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CLAUDINS 1, 3, 4, 7 AND 10-YEAR SURVIVAL IN TRIPLE-NEGATIVE BREAST TUMORS

Claudinas 1, 3, 4, 7 e sobrevida de 10 anos em tumores de mama triplo-negativos

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ABSTRACT

Purpose: Breast cancer is a major cause of morbidity and mortality and is known to be a heterogeneous disease. The clinical and molecular characterization of its subtypes is critical to guide its prognosis and treatment. The study of the expression of Claudins (CLDN) might help in the characterization of these tumors. This study investigated the association of expression of CLDN-1, CLDN-3, CLDN-4 and CLDN-7 with 10-year survival in a series of triple-negative breast cancers. **Methods:** Eighty triple negative tumors were analyzed by automated immunohistochemistry for CLDN-1, CLDN-3, CLDN-4 and CLDN-7. The immunohistochemical expression was assessed by the H-Score (intensity multiplied by the percentage of staining on membrane). The associations between the expression of CLDN and 10-year survival were evaluated by Kaplan-Meier curves and Cox regressions. **Results:** Positive expression (H-score ≥50) of CLDN-1, CLDN-3, CLDN-4 and CLDN-7 were observed in 41.3, 77.5, 67.5 and 18.8% of the cohort, respectively. Patients with positive CLDN-1 expression had a significant lower survival than their counterparts [HR=2.37 (95%CI 1.19–4.72)]. Further, CLDN-3 was inversely associated with overall survival. Patients with positive expression of CLDN-1 and negative expression of CLDN-3 had a HR 10.4 (95%CI 3.40–31.8) higher than patients with negative expression of CLDN-1 and positive expression of CLDN-3. Neither CLDN-4 nor CLDN-7 expression was associated with 10-year survival. **Conclusions:** Differential expression of CLDN can help in clinicopathological characterization of triple-negative tumors. Moreover, CLDN-1 and CLDN-3 appear to be important prognostic factors for these tumors.

KEYWORDS: Breast cancer; claudins; survival analysis; pathology; gynecology.

RESUMO

Objetivo: O câncer de mama é uma das principais causas de morbidade e mortalidade, conhecido por ser uma doença heterogênea. A caracterização clínica e molecular de seus subtipos é fundamental para orientar seu prognóstico e tratamento. O estudo da expressão de claudinas (CLDN) pode auxiliar na caracterização desses tumores. Este estudo investigou a associação da expressão de CLDN-1, CLDN-3, CLDN-4 e CLDN-7 com 10 anos de sobrevida em uma série de cânceres de mama triplo-negativos. **Métodos:** Oitenta tumores triplo-negativos foram analisados por imuno-histoquímica automatizada para CLDN-1, CLDN-3, CLDN-4 e CLDN-7. A expressão imuno-histoquímica foi avaliada pelo escore H (intensidade multiplicada pela porcentagem de coloração na membrana). As associações entre a expressão de CLDN e a sobrevida em 10 anos foram avaliadas pelas curvas de Kaplan-Meier e regressões de Cox. **Resultados:** Foi observada expressão positiva (escore H ≥ 50) de CLDN-1, CLDN-3, CLDN-4 e CLDN-7 em 41,3, 77,5, 67,5 e 18,8% da coorte, respectivamente. Pacientes com expressão positiva de CLDN-1 tiveram uma sobrevida significativamente menor do que suas contrapartes [HR = 2,37 (IC 95% 1,19-4,72)]. Além disso, o CLDN-3 foi inversamente associado à sobrevida global. Pacientes com expressão positiva de CLDN-1 e expressão negativa de CLDN-3 tiveram uma FC 10,4 (IC 95% 3,40–31,8) vezes maior do que pacientes com expressão negativa de CLDN-1 e expressão positiva de CLDN-3. Nem a expressão de CLDN-4 nem de CLDN-7 foi associada a uma sobrevida de 10 anos. **Conclusões:** A expressão diferencial de CLDN pode ajudar na caracterização clinico-patológica de tumores triplo-negativos. Além disso, CLDN-1 e CLDN-3 parecem ser importantes fatores prognósticos para esses tumores.

