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Simple Summary: The expression of the mechanosensory Piezo2 channel has been already described in different malignant tumors. There is discordance in the literature regarding breast carcinoma, being its expression described either as decreased or increased in neoplasms with respect to benign tissue. A retrospective cohort of 125 patients whose breasts were resected for carcinoma was chosen to determine relationship between Piezo2 and different clinical and histological variables. A significant association was found with the Ki67 proliferation index, with a tendency of most proliferative tumors to be negative for Piezo2.

Abstract: In the last decade a group of Ca2+ channels called Piezo were discovered, demonstrating a decisive role in the cellular response to mechanical stimuli and being essential in the biological behaviour of cells regarding the extracellular compartment. Several investigations have suggested a potential role in carcinogenesis with a tumor supressor role in some cases, but increased expression in several high grade neoplasms. Regarding Piezo2 expression in mammary gland neoplasms, early results suggested a protective role of Piezo2, but posterior works resulted in a relationship between Piezo2 expression and the highly-aggressive triple negative phenotype of breast carcinoma. A cohort of 125 patients with clinical follow up was chosen to study Piezo2 expression and clarify its clinical implications, using the same immunohistochemical valuation performed for other breast carcinoma parameters. Fisher exact was chosen to identify potential relationships between the different variables. A significant association was found with the Ki67 proliferation index, but not with mitoses. The tendency of most proliferative tumors was to have a diminished score for Piezo2. A similar association was found between Piezo2 expression and perineurial invasion.

Keywords: piezo2; piezo channels; mechano-signaling; breast cancer; immunohistochemistry

1. Introduction

1.1. Piezo channels

Piezo represent a new class of mechanosensitive channels, responding to mechanical stimuli by permitting Ca2+ ions pass though the cell membrane, influencing the cell biology [1,2]. Thanks to their aperture, these channels allow for Ca2+ ions entrance in the cells to transform tactile stimuli in action potentials [3]. Piezo family consists in two ion channels with marked homology named Piezo1

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and Piezo2 [4]. Mechanical forces stimulate these channels by pulling and pushing the cell membrane from the extracellular matrix [5], which are tethered on the other side to the cell actin cytoskeleton [6,7]. Furthermore, integrin activation on cell surface and adhesion to other cells and extracellular matrix depends on Piezo channels [8].

Although Piezo2 is specifically related to mechanoreception in the Peripheral Nervous System [9,10], Piezo1 channels demonstrate a more ubiquitous distribution and a polymodal behaviour; they are essential for vascular and erythrocyte function, for osteoclastogenesis and urinary excretion [2,4]. In this line, they are supposed to participate in biological processes associated to mechanical stimulation like vascular shear, urinary flow regulation, bladder distension, volume regulation, elongation and cellular growth, migration and proliferation [2,4].

1.2. Piezo and Cancer

Piezo mechanoreceptors were found in several tissues and neoplasms, but their function is not fully understood [2]. Mechanical stimuli may influence cancer biology affecting both neoplastic cells and their environment, by altering cell migration, proliferation, matrix remodeling and metastatic behavior [10]. In addition, Piezo channels have specific agonists (Yoda I) and antagonists (GsMTx4) that allow a precise study of this promising target [11].

Both Piezo1 and Piezo2 have diminished expression in lung neoplasms with respect to benign lung tissue, and a correlation was found between elevated Piezo mRNA expression and improved survival of non-small cell lung carcinoma [12]. An opposite behaviour was found in urinary bladder and colonic human and murine neoplasms [13,14]. In gliomas Piezo1 expression is increased in poorer prognosis neoplasms [15,16]. Moreover, increased Piezo1 expression was observed in metastatic colon carcinoma [17], prostate carcinoma [18] and oral squamous carcinoma [19].

One of the first known function of Piezo channels was cellular adhesion though integrin activation [8], and this may explain why carcinomas, less cohesive and stationary than benign tissue, have shown in some occasions diminished Piezo expression, and higher grade carcinomas (generally even less cohesive and indifferent to pressure made by surrounding cells and tissue) have much less expression [12]. The increased expression described in aggressive vesical neoplasms [13], high grade gliomas [15,16] and others may be more complex to explain; in this regard it has been suggested that Piezo2 accelerates the cell cycle though activation of Akt/mTOR, enhancing the growth of the neoplasm [18].

