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Article

# Inositol (1,4,5)-Trisphosphate Receptors in Invasive Breast Cancer: A New Prognostic Tool?

Arthur Foulon <sup>1,2,\*</sup>, Pierre Rybarczyk <sup>2,3</sup>, Nicolas Jonckheere <sup>4</sup>, Eva Brabencova <sup>5</sup>, Henri Sevestre <sup>2,3</sup>, Halima Ouadid-Ahidouch <sup>2</sup> and Lise Rodat-Despoix <sup>2</sup>

<sup>1</sup> Centre de Gynécologie-Obstétrique, Université Picardie Jules Verne, CHU Amiens Picardie, F-80089 Amiens, France

<sup>2</sup> Laboratoire de Physiologie Cellulaire et Moléculaire, UR-UPJV-4667, Université Picardie Jules Verne, F-80090 Amiens, France; rybarczyk.pierre@chu-amiens.fr (P.R.); henrisevestre@gmail.com (H.S.); halima.ahidouch-ouadid@u-picardie.fr (H.O.-A.); lise.despoix@u-picardie.fr (L.R.-D.)

<sup>3</sup> Service d'Anatomie et Cytologie Pathologiques, Université Picardie Jules Verne, CHU Amiens Picardie, F-80090 Amiens, France

<sup>4</sup> UMR9020-U1277, CANTHER-Cancer Heterogeneity, Plasticity and Resistance to Therapies, CNRS, Inserm, CHU Lille, Univ. Lille, F-59000 Lille, France; nicolas.jonckheere@inserm.fr

<sup>5</sup> Jean-Godinot Institute Cancer Center, Centre de Lutte Contre le Cancer de Reims et Champagne Ardenne, Centre de Ressources Biologiques, F-51100 Reims, France; eva.brabencova@reims.unicancer.fr

\* Correspondence: foulon.arthur@chu-amiens.fr

**Simple Summary:** The inositol-trisphosphate receptor (IP<sub>3</sub>R) is a key player in physiological and pathological intracellular calcium signaling. The objective of the present study was to assess the putative value of the three IP<sub>3</sub>R subtypes as prognostic biomarkers in breast cancer. We found that IP<sub>3</sub>R3 is the most strongly expressed subtype in breast cancer tissue. Furthermore, IP<sub>3</sub>R3 and IP<sub>3</sub>R1 are significantly more expressed in invasive breast cancer tissue than in non-tumor tissue. In contrast to IP<sub>3</sub>R1 and IP<sub>3</sub>R2, the expression of IP<sub>3</sub>R3 was positively correlated with prognostic factors including tumor size, regional node invasion, histologic grade, proliferation index, and hormonal status. By analyzing public databases, we found that the expression of all IP<sub>3</sub>R subtypes is significantly correlated with the overall survival and disease-free survival of patients with breast cancer. We conclude that relative to the other two IP<sub>3</sub>R subtypes, IP<sub>3</sub>R3 expression is upregulated in breast cancer and is correlated with prognostic factors. We strongly believe that our results will open up new perspectives with regard to the link between IP<sub>3</sub>R3s and breast cancer aggressiveness.

**Abstract:** Breast cancer is the leading cause of cancer death among women in worldwide and France. The disease prognosis and treatment differ from one breast cancer subtype to another, and the disease outcome depends on many prognostic factors. Deregulation of ion flux (especially Ca<sup>2+</sup> flux) is involved in many pathophysiology processes, including carcinogenesis. Inside the cell, the inositol-trisphosphate receptor (IP<sub>3</sub>R) is a major player in the regulation of the Ca<sup>2+</sup> flux from the endoplasmic reticulum to the cytoplasm. The IP<sub>3</sub>R3s (and particularly the IP<sub>3</sub>R3 subtype) are known to be involved in proliferation, migration, and invasion processes in breast cancer cell lines. The objective of the present study was to evaluate the potential value of IP<sub>3</sub>R3s as prognostic biomarkers in breast cancer. We found that expression levels of IP<sub>3</sub>R3 and IP<sub>3</sub>R1 (but not IP<sub>3</sub>R2) were significantly higher in invasive breast cancer of no special type than in non-tumor tissue from the same patient. However, the IP<sub>3</sub>R3 subtype was expressed more strongly than the IP<sub>3</sub>R1 and IP<sub>3</sub>R2 subtypes. Furthermore, the expression of IP<sub>3</sub>R3 (but not of IP<sub>3</sub>R1 or IP<sub>3</sub>R2) was positively correlated with prognostic factors such as tumor size, regional node invasion, histologic grade, proliferation index, and hormone receptor status. In an analysis of public databases, we found that all IP<sub>3</sub>R3s types are significantly associated with overall survival and progression-free survival in patients with breast cancer. We conclude that relative to the other two IP<sub>3</sub>R subtypes, IP<sub>3</sub>R3 expression is upregulated in breast cancer and is correlated with prognostic factors.

**Keywords:** inositol 1,4,5 trisphosphate; breast cancer; invasive prognostic marker



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## 1. Introduction

In 2020, around 2.2 million new cases of breast cancer (BC) were diagnosed worldwide [1]—making this disease a major public health problem that affects 1 in 9 women at some point in life. Despite recent and constant progress in diagnosis and management, BC is the second deadliest cancer in women. However, the implementation of BC screening programs and the development of systemic treatments have reduced mortality and the incidence of metastatic cancer. Ninety percent of BC deaths are due to metastases [2,3]. Invasive breast carcinoma of no special type (IBC-NST) is the most frequent histologic subtype. Many prognostic criteria are applied when choosing an adjuvant treatment in BC; these criteria are variously clinical (tumor size, axillary lymph node invasion, remote metastases, etc.) and histologic (the Scarff-Bloom-Richardson (SBR) grade, hormone receptor (HmR) status, human epidermal growth factor 2 (HER2) expression, and the Ki67 index) [4–11]. BC is a complex, heterogeneous disease. Perou et al. developed a molecular classification for BC, which included luminal A, luminal B, HER2, and triple-negative (TN) subtypes [12]. The luminal A and B subtypes generally spread more slowly and recur less frequently than the HER2 and TN subtypes [13–15]. Moreover, disease-free survival rates are higher for metastatic luminal BC than for metastatic HER2 or TN BCs [13].

At BC diagnosis, 5% of patients have metastases [16]. Ten to 15% of BC patients will develop remote metastases in the first three years after diagnosis [16,17]. A third of patients without axillary lymph node involvement will still develop metastases [17]. Early-stage (non-metastatic) BC is curable; treatments include local surgery, brachytherapy, and systemic therapies such as chemotherapy and hormone therapy. The choice of treatment for BC is now driven by the tumor's histologic and molecular characteristics. The current policy of care for BC is tending towards less aggressive, more targeted, more personalized treatments [18]. Indeed, the indication for systemic chemotherapy is based on the risk of recurrence risk. This risk is determined by the above-mentioned risk factors and the BC's molecular classification. This therapeutic de-escalation has been made possible by the development of new tools for determining the indication for adjuvant chemotherapy. Genomic tests can now evaluate the 10-year risk of BC recurrence, which in turn can guide the treatment choice [18].

Despite the development of these new tools, some BCs will recur locally or will form metastases. It is therefore essential to find novel treatments and prognostic markers for even more effective patient care. The heterogeneity of BC is now taken into account when developing new therapies and prognostic factors, with a view to making treatment even more personalized.

Calcium ions ( $\text{Ca}^{2+}$ , from outside the cell or from within the endoplasmic reticulum (ER)) drive the development of metastases. When  $\text{Ca}^{2+}$  channels are open, the cytosolic  $\text{Ca}^{2+}$  concentration rises by a factor of 5 to 10 (from 100 nM to 500–1000 nM). Inside the cell, inositol (1,4,5)-trisphosphate ( $\text{IP}_3$ ) generates part of this calcium signaling via the  $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ), which has three characterized subtypes:  $\text{IP}_3\text{R}1$ ,  $\text{IP}_3\text{R}2$ , and  $\text{IP}_3\text{R}3$ . The three subtypes show 60% to 80% homology and are expressed ubiquitously. Interestingly, it was found that each subtype has a specific calcium release signature: strong  $\text{Ca}^{2+}$  oscillations through  $\text{IP}_3\text{R}2$ , weaker oscillations through  $\text{IP}_3\text{R}1$ , and monophasic transients through  $\text{IP}_3\text{R}3$  [19].

The (dys)regulation of  $\text{IP}_3\text{Rs}$  expression and activity is involved in many oncogenic processes, including cancer cell growth, migration, proliferation, and survival.  $\text{IP}_3\text{R}1$  is involved in apoptosis resistance in prostate cancer cells [20].  $\text{IP}_3\text{R}2$  is overexpressed in acute myeloid leukemia, and the level of expression is significantly correlated with poorer overall survival [21].  $\text{IP}_3\text{R}3$  is involved in glioblastoma cell migration and invasion [22], gastric cancer cell proliferation [23], and many other cancers (pancreatic, colonic, and renal) [24].