PALAVRAS-CHAVE: Neoplasias de mama; claudinas; análise de sobrevida; patologia; ginecologia.

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INTRODUCTION

Breast cancer is the most frequent cancer among women in the world and the leading cause of death by cancer in women worldwide[1]. Breast cancer is recognized as a heterogeneous disorder with genotypic and phenotypic diversity[2,3]. This heterogeneity has been extensively studied in the recent decades due to the discovery of hormone receptors (estrogen receptor — ER, progesterone receptor — PR) and HER-2, which are currently important therapeutic targets in oncology[4-6]. Recently, through breast cancer's immunohistochemical classification, which has been considered an important prognostic tool[7], it was estimated that up to 23% of breast cancers are triple negative, *i.e.*, do not express any of these receptors[8].

The burden of triple-negative tumors is evident given that they respond poorly to chemotherapy and that still no targeted drug has been developed[9,10]. Thus, the identification and understanding of new proteins and biomarkers in this special kind of tumor would be helpful to classify this subtype more accurately and then to develop a more specific treatment to each subgroup[11]. In this perspective, tight junction proteins first identified by Furuse et al.[12] in 1998 called claudins (CLDN), whose family comprises 27 different members, have been investigated by several previous studies to be associated with various cancer types[13,14].

However, CLDN's role in breast cancer, especially in triple negative breast cancer, has not yet been fully established, neither its relationship with clinical outcomes nor overall-survival. Previous studies showed that a CLDN1-negative phenotype was associated with a high risk of recurrence and death among a cohort of 173 primary breast tumors[15]. Also, in a sample of 128 cases, in the triple-negative group, the positive expression of CLDN-3 and CLDN-4 was associated with poor clinicopathologic prognosis, while CLDN-1 was not related to any parameter under evaluation[16]. The elevated expression of CLDN-7 was also associated with shorter disease-free survival in breast cancer[17].

Despite the interest in CLDN has been increasing, the role of the proteins listed above are not well understood with regard to prognosis, especially of overall survival, and more studies are needed. Also, most evidences available come from high income countries. Thus, the purpose of this study was to investigate the association between the expression of CLDN-1, CLDN-3, CLDN-4 and CLDN-7 with 10 years' survival, in a series of triple-negative breast tumors from Brazil.

MATERIALS AND METHODS

Sample

The triple-negative tumor samples were selected from a sequential series of pathological reports obtained from patients that underwent diagnostic or surgery procedures or immunohistochemical (IHC) reactions at the Hospital de Clínicas de Porto Alegre between January 2001 and December 2006. This study was submitted and approved by the Research Ethics Committee of the Research and Postgraduate Group of the Hospital de Clínicas de Porto Alegre (GPPG 110263).

The original cohort consisted of 133 tumors paraffin blocks, of which 24 were excluded because their triple-negative nature was not confirmed (ER+=1, PR+=1, HER-2 undetermined=9 and HER-2 positive=13), 17 were excluded for pathological reasons (no tumor=14, in situ carcinoma=1, artefact=1 and bone marrow=1) and 12 were excluded because there was no data. The final cohort comprised 80 cases of human triple-negative breast cancer.

Tissue microarray

Tissue microarrays (TMA) were composed of 59 formalin-fixed, paraffin-embedded tumors. All samples were histologically reexamined and the tumoral regions of interest were selected for core punching. The cores were 2 mm in diameter. Small biopsy sample size (n=21) were analyzed individually and not submitted to TMA.