In any case, both Piezo1 and Piezo2 have a potential role both as oncoprotein and tumour suppressor protein [11]. When dealing with a tactile related protein such this, it´s unknown if tumor´s physical perceptions can change its fate. In this line, recent research shows that Piezo1 agonist Yoda1 is able to reduce macropinocytosis to impair cell nutrition [20].

1.3. Piezo and Mammary Gland Carcinoma

The presence of Piezo2 channel has already been noted in normal breast [21]. This antigen is present in epithelial cells and, although there was described tethering of Piezo channels to the cell´s actin cytoskeleton, there is no morphological evidence of coexpression between Piezo and actin ([6,22] supplementary material).

In any case, current evidence suggests that Piezo2 is not limited to nervous tissue, and probably has a wider function, like Piezo1. In this regard, it has demonstrated expression at least in vascular structures and the different epithelial glandular components; a role of Piezo2 channels has been proposed in the milk-flow induced response to duct shear and dilation though the gland, similarly to Piezo1 [21].

In breast carcinoma, Piezo1 and Piezo2 were studied in various different cell lines, with positive expression in all of them [22–24]. However, opposing results regarding Piezo1 and Piezo2 role in breast cancer exists in the literature [22,23,25]. The first study, employing cell lines of normal breast and breast cancer, found increased Piezo1 expression in tumor cells compared with normal cells [23]. The second study indicated that Piezo2 expression is reduced in malignant cells, showing lesser expression as more undifferentiated is the tumor [22]. Moreover, the most recent study, focused in

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triple negative breast cancer, found similar results to the Piezo1 study, as elevated PIEZO2 mRNA expression was correlated with worse prognosis and lung metastases, finding no significant relation with hormone-positive carcinomas [25]. With the last interpretation [25], Piezo2 might be a biomarker of worse prognosis, but with the previous interpretation would be a biomarker of better prognosis [22] . So, discordance exists in the bibliography regarding the prognostic relevance given to Piezo2 channels.

To clarify these apparently opposing effects and check the clinical relevance of Piezo2 channels in breast cancer, we retrospectively chose a consecutive cohort of breast cancer patients undergoing surgery with a clinical follow of 5 years. Piezo2 immunohistochemistry was performed in the cohort similar to the commonly performed Progesterone and Estrogen receptors. The study included the relationship between Piezo2 expression and several clinical and histological features, including the Nottingham score and the St. Gallen molecular phenotype classification [26–28].

2. Materials and Methods

2.1. Patients

A total of 125 consecutive patients undergoing surgery for breast carcinoma, treated between 2012 and 2013 in a single center were collected (Table 1). It mostly included patients with ductal adenocarcinoma, but other diagnoses were not excluded. 114 patients had invasive carcinoma and 11 patients had in situ carcinoma. Three (1 ductal in situ and 2 invasive ductal adenocarcinoma) samples were deemed invalid, because of insufficient material in the remaining paraffin block. The margins were complete in all the tumors, with no evidence of neoplastic remnants. If a patient presented two synchronous tumors, the highest grade one was considered. A total of 63 patients had lymph node metastases at diagnosis.

Table 1. Histology and stage of the cases.

Histology	Number	Percent	TNM Stage	Number	Percent
Ductal	105	84%	pTis	8	6.4%
Lobulillar	9	7.2%	pT1a	2	1.6%
Mucinous	4	3.2%	pT1b	18	14.4%
Tubulolobular	2	1.6%	pT1c	62	49.6%
Ductal / Lobulillar	1	0.8%	pT2	31	24.8%
Ductal + Tubular	1	0.8%	pT3	2	1.6%
Medullary	1	0.8%	pT4	1	0.8%
Micropapillary	1	0.8%			
Solid papillary	1	0.8%			

The histologic grade, according to the Nottingham classification, was available in a total of 109 invasive cases. According with this classification, a score of 1 to 3 was assigned to the different categories including pleomorphism, tubule formation and mitosis. These were added to obtain the grades (grade 1 with a score of 3-5; grade 2 with a score 6-7; grade 3 with a score 8-9). This information is quantified in Table 2.

Table 2. Nottingham grade and histologic features.