With regard to BC more specifically, the IP<sub>3</sub>R3 overexpression induced by estradiol promotes MCF-7 BC cell growth in vitro [25]. IP<sub>3</sub>R3 also regulates BC cell line proliferation via an interaction with BKCa voltage- and Ca<sup>2+</sup>-dependent K<sup>+</sup> channels [26]. Moreover, IP<sub>3</sub>R3 expression increases the migration capacity of human BC cells by shifting calcium oscillations towards a more sustained signature [27]. IP<sub>3</sub>R3 is also able to coordinate the remodeling of the profilin cytoskeleton organization through the ARHGAP18/RhoA/mDia1/FAK pathway [28]. In human BC more specifically, IP<sub>3</sub>R1 is not overexpressed. In contrast, Singh et al. found that IP<sub>3</sub>R2 and IP<sub>3</sub>R3 are more highly expressed in BC tissue than in non-tumor tissue [29]. In this context, we sought to (i) characterize the expression patterns of all IP<sub>3</sub>R subtypes within human BC tissue and (ii) evaluate the putative correlation between the IP<sub>3</sub>R3 expression level and the BC's proliferative/aggressive profile.

## 2. Materials and Methods

We conducted a prospective, observational study (named CARCINO study) at Amiens University Medical Center (Amiens, France). The study was approved by the local institutional review board (CPP Nord-Ouest II, Amiens, France; reference: ID-RCB 2015-A00537-42, dated July 2015). We included patients requiring surgery for an invasive BC with a greatest dimension > 15 mm. After resection, the pathologist collected one to three tumor tissue samples (size: 3 to 7 mm) depending on the tumor size. At least one sample per patient included was frozen immediately and stored at −80 °C for Western blot assays, any other samples were then used for immunohistochemistry. Informed consent was obtained from all subjects involved in the study.

### 2.1. Western Blot

The frozen tissue sample was mechanically dissociated in RIPA buffer (1% Triton 100×, 1% sodium deoxycholate, 150 mM NaCl, 2 mM EDTA, 5 mM PO<sub>4</sub>Na<sub>2</sub>/K, pH 7.2) supplemented with 0.8% protease inhibitor cocktail (Sigma Aldrich, IL, USA) in a special test tube for dissociation, using the GentleMACS™ system (Miltenyi Biotec, MA, USA) and the "Protein 01\_01" protocol. After centrifugation at 15,000× g and 4 °C for 15 min, the protein concentration in the supernatant was assayed using the BCA method (Bio-Rad, CA, USA) according to the manufacturer's instructions. Protein samples were then denatured for 10 min at 95 °C in Laemmli sample buffer. Protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond, Wis, GE Healthcare, Chicago, IL, USA). Membranes were blocked in 1% BSA in TBS-T (0.1% Tween 20, 50 mM Tris HCl buffer, 150 mM NaCl, pH 7.5). Next, the membranes were incubated overnight at 4 °C with mouse monoclonal anti-IP<sub>3</sub>R1 (1/500, Neuromab, CA, USA), rabbit monoclonal anti-IP<sub>3</sub>R2 (1/250, Santa Cruz, CA, USA), mouse monoclonal anti-IP<sub>3</sub>R3 (1/500, BD Biosciences, Switzerland), or goat polyclonal anti-actin (1/2500, Santa Cruz, CA, USA) primary antibodies diluted in 1% BSA in PBS-T. Actin primary antibody was used for loading control experiments. Membranes were then incubated for 1 h at RT with respective secondary antibodies (1/2500–1/5000; Santa-Cruz, CA, USA), developed using ECL substrate solution (ECL, RevelBolt Intense, Cell Signaling, Neve Yamin, Israel), exposed with the MF-ChemiBIS (DNR, bio-imaging systems, Neve Yamin, Israel) and analyzed using Quantity One software (Biorad, Hercules, CA, USA).

### 2.2. Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of BC tissue (thickness: 2 to 3 μm) were deparaffinized in xylene and then rehydrated in ethanol. The endogenous peroxidase activity was blocked before the antigen retrieval. The cell conditioning solution CC1 (BenchMark XT, Ventana, Rotkreuz, Switzerland) was then used for antigen retrieval.

Immunohistochemical staining was carried out on a BenchMark ULTRA system (Ventana, Rotkreuz, Switzerland) using antibodies against the three IP<sub>3</sub>Rs (1/50 for IP<sub>3</sub>R1 (Neuromab, CA, USA), 1/50 for IP<sub>3</sub>R2 (Santa Cruz, CA, USA) and 1/100 for

IP<sub>3</sub>R3 (BD Biosciences, Switzerland)). This was followed by avidin–biotin–peroxidase complex treatment. The signals were developed using a chromogenic reaction with 3,3'-diaminobenzidine tetrahydrochloride (iVIEW DAB Detection Kit, Ventana). The tissues were counterstained with hematoxylin. All antibodies were certified for immunohistochemical use. All experiments included a negative control (without the primary antibody).

The results were rated independently by two experienced investigators (PR and AF), using a Leica inverted microscope. The staining intensity score ranged from 0 to 3 (0 = no immunostaining; 1 = weak immunostaining; 2 = moderate immunostaining; 3 = strong immunostaining), and the percentage of stained cells was also recorded. An IP<sub>3</sub>R immunohistochemical (IH) expression score was then attributed for each tissue sample by multiplying the intensity score by the percentage of stained cells. The IH expression score therefore ranged from 0 (lowest) to 3 (highest).

We also used immunohistochemistry to assess the molecular subtype. This classification is essentially based on positivity for (and the percentage expression of) HmRs (the ER, in particular), HER2, and Ki67 (Table 1).

**Table 1.** Breast cancer molecular subtype. ER: estrogen receptor, PR: progesterone receptor.

	ER	PR	HER2	Ki67 Index
<b>Luminal A</b>	+	+	–	Low
<b>Luminal B Her2–</b>	+	+	–	High
<b>Luminal B Her2+ Her2</b>	+	+	+	High
<b>Triple negative</b>	–	–	–	High

### 2.3. Survival Analysis

Survival analysis was conducted using the SurvExpress online tool (Available in bioinformatica.mty.itesm.mx/SurvExpress). Expression levels of the individual genes (*ITPR1*, *ITPR2*, and *ITPR3*) and the combined signature were analyzed using SurvExpress and the optimized Maximize algorithm, which attributes a minimum *p*-value to a risk group. The hazard ratio (HR) [95% confidence interval (CI)] was also evaluated. Five datasets were used: the “Breast cancer recurrence data, 9 datasets from 7 authors” (1561 patients), “Breast cancer Meta-base: 10 cohorts 22K gene” (1888 patients), “Breast Invasive Carcinoma TCGA” (502 patients), “Miller Bergh Breast GSE3494-GPL96” (236 patients) and “BRCA-TCGA Breast invasive carcinoma—July 2016” (962 patients).

### 2.4. Statistical Analyses

In a descriptive analysis, normally distributed quantitative variables were quoted as the mean  $\pm$  standard error of the mean (SEM). Pairs of mean values were compared using a non-parametric Mann–Whitney test. A non-parametric Kruskal–Wallis test was used to compare means of more than two groups. The threshold for statistical significance was set to  $p < 0.05$ .