Immunohistochemistry

Immunohistochemical reactions were performed on 5 µm thick sections obtained from the TMA blocks. After deparaffination, antigen retrieval was performed using Dako PT Link (DAKO, Carpinteria, CA, USA) at 98º for 20 minutes. The reactions for CLDN-3, CLDN-4 and CLDN-7 were done under low pH, while CLDN-1 was recovered at a high pH, using EnVison FLEX Target Retrieval Solution (DAKO, Carpinteria, CA, USA). The slides were washed for 5 minutes in a commercial washing buffer (Wash Solution) and all immunohistochemical reactions were performed in an automated Dako Autostainer Link 48 (DAKO, Carpinteria, CA, USA). Sections were incubated with pre-diluted rabbit polyclonal CLDN1 (Cell Marque, USA) and rabbit polyclonal CLDN3, -4 and -7 (Spring, USA) for 30 minutes (CLDN1) or for 15 minutes (CLDN3, -4 and -7). EnVision kit (DAKO, Carpinteria, CA, USA) was used for visualization with the chromogen 2, 3-diamino-benzidine DAB (DAB Chromogen Solution, Dako, Carpinteria, CA, USA).

CLDN expression evaluation

The slides were evaluated by two independent experienced breast pathologists. Only the expression in the tumor-cell membrane was considered for these analyses. Both staining intensity and the percentage of stained membranes were evaluated. The brown staining intensity was scored as 0 (negative), 1+ (weak), 2+ (intermediate) and 3+ (strong) (Figure 1). Intensity and frequency of positive cells expressed in % were included in a scoring system called the H-score, used to evaluate the expression of CLDN, as previously described[18,19]. Briefly, H-scores derived

from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells [0–100%]; when multiplied, they generated a score ranging from 0–300. Primary categorical analysis was as follows: breast cancers with H-scores ≥50 were considered positive expressions of CLDN-1, CLDN-3, CLDN-4 and CLDN-7 and H-scores <50 were considered as negative expression[18].

Statistical analysis

Initially, for the association between the positive expression of each CLDN and 10-year survival, Kaplan-Meier survival curves were constructed considering the follow time from the date of surgery or biopsy-collected samples to the last registry of follow-up or death, and compared by the log-rank statistics. Phenotypes of CLDNs expressions were also evaluated. In terms to construct these phenotypes, a Spearman correlation was performed, and profiles correlated were considered. Then, crude Cox proportional hazard regressions were created to obtain proportional hazards ratios. Additionally, Cox proportional hazard regression adjusted to expression of other CLDN were conducted. Analyses were conducted using STATA, version 12.1

