabled the identification of distinct invasive breast cancer (IBC) molecular subtypes, such as luminal A, luminal B, tumors enriched with human epidermal growth factor receptor 2 (HER-2) and triple-negative tumors. Luminal A is a subtype positive for estrogen and progesterone receptors (ER and PR, respectively) and HER-2-negative, with low levels of Ki-67 expression. Luminal B is also positive for ER and PR, but negative or positive for HER-2, and with high levels of Ki-67. Triple-negative (or basal-like) tumors are ER- and PR-negative, as well as HER-2 negative. Finally, HER-2-enriched tumors are ER- and PR-negative, but HER-2-positive. These subtypes are useful in clinical management, since they bear distinct prognoses and predictive responses to targeted therapy (3-7). ER-positive tumors are associated with a better prognosis and are prone to respond well to hormone therapy (3-5) in terms of disease-free and overall survival (7,8), while patients with triple-negative tumors have a poor prognosis (6).

In clinical practice, immunohistochemical profiling including three important breast cancer markers (ER, PR and HER-2 status) is used to determine breast cancer subtypes (1,3-5,9), and this panel is currently considered the best choice for breast cancer predictive pathological evaluation (1). However, although molecular profiling provides important prognostic indicators, breast cancer risk stratification remains a challenge for certain subtypes, such as the triple-negative subtype, which is the most complex (3-6) and is usually associated with a more aggressive tumor behavior and worse prognosis (6).

Research efforts in molecular microarray sub-stratification identified new subcategories within triple-negative cancers (6,10). Among those, the 'claudin-low' subcategory has emerged as one of the most aggressive breast cancer profiles (11-13), being correlated to an enriched stem-cell component phenotype (11,12,14).

Claudins are cell adhesion molecules and components of the tight junction complex, which regulate permeability between cells and maintain cell-cell integrity (15,16), and their role in carcinogenesis and progression to metastasis currently constitutes an active research focus (17). Claudins are known to act as barriers for the diffusion of solutes between epithelial cells, participating in the regulation of water transportation, ions and certain macromolecules. Therefore, it is a reasonable hypothesis that claudins are likely to be involved in carcinogenesis (18).

The absence or low expression of claudins is associated with cancer development and reduced survival, due to the disruption of these tight junctions (19,20). The 'claudin-low' subtype of breast cancer is characterized by the reduced expression of claudin mRNAs, mainly claudins 3, 4 and 7 (11,12,14,20). Claudin expression deregulation has been previously implicated in cancer and is associated with metastasis and cancer progression (15,17), and low expression of claudins has been correlated to worse prognosis in breast cancer when assessed by RNA-based tests (14,17).

However, the claudin superfamily of integral membrane proteins is composed of numerous isotypes (15,16,18), and there is some heterogeneity in study results when claudin subtypes are evaluated. Claudin 1 has been identified as a novel survival factor in basal-like breast cancers, as downregulation of claudin 1 was shown to induce cell death in breast cancer cell lines (17). High claudin 4 expression has been associated with worse breast cancer-specific, recurrence-free and overall survival, even in patients receiving adjuvant tamoxifen therapy (21). In triple-negative breast cancer cases, downregulation of claudin 7 (13,22) or a combination of high expression of CLDN4 with low expression of CLDN7, were identified as predictive factors of worse prognosis (19).

In this context, the aim of the present study was to verify whether the addition of claudin immunoexpression evaluation to the immunohistochemical profile standard panel for patients with breast cancer would improve its prognostic assessment potential. Our objective, therefore, was to determine whether the expression of claudins 4 and 7 (the main claudins specifically expressed in human breast tissue) in tumor microarray (TMA) analysis would be associated with survival and prognostic outcomes in a large cohort of patients with breast tumors of different molecular subtypes (luminal A, HER-2 and triple-negative).

Materials and methods

Design, setting and ethics. This diagnostic study was conducted in a large referral center for cancer treatment in Brazil, a philanthropic hospital, following approval of the local Ethics Committee. The hospital obtained informed consent forms from all patients and controls for using archived tissues for research at any time. Patient anonymity was guaranteed.

Tumor samples and clinical data. The study used archived tumor samples from all consecutive female patients who were diagnosed with IBC of no specific type (not otherwise specified, NOS) between January 1980 and December 2001 in Hospital A.C. Camargo (São Paulo, SP, Brazil). Patient characteristics are presented in Table I. Among these tumor samples, formalin-fixed and paraffin-embedded samples with paraffin blocks suitable for immunohistochemistry analysis, with a registry of clinical data in the medical records and follow-up information, were selected.

From these cases, four TMA blocks were built for analysis, as described below. Two experienced pathologists (AFL and FAS) reviewed all cases to confirm the diagnosis, histological grade and the nuclear grade according to the criteria of the Nottingham system (23), and cases were individually discussed to reach consensus when necessary.