Nottingham grade	Frequency	Percent	Tubule formation	Frequency	Percent
Grade 1	32	29.4%	Score 1	8	7.3%
Grade 2	43	39.5%	Score 2	20	18.4%
Grade 3	34	31.2%	Score 3	81	74.3%
Pleomorphism	**Frequency**	**Percent**	**Mitoses**	**Frequency**	**Percent**
Score 1	13	11.9%	Score 1	66	60.6%
Score 2	68	62.4%	Score 2	26	23.9%
Score 3	28	25.7%	Score 1	17	15.6%

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Immunohistochemistry was available in most neoplasms (Table 3) and the St. Gallen subtypes were determined according to the immunohistochemical profile regarding hormone receptors, Her2 and Ki67 proliferation index (Table 4).

Table 3. Immunohistochemistry of breast carcinoma.

Estrogen receptors	Frequency	Percent	Progesterone receptors	Frequency	Percent
Positive	106	84.8%	Positive	85	68%
Negative	19	15.2%	Negative	45	32%
Hormonal receptors	**Frequency**	**Percent**	**Her2**	**Frequency**	**Percent**
Positive	108	84.4%	Positive	36	29%
Negative	17	13.6%	Negative	88	71%

The follow-up of the patients was, at least, 5 years after diagnosis. Possible clinical events were related with clinical progression of the neoplasm (relapse, metastases…).

Table 4. St. Gallen classification.

St. Gallen Classification	Frequency	Percent
Luminal A	69	55.2%
Luminal B Her2-negative	15	12%
Luminal B Her2-positive	24	19.2%
Her2+ non luminal	11	8.8%
Basal like	6	4.8%

2.2. Immunohistochemical Assay

The immunohistochemistry was individually performed over the same paraffin block initially selected for immunohistochemistry at diagnosis, generally including neoplastic and benign breast tissue. A tissue microarray was considered, but finally avoided in order to identify possible differences of expression.

Deparaffinized and rehydrated 5 µm sections were processed for detection of Piezo1 and Piezo2 using the EnVision antibody complex detection kit (Dako®, Copenhagen, Denmark) following the supplier's instructions. Briefly, endogenous peroxidase activity was inhibited (3% H2O2 for 15 min), and non-specific binding was blocked (10% bovine serum albumin for 20 min). Sections were then incubated overnight at 4 °C with the primary antibody. The antibody against Piezo2 was polyclonal raised in rabbit (Sigma-Aldrich®, Madrid, Spain), and recognizes the following amino acid sequence: FEDENKAAVRIMAGDNVEICMNLDAASFSQHNP (manufacturer's notice); it was used diluted to 1:200. Subsequently, the sections were incubated with anti-rabbit EnVision system-labelled polymer (Dako®, Copenhagen, Denmark) for 30 min. Finally, the slides were washed with buffer solution, and the immunoreaction was visualized with diaminobenzidine as a chromogen, washed, dehydrated, and mounted with Entellan (Merck®, Dramstadt, Germany). To ascertain structural details, the sections were counterstained with Mayer's haematoxylin.

Quantification of the immunohistochemical expression of Piezo channels was performed according to Allred score, commonly used in evaluating breast hormonal receptors [26,29]. This score assesses intensity of stain (0-3 points: negative, +, ++ or +++) and the proportion of positive neoplastic cells (0-5 points: negative, <1%, 1-10%, 11-33%, 34-66%, >66%). Both components are added for a total of 0-8 points. Two different pathologists independently examined all the samples and reached a consensus afterwards. Images of the immunohistochemical results were taken with a Nikon Eclipse Ci microscope paired with a Nikon DS-Ri2 camera and employing Nikon NIS Elements software (Nikon®, Tokio, Japan).

2.3. Data Analysis

The statistical analysis was performed using the Stata package (2013; StataCorp, College Station, Texas, USA). Categorical variables are reported as percentages. Normally distributed continuous variables are summarized using means with standard deviations. Non-normally distributed

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continuous variables are reported as medians and interquartile ranges. The Pearson $\chi 2$ test and Fisher exact tests were used to assess differences between categorical values as warranted. Interrater agreement was assessed using Cohen`s kappa. The kappa result was interpreted as follows: 0-0.20 indicate no agreement, 0.21-0.39 a minimal agreement, 0.40-0.59 a weak agreement, 0.60-0.79 a moderate agreement, 0.80-0.90 a strong agreement and any value above 0.90 an almost perfect agreement [30].