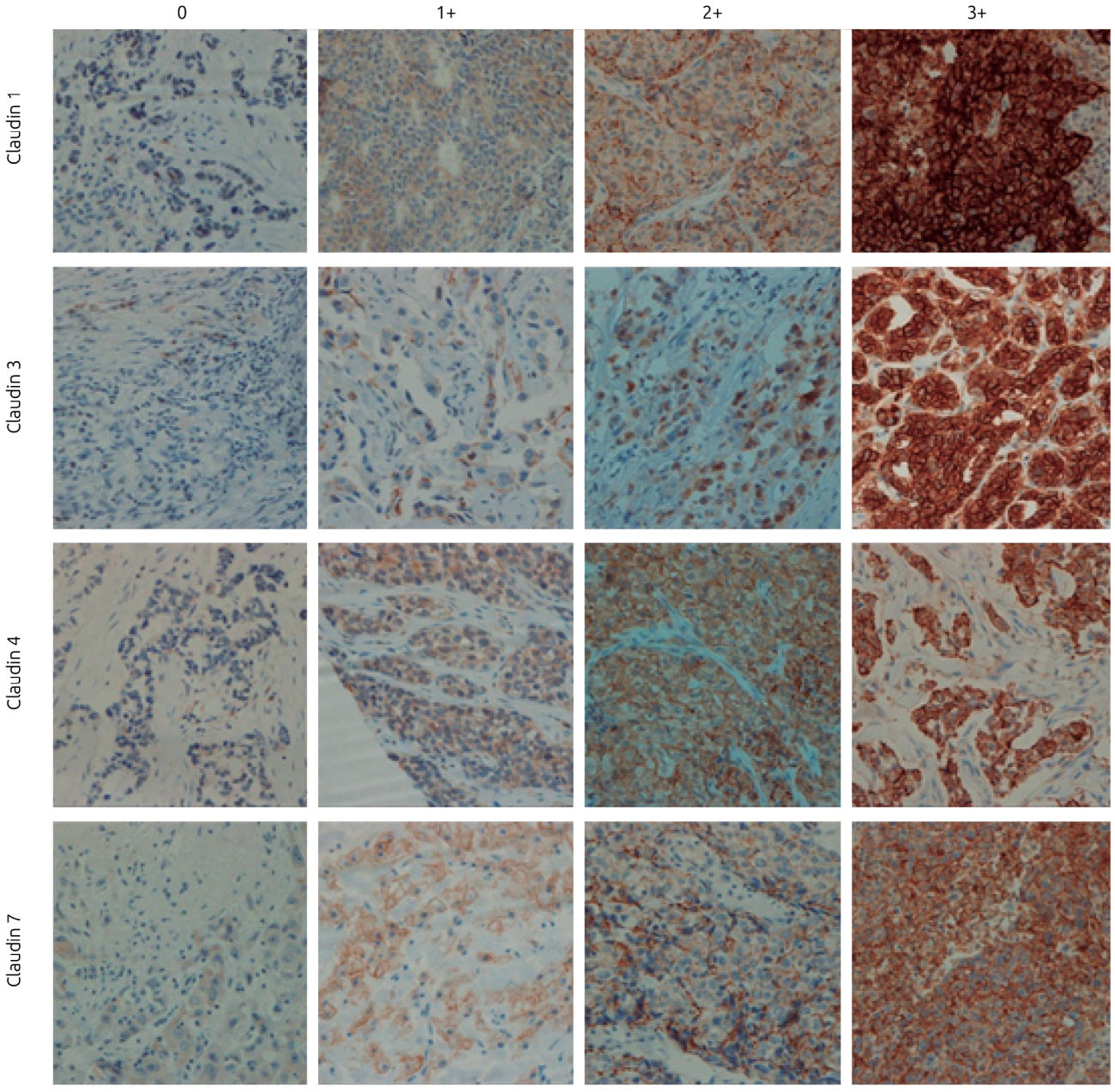

Figure 1. Representative immunostaining of triple-negative breast tumors with claudins 1, 3, 4, 7 antibodies.

RESULTS

The sample's clinicopathological characteristics are summarized in Table 1. The mean age of patients was 54.5 years; 93.8% of the tumor samples were invasive ductal carcinomas. Most of the sample was composed by grade-3 tumors (51.3%), more than 2 cm (85.0%) and with Ki-67 proliferation rate above 14% (47.5%). Half of patients underwent surgery + radiotherapy + chemotherapy protocols. Most of the tumor tissues were obtained from primary disease (83.8%) while some derived from secondary disease (16.3%). In this cohort, a total of 58.8% of the sample was positive expressors (H-scores ≥50) of CLDN1, 77.5% of CLDN3, 67.5% of CLDN4, 18.8% of CLDN7 (Table 2). Only eight patients had negative expression of all, and six patients had positive expression for all CLDN evaluated. More than 70% of the sample had positive expression for at least two CLDNs.

Table 1. Clinicopathological characteristics of the sample.