## 3. Results

### 3.1. Study Population

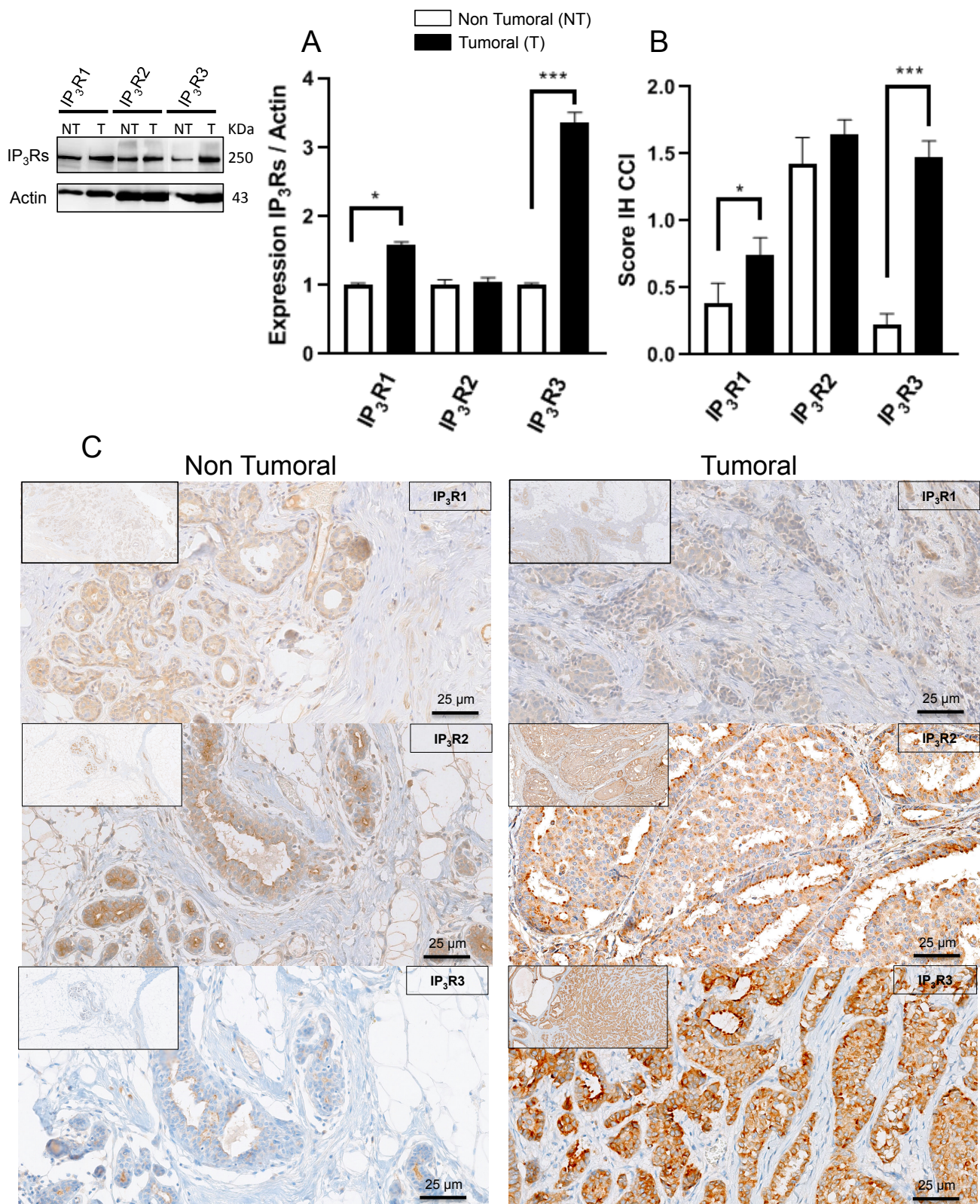
Between 1 November 2015 and 1 November 2018, 52 patients with IBC-NST treated at Amiens University Medical Center were included in the study. An additional 15 IBC-NST samples from patients at the Jean Godinot Institute cancer center (Reims, France) were included. Non-tumor tissue from the same patient was available for each BC tissue sample. The clinical and histologic characteristics are summarized in Table 2.

**Table 2.** Characteristics of the cohort of Invasive breast carcinoma of no special type (IBC-NST) samples from the CARCINO study and the Jean Godinot Institute cancer center. Data are quoted as the mean  $\pm$  SD or n (%). BMI: body mass index, T: tumor size (T1:  $\leq$ 20 mm; T2: 20–50 mm; T3: 50 mm). N: regional lymph nodes (N0: no lymph node invasion, N+: lymph nodes invaded). HmR: hormone receptor. SBR: Scarff-Bloom-Richardson.

		CARCINO IBC-NST Samples (n = 52)	Jean Godinot Institute IBC-NST Samples (n = 15)
		n (%)	n (%)
Age		57 $\pm$ 1.7	65.3 $\pm$ 3
BMI		27.1 $\pm$ 0.75	28.7 $\pm$ 2.4
TNM	T1	16 (30.7)	4 (26.7)
	T2	31 (59.6)	8 (53.3)
	T3	5 (9.7)	3 (20)
	N0	25 (48.1)	8 (53.3)
	N+	27 (51.9)	7 (46.7)
HmR+		43 (82.7)	12 (80)
HER2+++		12 (23.1)	2 (13.3)
Triple-negative		7 (13.5)	2 (13.3)
SBR grade	1	6 (11.5)	2 (13.3)
	2	26 (50)	3 (20)
	3	20 (38.5)	10 (66.7)
Ki67 > 20%		26 (50)	10 (66.7)

### 3.2. IP<sub>3</sub>Rs and Invasive Breast Carcinoma of No Special Type

First, we used Western blotting to evaluate the expression of each IP<sub>3</sub>R subtype in IBC-NST samples and non-tumor tissue samples. We found that both IP<sub>3</sub>R1 and IP<sub>3</sub>R3 expression were significantly higher in BC tissue than in non-tumor tissue (IP<sub>3</sub>R1; 1.59  $\pm$  0.04 ( $N = 26$ ) vs. 1  $\pm$  0.03 ( $N = 12$ ), respectively;  $p = 0.02$ ; IP<sub>3</sub>R3: 3.37  $\pm$  0.14 ( $N = 29$ ) vs. 1  $\pm$  0.02 ( $N = 12$ ), respectively;  $p < 0.0001$ ) (Figure 1A). IP<sub>3</sub>R3 expression was three times greater in BC tissue than in non-tumor tissue. In contrast, there was no difference in IP<sub>3</sub>R2 expression between BC tissue and non-tumor tissue (1.05  $\pm$  0.06 ( $N = 25$ ) vs. 1  $\pm$  0.08 ( $N = 13$ ), respectively;  $p = 0.8$ ) (Figure 1A). The same results were obtained when considering the IH expression score. Indeed, the IP<sub>3</sub>R1 IH expression score was significantly higher in BC tissue than in non-tumor tissue (0.75  $\pm$  0.14 ( $N = 40$ ) vs. 0.39  $\pm$  0.14 ( $N = 18$ ), respectively;  $p = 0.05$ ); the same was true for the IP<sub>3</sub>R3 IH expression score (1.48  $\pm$  0.12 ( $N = 41$ ) vs. 0.22  $\pm$  0.08 ( $N = 17$ ), respectively;  $p < 0.0001$ ) (Figure 1B). In contrast, there was no difference in the IP<sub>3</sub>R2 IH expression score between BC tissue and non-tumor tissue (1.64  $\pm$  0.11 ( $N = 30$ ) and 1.43  $\pm$  0.19 ( $N = 18$ ), respectively;  $p = 0.3$ ) (Figure 1B). Taken together, our results showed that IP<sub>3</sub>R1 and IP<sub>3</sub>R3 are overexpressed in IBC-NST, but not IP<sub>3</sub>R2.



**Figure 1.** IP<sub>3</sub>R expression in BC tissue and non-tumor tissue. The relative expression levels of IP<sub>3</sub>R1 and IP<sub>3</sub>R3 are significantly higher in BC tissue than in non-tumor tissue; this difference was not observed for IP<sub>3</sub>R2 (A). The same results were obtained when considering the IH expression score (B). (A) IP<sub>3</sub>R relative expression in IBC-NST, in a Western blot (T: tumor tissue; NT: non-tumor tissue). (B) The IP<sub>3</sub>R IH expression score in IBC-NST (T: tumor tissue; NT: non-tumor tissue). (C) A representative IH image (magnification: 200 X; insert: 800 X). \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

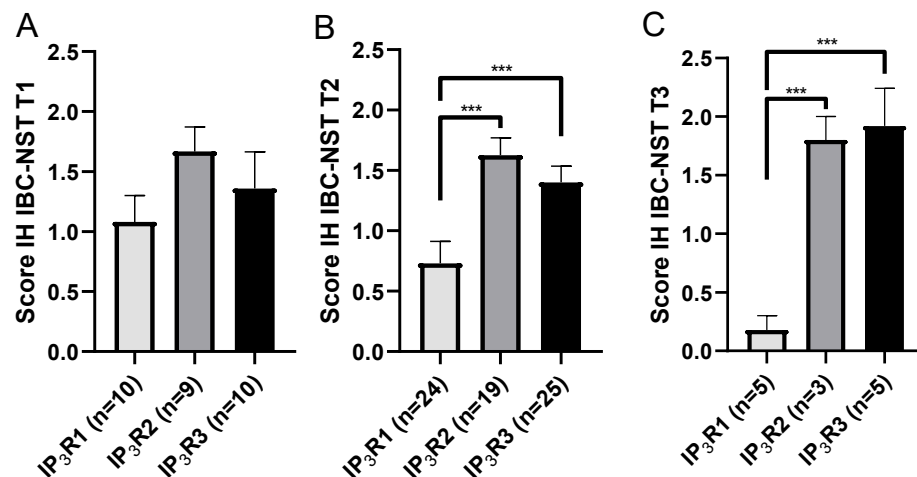
### 3.3. IP<sub>3</sub>R Expression and Predictive Factors

After having found, in Western blot and in IH, that IP<sub>3</sub>R1 and IP<sub>3</sub>R3 were overexpressed in IBC-NST, we next sought to establish a link between IP<sub>3</sub>R expression on the one hand and predictive factors for survival and recurrence in BC patients on the other.