3. Results

3.1. Immunohistochemistry

Available normal breast tissue usually showed intense, generally scattered, Piezo2 expression in glandular cells (Figs 1a-c). On the other side, results in neoplastic tissue were commonly slightly less intense, and the expression was commonly homogeneous in either proliferative conditions (Fig. 1d) and neoplasms (Figs. 1e,f).

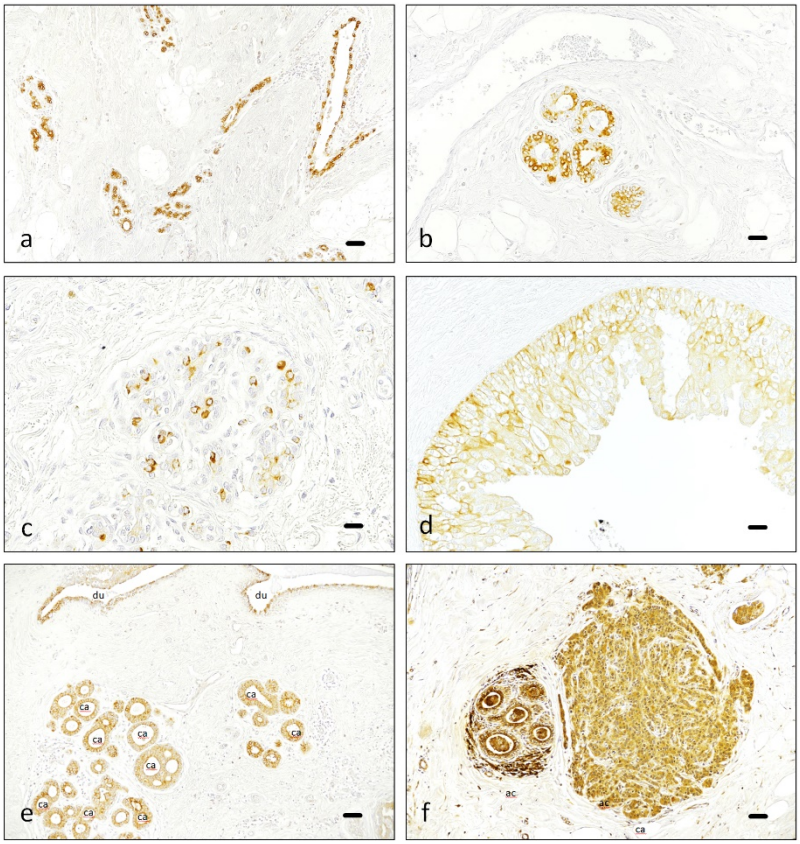

Figure 1. Piezo2 expression in non neoplastic conditions. In most tubules, Piezo 2 expression is positive in part of the ductal epitelial cells (a). Acini are also partially positive (b). Benign acini sometimes demonstrate intense expression in few of the cells (c). Benign hyperplastic regions tend to be more homogeneous in intensity (d). In situ (e) and invasive carcinoma (f) are usually uniformly positive (ca in images e and f), contrasting with the scattered positive cells in normal ducts (du in image e) and acini (ac in image f). Scale bar 100 µm (a,e,f), 50 µm (b,d), 25 µm (c).

Neoplastic tissue was evaluated by two pathologists (Fig. 2). A 90.5% agreement was observed between both in the first examination, with a Cohen´s kappa of 0.59 (95% CI 0.30-0.87) (Suppl. Mat.). An assessment of conflictive cases showed most divergent opinions were related with the interpretation of background staining.