Variables	N	%
Age		
<50	26	35.6
≥50	47	64.4
Histological type		
Ductal	75	94.9
Lobular	1	1.3
Atypical Medullary	1	1.3
Metaplastic	1	1.3
Mixed Invasive	1	1.3
Tumor size		
≤2 cm	6	5.0
2 cm	34	85.0
Histological grade		
NA	11	15.1
I	2	2.7
II	19	26.0
III	41	56.2
Necrosis		
Negative	41	51.3
Positive	33	41.3
Ki67		
≤10	14	17.5
>10	38	47.5
p53		
Negative	27	33.8
Positive	26	32.5
Primary treatment		
Surgery	6	7.5
Surgery+Radiotherapy	10	12.5
Surgery+Chemotherapy	14	17.5
Surgery+Radiotherapy+Chemotherapy	40	50.0
Palliative	1	1.3
Material analysed		
Primary Biopsy	19	23.8
Primary Surgery	48	60.0
Secondary Biopsy	5	6.3
Secondary Surgery	8	10.0

From 80 patients initially part of our study, information about the main outcome was available for only 66, half of whom(n=33) were dead 10 years after cancer diagnosis. Hazard ratio to death in 10-years is presented in Table 3. In crude analyses, only positive expression of CLDN1 was associated with a higher risk of death [HR=2.37 (95%CI 1.19–4.72)] as compared to negative expression participants. Otherwise, in the adjusted analyses, the effect associated with the expression of CLDN1 was more marked, while positive expression of CLDN3 was associated with a lower hazard risk [HR=0.25 (95%CI 0.07-0.70)] (Table 3). Kaplan–Meier curves were constructed for survival analysis, and the log rank test was used to compare curves. We were able to demonstrate that a high H-score of CLDN-1 was associated with poor overall survival (p=0.014) (Figure 2). Even though in 10 years the death rate was not different among patients with positive and negative expression of CLDN 3, a tendency for a better outcome, especially in a 5-year period was observed [HR=0.36 (95%CI 0.16-0.83); p=0.017]. No statistical difference was observed in the 10-year survival of patients with low or high H-score of CLDN-4 and CLDN-7.

Figure 3 and Table 4 present, respectively, survival curves and hazard ratio associated with each phenotype studied. Patients with positive expression of CLDN-1 and negative expression of CLDN3 presented a ten-times higher hazard [HR=10.41 (95%CI 3.40-31.8)] than the opposed group. All patients presenting this phenotype were dead up to 48 months after their diagnosis (Figure 3). Also, the hazard to death in 10 years was higher among patients with the phenotype characterized by positive expression of CLDN-4 and negative expression of CLDN-3 [HR=5.31 (95%CI 1.06-26.4)] (Table 4).

DISCUSSION

This was one of the first studies to assess the expression of CLDN1, CLDN3, CLDN4, and CLDN7 in a relatively large sample

Table 2. Protein expression pattern and median H-Scores of claudins.

	Mean (SD)	Median (25–75)	H-score ≥50 (%)
CLDN1	81.4 (95.7)	30 (10–145)	41.3
CLDN3	149.6 (100.7)	160 (60–240)	77.5
CLDN4	118.6 (95.8)	100 (25–180)	67.5
CLDN7	28.8 (54.7)	0 (0–30)	18.8

SD: standard deviation.

(n=80) of triple-negative breast tumors in Brazil. Tissue microarrays and an automated system were used to process all the samples, trying to minimize human errors of the IHC technique and enhance the methods' reproducibility. In this study we investigated the association between the expression of CLDN-1, CLDN-3, CLDN-4 and CLDN-7 and 10-year survival in a series of triple-negative breast tumors. A significant association was found between the positive expression of CLDN1 and a worse overall survival rate. Also, a tendency towards a better overall survival rate in patients with a high CLDN3 H-Score was observed. Further, in our study we showed that the expression of CLDN, especially CLDN1 and CLDN3, might play an important role, independently of each other, in the carcinogenesis process of triple-negative breast tumors.

Table 3. Hazard ratio to death in 10 years.