TMA construction. The construction of TMAs followed standard procedures, as previously described (24). Briefly, 1-mm diameter cylinders from selected tumor areas of the donor paraffin blocks were extracted and inserted in order into the receptor blocks (Beecher Instruments, Silver Spring, MD, USA). Each case was sampled twice and all cylinders were distributed in four new blocks and stored at 4˚C. Subsequently, 4-μm sections were prepared for each marker for immunohistochemistry analysis. Healthy breast tissues were also collected and confirmed as negative for breast cancer on microscopic analysis to be used as controls (n=4, aged 50-75 years). These negative controls were selected among patients who had undergone plastic surgery for aesthetic purposes, and whose examination results excluded cancer.

Immunohistochemical staining. Each TMA slide was stained with the following antibodies: Anti-ER (clone SP1; rabbit monoclonal, monoclonal, anti-human estrogen receptor, clone SP1; cat. no. MA1-39540; 1:50; Thermo Fisher Scientific, Inc., Waltham, MA, USA), anti-PR (clone PgR636; monoclonal mouse anti-human progesterone receptor; Clone PgR 636; cat. no. M3569; 1:500; Dako Agilent Technologies, Inc., Santa Clara, CA, USA), anti-HER-2 (polyclonal rabbit anti-human; c-erbB-2 Oncoprotein; cat. no. A048529-2; 1:2,000; Dako; Agilent Technologies, Inc), claudin 7 (polyclonal rabbit anti human; cat. no. PA1-37474; 1:400), claudin 4 (claudin 4 monoclonal antibody anti human clone 3E2C1; cat. no. 32-9400; 1:200, and claudin 4 polyclonal antibody anti human clone ZMD.306; cat. no. 36-4800; 1:200) (all from Invitrogen; Thermo Fisher Scientific Inc.), CD44 (monoclonal antibody anti human clone DF1485, 1:100; cat. no.: MAB5315; 1:100; Abnova, Taipei, Taiwan) and CD24 (monoclonal antibody anti human clone C-20; 1:100; cat. no. SC7034 clone C-20; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Table I. Clinical and pathological characteristics of 803 breast cancer patients.

Characteristics	Patient no. (%)
Age, years [median (range)]	54 (24-96)
Hormonal status	
Postmenopausal	317 (39.5)
Pathological staging	486 (60.5)
II	395 (49.2)
III	350 (43.2)
IV	58 (7.2)
Histological grade	
I	103 (12.2)
II	480 (59.8)
III	218 (27.1)
Missing data	2 (0.2)
Nuclear grade	
1	6 (0.7)
2	249 (31.0)
3	546 (68.0)
Tumor size	
T1+T2	394 (49.1)
T3+T4	409 (50.9)
Lymph node metastasis	
pN0	268 (33.1)
pN+	524 (65.3)
Missing data	12 (1.1)
Molecular subgroup of the primary tumor	
Luminal A	500 (62.3)
HER-2	114 (14.2)
Triple-negative	179 (22.3)
Missing data	10 (1.2)

Luminal A, ER- and/or PR-positive, HER-2-negative; HER-2, HER-2 positive; triple-negative, ER-, PR- and HER2-negative; pN0, no regional lymph node metastasis identified histologically; pN+, malignant cells in regional lymph nodes.

In order to standardize the immunohistochemical staining for the primary antibodies, the antigen retrieval method (equipment for humid heat, pH and type of buffer), the dilution of primary antibodies and the visualization system were optimized. Paraffin-embedded sections and breast tumor array sections were deparaffinized in xylene and rehydrated through graded ethanols.

For deparaffinization, the slides containing the histological sections were kept in the oven for 24 h at 60˚C and then were immersed in xylene at 60˚C for 20 min. They were then immersed in xylol at room temperature for 20 min and then rehydrated in ethanol in decreasing concentrations: 100% for 30 sec, 85% for 30 sec and 70% for 30 sec.

Following deparaffinization and rehydration of the TMA sections, antigen retrieval was performed in a pressure cooker (until boiling, which occurs at ~60˚C in São Paulo) using sodium citrate buffer (pH6) as retrieval solution. Following primary antibody incubation (for 0 min at 25˚C) and a polymer-peroxidase (Leica Microsystem GmbH, Wetzlar, Germany) amplification step was performed. No secondary antibody was employed. Antigen detection was carried out in a solution containing 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 6% H_2O_2.

Counterstaining with Harris hematoxylin was performed, at 25˚C for 30 sec including positive controls in each staining reaction. The positive controls consisted of normal breast and neoplastic tissue known to express each of the antigens of interest. The primary antibody was omitted as a negative control in the same sample. All slides were observed in a light microscope (DM300; Leica Microsystems GmbH).

Immunohistochemistry analysis. In order to classify the tumors into breast cancer subtypes, ER and PR were evaluated according to Allred scoring (25). Immunoreactivity ≤2 (or <1% of positive tumor cells) was considered as a negative result for ER and PR.