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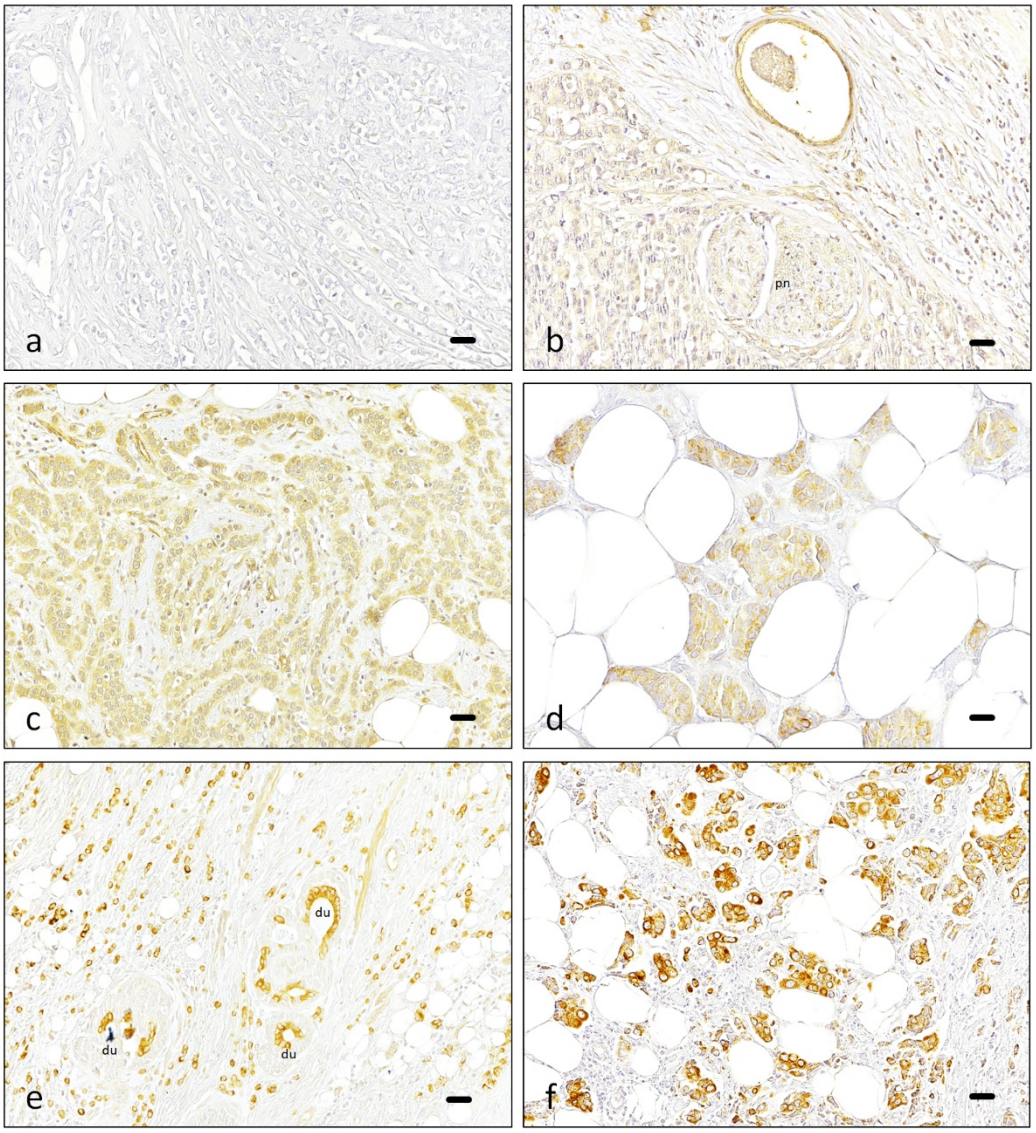

Figure 2. Piezo2 expression in neoplastic conditions. Images illustrating the different scores applied into the neoplastic cells: Negative (a), 1+ (b), 2+ (c, d) and 3+ (e, f). Sometimes, additional features were noted, like perineurial invasion (pn in b) or remnants of benign mammary tissue inside the neoplasm (du in e). Scale bar 50 μm (a,b,c,e,f), 25 μm (d).

The majority of cases were positive for Piezo2, with variable pattern and intensity, being the score 8 the most frequent category, with intense and either complete or nearly complete expression in the neoplasm (Table 5).

Table 5. Piezo2 expression in infiltrating carcinoma.

Cells expressing	<1% (1)	1-10% (2)	11-33% (3)	34-66% (4)	>66% (5)
Low expression (1)	1	2	9	14	17
Medium expression (2)	0	0	6	6	17
Intense expression (3)	0	0	0	3	36

Percentage of Piezo2 expressing tumor cells is depicted in the columns. The intensity of the expression is depicted in the rows. The score granted in specified between parentheses.

In addition, a particular staining pattern was observed when the epithelial cells showed intense secretory activity (luminal or apical "snouts", usual in apocrine-type secretion). This activity was accompanied sometimes by intense Piezo2 expression, both in benign (Figs. 3a,b) and malignant

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tissue (Fig. 3c). However, when normal mammary gland tissue was noted as "apocrine" and snouts were less apparent, Piezo2 staining was commonly faint (Fig. 3d).