Outcomes	Crude analysis		Adjusted analysis*	
	HR (95%CI)	p	HR (95%CI)	p
Death				
CLDN-1	2.37 (1.19–4.72)	0.014	2.92 (1.40–6.09)	0.004
CLDN-3	0.54 (0.26–1.13)	0.104	0.25 (0.07–0.70)	0.008
CLDN-4	1.14 (0.54–2.39)	0.738	2.08 (0.79–5.50)	0.139
CLDN-7	1.09 (0.49–2.42)	0.836	1.05 (0.46–2.38)	0.911

*adjusted to other CLDN.

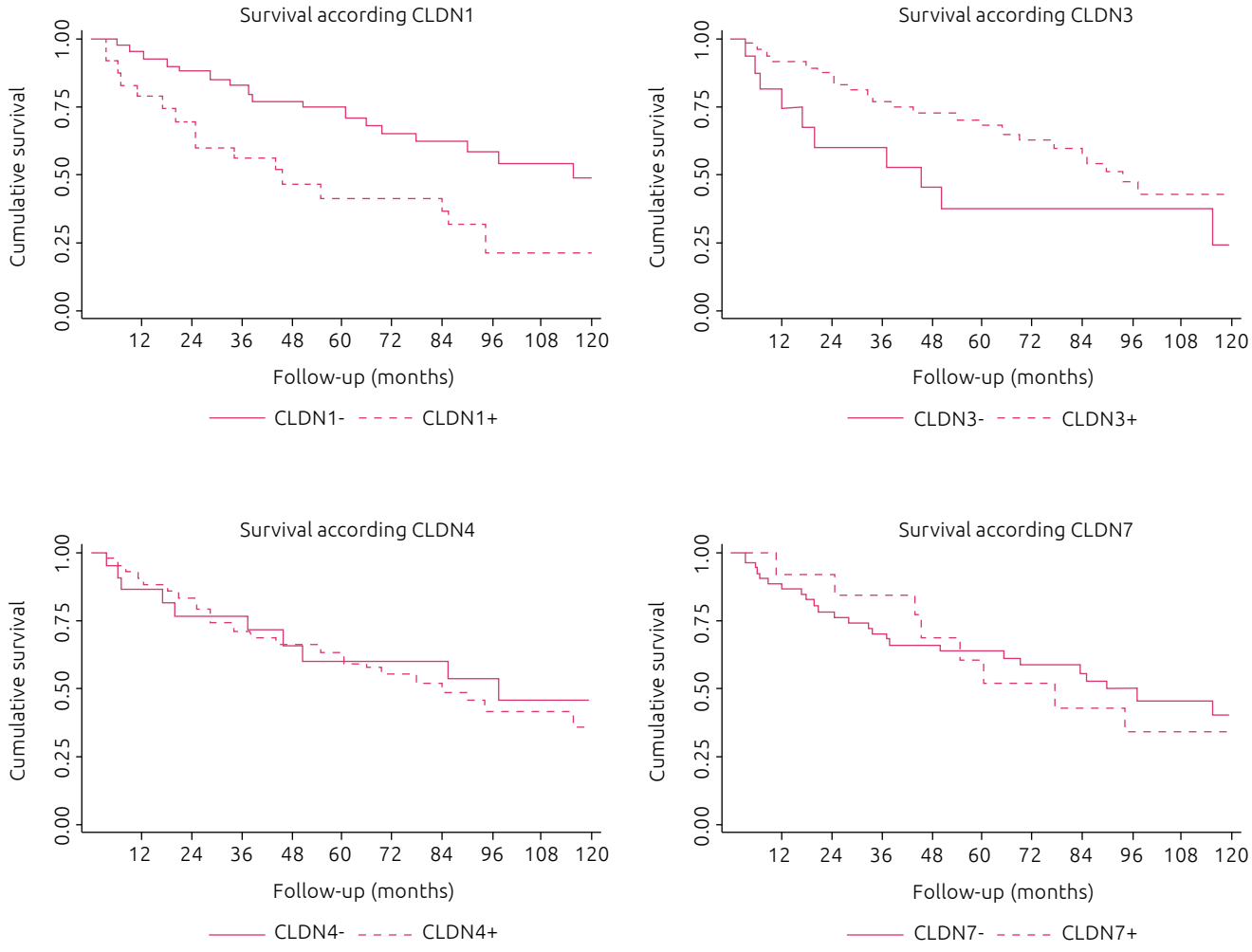

Figure 2. Kaplan–Meier curve for survival of negative and positive H-scores of CLDN1, CLDN3, CLDN4 and CLDN7.