### 3.4. IP<sub>3</sub>R Subtype IH Expression and Tumor Size

Tumor size is a major risk factor for local or remote BC recurrence; the larger the tumor, the greater the risk [30–33]. We compared the IH expression score for the three subtypes, as a function of the tumor size. We also studied the IH expression of IP<sub>3</sub>R2, even though its expression levels were similar in the paired tumor and non-tumor samples.

In BCs less than 20 mm in size (T1), there were no statistically significant differences in the IP<sub>3</sub>R1, IP<sub>3</sub>R2, and IP<sub>3</sub>R3 IH expression scores (IH expression score in T1 BCs:  $1.08 \pm 0.22$  ( $N = 10$ ) for IP<sub>3</sub>R1;  $1.67 \pm 0.21$  ( $N = 9$ ) for IP<sub>3</sub>R2 and  $1.36 \pm 0.3$ ;  $p = 0.33$  ( $N = 10$ ) for IP<sub>3</sub>R3) (Figure 2A). In T2 BCs (20–50 mm) and T3 BCs (>50 mm), the IP<sub>3</sub>R2 and IP<sub>3</sub>R3 IH expression scores were significantly higher than the IP<sub>3</sub>R1 IH expression score (IH expression score in T2 BCs:  $0.73 \pm 0.18$  ( $N = 24$ ) for IP<sub>3</sub>R1 vs.  $1.63 \pm 0.14$  ( $N = 19$ );  $p = 0.0001$  for IP<sub>3</sub>R2 and  $1.4 \pm 0.14$  ( $N = 25$ );  $p = 0.004$  for IP<sub>3</sub>R3. IH expression score in T3 BCs:  $0.18 \pm 0.12$  ( $N = 5$ ) for IP<sub>3</sub>R1 vs.  $1.8 \pm 0.2$  ( $N = 3$ );  $p = 0.04$  for IP<sub>3</sub>R2 and  $1.92 \pm 0.32$  ( $N = 5$ );  $p = 0.008$  for IP<sub>3</sub>R3) (Figure 2B,C). The IH expression of IP<sub>3</sub>R2 was independent of tumor size. Thus, large tumor size was more closely related to IP<sub>3</sub>R3 expression than to IP<sub>3</sub>R1 expression.



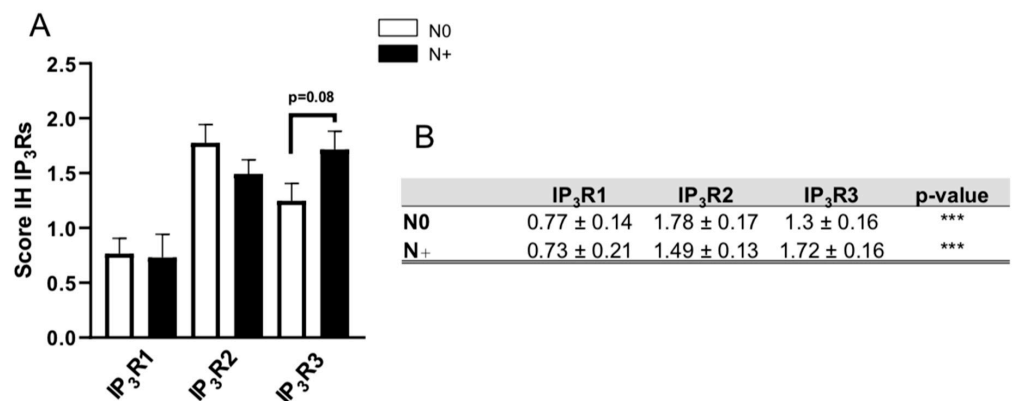
**Figure 2.** The IP<sub>3</sub>R IH expression score, as a function of tumor size (A–C). The IP<sub>3</sub>R IH expression scores did not differ significantly in T1 BC (A). The IP<sub>3</sub>R2 and IP<sub>3</sub>R3 IH expression scores were significantly higher than IP<sub>3</sub>R1 score in T2 and T3 BCs (B,C). \*\*\*  $p < 0.001$ .

### 3.5. IP<sub>3</sub>Rs and Lymph Node Involvement

Regional lymph node involvement is a risk factor for local and remote recurrence [30,31]. We compared IBC-NST with (N+) and without (N0) regional lymph node involvement with regard to the IH expression score for each of the three subtypes.

Only the IP<sub>3</sub>R3 IH expression score was greater (by a factor of 1.3) in N+ BCs ( $1.72 \pm 0.16$  ( $N = 20$ )) than in N0 BCs ( $1.3 \pm 0.16$  ( $N = 21$ );  $p = 0.08$ ) (Figure 3A). The IP<sub>3</sub>R1 and IP<sub>3</sub>R2 IH expression scores were similar when comparing N0 and N+ samples. In N0 BCs, the IH expression score was  $0.77 \pm 0.14$  ( $N = 20$ ) for IP<sub>3</sub>R1 vs.  $1.78 \pm 0.17$  ( $N = 16$ ;  $p < 0.0001$ ) for IP<sub>3</sub>R2, and  $1.3 \pm 0.16$  ( $N = 21$ ;  $p = 0.03$ ) for IP<sub>3</sub>R3. In N+ BCs, the IH expression score was  $0.73 \pm 0.21$  ( $N = 20$ ) for IP<sub>3</sub>R1, vs.  $1.49 \pm 0.13$  ( $N = 14$ ;  $p = 0.001$ ) for IP<sub>3</sub>R2, and  $1.72 \pm 0.16$  ( $N = 20$ ;  $p = 0.001$ ) for IP<sub>3</sub>R3. (Figure 3B). Moreover, the IP<sub>3</sub>R1 IH expression score was significantly lower than the IP<sub>3</sub>R2 and IP<sub>3</sub>R3 IH expression scores in both N0 and N+ BCs (Figure 3B). Thus, IP<sub>3</sub>R3 appeared to be specifically related to lymph node involvement in IBC-NST.

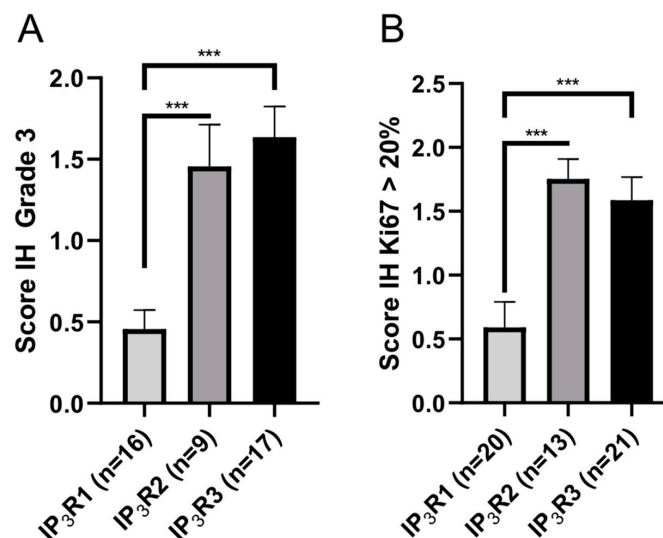




**Figure 3.** The IP<sub>3</sub>R IH expression scores as a function of lymph node status (A,B). \*\*\*  $p < 0.001$ .

### 3.6. IP<sub>3</sub>Rs, Histology Grades, and the Ki67 Proliferation Index

The Ki67 proliferation index and the histologic (SBR) grade are risk factors for BC recurrence: the higher the index or grade, the greater the risk of recurrence [5,30–33]. We therefore compared the SBR histologic grade and Ki67 index with regard to the three subtypes IH expression scores. We found that the IH expression scores for IP3R2 and IP3R3 were significantly higher than that for IP3R1 in grade III SBR samples and samples with a Ki67 index greater than 20% (Figure 4A,B). The IH expression score in grade 3 samples was  $0.46 \pm 0.12$  ( $N = 16$ ) for IP3R1 vs.  $1.47 \pm 0.26$  ( $N = 9$ ;  $p = 0.0006$ ) for IP3R2 and  $1.64 \pm 0.19$  ( $N = 17$ ;  $p < 0.0001$ ) for IP3R3. (B) The IH expression score in samples with a Ki67 index  $> 20\%$  was  $0.59 \pm 0.2$  ( $N = 20$ ) for IP3R1 vs.  $1.75 \pm 0.15$  ( $N = 13$ ;  $p < 0.0001$ ) for IP3R2 and  $1.59 \pm 0.18$  ( $N = 21$ ;  $p < 0.0001$ ) for IP3R3 (Figure 4A,B). In both settings, there was no difference between the IP3R2 and IP3R3 IH expression scores. Given that IP3R2 is not overexpressed in BC tissue, IP3R3 expression is thus closely related to BC with a poor prognosis.