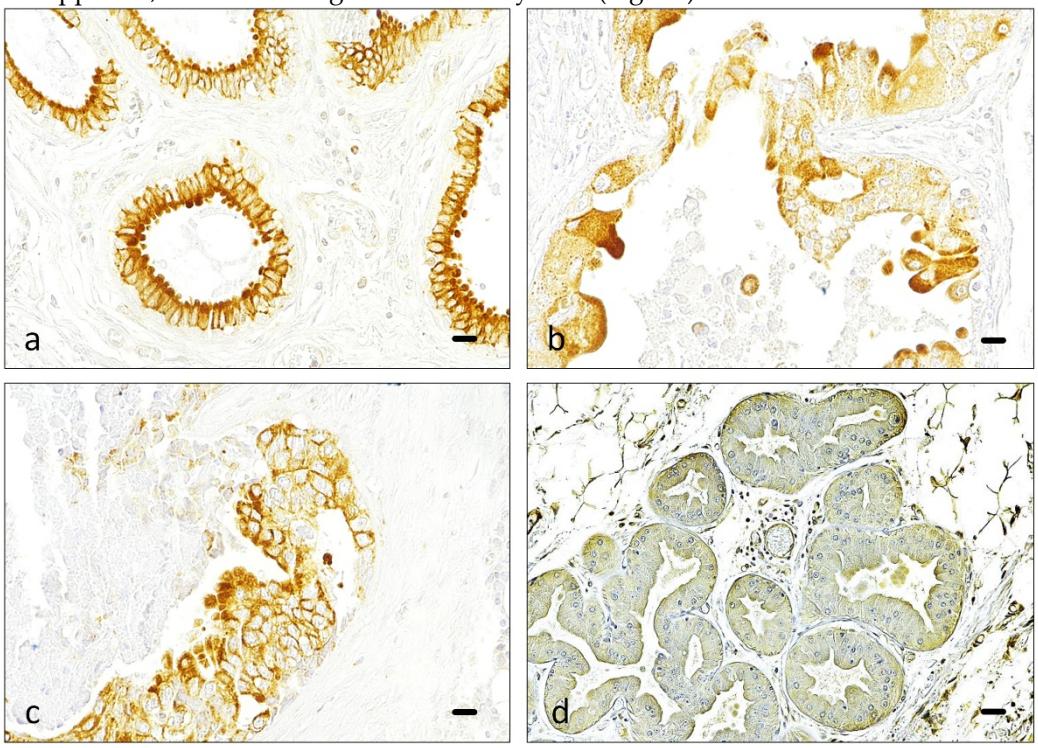

Figure 3. Piezo2 luminal expression pattern. When mammary secretion is apparent in the form of apical "snouts", Piezo2 expression is intense in this region of the cell cytoplasm (a,b). When the neoplastic tissue maintains good differentiation and snouts are recognizable, Piezo2 expression is also highlighted in the luminal region of the cell (c). Areas of defined apocrine metaplasia are mostly negative with Piezo2 (d). Scale bar 50 μm (a,c,d), 25 μm (b).

3.2. Data Analysis

Immunohistochemical results indicate that benign breast tissue demonstrated greater intensity of staining for Piezo2 than neoplastic tissue, with a median of 2 for neoplastic breast tissue and a median of 3 for normal mammary glands (Table 6). With non-parametric tests, the group of neoplastic tissue presented greater values (p=0.004). Only three cases had apocrine metaplasia in normal mammary glands, all of them with an intensity of 1 for Piezo2.

Table 6. Piezo2 intensity in normal breast and cancer.

Normal gland			Cancer		
Value	**Frequency**	**Percent**	**Value**	**Frequency**	**Percent**
Score 0	1	1.6%	Score 0	5	8.2%
Score 1	16	26.2%	Score 1	22	36.1%
Score 2	12	19.7%	Score 2	18	29.5%
Score 3	32	52.5%	Score 3	16	26.2%

Fisher´s exact revealed no significant correlation between Piezo2 expression and survival, tumor´s histology, stage, tubule formation, pleomorphism, mitoses, infiltration or lymph node invasion. No relationship was either observed between Piezo2 and Estrogen Receptors, Progesterone Receptors or Her2.