Luminal A was considered as ER- and PR-positive and HER-2-negative, with low levels of Ki-67 expression (<14%); luminal B was considered as positive for ER and PR, positive for HER-2 (3+/3), with a Ki-67 index >14%; triple-negative (or basal-like) tumors were ER-, PR- and HER-2-negative.

For HER-2 samples, the presence of reactivity in <10% of the tumor cells was scored as 0, and cases in which there was barely perceptible focal membranous staining were scored as 1; weak to moderate staining observed in >10% of the tumor cells was scored as 2, and a strong complete membranous staining continuously in >10% of the tumor cells was scored as 3. We considered the result to be positive only if the score was 3, according to American Society of Clinical Oncology of American Pathologists recommendations update (26,27).

In the CD24/44 evaluation, the specimens with <10% of positive cells were considered as CD24/44-negative, and as positive when the membranes were stained in a distinct, thin pattern, limited to the membrane, without cytoplasmic or nuclear reactivity for CD44, CD24, claudins 4 and 7. CD24 is mainly detected in the cytoplasm. A HercepTest model was used for reporting results, and the scoring was as follows: 0, totally negative; 1+, 1-10% positive neoplastic cells; 2+, moderate staining in 10-30% of neoplastic cells; and 3+, >30% of strongly reactive neoplastic cells. Normal breast tissue usually exhibits a 2+ pattern of claudin 7 expression and 1+ or 0 for CD24 and CD44. Therefore, 3+ was considered as overexpression of these markers in the present study.

As regards the claudin expression detection and classification methods, our analysis was based on the Lanigan *et al* study (21), which reported a 0-3+ pattern of claudin expression, and also previous studies of our group (28), stating that 'CD44 and claudin-7 were considered positive when membranes were stained in a distinct and delicate pattern without reactive cytoplasm or nuclei. We used a HercepTest model for reporting results and the scoring was: 0, totally negative; 1+, 1-10% positive neoplastic cells; 2+, moderate staining in 10-30% of neoplastic cells; and 3+, >30% of strongly reactive neoplastic cells. CD24 was detected mainly in the cytoplasm and scoring was conducted as for CD44.' This assay was performed once per patient.