Some limitations should be pointed. Unfortunately, we were unable to obtain complete medical data of all patients. Many medical records were lost in the process and some patients were lost to follow-up. However, when baseline characteristics were compared between our original cohort and analytical, no statistical differences were observed, fact that minimize the likelihood of any bias related to losses has influenced our results. Another limitation of the study was that not all of the samples, but most of them (83.8%), were from the primary disease. It is known that a recurrent tumor can change its primary characteristics as well as its hormone receptors and Her-2. We performed analyses including only primary disease patients and the results were similar (data not showed). Lastly, our limited sample size might be a limitation, given that some comparisons and results could have been influenced by the absence of statistical power.

To compare the characteristics of our triple-negative sample with other studies must take into account the definition of triple negative. Some authors consider the triple-negative subtype as part of the basal-like subtype[11,20,21], and some consider it an independent group[5,9,22]. Triple-negative samples were considered in this study ER-, PR- and HER2- cases, as described by Sorlie et al.[23], Chen et al.[24] and Gucalp and Traina[25]. Also, the profile of the patients in our sample were, in average, worse when compared to other studies[5,6,10,26]. This can be explained by the fact that breast cancer screening programs in Brazil are still inefficient, and when patients get to the treatment, the disease is already in a more advanced stage. If the mean age of our patients at the diagnosis of the primary disease is observed, it will confirm our hypothesis that the patients are diagnosed in more advanced stages.

In our study, high H-Score of CLDN1 was an important predictor of overall survival. This finding was not in agreement with the results reported by previous studies[27,28], where a low expression of CLDN1 was related to the worst outcome. However, Blanchard et al.[18] observed that the high expression of CLDN1 was related to basal-like tumors. Considering that triple-negative tumors share characteristics with basal-like