In contrast, a clear relation was identified between Piezo2 expression and Ki67, with p-values under 0.05, with higher Piezo2 expression in proliferative tumors. This finding was significant categorizing Ki67 in two categories (≥20% and <20%) and in three categories (≥30%, >5% and <30%, and ≤5%). In the first case, the Fisher´s exact was 0,01 and in the second one, it was 0,02. The

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relationship was confirmed when the expression of Piezo2 was divided in two categories (<2 and ≥2), also categorizing Ki67 in the previous two categories (p = 0.01). Results are summarized in Figure 4.

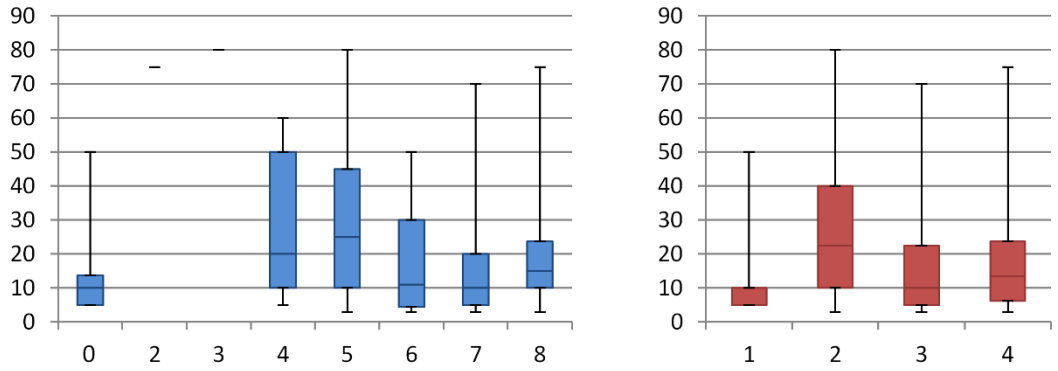

Figure 4. Piezo2 expression in relation to Ki67 proliferation index. Left diagram illustrates all the cases, with Ki67 proliferation index illustrated in the vertical axis and global Allred score for Piezo2 is illustrated in the horizontal axis. Right diagram illustrates only the infiltrative cases, with Ki67 in the vertical axis and the Piezo2 intensity score in the horizontal one.

A similar relationship was found regarding perineurial invasion, with a Fisher´s exact test´s p value of 0,01 (Figure 5). The number of cases noted as positive for perineurial infiltration was 15 and the number without it was 97.

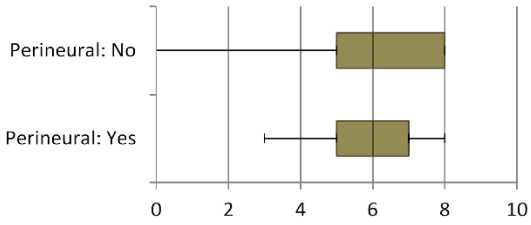

Figure 5. Piezo2 expression in relation to perineurial infiltration. The global Allred score for Piezo2 is illustrated in the horizontal axis.

4. Discussion

In our experiment, most patients were positive for Piezo2, which is in accordance with the literature [22]. The experiment also demonstrated more intense expression of Piezo2 in benign breast tissue than in neoplastic tissue. The apparent contradiction with the previous report for Piezo1 suggesting its correlation with neoplastic aggressive phenotypes, might be partly explained by a limited representativeness of the MCF-7 breast cancer cellular line employed in the older study [23,31], but other studies have produced similar results, indicating association between PIEZO1 and poor prognosis [32,33], probably by the induction of a compression-enhanced invasive phenotype and matrix degradation of cancer cells through Piezo1 channels [34,35]. Moreover, this MCF-7 line was also employed in the first study of Piezo2 expression with opposite results to Piezo1 regarding the prognostic significance [22]. Although both Piezo channels have high homology, their functional response is different [35], and for this reason Piezo1 determines poor prognosis in the MCF-7 line while Piezo2 determined the opposite results in the same cellular line [22,23].

Although we found stronger Piezo2 expression in benign breast tissue, we also detected a tendency of more Piezo2 expression in the more proliferative neoplasms. This may be the key point to explain the apparent discordance mentioned in the introduction regarding the different studies employing cellular lines to study Piezo2 expression [22]. With this interpretation, Piezo2 would have diminished expression in carcinomas, but its expression would be enhanced in highly proliferative carcinomas, measured by the Ki67 immunostaining, in line with the findings of Katsuta et al. [25].