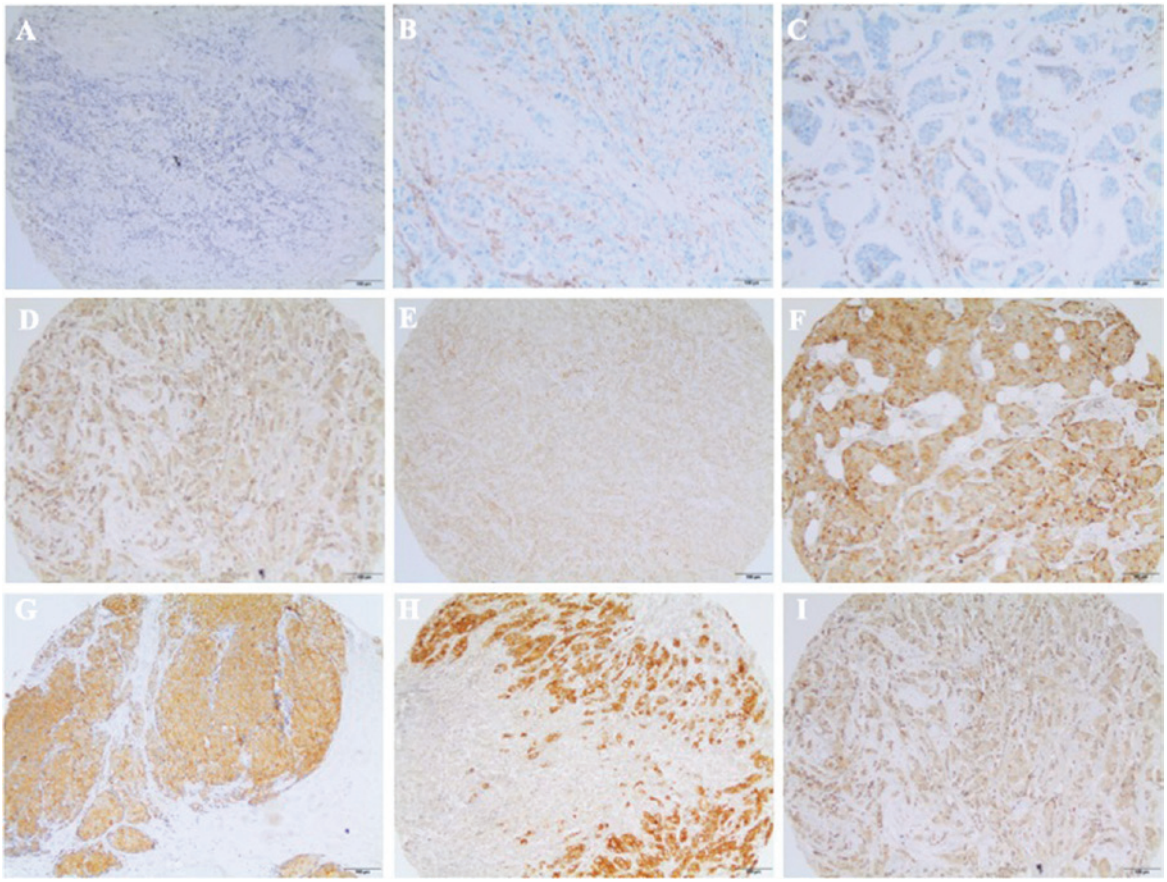

Figure 1. Examples of CD24, CD44 and claudin 4 and 7 immunostaining in mammary invasive carcinomas (scale bar, 100 μm). (A) Claudin-negative; (B) CD24-negative; (C) CD44-negative; (D) claudin 4 2+; (E) claudin 7 2+; (F) CD24-positive; (G) claudin 4 3+; (H) claudin 7 3+; (I) CD44-positive.

Statistical analysis. Data were presented as numbers (n) and percentage of cases. Associations between the immunohistochemical expressions and important clinicopathological variables were investigated using the Chi-squared test (χ^2). Survival probabilities were estimated by the univariate Kaplan-Meier method for univariate analysis and survival curves were compared using the log-rank test (Mantel-Haenszel method). SSPS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the analyses.

Results

Clinicopathological characteristics of the patients. During the study period, 880 patients with IBC were identified in our archives, and tissue samples and follow-up data were available for 803 of those patients. Further analysis of immunostained samples allowed for inclusion of 793 suitable cases in this study. The distribution of cases according to the clinical and pathological variables and ER, PR and HER-2 immunoexpression is shown in Table I. The present series included 500 cases with a luminal profile, 114 with HER-2 expression, and 179 triple-negative cases. In all these IBC cases, treatment involved mastectomy, radiotherapy and axillary lymph node dissection. ER-positive patients received hormone therapy, and the remaining were treated with chemotherapy. The median follow-up period was 70 months. At the final follow-up (July, 2007), 425 patients remained alive and 378 had succumbed to the disease.

Claudin expression in primary breast carcinomas. The claudin status according to immunohistochemistry expression is presented in Table II. The majority of cases (62.6%) exhibited preserved claudin 4 or 7 expression and 46% were claudin 7-positive. Over 73% of the cases were positive for claudins 4 or 7, and only 15.7% were negative for both claudins. Claudin 4 staining was restricted to epithelial cells and concentrated at the cell membrane, whereas claudin 7 exhibited a non-diffuse and punctate distribution. Examples of CD24, CD44 and claudin 4 and 7 immunostaining are shown in Fig. 1.

CD24/44 expression analysis revealed only 10.2% of cases with stem cell profile (CD44-positive/CD24-negative). The pattern of CD44 expression was mainly membranous, whereas CD24 was expressed predominantly in the cytoplasm.

No associations were observed between claudin 7 expression and the presence of ER/PR and HER-2. Claudin 7 expression was also not found to be associated with clinical or pathological variables, such as stage, tumor size or lymph node status in TMA analysis (Tables III and IV).

However, claudin 4 expression exhibited a direct association with histological and nuclear grade. The same progressive accumulation of claudin 4-positive cases was associated with lymph node status, meaning that, proportionally, more claudin 4-positive cases were concentrated at the positive lymph node category (Table III).

Among the ER-positive cases, which comprised the majority of the patients, 60.5% were claudin 4-positive.

However, almost 70% of the ER-negative cases were also associated with preserved claudin 4 expression (P=0.01). Similarly, although 53.7% of the PR-positive cases were claudin 4-positive, a high number of tumors (67.1%) stained positive in the PR-negative samples (P=0.037). However, positive claudin 4 expression was more prevalent among HER-2 positive tumors (P<0.001) (Tables III and IV).

The frequency of claudin 4 and 7 expression in several combinations of CD44 and CD24 phenotypes was next analyzed. Claudin 4 expression exhibited no differences in frequency between possible combinations, while claudin 7 positivity was associated with the $CD44^+/CD24^+$ and $CD44^-/CD24^+$ phenotypes in the sample (P=0.001) (Tables III and IV).

The expression of claudins 4 and 7 in breast carcinomas was examined according to previously defined subtypes of breast cancer patients. Claudin 4 was less expressed in the luminal A and triple-negative subtypes and exhibited the highest frequency of positive expression in HER-2-enriched tumors, whereas claudin 7 positivity was not associated with any of the breast cancer subtypes (Tables IV and V). When the presence of the $CD44^+/CD24^-$ phenotype was investigated, we observed that the stem cell phenotype was not related to the defined subgroups.