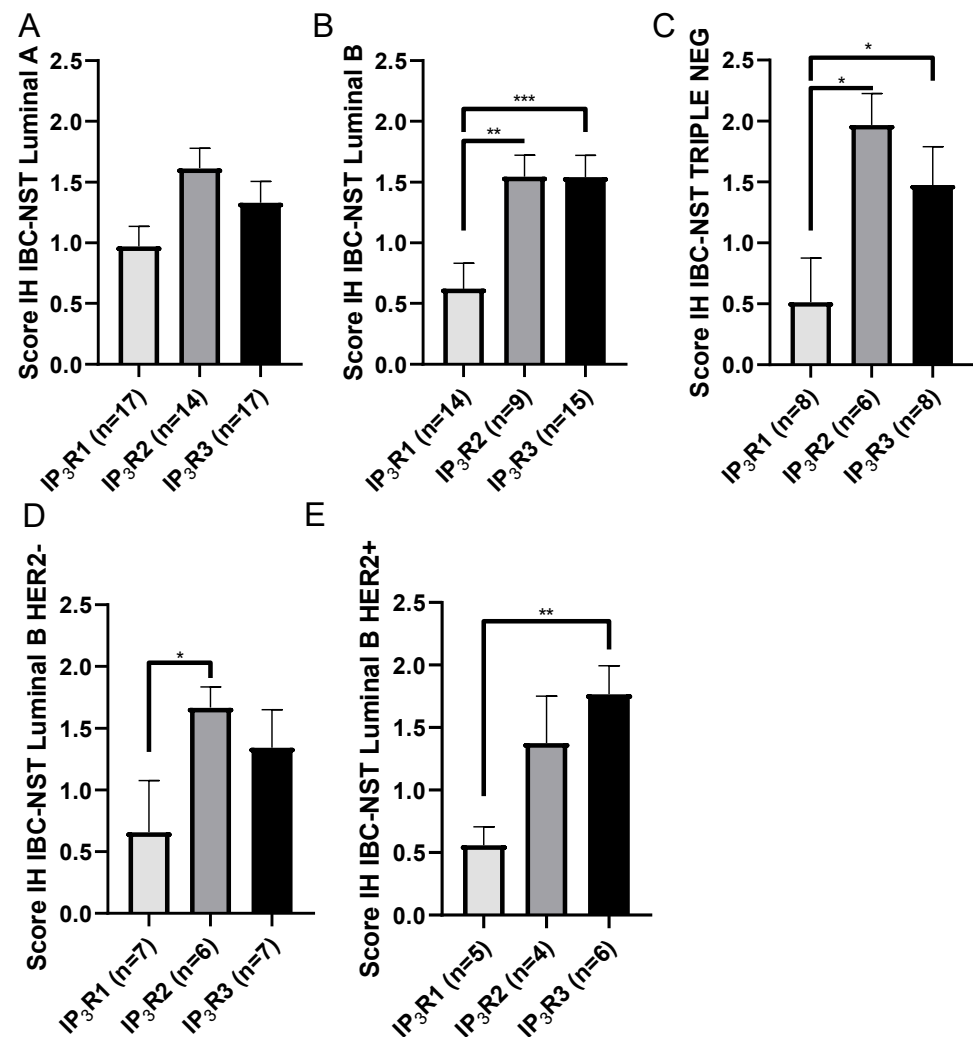


**Figure 4.** The IP<sub>3</sub>R IH expression scores in SBR grade 3 IBC-NST (A) and IBC-NST with a high Ki67 proliferation index (B). \*\*\*  $p < 0.001$ .

### 3.7. IP<sub>3</sub>Rs and the Molecular Classification of BCs

At present, clinicians typically classify carcinomas into five subtypes (luminal A, luminal B HER2<sup>-</sup>, luminal B HER2<sup>+</sup>, HER2, and TN) on the basis of histologic and molecular characteristics, which notably include expression of the estrogen receptor (ER), progesterone receptor (PR), HER2, and Ki67. Tumors that are ER<sup>+</sup> and/or PR<sup>+</sup> are referred to as HmR<sup>+</sup>. The prognosis, disease aggressiveness, and risk of remote metastasis and relapse vary from one subtype to another. In fact, the luminal A subtype has the best prognosis [34].

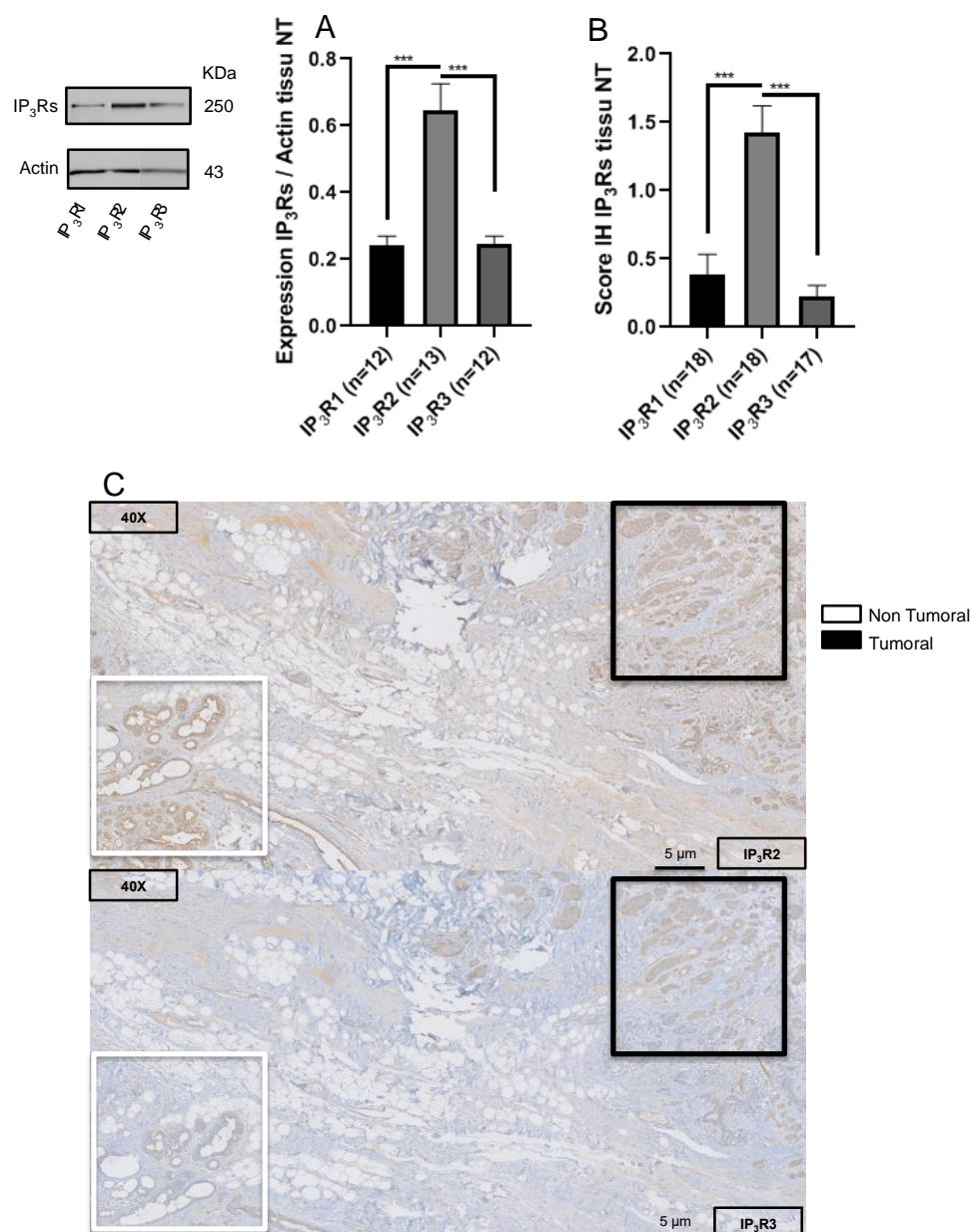
We assessed the three IP<sub>3</sub>R IH expression scores in the five BC molecular subtypes. In luminal A BC, there were no significant differences in the IH expression scores for the three IP<sub>3</sub>R subtypes (IH expression scores in luminal A BC:  $0.97 \pm 0.17$  ( $N = 17$ ) for IP<sub>3</sub>R1, vs.  $1.61 \pm 0.16$  ( $N = 14$ ) for IP<sub>3</sub>R2 and:  $1.33 \pm 0.17$  ( $N = 17$ ) for IP<sub>3</sub>R3;  $p = 0.2$ ) (Figure 5A). In luminal B and TN samples, the IH expression score was significantly lower for IP<sub>3</sub>R1 than for IP<sub>3</sub>R2 and IP<sub>3</sub>R3 (IH expression scores in luminal B BC:  $0.62 \pm 0.21$  ( $N = 14$ ) for IP<sub>3</sub>R1, vs.  $1.54 \pm 0.18$  ( $N = 9$ ;  $p = 0.002$ ) for IP<sub>3</sub>R2 and  $1.54 \pm 0.18$  ( $N = 15$ ;  $p = 0.0002$ ) for IP<sub>3</sub>R3 and IP<sub>3</sub>R1 IH expression scores in TN BC:  $0.51 \pm 0.36$  ( $N = 8$ ) for IP<sub>3</sub>R1 vs.  $1.97 \pm 0.26$  ( $N = 6$ ;  $p = 0.01$ ) for IP<sub>3</sub>R2 and  $1.48 \pm 0.32$  ( $N = 8$ ;  $p = 0.02$ ) for IP<sub>3</sub>R3) (Figure 5B,C). In luminal B HER2- samples, the IH expression score was significantly higher for IP<sub>3</sub>R2 than for IP<sub>3</sub>R1 (IH expression scores in luminal B HER2-:  $0.66 \pm 0.42$  ( $N = 7$ ) for IP<sub>3</sub>R1, vs.  $1.67 \pm 0.17$  ( $N = 6$ ;  $p = 0.04$ ) for IP<sub>3</sub>R2 and  $1.3 \pm 0.31$  ( $N = 7$ ) for IP<sub>3</sub>R3). In luminal B HER2+ samples, the IH expression score was significantly higher for IP<sub>3</sub>R3 than for IP<sub>3</sub>R1 (IP<sub>3</sub>R1 IH expression scores in luminal B HER2+:  $0.56 \pm 0.15$  ( $N = 5$ ) for IP<sub>3</sub>R1, vs.  $1.77 \pm 0.23$  ( $N = 6$ ;  $p = 0.004$ ) for IP<sub>3</sub>R3 and  $1.38 \pm 0.38$  ( $N = 4$ ) for IP<sub>3</sub>R2) (Figure 5D,E). There were no differences between the IP<sub>3</sub>R2 and IP<sub>3</sub>R3 IH expression scores in any of the subtypes. This finding suggests that IP<sub>3</sub>R3 is associated with a more aggressive disease profile for IBC-NST.