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Ki67 is a nuclear protein, characteristically expressed in proliferating vertebrate cells. This marker reacts with cells that are not in G0 phase of the cell cycle, and is commonly used in clinical cancer histopathology to assess the proliferation index [36–38]. For example, Ki67 proliferation index is determinant in GIST [39] and neuroendocrine tumors [40,41]. Moreover, it is a valuable prognostic factor in other tumors like gliomas [42], lymphoma [43], sarcomas [44], melanoma [45] or carcinomas [46–48].

Regarding breast carcinoma, although inconsistencies have been described between elevated Ki67 index and high mitosis accounts [37,49], Ki67 remains as a strong prognostic factor and is closely linked with mitotic count [50,51]. It has been described as the most powerful IHC prognostic indicator of early breast cancer in univariate analyses, probably due by its correlation of tumor grade [52]. Furthermore, mitosis and Ki67 relationship in breast varies dynamically, and the mentioned inconsistencies were related to certain cancer subtypes [53]. An explanation of this behavior may be the parallel relationship existing between the immune response against cancer cells and the actual proliferation of neoplastic cells, both requiring the existence of Ki67 [38]. Piezo1 also has been described to affect the immune response, by the enhancement of myeloid-derived suppressor cells in cancer and infectious disease, inhibiting immune responses and, thus, promoting cancer proliferation [54].

Although mitosis are one of the three items included in the Nottingham histological grading system, Ki67 has a predictive role and has even been suggested as a criterion to select the therapy in some breast carcinomas [55]; in addition Ki67 evaluation is critical, according to St. Gallen Consensus, differentiating Luminal A and Luminal B molecular subtypes [37]. We have found a significant relationship between Piezo2 expression and the Ki67 proliferation index, considering Ki67 under two or three categories. The categorization of Ki67 under two categories was employed by many authors and is associated with the St. Gallen breast cancer categories and overall survival [56,57]. Although the cut-point selection ranges from 15% to 20%, in the practice Ki67% is generally reported in 5% intervals so, actually, breakpoints of >15% and ≥20% are the same in the clinical practice and are coherent with the St. Gallen Consensus. The categorization of Ki67 under three categories is probably the best option for early breast cancer, as long as renders more predictive value to the extreme categories <5% and >30% [37,50,55]. For this reason, two Ki67 categorizations were employed, in the statistical analysis, both of them being significant. In any case, uncertainty remains surrounding the selection of relevant cut-off points for Ki67 [37].

The main limitations in the present study are the sample size and, particularly, the tuning of the immunohistochemistry for Piezo2. Although our group has experience regarding this immunohistochemical technique, the background staining prevented a high concordance between observers. In any case, certain variability in the scoring by histopathologists appears when different individuals make interpretations over a slide [37]. Moreover, no specific threshold has been established for this technique. For this reason, this can be only considered a preliminary study. Other consideration to be made is related with the retrospective nature of the investigation, but we think that this approach was necessary to find some preliminary results that may guide a subsequent prospective study.

5. Conclusions

This is the first study assessing Piezo2 expression in breast carcinoma employing clinical data of a cohort of patients. Including the above-mentioned limitations, normal breast showed enhanced Piezo2 expression than neoplastic tissue. On the other hand, a significant positive relation was demonstrated between Piezo2 expression, elevated Ki67 proliferation index and perineurial invasion. Moreover, there is a morphological shift from the single cell expression of benign tissue to a more generalized expression in neoplasms.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Expression of Piezo2 and Actin in mammary gland tissue; Table S1: Statistical charts.

Author Contributions: Conceptualization, J.F., Y.G.-M. and O.G.-S.; methodology, A.R.-F., Y.G.-M. and F.J.G.-A.; software, F.J.G.-A. and J.F.; validation, J.F., F.J.G.-A. and O.G.-S.; formal analysis, A.R.-F., M.A.G.-M. and J.F.; investigation, Y.G.-M., S.M.-G. and R.S.-B.; resources, J.F. and A.R.-F.; data curation, F.J.G.-A. and J.F.; writing—original draft preparation, R.M-S.; writing—review and editing, J.F. and F.J.G.-A.; visualization, J.F.; supervision, O.G.-S. and J.F.; project administration, J.F.; funding acquisition, J.F. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Patient consent was waived due to the retrospective nature of the study over archive material.

Conflicts of Interest: The authors declare no conflicts of interest.

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