We then investigated the possible prognostic value of claudin 4 and 7 expression. Considering all patients, claudin immunoexpression profile was not able to distinguish between patients with better or worse prognosis. As seen in Tables VI and VII, claudin 4 and 7 expression profiles were not associated with overall survival or disease-free interval. Among triple-negative cases (Table VIII) and luminal cases (Table IX), the immunoexpression of claudin 4 and 7 did not indicate clinical evolution; this was also true for the HER-2 subgroup (Table X; P=0.09).

Table II. Frequency of claudin 4 and 7 and CD44/CD24 protein expression in primary breast carcinomas.

Biomarkers	Patient no. (%)
Claudin 4	
Negative	288 (35.9)
Positive	503 (62.6)
Missing data	12 (1.5)
Claudin 7	
Negative	355 (44.2)
Positive	369 (46.0)
Missing data	79 (9.8)
CD44/CD24	
-/-	285 (35.5)
+/+	126 (15.7)
-/+	256 (31.9)
+/- (stem cells)	82 (10.2)
Missing data	54 (6.7)

Discussion

In the present study, 803 IBCs were evaluated for claudin 4 and 7 immunohistochemical expression, in search for the possible role of these claudins as prognostic indicators, beyond breast cancer subtype profiling. The distribution of subtypes in our sample (62% luminal, 14% HER-2-positive and 22% triple-negative) reflected the distribution in the literature (4,29). However, in the present study, what is referred to as 'claudin-low' status (herein defined by the low expression of claudins 4 and 7) was not found to be associated with the triple-negative-like IBC subtype. We consider the status of any individual claudin to play a limited role in determining prognosis or providing predictive information in luminal A, HER-2 and triple-negative breast cancer subtypes, whereas it is of little value to the routine immunohistochemistry panel investigation for patients with breast cancer. Although suggested to act as a tumor supressor, high levels of claudin 1 have been observed in the aggressive triple-negative or basal-like cancer subtype (30,31); in addition, the downregulation of claudin 1 has been found to be associated with ER positivity and poor prognosis (31,32).

Indeed, the individual expression of claudins has been reported to be limited and controversial (33). Claudins 4 and 7 are the most commonly identified in breast tissue (33), and both have been reported as potential markers for clinical outcomes (21,31,34-37). In the present study, although the majority of the evaluated IBC cases exhibited preserved claudin 4 and 7 expression, (62 and 46%, respectively) the distribution of the claudin-low or negative cases did not correspond to the triple-negative molecular profile. Only 15.7% of the patients in this study were negative for both claudins, and this frequency is consistent with that reported in the literature (11,14,38,39).

Claudin 4-negative cases (36% of our total cases) were more common among the luminal profile cases (ER^+, PR^+ and $HER\text{-}2^-$), with a smaller amount in the triple-negative subtype, followed by the HER-2 expression enriched subgroup. The same pattern was verified on negative claudin 7 results, although without statistical significance. The most important finding in this study is the inverse association between claudin 4 and ER/PR expression, which indicates that he majority of claudin-negative cases (71.3%) were actually clustered among the ER-positive cases, or luminal subtype. Szasz *et al* (40) also suggested that claudin 4 was less expressed in luminal cancers.

Myal *et al* (41) proposed a 'claudin-high' subtype, which comprises ER-negative tumors exhibiting high claudin 4 expression. Some studies (21,30,36,39) reported that claudin 4 was associated with shorter disease-free survival in the luminal cancer group, an association not confirmed in our series. However, this feature must be further investigated in multivariate analyses in order to estimate the possible contribution of other prognostic variables in the association.

However, Lu *et al* (13) defined different criteria for considering cases as 'claudin-low': For these authors, five subtypes of claudins (1,3,4,7 and 8) should be altered for the status to be named 'claudin-low', and the authors reported that only 3% of claudin-low tumors belonged to the luminal phenotype. However, in the evaluation of other claudin-low characteristics in our study, such as the cancer stem cell-like phenotype in the negative cases, no association was found between the absence of claudin 4 and $CD44^+/CD24^-$ and unfavorable prognostic parameters, including overall and disease-free survival.

Unexpectedly, previous results associating low claudin 4 expression with a better prognosis, particularly in

Table III. Correlation between the expression of biomarkers in breast carcinoma and prognostic factors.