**Figure 5.** IP<sub>3</sub>R expression score as a function of the BC molecular subtype. (A) Luminal A, (B) luminal B, (C) triple negative, (D) luminal B HER2-, and (E) luminal B HER2+ BC. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

### 3.8. Expression of IP<sub>3</sub>Rs in Non-Tumor Tissue

We also compared the expression of IP<sub>3</sub>Rs in the non-tumor tissue. Our results, obtained both by IH and Western blot, show that IP<sub>3</sub>R2 is strongly expressed in contrast to IP<sub>3</sub>R1 and IP<sub>3</sub>R3. IH expression score: IP<sub>3</sub>R2 =  $1.43 \pm 0.19$  ( $N = 18$ ) vs.  $0.39 \pm 0.14$  ( $N = 18$ );  $p = 0.0001$  for IP<sub>3</sub>R1 and  $0.22 \pm 0.08$  ( $N = 17$ );  $p < 0.0001$  for IP<sub>3</sub>R3. The IP<sub>3</sub>R2 relative expression score is  $0.65 \pm 0.08$  ( $N = 13$ ) vs.  $0.24 \pm 0.03$  ( $N = 12$ );  $p < 0.0001$  for IP<sub>3</sub>R1 and  $0.25 \pm 0.03$  ( $N = 12$ );  $p < 0.0001$  for IP<sub>3</sub>R3. Indeed, the relative expression and IH expression scores are significantly higher for IP<sub>3</sub>R2 than for IP<sub>3</sub>R1 or IP<sub>3</sub>R3 (Figure 6A,B). Thus, in contrast to IP<sub>3</sub>R3, IP<sub>3</sub>R2 appears as a highly expressed IP<sub>3</sub>R in non-tumoral as in tumoral breast tissue.

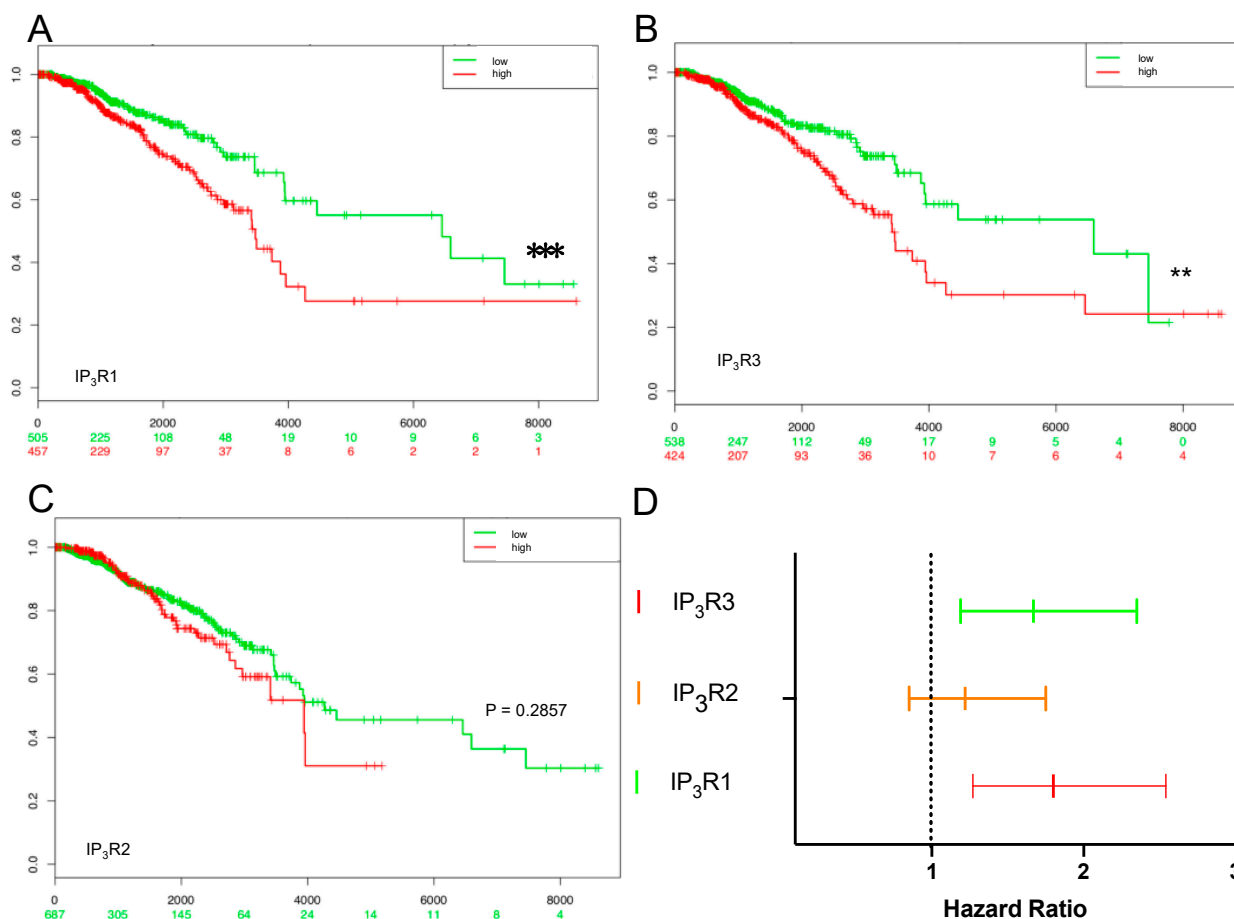


**Figure 6.** IP<sub>3</sub>R expression in non-tumor tissue. (A) Western blot analysis of the relative expression of IP<sub>3</sub>R1/2/3. (B) The IH expression score for IP<sub>3</sub>R1/2/3. (C) Representative IH images. \*\*\*  $p < 0.001$ .

### 3.9. IP<sub>3</sub>Rs and Patient Survival

We next sought to establish whether or not the expression of the three IP<sub>3</sub>R subtypes was correlated with the patients' survival. We compared survival data and the HR for

predefined populations, such as high-risk and low-risk groups for the three receptor subtypes (Figure 7). We used the SurvExpress website’s optimized algorithm to generate risk groups by sorting according to the prognostic index (a higher IP<sub>3</sub>R value for a higher risk) and splitting the patients into cohorts where the *p*-value was the lowest [35].