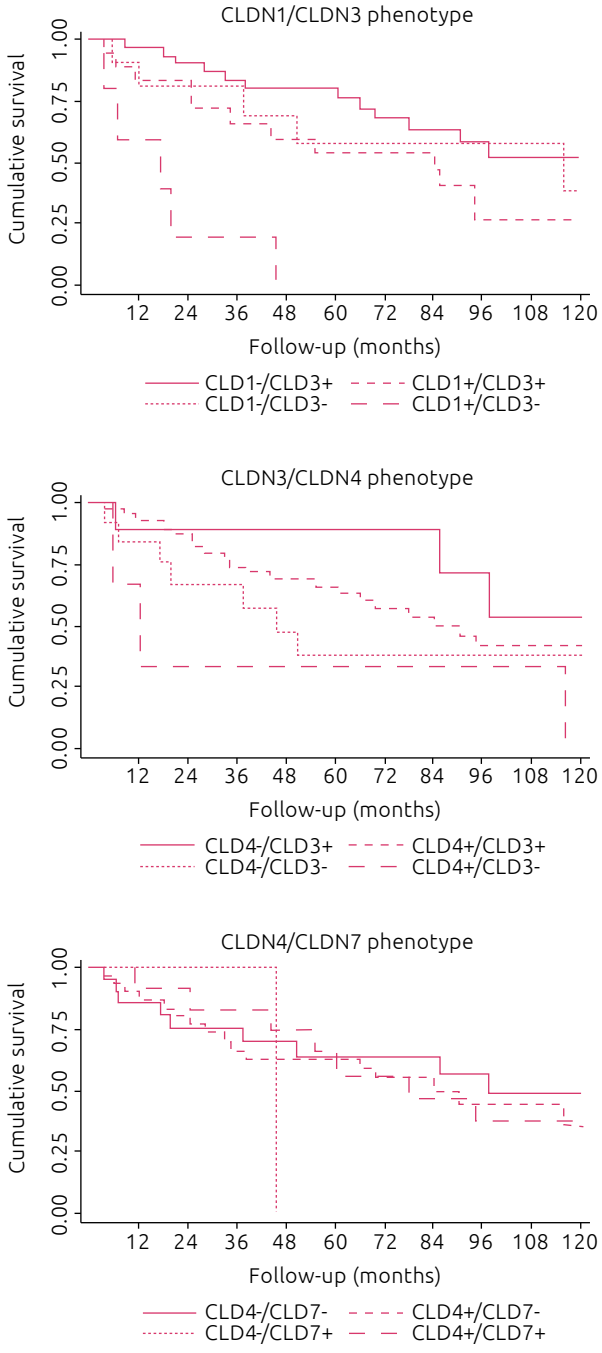

Figure 3. Kaplan–Meier curve for survival according to phenotypes.

Table 4. Description of phenotypes and hazard ratio to death in 10 years.

Phenotypes	N (%)	HR (95%CI)	p
CLDN 1 and 3			
$1^{-}/3^{+}$	36 (45.0)	1.00	0.005
$1^{+}/3^{+}$	26 (32.5)	1.95 (0.85–4.45)	
$1^{-}/3^{-}$	11 (13.8)	1.38 (0.49–3.92)	
$1^{+}/3^{-}$	7 (8.8)	10.41 (3.40–31.8)	
CLDN 3 and 4			
$4^{-}/3^{+}$	12 (15.0)	1.00	0.216
$4^{+}/3^{+}$	50 (62.5)	1.81 (0.54–6.12)	
$4^{-}/3^{-}$	14 (17.5)	2.59 (0.67–10.0)	
$4^{+}/3^{-}$	4 (5.0)	5.31 (1.06–26.4)	
CLDN 4 and 7			
$4^{-}/7^{-}$	24 (30.0)	1.00	0.822
$4^{+}/7^{-}$	41 (51.3)	1.25 (0.55–2.83)	
$4^{-}/7^{+}$	2 (2.5)	2.92 (0.36–23.5)	
$4^{+}/7^{+}$	13 (16.3)	1.15 (0.43–3.10)	

subtype, our results might be explained by the different profile of our sample in relation to other studies'[27,28]. Even using a different criterion, in a recent study, Ma et al, analyzing a cohort including 173 triple-negative breast cancer patients, found that in TNBC, the CLDN1-negative phenotype expression was strongly suggested to be an independent adverse prognostic factor in this heterogeneous subtype of breast cancer. We performed additional analyses in terms to compare our results with Ma's study and the results were in the same direction of those presented with H-score (see Supplementary Data)[15].

To our knowledge, this was the first time that the low H-Score of CLDN3 showed a tendency associated with worse overall survival. Several studies have evaluated the potential therapeutic effect of Clostridium perfringens enterotoxin (CPE)[29]. This enterotoxin is a specific ligand of CLDN3 and CLDN4. CPE has the ability to lyse the cells that overexpress CLDN3 and CLDN4. Experimental studies have demonstrated sensitive and specific tumor cytolysis, including breast cancer and brain metastasis[30]. Thus, CLDN identification in breast cancer can guide therapy in the future.

In conclusion, differential expression of CLDN can help in clinic-pathological characterization of triple-negative tumors. Furthermore, CLDN1 and CLDN3 appear to be prognostic factors for these tumors. Finally, the study of CLDN can bring perspectives for the use of molecules with targeted therapy effect.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version: Suplementary Material.

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