	Markers			
	Claudin 4		Claudin 7	
Clinicopathological data	Negative	Positive	Negative	Positive
Histological grade, n (%)				
I	37 (36.6)	64 (63.4)	47 (50.0)	47 (50.0)
II	197 (41.6)	197 (58.4)	214 (49.9)	215 (50.1)
III	54 (25.2)	160 (74.8)	94 (47.0)	106 (53.0)
	P<0.001		P=0.78	
Nuclear grade, n (%)				
1	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)
2	112 (45.7)	133 (54.3)	118 (51.8)	110 (48.2)
3	173 (32.2)	365 (67.8)	235 (48.0)	255 (52.0)
	P=0.001		P=0.47	
Tumor stage, n (%)				
T1 + T2	144 (37.1)	244 (62.9)	175 (48.7)	184 (51.3)
T3 + T4	144 (35.7)	259 (64.3)	180 (49.3)	185 (50.7)
	P=0.71		P=0.88	
Lymph nodes, n (%)				
Negative	110 (41.7)	154 (58.3)	118 (49.4)	121 (50.6)
Positive	175 (34.0)	340 (66.0)	232 (48.8)	243 (51.2)
	P=0.04		P=0.94	
Pathological stage, n (%)				
II	147 (38.0)	240 (62.0)	179 (49.7)	181 (50.3)
III	118 (34.0)	229 (66.0)	158 (49.5)	161 (50.5)
IV	23 (40.4)	34 (59.6)	18 (40.0)	27 (60.0)
	P=0.44		P=0.46	
Estrogen receptor, n (%)				
Negative	82 (30.1)	190 (69.9)	130 (52.6)	117 (47.4)
Positive	204 (39.5)	313 (60.5)	224 (44.2)	251 (52.8)
	P=0.01		P=0.18	
Progesterone receptor, n (%)				
Negative	143 (32.9)	231 (67.1)	198 (49.6)	201 (50.4)
Positive	143 (40.3)	212 (53.7)	156 (48.3)	167 (51.7)
	P=0.037		P=0.76	
HER-2, n (%)				
Negative	262 (37.9)	429 (62.1)	301 (48.9)	314 (51.1)
Positive	21 (18.4)	93 (81.6)	51 (49.5)	52 (50.5)
	P<0.001		P=0.92	
CD44/CD24, n (%)				
-/-	111 (39.4)	171 (60.6)	168 (64.4)	171 (60.6)
+/+	47 (37.9)	77 (62.1)	47 (37.3)	79 (62.7)
-/+	80 (32.0)	170 (68.0)	83 (33.5)	165 (66.5)
+/-	25 (30.5)	57 (69.5)	52 (65.8)	27 (34.2)
	P=0.22		**P<0.001**	

HER-2, human epidermal growth factor receptor 2; P-values were two-sided; bold print indicates statistical significance.

patients with luminal subtypes of breast cancer, have been reported (40). Paradoxically, claudin 4 positivity in our series was associated with poor prognostic factors, such as higher tumor grade, presence of positive lymph nodes and

Table IV. Association between claudin 4 and claudin 7 expression, molecular subgroups and CD44/CD24 expression in invasive ductal breast carcinomas.

	Claudin 4-positive				Claudin 7-positive			
Variables	N (%)	OR	CI (95%)	P-value	N (%)	OR	CI (95%)	P-value
Molecular subgroup								
Luminal A	293 (58.6)	-	-		239 (65.3)	-	-	
HER-2	93 (18.6)	3.0	1.8-4.99	**<0.001**	52 (14.2)	0.91	0.59-1.40	0.68
Triple-negative	114 (22.8)	1.22	0.86-1.76	0.26	75 (20.5)	0.77	0.54-1.10	0.16
CD44/CD24								
-/-	171 (36.0)	-	-	-	93 (25.5)	-	-	
+/+	77 (16.2)	1.06	0.69-1.64	0.78	79 (21.7)	3.04	1.95-4.72	**<0.001**
-/+	170 (35.8)	1.38	0.96-1.97	0.08	165 (45.3)	3.59	2.49-5.17	**<0.001**
+/-	57 (12.0)	1.48	0.87-2.51	0.15	27 (7.4)	0.94	0.55-1.59	0.81

OR, odds ratio; CI, confidence interval; luminal A, ER- and/or PR-positive, HER-2-negative; HER-2, HER-2-positive; triple-negative, ER-, PR- and HER-2-negative. P-values were two-sided; bold print indicates statistical significance.

Table V. Distribution of protein expression pattern according to molecular groups of locally advanced invasive ductal breast carcinomas.

Variables	Luminal A	HER-2	Triple-negative	P-value
Claudin 4, n (%)				
Negative	199 (70.3)	21 (7.4)	63 (22.3)	**0.001**
Positive	293 (58.6)	93 (18.6)	114 (22.8)	
Claudin 7, n (%)				
Negative	214 (60.8)	51 (14.5)	87 (24.7)	0.37
Positive	239 (65.3)	52 (14.2)	75 (20.5)	
CD44, n (%)				
Negative	338 (61.3)	75 (13.6)	138 (25.0)	**0.02**
Positive	141 (68.1)	34 (16.4)	32 (15.5)	
CD24, n (%)				
Positive	228 (62.3)	58 (15.8)	80 (21.9)	0.62
Negative	242 (64.0)	51 (13.5)	85 (22.5)	
CD44/CD24				
+/+	171 (60.4)	45 (15.9)	67 (23.7)	0.11
+/-	84 (68.4)	21 (17.1)	18 (14.6)	
-/+	157 (61.8)	30 (11.8)	67 (26.4)	
-/-	56 (68.3)	13 (15.9)	13 (15.9)	

Luminal A, ER- and/or PR-positive, HER2 negative; HER-2, HER2-positive; triple-negative, ER-, PR- and HER-2-negative. P-values were two-sided; bold print indicates statistical significance.