**Figure 7.** Analysis of the association between IP<sub>3</sub>R expression and overall survival, after application of the SurvExpress tool to the “BRCA-TCGA Breast invasive carcinoma—July 2016” database (comprising 962 BC samples). (A,B) IP<sub>3</sub>R1 expression and IP<sub>3</sub>R3 expression were significantly associated with poor overall survival ( $p = 0.0009$  and  $p = 0.003$ ). (C) IP<sub>3</sub>R2 expression was not significantly associated with poor overall survival ( $p = 0.286$ ). (D) The HR for each IP<sub>3</sub>R appears in an analysis of the “Breast invasive carcinoma—July 2016” database. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

We chose to analyze five databases (listed in the “Survival analysis” section of the Methods) with the largest number of documented patients. In the “BRCA-TCGA Breast invasive carcinoma—July 2016” database, IP<sub>3</sub>R1 and IP<sub>3</sub>R3 expression were significantly associated with poor overall survival (Figure 7A,B). The results for IP<sub>3</sub>R2 were not statistically significant (Figure 7C). IP<sub>3</sub>R1 gave a higher HR (1.8; 95%CI [1.27–2.54];  $p = 0.0009$ ) than IP<sub>3</sub>R3 (1.67; 95%CI [1.19–2.35];  $p = 0.003$ ) or IP<sub>3</sub>R2 (1.22; 95%CI [0.85–1.75];  $p = 0.286$ ) (Figure 7D). IP<sub>3</sub>R1 and IP<sub>3</sub>R3 expression were significantly associated with poor overall survival in 1358 and 1700 patients, respectively (Table 3), whereas IP<sub>3</sub>R2 expression was associated with poor overall survival in only 434 patients (Table 3).

**Table 3.** Statistically significant correlations between IP<sub>3</sub>R subtype expression and survival. IP<sub>3</sub>R3 and IP<sub>3</sub>R1 expression levels were significantly associated with worse overall survival in three datasets (comprising 1358 patients (A) and 1700 patients (B), respectively), and IP<sub>3</sub>R2 was significantly associated with worse overall survival in two datasets (comprising 434 patients) (C). Expression of the three IP<sub>3</sub>R subtypes was significantly associated with worse recurrence-free survival in two datasets (comprising 3449 patients) (D). HR: hazard ratio. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

<b>A</b>			
Datasets—IP <sub>3</sub> R3 and Overall Survival	N: Low-Risk Group vs. High-Risk Group	HR [95%CI]	<i>p</i> -Value
Breast—Breast cancer recurrence data, 9 datasets from 7 authors	198; 92 vs. 106	1.74 [1.23–2.46]	**
Breast—Breast Cancer Metabase:10 cohorts 22K genes	198; 86 vs. 112	1.44 [1.02–2.02]	*
Breast—BRCA-TCGA Breast Invasive Carcinoma—July 2016	962; 538 vs. 424	1.67 [1.19–2.35]	**
<b>B</b>			
Datasets—IP <sub>3</sub> R1 and Overall Survival	N: Low-Risk Group vs. High-Risk Group	HR [95%IC]	<i>p</i> -Value
Breast—Breast Invasive Carcinoma TCGA	502; 376 vs. 126	1.87 [1–3.19]	*
Breast—Miller Bergh Breast GSE3494—GPL96	236; 45 vs. 191	4.64 [1.45–14.87]	**
Breast—BRCA—TCGA Breast invasive carcinoma—July 2016	962; 505 vs. 457	1.8 [1.27–2.54]	***
<b>C</b>			
Datasets—IP <sub>3</sub> R2 and Overall Survival	N: Low-Risk Group vs. High-Risk Group	HR [95%IC]	<i>p</i> -Value
Breast—Breast Cancer Metabase:10 cohorts 22K genes	198; 167 vs. 31	1.61 [1.03–2.53]	*
Breast—Miller Bergh Breast GSE3494—GPL96	236; 45 vs. 191	4.64 [1.45–14.87]	**
<b>D</b>			
Datasets—Disease-Free Survival	N: Low-Risk Group vs. High-Risk Group	HR [95%IC]	<i>p</i> -Value
IP <sub>3</sub> R3—Breast cancer recurrence data, 9 datasets from 7 authors	1561; 967 vs. 594	1.28 [1.08–1.51]	**
IP <sub>3</sub> R3—Breast Cancer Metabase:10 cohorts 22K genes	1888; 1407 vs. 481	1.25 [1.05–1.49]	*
IP <sub>3</sub> R2—Breast cancer recurrence data, 9 datasets from 7 authors	1561; 1194 vs. 367	1.27 [1.05–1.53]	*
IP <sub>3</sub> R2—Breast Cancer Metabase:10 cohorts 22K genes	1888; 1614 vs. 274	1.36 [1.11–1.67]	**
IP <sub>3</sub> R1—Breast cancer recurrence data, 9 datasets from 7 authors	1561; 842 vs. 719	1.43 [1.21–1.69]	***
IP <sub>3</sub> R1—Breast Cancer Metabase:10 cohorts 22K genes	1888; 1113 vs. 775	1.48 [1.27–1.73]	***

Whenever possible, we evaluated the association between IP<sub>3</sub>RS expression and disease-free survival (Table 3); data were available for the “Breast cancer recurrence data, 9 datasets from 7 authors” and “Breast cancer Meta-base: 10 cohorts 22K gene” databases, comprising a total of 3449 samples. IP<sub>3</sub>R expression was significantly associated with poor disease-free survival (Table 3).

#### 4. Discussion

Our present results evidenced different IP<sub>3</sub>R subtype expression profiles in BC. IP<sub>3</sub>R3 was most overexpressed in IBC-NST and was correlated with clinical features such as

tumor size and grade, regional node invasion, proliferation index, and HmR status. IP<sub>3</sub>R1 was also overexpressed, albeit to a lesser extent than IP<sub>3</sub>R3; however, the expression of IP<sub>3</sub>R1 was inversely correlated with tumor size and did not vary as a function of the other prognostic markers. IP<sub>3</sub>R2 was more strongly expressed than the other two subtypes in non-tumor tissue without being overexpressed in BC tissue compared to non-tumor tissue. Interestingly, IP<sub>3</sub>R2 expression remained high regardless of tumor size, lymph node involvement, histologic grade, tKi67 index, and BC molecular classification. Moreover, we found that high IP<sub>3</sub>R expression was significantly associated with worse overall survival among treated patients.

IP<sub>3</sub>Rs have a role in the pathophysiology of cancer. In myeloid acute leukemia, the IP<sub>3</sub>R2 expression level is correlated with worse overall survival [21]. IP<sub>3</sub>R1 is involved in resistance to apoptosis in prostate cancer cells [20] and in the epithelial–mesenchymal transition induced by epidermal growth factor in the MDA-MB-468 human BC cell line [36]. Kang et al. demonstrated that IP<sub>3</sub>R3 had prognostic value in glioblastoma in an animal model; the survival rate was higher when IP<sub>3</sub>R3 expression was inhibited [22]. IP<sub>3</sub>R3 is also involved in the peritoneal dissemination of gastric cancer cells [23]. Moreover, the IP<sub>3</sub>R3 expression level is correlated with the aggressiveness of colorectal cancer [37].

More specifically, IP<sub>3</sub>R3 is a key factor in many mechanisms of BC oncogenesis. It is involved in BC cell proliferation via estrogen-dependent stimuli [25] and an interaction with the BKCa potassium channel [26]. Moreover, the IP<sub>3</sub>R3 expression level influences the migration capacity of human BC cells by changing the calcium signature [27]. IP<sub>3</sub>R3 is also able to coordinate the remodeling of the profilin cytoskeleton via the ARHGAP18/RhoA/mDia1/FAK pathway [28]. However, a prognostic role for IP<sub>3</sub>R3 (or even a correlation with BC aggressiveness, as observed for glioblastoma or colorectal cancer) has not yet been demonstrated. Studying the tissue expression of the three IP<sub>3</sub>R subtypes is thus essential for understanding their mechanism of action. In that context, our results established for the first time a link between the level of expression of IP<sub>3</sub>R3 and the aggressiveness of IBC-NST. To the best of our knowledge, our study is the first to have established this link in BC—a link that is already known for cancers such as glioblastoma and gastric cancer. Although the prospective nature of our study consequently limited the number of samples eligible for this work, it enabled us to obtain precise data in terms of overall survival but also in terms of recurrence-free survival at 5 years. These data will be the subject of a future study.

Based on a SurvExpress analysis of five databases, we showed that expression of the three IP<sub>3</sub>R subtypes is negatively correlated with overall survival and disease-free survival. In BC, there are many clinical or histologic survival factors. All these criteria should be taken into account when selecting the primary treatment, neo-adjuvant or adjuvant chemotherapy, or a more targeted type of therapy such as hormone therapy. The negative correlation between IP<sub>3</sub>R subtype expression on the one hand, and overall survival and disease-free survival on the other, testifies to the receptor's significant involvement in breast carcinogenesis in general and metastatic invasion in particular. It is known that the mortality rate in BC is linked to the occurrence of remote metastases [38,39]. Moreover, our database analyses showed that receptor expression is inversely correlated with overall survival and disease-free survival—suggesting a genuine prognostic role for these receptors.

We found that IP<sub>3</sub>R1 and (especially) IP<sub>3</sub>R3 are expressed significantly more in BC tissue than in non-tumor tissue (Figure 1). IP<sub>3</sub>R3 expression appears to be correlated with all the known prognostic factors for BC. In non-tumor tissue, IP<sub>3</sub>R2 is expressed significantly more than IP<sub>3</sub>R1 and IP<sub>3</sub>R3; however, IP<sub>3</sub>R2 expression is also high in all BC subtypes. IP<sub>3</sub>R1 is overexpressed in BC but to a lesser extent than IP<sub>3</sub>R3. IP<sub>3</sub>R1 expression (relative to the two other subtypes) decreases with tumor size but is not correlated with other BC aggressiveness factors (lymph node involvement, histologic grade, and the Ki67 index). IP<sub>3</sub>R3 can thus be considered as a marker of aggressiveness in BC as its expression is correlated to the severity of BC.

Our data argue in favor of the involvement of all IP<sub>3</sub>R subtypes in breast carcinogenesis processes. Differences in expression between non-tumor tissue and tumor tissue are also observed in colorectal cancer. Indeed, IP<sub>3</sub>R3 is not detected in non-tumor tissue (in contrast to the other two subtypes) but is overexpressed in colorectal cancer tissue [37]. The IP<sub>3</sub>R subtypes are not expressed in all tissues and are expressed to differing degrees in given cell types. In rat hepatocytes, for example, IP<sub>3</sub>R3 is not expressed, IP<sub>3</sub>R1 is expressed diffusely in the cytoplasm, and IP<sub>3</sub>R2 is concentrated in the pericanalicular region [38]. These subcellular differences in location suggest that the three IP<sub>3</sub>R subtypes have distinct functions in the hepatocyte's calcium signaling activity [38]. The differences in expression of the three subtypes between BC tissue and non-tumor tissue suggest that each subtype has a different role in breast carcinogenesis. IP<sub>3</sub>R1 and IP<sub>3</sub>R3 are involved in the epithelial–mesenchymal transition in BC cells, whereas IP<sub>3</sub>R2 is not [36].

Next, we sought to establish a link between IP<sub>3</sub>R expression and other predictive factors of survival in BC. There were no significant differences in IP<sub>3</sub>R subtype expression in tissue from BCs smaller than 20 mm. However, the IH expression scores were significantly higher for IP<sub>3</sub>R2 and IP<sub>3</sub>R3 than for IP<sub>3</sub>R1 in tumors larger than 20 mm (i.e., T2 and T3). IP<sub>3</sub>R3 expression appears to be correlated with tumor size. The IP<sub>3</sub>R3 subtype is therefore linked to disease aggressiveness with regard to tumor size—a major factor in the decision to prescribe adjuvant treatment. Based on other prognostic criteria, patients with a small tumor size might not receive chemotherapy. Wallgren et al. showed that tumor size >20 mm was a risk factor for local or remote recurrence [4].

Regional lymph node invasion is a major risk factor for recurrence; the greater the number of invaded axillary lymph nodes, the greater the incidence of local or remote recurrence [4]. IP<sub>3</sub>R3 is the most highly involved subtype since it (but not IP<sub>3</sub>R1 or IP<sub>3</sub>R2) is upregulated in N+ tissue. IP<sub>3</sub>R3 expression may therefore be linked to a more aggressive profile with regard to the lymph node invasion criterion.

In SBR grade III IBC-NST, IP<sub>3</sub>R1 was expressed significantly less than the other two subtypes. These results suggest that IP<sub>3</sub>R3 expression is associated with the histologic prognosis and, again, more aggressive disease in patients with IBC-NST. Likewise, in tissue with a Ki67 proliferation index above 20%, IP<sub>3</sub>R1 was expressed significantly less than the other two subtypes. IP<sub>3</sub>R3, therefore, appears to be involved in IBC-NST with a high proliferation index and is linked to a more aggressive disease profile. The proliferation index and the histologic grade are predictive of a poor prognosis and are taken into account for certain treatment decisions.

Lastly, IP<sub>3</sub>R3 was expressed significantly more than IP<sub>3</sub>R1 in BCs overexpressing HmRs and HER2, whereas the expression of IP<sub>3</sub>R2 was significantly higher than that of IP<sub>3</sub>R1 in TN BC. Moreover, no differences between IP<sub>3</sub>R2 expression and IP<sub>3</sub>R3 expression were found in these two tumor subtypes. Overexpression of the HER2 receptor and the absence of HmR and HER2 expression are associated with a worse prognosis [10]. Our results therefore suggest that IP<sub>3</sub>R3 is associated with more aggressive IBC-NST profiles, as defined by positivity or negativity for HmRs and HER2.

Based on our results, IP<sub>3</sub>R3 expression appears to be associated with more aggressive IBC-NST profiles. We established that the level of IP<sub>3</sub>R3 expression was significantly higher in BC tissue than in non-tumor tissue. IP<sub>3</sub>R2 was not overexpressed in BC tissue but is strongly expressed in all tissues—suggesting that it is essential for the mammary gland's function. Therefore, our findings suggest that (i) IP<sub>3</sub>R3 is the subtype most significantly involved in breast carcinogenesis processes, and (ii) IP<sub>3</sub>R3 expression is linked to a more aggressive BC profile. Hence, IP<sub>3</sub>R3 might be a specific marker of BC aggressiveness. In contrast to the other two subtypes, IP<sub>3</sub>R3 is significantly involved in the proliferation and migration of human BC cells [26,27]. Furthermore, IP<sub>3</sub>R3 influences the morphology of BC cells. When IP<sub>3</sub>R3 expression is low, human BC cells take on a round shape that is much less conducive to migration and invasion [28]. Proliferation, migration, and invasion are three major factors in carcinogenesis. In BC, the prognosis is essentially linked to the presence of regional or remote metastases. When cancer cells lack migratory or invasive abilities,

they are unable to metastasize. IP<sub>3</sub>R3 might be involved in cell migration and invasion, with a role in the BC prognosis. IP<sub>3</sub>R3 is known to be involved in the carcinogenesis of other cancers. IP<sub>3</sub>R3 is thus overexpressed in cholangiocarcinoma, where it is involved in cell migration and proliferation [39]. It also has a major role in invasion processes in glioblastoma [22]. These two cancers have a very poor prognosis, which emphasizes IP<sub>3</sub>R3 putative involvement as a prognostic factor in certain cancers.

IP<sub>3</sub>Rs (mainly IP<sub>3</sub>R3) have a role in apoptosis. In B and T lymphocytes, elevated IP<sub>3</sub>R3 expression induces more apoptosis [40]; this is also the case in pancreatic cells [41] and hepatocytes [42]. In some cancers, IP<sub>3</sub>R3 becomes anti-apoptotic; this is the case in clear cell kidney cancers, where IP<sub>3</sub>R3 has anti-apoptotic activity and IP<sub>3</sub>R1 has pro-apoptotic activity [43]. This might also be the case in BC since the inhibition of IP<sub>3</sub>R expression in human BC cells induces an increase in apoptosis [29].

The current problem in BC management is the absolute necessity to not “over-treat” patients. The latest results from basic research and clinical studies argue in favor of therapeutic de-escalation and personalized medicine [18]. For example, the advent of genomic testing several years ago has enabled some patients with non-aggressive cancers to avoid chemotherapy. IP<sub>3</sub>R3’s involvement in overall survival and disease-free survival in BC remains to be defined. If it is proven that a low IP<sub>3</sub>R3 threshold in an invasive breast tumor is correlated with greater survival, this receptor might eventually become a complementary tool in the decision to initiate (or not) adjuvant treatment. IP<sub>3</sub>R3 expression could therefore be integrated into genomic-based calculations of the recurrence risk.

In conclusion, our results strongly suggest that IP<sub>3</sub>R3 can be considered a prognostic marker in BC. In the longer term, it might be possible to predict tumor aggressiveness and perhaps select an appropriate adjuvant treatment if the primary tumor strongly expresses IP<sub>3</sub>R3.

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