ER/PR negativity, which is in agreement with previous reports (13,21,31,40).

Some prior studies have demonstrated that immunohistochemical expression of claudin 4 was associated with the basal phenotype (30,42) and triple-negative subtype (43). However, our results indicated that claudin 4 was mostly found among cases with the HER-2 subtype, and this finding is in agreement with others reports (22). These findings were substantiated by a logistic regression analysis, which found claudin 4 positivity to be significantly associated with HER-2 positivity. However, our findings were not indicative of a correlation between claudin expression and unfavorable prognosis.

There was no statistically significant association between the expression of claudin 7 and any of the clinicopathological parameters evaluated in our cases, contradicting previous studies reporting increased claudin 7 expression in ER-positive

Table VI. Univariate analysis considering overall survival of breast cancer patients.

Variables	Number of patients	Median survival (years)	95% confidence interval	P-value
Pathological stage				
II	395	Not achieved	Not determined	**<0.001**
III	350	6.0	4.9-7.1	
IV	58	2.0	1.4-2.6	
HER-2				
Negative	679	12.0	9.8-14.2	**0.008**
Positive	114	5.0	0.6-9.4	
Estrogen receptor				
Negative	275	6.0	4.2-7.8	**<0.001**
Positive	525	14.0	12.0-15.9	
Progesterone receptor				
Negative	440	8.0	6.1-9.9	**<0.001**
Positive	360	16.0	12.8-19.2	
Molecular subgroups				
Luminal A	500	14.0	12.0-15.9	**<0.001**
HER-2	114	5.0	0.6-9.4	
Triple-negative	179	6.0	3.8-8.2	
Claudin 4				
Negative	288	10.0	7.2-12.8	0.49
Positive	503	12.0	9.7-14.3	
Claudin 7				
Negative	355	11.0	8.6-13.4	0.97
Positive	369	12.0	9.3-14.7	
CD44/CD24				
-/-	285	13.0	10.3-15.8	0.65
-/+	256	11.0	8.4-13.6	
+/-	82	9.0	5.4-12.6	
+/+	126	12.0	9.5-14.5	

HER-2, human epidermal growth factor receptor 2; P-value was determined by the log-rank test; bold print indicates statistical significance.

and decreased expression in ER-negative breast tumors, or an association with high histological grade (13,34,44,45). Preliminary results of our group were previously reported in a smaller number of cases, showing that claudin 7 was associated with shorter recurrence-free interval (28). However, in the present study, which included a larger number of cases, there was no observed association of claudin 7 with worse prognosis in the univariate analysis. Other authors demonstrated a distinct prognostic role for claudins 1, 4 and 7 in other studies including a smaller number of patients (13,21,37,40).

If the triple-negative subtype is prone to exhibit lack of adhesion molecules, particularly claudins, it may be hypothesized that epithelial-to-mesenchymal transition, frequently described in this context, may explain this phenomenon. The focus of this study has been on claudins 4 and 7, and it was concluded that the absence of these two distinct types of claudins did not confer a worse prognosis or lower survival rate in our series. It appears that cell adhesion abnormalities and aggressive behavior are not dependent on one single class of these proteins (46).

Triple-negative tumors are difficult to subclassify, as they are a very heterogeneous group. Since Hennessy *et al* (11) identified a potentially highly aggressive subtype of triple-negative breast cancers as 'claudin-low', efforts have been focused on differentiating those cases from the less severe triple-negative cancers. Recently, ER, PR and HER-2, the immunohistochemically detected tumor markers that are known to be of great value in the treatment of breast cancer, were incorporated into the AJCC Breast TNM Staging System (47) to refine prognosis. It would be very useful if claudin protein expression, based on reproducible immunohistochemical assays, could accurately identify the cases with worse prognosis among all triple-negative tumors. However, it appears that the protein expression in paraffin-embedded tissues does not reflect the claudin RNA level determined by microarray and, therefore, is not considered a safe evaluation for prognosis.

As the majority of data on claudin-low breast cancers were reported among triple-negative cases, particularly in metaplastic carcinomas, we attempted to determine whether claudin expression at the protein level can differentiate cases

Table VII. Univariate analysis considering disease-free survival of breast cancer patients.

Variables	Number of patients	Median survival (years)	95% confidence interval	P-value
Pathological stage				
II	395	21.0	ND	**<0.001**
III	350	5.0	4.0-6.0	
HER-2				
Negative	632	16.0	9.8-14.2	**0.009**
Positive	106	8.0	Not determined	
Estrogen receptor				
Negative	248	8.0	2.1-13.8	**<0.001**
Positive	497	Not achieved	Not determined	
Progesterone receptor				
Negative	403	10.0	5.7-14.3	**<0.001**
Positive	342	NA	Not determined	
Molecular subgroups				
Luminal A	474	Not achieved	Not determined	**<0.001**
HER-2	106	8.0	Not determined	
Triple-negative	158	10.0	3.7-16.3	
Claudin 4				
Negative	265	14.0	9.9-18.1	0.76
Positive	469	21.0	8.0-34.0	
Claudin 7				
Negative	337	Not achieved	Not determined	0.18
Positive	342	16.0	Not determined	
CD44/CD24				
-/-	271	Not achieved	Not determined	0.93
-/+	231	Not achieved	Not determined	
+/-	78	16.0	4.8-27.2	
+/+	120	16.0	9.9-22.0	

HER2, human epidermal growth factor receptor 2; P-value was determined by the log-rank test; bold print indicates statistical significance.

Table VIII. Univariate analysis considering triple-negative breast cancer patients.

Variables	Number of patients	Median survival (years)	95% confidence interval	P-value
Overall survival				
Claudin 4				0.56
Negative	63	5.0	2.9-7.08	
Positive	114	6.0	2.3-9.6	
Claudin 7				0.82
Negative	87	6.0	3.13-8.87	
Positive	75	16.0	3.0-10.95	
Disease-free survival				
Claudin 4				0.35
Negative	57	6.0	2.2-9.8	
Positive	99	15.0	7.3-22.6	
Claudin 7				0.79
Negative	79	15.0	3.23-26.8	
Positive	63	Not achieved	Not determined	

P-value was determined by the log-rank test.

Table IX. Univariate analysis considering luminal breast cancer patients.

Variables	Number of patients	Median survival (years)	95% confidence interval	P-value
Overall survival				
Claudin 4				0.39
Negative	199	11.0	7.8-14.2	
Positive	293	14.0	11.8-16.3	
Claudin 7				0.82
Negative	214	13.0	10.3-15.8	
Positive	239	Not achieved	Not determined	
Disease-free survival				
Claudin 4				0.70
Negative	185	16.0	11.9-20.0	
Positive	281	Not achieved	Not determined	
Claudin 7				0.28
Negative	206	Not achieved	Not determined	
Positive	229	Not achieved	Not determined	

P-value was determined by the log-rank test.

Table X. Univariate analysis considering HER-2-positive breast cancer patients.

Variables	Number of patients	Median survival (years)	95% confidence interval	P-value
Overall survival				
Claudin 4				0.51
Negative	21	4.0	2.7-5.3	
Positive	93	10.0	3.4-16.6	
Claudin 7				0.27
Negative	51	11.0	Not determined	
Positive	52	4.0	0.0-8.0	
Disease-free survival				
Claudin 4				0.88
Negative	20	4.0	Not determined	
Positive	86	8.0	Not determined	
Claudin 7				0.09
Negative	50	Not achieved	Not determined	
Positive	48	3.0	1.4-4.5	

HER-2, human epidermal growth factor receptor 2; P-value was determined by the log-rank test.

with worse prognosis within breast cancer molecular subtypes. Our results demonstrated that claudin 4 and 7 status was not indicative of a better or worse outcome within breast cancer subtypes. Some of the reports linking metaplastic carcinoma to claudin expression were based on immunohistochemistry results (22). Moreover, by assessing the presence of a given protein by immunohistochemistry within the tumor, the result is ensured to reflect neoplastic cells rather than normal tissue contamination. This is particularly important for claudin identification, since the claudin-low phenotype or the absence or claudin expression as a result will not be affected by the presence of stromal desmoplastic tissue samples. Unavailability of immunohistochemistry data may hinder the evaluation of the molecular subtypes, as the materials may not be sufficient for TMA analysis (20).

In summary, our results indicated that, despite claudin 4 positivity being correlated with poor prognostic factors, such as increased tumor grade, presence of positive lymph nodes and ER/PR negativity, claudin-low cases (including low expression of claudins 4 and 7) evaluated by immunohistochemistry did not correspond to triple-negative breast cancer molecular profile. Therefore, individual evaluation of claudin expression alone may not allow for an accurate correlation with the triple-negative subtype of the disease.

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Availability of data and materials

All the data reported in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

AFL, FAS and MMB designed the study and wrote the manuscript. AFL, FSP, SN, RMR collected and analyzed data. All the authors have reviewed the manuscript critically and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was conducted following approval of the local Ethics Committee. The hospital obtained informed consent forms from all patients for using archived tissues for research at any time. Patient anonymity was guaranteed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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